ELECTRONIC LETTER

Genetic influences on the circulating cytokines involved in osteoclastogenesis

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J Med Genet 2004;**41**:e76 (http://www.jmedgenet.com/cgi/content/full/41/6/e76). doi: 10.1136/jmg.2003.014373

The tumour necrosis factor family molecule RANKL (receptor activator of nuclear factor-kB ligand), its cellular receptor RANK, and the decoy receptor, osteoprotegerin (OPG) represent a novel cytokine triad with pleiotropic effects on bone metabolism, the immune system, and endocrine functions.[1] As illustrated in figure 1, RANKL is expressed on the osteoblast/stromal cell surface, binds to its receptor-RANK on the surface of haematopoietic precursor cells and, in the presence of macrophage colony stimulating factor (M-CSF), stimulates differentiation, fusion, activation, and survival of osteoclasts.[2 3] These four molecules, acting in accord, are the major regulators of osteoclast formation and function. It has been claimed that binding M-CSF and RANKL to their respective receptors is the necessary and sufficient condition to initiate osteoclastogenesis.[4] On the other hand, RANKL is abundantly produced by activated T lymphocytes, and it prevents apoptosis and prolongs survival of dendritic cells, which also express large amounts of the receptor for RANKL (fig 1). This dual function of the RANKL/RANK/OPG system explains why autoimmune diseases, cancers, leukemias, asthma, chronic viral infections, and periodontal disease result in systemic and local bone loss.

Numerous reports now implicate the altered RANKL/RANK/OPG system physiology as a factor in the development and severity of many diseases. Three such maladies for which a growing volume of literature exists are rheumatoid arthritis, osteoporosis, and skeletal metastases. Neoplastic cells in giant cell tumours[5] and multiple myeloma[6] express RANKL as well as other osteolytic factors, and the OPG/RANKL ratio was significantly lower in patients with these osteolytic tumours than in those with non-lytic neoplasms.[7–9] However, patients with some lytic solid tumours, including advanced prostate cancer[10] and Hodgkin's disease,[8] have higher serum OPG concentrations than do healthy individuals, with the levels often more elevated in subjects with metastatic rather than localised disease.[10] It has been shown that compared with healthy subjects or patients with other types of tumour, patients with myeloma bone disease have lower OPG levels in their serum (or within the bone microenvironment), and that these low OPG serum levels are inversely correlated with the severity of the disease. One study even suggests that serum levels of OPG could serve as a cardiovascular risk factor.[11] Further support for OPG being a vascular regulator comes from an epidemiological study conducted by Browner and colleagues,[12] wherein the serum OPG was 30% higher in women with diabetes than in non-diabetic women, and was also associated with increased cardiovascular mortality.

By studying well characterised patient cohorts with endocrine and immune diseases such as Cushing's syndrome, acromegaly, growth hormone deficiency, HIV infection, and common variable immunodeficiency (CVI), the investigators reported significantly increased OPG serum levels in both groups with persistent immune activation (HIV, CVI).[13] All these data provide further evidence that the immune system and bone metabolism may be linked through the RANKL/OPG system.

Key points

- OPG and M-CSF plasma levels were measured in 566 healthy individuals belonging to 126 nuclear and more complex ethnically homogeneous Caucasian families.
- Analysis showed that the additive genetic component explained a very substantial portion of the OPG and M-CSF variations, but little of the sRANKL variation.
- Common familial environment factors made a significant contribution to the OPG and M-CSF variations, but were undetectable for sRANKL.
- The strong impact of genetic factors on the variation of OPG and M-CSF suggests that profound deviations in these cytokine levels may be a consequence of anomalies in the corresponding genes, whereas with sRANKL they may be due to environmental influences.

It is obvious that the assessment of local and systemic levels of RANKL/OPG/M-CSF, the clarification of genetic and environmental factors affecting the production of these cytokines, and the evaluation of the role of these factors as possible biochemical markers for risk stratification in metabolic bone diseases, may represent an important area of research in normal and pathological osteoclastogenesis. Yet, despite a growing body of evidence on the paramount importance of the RANK-OPG axis, and of M-CSF, and despite data suggesting strong genetic effects on the circulating levels of calciotropic hormones[14] and several other factors of bone metabolism,[15] there are still virtually no published data on the genetic regulation of the above mentioned cytokines. A few studies carried out on transgenic mice and/or cell culture[16–18] suggest that the RANKL gene may be mapped to chromosome 13q14, the OPG gene to chromosome 8q24, and the M-CSF gene to chromosome 1p21–p31. However, whether (and to what extent) these genes affect circulating levels of the corresponding molecules has yet to be ascertained.

The present paper is the first to report the results of a family based, quantitative genetic analysis of the variation in circulating levels of sRANKL, OPG, and M-CSF. An attempt has been made to evaluate the magnitude of the genetic and environmental effects on the variation in each of these molecules in apparently healthy individuals from an ethnically homogeneous Caucasian population.

MATERIALS AND METHODS

Participants

Participants included 566 apparently healthy individuals, 291 men and 275 women, aged from 18 to 80 years, from 126

nuclear and more complex families each of three generations, from Chuvasha (Bashkortostan Autonomic Region, Russian Federation). Chuvashians are descendants of Bulgars who moved to the Volga river valley centuries ago, and who live there in numerous small villages. Details of the study design and data collection methods have been published recently elsewhere.[19] The studied population is an agricultural community characterised by a demographically stable structure with traditional relations between family members, who share very similar ecological and socio-economic conditions. Subjects who participated in the study had no chronic or acute infection, haematological, metabolic, or other diseases, and no amenorrhoea; nor were they receiving prescription medication or steroidal anti-inflammatory drugs on a regular basis, nor consuming vitamin, mineral, or other dietary supplements.[19] Participants were unaware of the specific hypotheses tested, and signed a document of informed consent to the study, which was conducted with the approval of the Ethics Committee of Tel Aviv University.

Blood sampling and biochemical assays

We took venous blood samples after the participants had fasted overnight. Within one hour of collection, samples were centrifuged to obtain plasma which was frozen in aliquots and stored at −70℃ until analysis. Development enzyme linked immunoassay (ELISA) kits (Quantikine, R&D Systems, Minneapolis, USA) were used to measure OPG/sRANKL soluble forms in 566 and 545 individuals, respectively. The detection limit was 40 pg/ml for OPG and 20 pg/ml for sRANKL. For M-CSF (in 420 individuals) we used commercial ELISA by the same manufacturer. The minimum detectable dose of M-CSF was less than 9 pg/ml. The intra-assay coefficients of variation were less than 8% for OPG and RANKL and about 4% for M-CSF, and the interassay coefficient of variation was between 6% (M-CSF) and 10% (OPG/RANKL). The plasma levels of the sex hormones, testosterone and oestradiol, were also measured. Total testosterone (TESTO) and oestradiol (ESTR) values were determined by standard radioimmunoassay procedure, using TESTO-CT2 and ESTR-US-CT RIA kits (CIS Bio International, ORIS Group, France). The further technical information on all assays used in this study has recently been published elsewhere.[15 20]

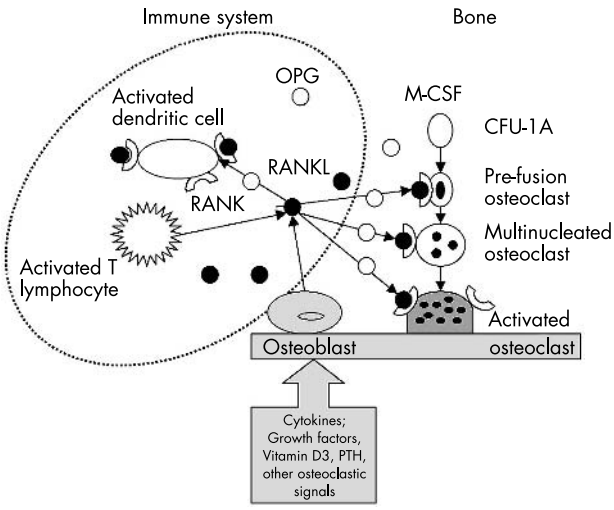

Figure 1 Schematic representation of the cellular interactions linking the immune system with bone homeostasis. RANK, receptor activator of nuclear factor kB; RANKL, RANK ligand; OPG, osteoprotegerin; M-CSF, macrophage colony stimulating factor; CFU-M, colony forming unit macrophage; PTH, parathyroid hormone. RANKL, expressed by activated T cells as well as osteoblasts under the influence of many proresorptive stimuli, binds to its specific membrane bound receptor RANK, thereby promoting osteoclast or dendritic cell differentiation, activation, and survival. OPG downregulates osteoclastic resorption by acting as a soluble sink for RANKL.

Quantitative genetic analysis of pedigree data

Before the model based genetic analysis of the data, we examined the possible extent of the familial aggregation of each of the studied cytokines. The data were first adjusted for the respective significant covariates, using multiple regression analysis to take into account family size and structure; then correlations between the mean of both parents (mid-parent) and the mean of the offspring (midchild) at each of the cytokines was computed.

Techniques for genetic model fitting were used to obtain estimates of genetic and environmental factors. Model fitting is based on the quantitative genetic theory that defines a phenotype as the sum of effects of both genotype and environment.[21] The reported phenotypes are assumed to be linear functions of three underlying factors: additive genetic variance V_{AD}; common familial environmental (CE) factors V_{CE}, that could be shared by both spouses, parents, and siblings; and unique environmental effects (V_{RS}) that reflect the amount of unexplained residual variation of the trait. Therefore, the total for phenotypic variance V_{PH} equals $V_{AD} + V_{CE} + V_{RS}$. In addition, the general model included regression parameters that estimated the effects of age and other potential covariates on each dependent variable in men and women separately. A maximum likelihood ratio test was used for comparing between the general model and a more limited model, containing one or more parameters constrained to the expected value. Division of each significant component of variation by the total variance gives the different standardised components of variance. For example—the heritability ($h^2 = V_{AD}/V_{PH}$) can be defined as the proportion of overall phenotypic variation attributable to additive genetic factors. This eventually leads to a model in which the pattern of variation is explained by as few factors (components) as possible. The best fitting and most parsimonious model was obtained after excluding all non-significant parameters from the general model. All the above computations were carried out using the FISHER statistical package.[22]

To distinguish between the genetic and environmental sources of covariation between the pairs of traits, at the next

Table 1 General characteristics and concentrations of sRANKL, OPG, and M-CSF for men and women in Chuvasha pedigrees

	Men (n = 291)	Women (n = 275)
Variable	Mean ± SD (range)	Mean ± SD (range)
Age (years)	44.64±16.65 (18.0–80.0)	44.23±16.40 (18.0–76.0)
Weight (kg)	64.82±11.50 (41.1–100.3)	60.53±13.09 (35.5–100.6)
Height (m)	1.66±0.07 (1.48–1.89)	1.55±0.08 (1.39–1.77)
BMI (kg/m^2)	23.35±3.68 (16.28–36.4)	25.34±5.32 (15.78–44.39)
TEST (nmol/l)	16.05±6.68 (0.60–38.70)	1.62±4.05 (0.10–34.90)
ESTR (pmol/l)	63.74±43.03 (0.10–308.10)	178.59±183.51 (0.70–779.20)
OPG (pg/ml)	1575.46±457.18 (674.20–2947.20)	1531.95±393.14 (685.0–2958.4)
M-CSF (pg/ml)	889.03±368.92 (187.30–2014.60)	809.46±401.85 (222.30–2011.50)
sRANKL (pg/ml)	1013.84±767.81 (40.60–3402.30)	1209.16±885.80 (51.20–3495.20)

n, number; BMI, body mass index; TEST, testosterone; ESTR, estradiol; OPG, osteoprotegerin; M-CSF, macrophage colony stimulating factor; sRANKL, soluble receptor activator for nuclear factor kappaB ligand.

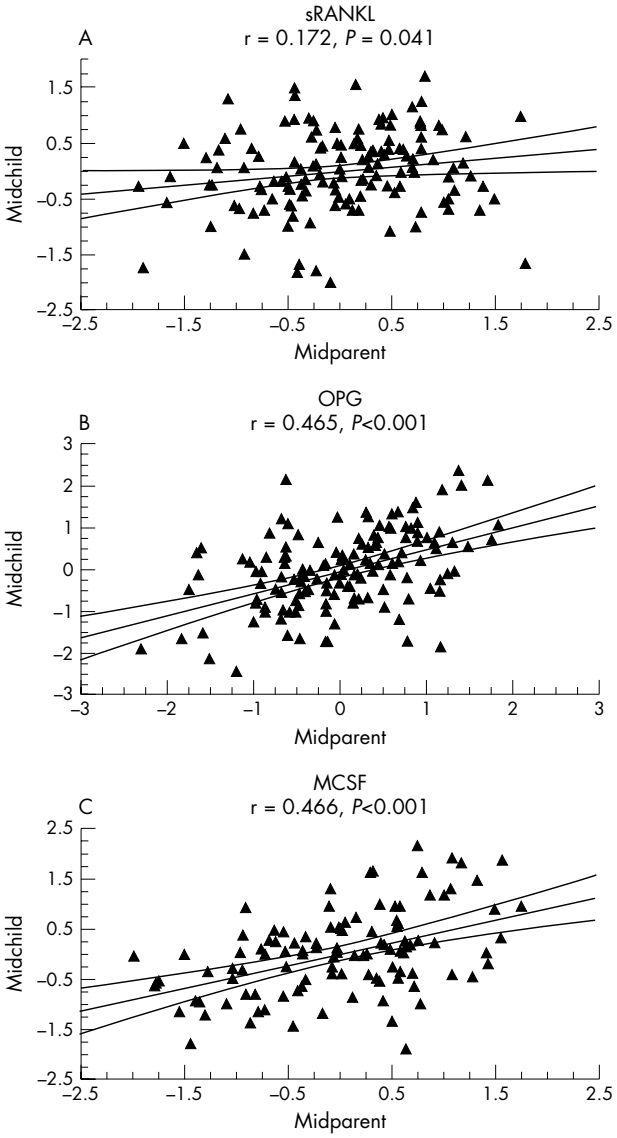

Figure 2 The midparent/midchild correlations of circulating OPG, M-CSF, and sRANKL in both sexes, adjusted for corresponding significant covariates: (a) sRANKL, (b) OPG, (c) M-CSF, respectively.

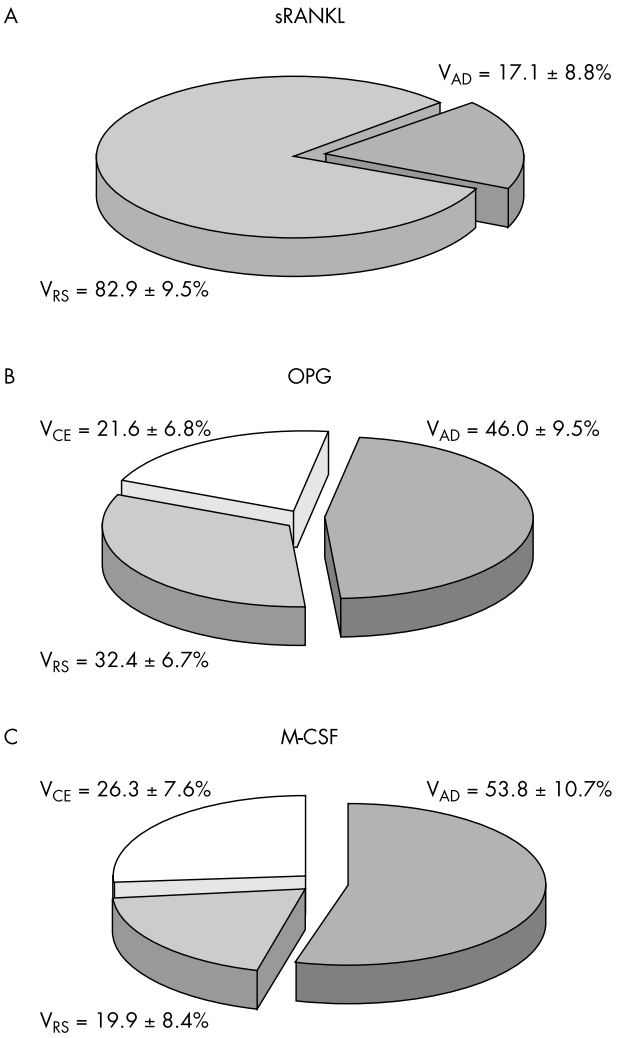

Figure 3 Diagrams showing relative contribution of genetic (AD) and environmental (CE and RS) effects to variation of the studied traits in Chuvasha pedigrees. V_{AD} and V_{CE} are the estimated percentage of total variation ($\pm$ standard error) attributable to genetic and common household influences, and V_{RS} of the unique environmental influence: (a) sRANKL, (b) OPG, and (c) M-CSF, respectively.

stage of the analysis a bivariate variance component model was fitted to variables that showed significant phenotypic correlation.[21] The bivariate mixed model was undertaken employing the same FISHER package.[22] The program calculates additive genetic (r_G) and environmental (r_E) correlations.

Preliminary descriptive statistical analysis revealed significant skewness of all trait distribution. To obtain normal distributions, avoiding bias in the maximum likelihood model fitting results, we log transformed all data before the genetic analysis. In the female sample, all correlations were first evaluated separately in premenopausal and in postmenopausal women. The definition of menopausal status was based on subdivision of the female sample into two age categories, younger and older than 50 years, and then on amenorrhoea in the older women during the six months before data collection. The second category was accepted by us as the postmenopausal group. We also measured oestradiol and progesterone plasma levels, and tested these against postmenopausal status. Since no differences in the pattern and extent of correlations were found, the data were combined and assessed for the entire group of women. The significant influence of these variables on the dependent variables was tested by investigating whether their regression coefficients could be set to zero without a large reduction in fit of the full model of additive genetic, common environmental, and unique environmental variance.

RESULTS

Table 1 shows the general characteristics and mean concentrations of the studied variables for men and women separately. The data for the sRANKL/OPG/M-CSF system and sex steroids are presented before log transformation and in the original units. The plasma concentrations of cytokine molecules in the entire sample ranged from 0.674 ng/ml to 4.929 ng/ml for OPG, from 0.105 ng/ml to 4.468 ng/ml for sRANKL, and from 0.187 ng/ml to 7.604 ng/ml for M-CSF. The mean concentrations of sRANKL and M-CSF were significantly different in men and women (Mann–Whitney *U* test, $p<0.05$); but there were no significant differences in

OPG circulating levels between the sexes. The distributions of all the measured biochemical indices in both genders showed statistically significant deviations from normal, and were consequently subjected to logarithmic transformation. After the transformation, the *d*-values associated with the Kolmogorov–Smirnov's test for normality of distribution ranged between 0.016 and 0.027 ($p>0.10$, in all instances), unequivocally indicating normal distribution of each of the dependent variables. In the following analysis, only log transformed data were used. Individuals who had very high circulating levels of the studied traits (>4SD) were excluded from genetic analysis. This amounted to excluding 20 participants for RANKL, 11 participants for OPG, and three participants for M-CSF.

The examination of age and sex influences on each of the three studied variables showed that plasma levels of sRANKL and M-CSF were significantly different between men and women. Constraining the sex specific regression parameters to equal one another was rejected by the likelihood ratio test, with the p value ranging from <0.05 to >0.01. For OPG and M-CSF levels, strong dependence on age was observed, $p<0.001$. The extent of M-CSF correlation with age was significantly greater in women ($r = 0.29$; $p<0.001$) than in men ($r = 0.17$; $p = 0.012$). It was about the same in both genders for OPG ($r = 0.42$–0.43; $p<0.001$), whereas the sRANKL levels did not correlate with age. There was no association between the circulating levels of cytokines and anthropometric traits (body weight, height, and body mass index).

Impressive and statistically highly significant ($p<0.001$) midparent/midchild correlations were observed in the OPG and M-CSF levels adjusted for age and/or sex (fig 2B and C), whereas for sRANKL this correlation was only marginally significant ($p = 0.04$) and very small (fig 2A). To test the effect of the putative genetic and common environmental factors, we next analysed the data using the quantitative genetic model fitting and the variance decomposition analysis approach. Here we estimated simultaneously the potential effects of the various familial components and corresponding covariates, namely menopausal status, gender, and age. Since the inclusion of the menopausal status, as well as number of years after menopause, did not alter the results of the analysis, and for the sake of simplicity, we offer here only the results obtained in the total cohort of women.

Figure 3 provides variance component estimates and their standard errors in the respective best fitting and most parsimonious models. As expected from the familial correlations, the additive genetic component explained a very substantial portion of the OPG ($45.9 \pm 9.5\%$) and M-CSF ($53.8 \pm 10.7\%$) variation, whereas only $17.1 \pm 8.8\%$ of the sRANKL variation was attributable to genetic influences. Common familial environment factors made a significant contribution to the OPG ($21.6 \pm 6.8\%$) and M-CSF ($26.3 \pm 7.6\%$) variations, but were undetectable for sRANKL.

Bivariate genetic analysis revealed only modest, albeit reliable, phenotypic correlation between OPG and M-CSF ($r = 0.191 \pm 0.048$; $p<0.001$). However, our estimates of the genetic and environmental effects were inconclusive. Each of the parameters (r_G and r_E) separately could be constrained to zero without significant loss ($p>0.05$) of the model fit to data. However, the constraint of both parameters simultaneously to zero was not acceptable by the likelihood ratio test ($\chi^2 = 6.8$; $p<0.01$, $df = 2$).

DISCUSSION

A number of studies have highlighted the clinical importance of circulating levels of OPG, RANKL, and M-CSF and the involvement of these cytokines in bone remodelling. Discovery of these molecules has yielded insight into the interplay between regulatory humoral and local factors and intracellular signals controlling the formation of osteoclasts. An array of bone metabolites, namely calciotropic hormones (including PTH and Vitamin D), growth factors (including IGF-1, IGFBPs, and TGF_β), and cytokines (including TNF_α and ILs), targeting primarily cells derived from mesenchyme in the bone microenvironment, control the expression of M-CSF, RANKL, and OPG.[23 24] The latter have in fact been identified as major regulators of proliferation, differentiation, fusion, and apoptosis of osteoclasts.[4 25]

The decisive role of these cytokines in the regulation of bone metabolism has been demonstrated by the extreme skeletal phenotypes (osteoporosis as against osteopetrosis) in mice with altered expression of these molecules.[26] Interestingly, deletion of the RANK gene leads to the exact phenocopy of RANKL deficient mice, which exhibit severe osteopetrosis and also display lymph node agenesis and impaired splenic structures, thus reconfirming a long suspected link between the immune system and bone metabolism.[27] Regrettably, however, there is little published information on the genetic regulation of OPG/sRANKL/M-CSF. What little data have been published suggest that the OPG and RANKL genes may be associated with osteoporosis and with vascular morphology and function,[28] yet there are so far no data available on the genetic determination of their circulating levels. On the other hand, there is a growing body of evidence that the circulating levels of bone metabolites, including calciotropic hormones, some growth factors, and some cytokines, are determined to a considerable extent (between 30% and 80%) by genetic factors.[29 30]

The present study is the first comprehensively to quantify putative genetic and environmental effects on the plasma levels of M-CSF, OPG, and sRANKL. High heritability values were obtained for the first two factors, but low h^2 was estimated for sRANKL. These findings were consistent in two types of analyses carried out, namely model based variance decomposition (fig 3) and midparent/midchild correlations (fig 2). In addition, a substantial proportion of OPG (21.6%) and M-CSF (26.3%) variation (adjusted for age and gender) was attributable to common family environment.

Our analysis of plasma levels of sRANKL yielded no correlation with any of the two studied molecules, and merely a small additive genetic effect (≈17%) with a large standard error (fig 3). All other familial resemblance components, when constrained to zero, failed to lead to significant loss in data fit ($p>0.10$). Considering that RANKL exists in two biologically active forms—a membrane bound and a soluble form—one possible reason for our failure to evince higher values of heritability could be that the fluctuation of only the soluble form may not reflect the total variation in RANKL. Perhaps sRANKL may not be a very useful indicator of osteoclastogenesis, and this would also explain why it showed no correlation with either OPG or M-CSF.

Contrariwise, the strong impact of genetic factors on variation of the other two cytokines is of great importance, for this finding suggests the need for further efforts to unravel the specific encoding genes influencing variation and determining circulatory levels of OPG and M-CSF. With recent advances in the fields of molecular genetics and genetic epidemiology, it now becomes possible to assess to what extent the interindividual differences in levels of the studied cytokines can be attributed to the effects of single or multiple polymorphisms. It is interesting that our data suggest also the existence of some environmental factor(s) shared by family members, that significantly affect OPG and M-CSF levels. The nature of this agent is still enigmatic, but a common environmental effect has been detected in and reported for other circulating molecules, such as vitamin D[14]

and some haemostatic molecules.[31] The need to elucidate the nature of these environmental factors and to identify the specific genes should stimulate future research into the genetic regulation of the molecules involved in osteoclastogenesis.

ACKNOWLEDGEMENTS

This study was supported jointly by the Israel National Science Foundation (grant # 544/00-1) and by a postdoctoral fellowship grant kindly provided by UNESCO and Israel (the Ministry of Education, the Council for Higher Education's Planning and Budgeting Committee, the Ministry of Foreign Affairs, and the Israel National Commission for UNESCO).

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