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Review Article

Hylonome

Quantitative genetics of circulating molecules associated with bone metabolism: A review

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Abstract

This paper reviews recent advances in the studies of various biochemical factors (biomarkers) involved in bone metabolism and remodeling. The collected data in this area suggest the existence of complex and multilevel relationships between calciotropic hormones, various cytokines and growth factors. The paper summarizes the data on the magnitude of the familial and genetic effects on the interindividual variation in circulating levels of many of these biomarkers. The majority of the cited heritability estimates are well above 20%, reaching up to 80% for some cytokines (e.g., TNF-α and VEGF). These estimates point to potential targets for the identification of novel quantitative trait loci involved in the control of the respective molecules variation. This information is of particular importance, because the available data on the association between specific genes/polymorphisms and the respective circulating molecules variation is still very limited. The paper also provides recent findings on the genetics of co-variation between the circulating levels of various biomarkers. It shows that only in a few instances, such as for example, between IGF-I and IGFBP-3, and IGFBP-1 and leptin, significant and substantial genetic (and environmental) correlations were found. It appears that despite the prominent strong genetic effects on variation of each of the numerous biomarkers, the pleiotropic effects are rather limited. We consider briefly some important new data obtained using the gene expression approach and microarray technique. The data, for instance, indicate that the genetic effects on bone metabolism appear to be an open system, which can be activated or modulated by external factors such as drugs, e.g., PTH. Extensive molecular genetic studies in this area are both timely and imperative to detect the specific genes affecting variation (and co-variation) of the circulating factors associated with bone metabolism.

Keywords: Cytokines, Growth Factors, Systemic Hormones, Candidate Genes, Gene Expression, Heritabilty Estimates, Genetic Correlation

Introduction

Bone formation commences in the embryonic stage and continues throughout childhood and adolescence, when the skeletal system reaches maturity. In the adult skeleton, bone continues to remodel throughout the remaining life and adapts its physical and physiological properties to the mechanical stimuli placed upon it. Remodeling is essential for the maintenance of normal bone structure and for calcium metabolism. The process is such that the entire adult skeleton in the human body is replaced roughly every 10 years[1]. Bone remodeling and metabolism are based on self-regulating cellular events, that occur through the coupling of bone formation by osteoblasts (OB) with bone resorption by osteoclasts (OC). OB play a central role in bone formation by differentiation into bone cells (osteocytes) and by synthesizing multiple bone matrix proteins. Additionally, OB regulate OC maturation and activity through soluble factors as well as cognate interaction, which lead to bone resorption. OC, in turn, probably regulate the rate of proliferation and differentiation of OB via a few OC-derived molecules[2], which may have a specific paracrine mode of action on OB cells. The development and functions of OB and OC are controlled by the combined action of numerous and diverse molecules produced locally, as well as by systemic hormones. Local factors include cytokines, growth factors, nitric oxide, as well as signals resulting from mechanical loading and cell-

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Abbreviations (presented in alphabetical order)	
BGP	Osteocalcin or bone Gla-protein
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BSAP	Bone specific alkaline phosphatase
BS	Bone size
c-fms	Macrophage colony stimulating factor receptor
COLIA1	Collagen 1A1
EGF	Epidermal growth factor
EGFR	EGF receptor
ERα	Estrogene receptor alpha
ESC	Endothelial stromal cells
HGF	Hepatocyte growth factor
ICAM-1	Intercellular adhesion molecule-1
IGF-1	Insulin-like growth factor 1
IGFBP-1 and 3	Insulin-like growth factor binding protein-1 and 3
IL-1 and IL-6	Interleukin-1 and 6
M-CSF	Macrophage colony-stimulating factor
MSC	Mesenchymal stem cells
OB	Osteoblasts
OC	Osteoclasts
OPG	Osteoprotegerin
PDGF BB	Platelet-derived growth factor BB
PICP	Carboxyterminal propeptide of type 1 collagen
PTH	Parathyroid hormone
SCL	Sclerostin
sRANKL	Soluble receptor activator of nuclear factor-kB ligand
TGF-β1	Transforming growth factor-beta 1
TNF-α	Tumor necrosis factor-alpha
VCAM-1	Vascular cells adhesion molecule-1
VEGF	Vascular endothelial growth factor
Vit D	Vitamin D
25(OH)D	25-hydroxyvitamin-D

cell communication[3]. Martin and Rodan[1] suggested that molecular mediators of bone remodeling can be divided into: (1) major endocrine factors, such as sex steroids, parathyroid hormone (PTH) and vitamin D; (2) paracrine/autocrine factors as, *inter alia*, insulin-like growth factors (IGFs) and IGF-binding proteins, transforming growth factor-β family (TGF-β), interleukins (e.g., IL-1, IL-6), macrophage colony-stimulating factor (M-CSF) or tumor necrosis related factors (TNF-α, RANK-Ligand, OPG); and (3) matricrine factors, such as collagen type 1, (PICP) and osteocalcin (BGP). There are however, some additional molecules that are also important in bone metabolism, especially the adhesion factors involved in OB - OC interactions, e.g. intercellular and vascular cell adhesion molecules[4]. Noteworthy also is the vascular endothelial growth factor, (VEGF) in as much as vascularization plays a crucial role in bone development and remodeling[5].

Interactions between cytokines and growth factors involved in bone remodeling

Figure 1 schematically shows the interactions between OB and OC in bone remodeling, and the main molecules involved in this process. One can see that the process starts with maturation of OB following the mesenchymal stem cells exposure to bone morphogenetic proteins (BMP) produced by the endothelial stromal cells (ESC). OB express the receptor activator of the nuclear factor kB ligand (RANKL) on their surface and secrete soluble M-CSF. Calciotropic hormones, growth factors, and pro-inflammatory cytokines, such as ILs and TNF-α, affect the expression of RANKL and M-CSF. The latter, in turn, interact with their corresponding receptors, RANK and c-fms on the surface of OC precursors, thus promoting OC differentiation and final maturation[2,6]. It has been reported that pro-inflammatory cytokines also directly affect OC precursors and mature cells, initiating signals prompting cell fusion and survival; furthermore, osteoclastogenesis can be blocked by osteoprotegerin (OPG), which acts as a potent inhibitor of osteoclastogenesis by binding RANKL, and can be produced by a variety of cells, including OB lineage cells[7]. One should further note that osteoclastogenesis is also affected strongly by cytokines, growth factors and systemic hormones and not only via OB[8,10] (Figure 1).

Figure 1 reveals that OB express intercellular and vascular cell adhesion molecules, namely ICAM-1 and VCAM-1, respectively. These molecules are important for cellular adhesion of OB whereby the latter communicate with opposing cells in the bone milieu, a process that leads to OB activation[4,9]. Moreover, the adhesion molecules on the OB, not only function as a glue with opposing cells, but also transduce activation signals that facilitate production of bone resorbing cytokines. In this regard, ICAM-1 is especially involved with RANKL in OC maturation, and contributes to the efficient cognate interaction of OB with OC precursors. Both ICAM-1 and VCAM-1 are ligands for cellular receptors like beta integrins, which are major adhesion receptors mediating interactions between OB and extracellular matrix. The adhesive interactions increase the secretion of members of the epidermal growth factor family, such as epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) by osteoblasts[10,11]. VEGF, for instance, can act directly on OB[5]. Inhibition of VEGF decreases primary OB differentiation, diminishes cartilage formation and delays cartilage resorption during ectopic bone formation induced by BMP. OC are responsive to VEGF, which stimulates their recruitment, survival and activity. VEGF is also involved in local bone metabolism. It affects OB and chondrocyte proliferation and promotes OB survival[12]. Its receptor is expressed in chondroblasts of the developing ossification centers and also in OB and OC[13]. There are reasons to believe that in the absence of this receptor expression, accelerated differentiation of chondrocytes could possibly decrease proliferation of OB[14].

While there is a large body of evidence suggesting the OB regulation of OC, there are also some recent claims for the

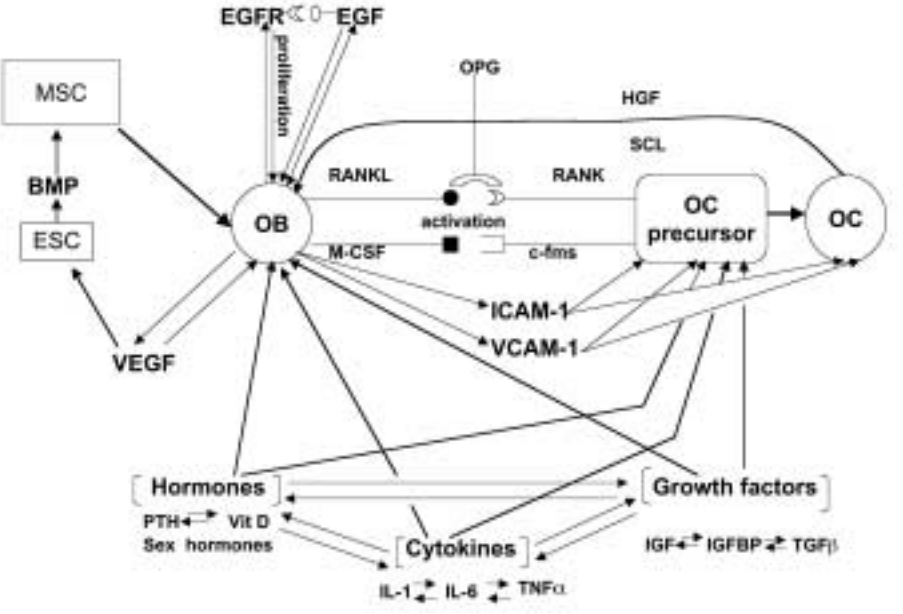

Figure 1. Schematic representation of the interaction of various molecules in the process of bone remodeling. The diagram is according to references: #1-4,7,10. The presented scheme shows osteoblasts (OB) differentiating from mesenchymal stem cells (MSC) and their stimulating effect on osteoclasts (OC) differentiation and maturation. It also shows possible reciprocal regulation of OB by OC, and the systemic hormones and other molecular factors that may directly influence both OB activation and OC precursors' rate of maturation. The molecules' definition is given in the Table with abbreviations, on the preceding page 3.

existence of a reciprocal effect (Figure 1). Thus, Phan et al.[2], in their review paper, suggested a number of potential mechanisms. For example, they indicated that hepatocyte growth factor (HGF) expressed by OC is able to influence DNA synthesis and cellular proliferation in OB. Moreover, it is noted that there are data demonstrating that HGF, together with vitamin D, could induce the proliferation and differentiation of bone marrow stromal cells. The control of OB differentiation may also be achieved by several other molecules expressed by OC cells, such as sclerostin and platelet-derived growth factor BB (PDGF BB)[2]. Thus, sclerostin has been demonstrated to inhibit osteoblastogenesis[15]. Kubota et al.[16] showed that PDGF BB proteins exert an inhibitory effect on OB growth and differentiation. Yet, there are also several observations implying that the aforementioned molecules are not exclusively produced by OC, but can also be expressed by other types of cells[2].

Contribution of the systemic hormones to bone remodeling

Regulation of calcium homeostasis and bone remodeling involves a delicate balance of interactions between systemic hormones, in particular between the parathyroid hormone (PTH) and vitamin D. Both hormones have multiple functions via their influence on various target tissues, but their major role is to maintain calcium-phosphate homeostasis and to regulate bone metabolism on a systemic level[17]. PTH acts directly on cells of the OB lineage, thereby influencing OB differentiation and function, and hence bone formation[18]. PTH promotes bone formation in both trabecular and cortical bone, and these actions are associated with increased trabecular thickness and increased bone strength. However, it is involved also in OC differentiation and activation. Current evidence indicates that PTH-induced bone resorption and calcium mobilization occurred by altering the expression of RANKL on the OB cell surface[19]. The binding of PTH by OB receptors promotes the activation of RANKL on the RANK receptor present on the surface of precursors and mature OC. This effect may be mediated via the production of growth factors (e.g., IGF-1 and TGF-β) by bone cells in response to PTH. As for vitamin D, most of its effects are attributed to the $1,25(OH)_2D_3$ metabolite, which stimulates synthesis of the main non-organic component of bone osteocalcin through interaction with a vitamin D response element in the osteocalcin gene[20]. It inhibits osteoclastogenesis by decreasing the pool of OC precursors in bone marrow[21], and exerts a direct effect on OB proliferation and expression of specific bone proteins and growth factors. Vitamin D also likely modifies the expression of RANKL[19,22].

In recent decades, several observations have shown that obesity protects from osteoporosis[23,24]. From an endocrinological standpoint, these observations suggest that bone mass/size and fat mass may share common regulatory factor(s). One such candidate factor could be a circulating leptin that is strongly linked to various characteristics of human obesity and adipose tissue. Indeed, several human popula-

Biochemical factor		Parent-Offspring or Twin Correlation	Genetic (h^2) and Common Family Environment (c^2)effect	Ref. #
Systemic Hormones				
25-hydroxyvitamin-D	F	r=0.24, p<0.001	h^2=53.31±9.88% c^2=13.14±6.51%	45
25(OH)D	T	r=0.85, p<0.001, MZ r=0.58, p<0.001, DZ	h^2=43%, CI 28-57% c^2=27%, CI 22-31%	44
Parathyroid hormone	F	r=0.27, p<0.001	h^2=37.5±2.75% c^2=25.0±7.86%	45
PTH	T	r=0.61, MZ, p<0.01 r=0.34, DZ, p<0.01	h^2=60%, CI 54-65% c^2=NS	44
Leptin	F	r=0.17, p<0.01	h^2=45.90±11.16% c^2=NS	27
	T	Data not available	h^2=63.8% (p=0.012)	56
	T		h^2=34.0±16.9% (Men) h^2=45.0±15.6% (Women), c^2=NS	57
	T	Data not available	h^2=28%, (p<0.05), free leptin h^2=73%, (p<0.01), bound leptin	58
Paracrine / Autocrine Factors	**A. Growth factors**			
Insulin-like growth factor 1	F	r=0.27, p<0.001	h^2=49.2±10.1% c^2=NS	50
IGF-1	T	r=0.41, p<0.01, MZ r=0.12, p<0.22. DZ	h^2=38, (p<0.05)	59
	T	Data not available.	h^2=63%, (p<0.05)	60
Insulin-like growth factor binding protein-1	F	r=0.20, p<0.001	h^2=23.3±7.8% c^2=23.2±7.8%	50
IGFBP-1	T	Data not available	h^2=36%, (p<0.05)	60
Insulin-like growth factor binding protein-3	F	r=0.14, p=0.007	h^2=57.82±10.64% c^2=0	61
IGFBP-3	T	r=0.65, p<0.001, MZ r=0.23, p<0.06, DZ	h^2=60%, (p<0.01)	59
Epidermal growth factor **EGF**	F	r=0.26, p<0.001	h^2=48.4 ± 9.6% c^2=32.6 ± 8.1%	62
Vascular endothelial growth factor **VEGF**	F	r=0.31, p<0.001	h^2=79.9±6.9% c^2=20.1±5.7%	62
Transforming growth factor-beta 1	F	r=0.23, p<0.001	h^2=40.23±10.43% c^2=43.35±14.45%	63
	T	Data not available	h^2=54%, CI 54-65%	64
TGF-β	F	r=0.09±0.06	h^2=27.9±9.3% h^2=47%, (p<0.01)	65 66

B. Other Cytokines				
Interleukin-6	F	r=0.09, p=0.070	h^2=24.13±10.17% c^2=NS	43
IL-6	T	Data not available	h^2=17%, CI 11-28% c^2-NS	42
Tumor necrosis factor-alpha	F	r=0.11, p=0.036	h^2=82.34±6.74% c^2=17.66±5.46%	43
	T	Data not available	h^2=26%, CI 1-34% c^2-NS	42
TNF-α	F	r=0.33±0.07	h^2=67.9±11.2% h^2=81.6%, (p<0.01)	65 66
	F	Data not available	h^2=60%, p<0.05	67
Soluble receptor activator of nuclear factor-kB ligand	F	r=0.13, p=0.005	h^2=17.7±8.8% c^2=NS	68
	T	Data not available	h^2=82%, p<0.01	41
SRANKL				
Osteoprotegerin	F	r=0.28, p<0.001	h^2=46.0±9.5% c^2=21.6±6.8%	68
OPG	T	r=0.35, p<0.05, MZ r=0.32, p<0.05, DZ	h^2=6%, p<0.05	41
Macrophage colony stimulating factor	F	r=0.30, p<0.001	h^2=53.8±10.7% c^2=26.3±7.6 %	68
M-CSF				
Matricine Factors				
Osteocalcin or bone Gla-protein	F	r=0.22, p<0.001	h^2=38.41±12.98% c^2=NS	69
BGP	T	r=0.64, MZ r=0.47, DZ	h^2=29%, CI 14-44% c^2=34, CI 28-40%	44
Carboxyterminal Propeptide of type 1 Collagen	F	r=0.22, p<0.001	h^2=55.54±14.71% c^2=NS	69
PICP				
Bone specific alkaline posphatase	T	r=0.71, p<0.01, MZ r=0.55, p<0.01, DZ	h^2=74%, CI 67-80%	44
BSAP	T	r=0.60±0.09, MZ r=0.27±0.013, DZ	h^2=63%, p<0.001	70
Adhesion Molecules				
Intercellular adhesion molecule-1	F	r=0.32, p<0.001	h^2=49.40±7.54% c^2=13.99±5.95%	71
ICAM-1	T	Data not available	h^2=55, CI 34-65% c^2=NS	42
	T	Data not available	h^2=24%	72
Vascular cells adhesion molecule-1	F	r=0.38, p<0.001	h^2=51.76±7.33% c2 = 26.88 ± 10.32%	71
VCAM-1				

Table 1. Familial influences on the variation of the circulating levels of the selected molecules (MZ and DZ are mono- and dizygotic twins, respectively; F and T are family and twin-based sample in the study; CI is 95% confidence interval).

tion-based studies have revealed that circulating leptin levels are associated with BMD of various skeletal sites, especially in women[25,26], or else with bone size and proportions[27].

A central regulation of body weight and bone remodeling (via hypothalamus) by leptin has been proposed to explain the above correlation[28,29]. Leptin is produced mainly by white adipose tissue (adipocytes), and is subsequently circulated in the plasma. After crossing the blood-brain barrier, it acts in the hypothalamic nuclei to regulate food intake, energy expenditure, growth and sexual maturation. It exerts negative effects on bone formation via a hypothalamic pathway mediated downstream by the sympathetic nervous system. However, leptin may also affect bone remodeling in adults by stimulating the OPG-RANKL pathway[29]. Moreover, leptin may directly affect the secretion of the human monocytes of receptor antagonists of some interleukins (e.g., IL-1) that, in turn, may influence IL-1 dependent bone turnover[30]. Furthermore, it has been shown that circulating leptin may exert a significant effect on various markers of bone metabolism (such as BGP and PICP) and on insulin-like growth factor-I (IGF-I) and its binding proteins (IGFBP)[31].

Another novel adipocyte -derived hormone that is likely to influence bone turnover and bone mass is adiponectin[32]. This adipocytokine is specifically and markedly expressed in human adipose cells and is the only adipospecific protein currently known to be negatively regulated in obesity[33]. Adiponectin is structurally similar to tumor necrosis factor alpha (TNF-α) and has been linked to bone homeostasis via its translation and secretion, as well as by expression of its receptors in bone-forming cells[34]. Yet, the mechanisms involved in this relation remain poorly understood, and we shall therefore refrain from further discussion of this molecule.

In summary, the above-cited findings clearly suggest the existence of complex and multilevel relationships between various biochemical factors potentially involved in bone metabolism. The questions that arise from these findings, however, include the following: (1) What is the extent of involvement of genetic effects in determining the variability in circulating levels of various biochemical factors? (2) Are the latter correlated in any way and to what extent do common/pleiotropic genetic and familial effects determine these correlations? (3) What are the possible specific genes/polymorphisms that affect plasma variation of the above molecules?

Genetic determination of circulating levels of biochemical factors

It should be stressed that many of the above-mentioned factors, and in particular systemic cytokines, are not skeletal specific molecules, but are rather produced by a variety of non-skeletal tissues. Hence, they therefore may not necessarily reflect local cytokines' production or action within the bone microenvironment[35]. This notwithstanding, significant correlations have been found between the serum levels of several biochemical factors and bone characteristics[27,36-39].

Although to date, very little is known on the inheritance of the specific biochemical indices related to bone metabolism, there is nonetheless a growing body of publications on this subject. Table 1 summarizes what is presently known on the contribution of familial effects to interindividual differences among circulating biochemical indices involved in bone remodeling. The provided data is derived from primarily nuclear families and twin studies. It is well established that circulating levels of many of these indices depend on the age and sex of the individual and sometimes on other co-variates, such as body mass[40], and therefore, we present herein the data adjusted for known significant confounding variables. The majority of the quoted studies have also used model-fitting methods of analysis, utilizing the maximum likelihood ratio test and evaluating both putative genetic effects and common familial environment influences.

Table 1 shows that all the studied biochemical indices displayed statistically significant familial effects, of which putative genetic effects were detected in the variation of all indices. One can see that the majority of h^2 estimates are well above 20%, with the highest estimates obtained for TNF-α (>80%) and VEGF (~80%). The lowest genetic effect was recorded for OPG (6%, by Abrahamsen et al.[41]) and IL-6 (17.0% by de Maat et al.[42], and 24.1% by Pantsulaia et al.[43]). Note, however, that h^2 estimates for OPG obtained by our team[68] were much higher (46.0±9.5%), than in the study[41]. The latter, however, provides substantial estimates for correlations between DZ and MZ twins (Table 1), suggesting a considerable effect of common twin environment on OPG variation. This finding is in agreement with our data[68] indicating that some 22% of OPG variation can be attributable to a shared household environment.

From Table 1, it is also clear that significant common family environment (c^2) influences on the variation of the circulating levels of the listed biochemical factors are almost the rule, and certainly not an exception. The findings were often in agreement in various studies, e.g., 25(OH)D[44,45]. In general, significant c^2 estimates varied between 13% for 25(OH)D and 34% for osteocalcin and even 43.3% for TGF-β. It is certainly of great interest to ascertain the nature of the environmental factors that exert such a substantial effect on the variation of plasma/serum concentrations of different molecules in family members. On the other hand, high heritability estimates, such as for leptin, IGFBP3, EGF, VEGF and others (Table 1), may point to potential targets for the identification of novel quantitative trait loci involved in the control of the respective molecules variation. Currently it is not clear what these loci are (Table 2), and whether and to what extent the same genes also influence the circulating levels of different biochemical factors presumably involved in the same function(s) or processes.

Genetic component in co-variation between biochemical factors of bone metabolism

There are extremely limited data on this subject, but below we cite several studies affirming that correlations between different circulating molecules are possible.

Chromosomal Location of the Gene (according to OMIM)	Genetic Effect on:	
	Circulating Levels of the Biochemical Factor	Bone Characteristics
PTH 11p15.3-p15.1	No association (383 healthy unrelated postmenopausal Japanese women)[74].	No association with BMD or BMC (TDT; 1,263 subjects from 402 Chinese nuclear families)[75].
		No association with hip or spine BMD (TDT; 630 subjects from 53 human pedigrees)[76].
		Association with bone radiogrammetric dimensions (91 healthy unrelated premenopausal Caucasian women)[77].
		Association with BMD (383 healthy unrelated post-menopausal Japanese women)[74].
IL-6 7p21	Association (promoter region polymorphism) with IL-6 plasma level, 102 healthy subjects)[78].	Association with BMD (335 Korean premenopausal women)[80].
	Gender- , age- , and BMI- specific association with IL-6 plasma level (171 healthy families)[79].	Association with BMD (470 postmenopausal Japanese women)[81].
		No linkage or association with hip or spine BMD (420 Caucasian and 124 African-American sister pairs)[82].
RANKL 13q14	Data not available.	Data not available.
OPG 8q24	No association (unrelated postmenopausal Danish women: 66 women with lower forearm fracture, 41 women with hip fracture, and 206 age-matched controls)[83].	Association with BMD and fracture risk (66 women with lower forearm fracture, 41 women with hip fracture, and 206 age-matched controls)[83].
		Association with BMD (1,094 women and 1,127 men)[84].
		No association with BMD (864 women, all 75 years old)[85].
M-CSF 1p13-p21	Data not available.	Data not available.
Osteocalcin 1q25-q31	No association (1,366 non-identical twin sisters aged 18-75 years)[86].	No association with BMD (388 pre-menopausal and 169 postmenopausal Chinese women)[87].
	No association (261 pre- and perimenopausal women)[76].	Linkage and association with BMD (1366 non-identical twin sisters aged 18-75 years)[86].
		Linkage and association with BMD (630 subjects from 53 human pedigrees)[76].
		No association with BMD (261 pre- and perimenopausal women)[76].
Leptin 7q31.3	Association (promoter region polymorphism) with leptin plasma level (39 non-obese female subjects)[88].	Data not available.
	No association (205 obese patients; mean age, 46.9+/-14.23 yr)[89].	
IGF-1 12q22-q24.1	No association (1,041 incident breast cancer cases and 1,086 randomly selected, age frequency-matched controls)[90].	Sex-specific association with height (in men; total 9,278 individuals, mean age 69,1 ± 9,7)[91].
	Association (promoter region polymorphism) with IGF-1 plasma level (9,278 individuals, mean age 69,1±9,7)[91].	Association with bone geometry (elderly 2,372 men and 3,114 women)[95].
	No association (promoter region polymorphism) (441 white healthy women)[92].	Association with BMD (for postmenopausal women) (5,648 individuals)[96].
	No association (promoter region polymorphism; 113 healthy individuals including 60 men and 53 women)[93].	No association with height (640 individuals aged 25 years)[94].
	Association (promoter region polymorphism and IGF-1 plasma levels in 640 individuals aged 25 years)[94].	Association with BMD (300 postmenopausal Korean women)[97].
IGFBP-1 7p13-p12	Data not available.	Data not available.
IGFBP-3 7p13-p12	Association (promoter region polymorphism and IGFBP-3 plasma levels in women with incident breast cancer risk and matchedcontrols, total: 943 ind.)[98].	Data not available.
	Association with IGFBP-3 plasma levels (390 healthy Chinese women)[99].	
TGF-β 1 19q13.1	Association with TGF-β 1 plasma levels (102 estrogen-deficient postmenopausal women)[100].	Association with BMD (102 estrogen-deficient postmenopausal women)[100].
	Association with TGF-β 1 plasma levels (170 pairs of female twins, average age 57.7 years)[64].	Association with BMD (287 postmenopausal women)[101].
	Association with TGF-β 1 plasma levels (287 postmenopausal women)[101].	Association with BMD (296 osteoporotic patients with vertebral fractures and 330 normal individuals)[102].

TNF-α **6p21.3**	No association (177 postmenopausal Japanese women)[103].	Association with BMD (177 postmenopausal Japanese women)[103].
	Gender-, age-, and BMI-specific association with TNF-α plasma level (171 healthy families)[79].	Association with BMD and bone area (97 girls, aged 16.9+/-1.2 years)[104].
	No association (97 girls, aged 16.9+/-1.2 years)[104].	
EGF **4q25**	Data not available.	Data not available.
VEGF **6p12**	Association with VEGF plasma levels (23 healthy men aged 18-36)[105].	Data not available.
	Association with VEGF plasma levels (21 non-smoking postmenopausal controls)[106]. Association with VEGF plasma levels (64 healthy Japanese subjects, aged 22–30 years)[107].	
ICAM-1 **19p13.3 - 19p13.2**	Association with ICAM-1 plasma levels (413 children aged 6-21 years and 363 adults aged 38-55 years)[108].	Data not available.
VCAM-1 **1p32 - 1p31**	Data not available.	Data not available.

Table 2. Association between the biomarker genes and bone metabolism-associated phenotypes.

Correlations between calciotropic hormones, bone mass and estrogen receptor-alpha (ERα) gene

In one study[45], 517 individuals (males and females, aged 18-80) were assayed for plasma levels of PTH and 25(OH)D and radiographic hand BMD. The variation of all variables was adjusted for sex and age differences. Subsequently, each individual was genotyped for two polymorphic sites at the ERα gene (PvuII and XbaI) and one DNA polymorphism (SpI) at the COLIA1 gene[46].

The correlation between the PTH and 25(OH)D levels in the total sample was low, regardless of sex and only marginally significant (-0.14 - -0.20, $p<0.05$). Quantitative genetic analysis showed that this correlation was unlikely to be attributable to pleiotropic genetic effects, in that the corresponding genetic correlations[47] were not significant, $p>0.20$[45]. As for the correlations of these two hormones with hand BMD, they too were not very high, but were still significant and in the expected directions, namely, -0.149 ($p<0.05$) and –0.254 ($p<0.01$) with PTH, and 0.212 and 0.162 ($p<0.05$ for both) with 25(OH)D, in males and females, respectively. Interestingly, however, the results of bi-variate genetic analysis revealed reliable genetic correlation ($r=-0.461\pm0.153$) between circulating levels of PTH and BMD, but not between the 25(OH)D and BMD.

From our study[46] we learned that the Px-haplotype of the ERα gene (resulting from the combined PvuII and XbaI RFLPs in intron 1) was significantly associated with low hand BMD, particularly in elderly women (≥50 years). Moreover, the combination of this haplotype with the "s" allele of the COLIA1 gene further decreased BMD in these women. It was therefore of interest to ascertain whether the genetic effects of these genotypes on BMD are possibly mediated through circulating levels of calciotropic hormones. Indeed, comparison of the same elderly women, carrying Px haplotype and "s" allele, with women lacking both these genetic markers, revealed remarkable differences between the two groups: as shown in Figure 2, the Px carriers had significantly lower BMD and 25(OH)D levels, and much higher PTH than the non-carriers. PICP and BGP also tended toward higher levels in the carrier's group. These findings suggest that ERα and COLIA1 may be involved in bone turnover that, in turn, creates differences in BMD between the respective genotypes. It should be mentioned, however, that there is a substantial heterogeneity in the results concerning the association between the ERα gene and BMD. The recent meta-analysis of individual level data from eight European centers showed that it is more likely a susceptibility gene for bone fractures, rather than for low BMD[73].

Genetic correlations between growth factors and some other molecules

The major goal of the studies exploring the nature of covariation between different variables has been to assess the potential pleiotropic genetic and environmental influences on these variables. One useful approach to evaluate the contributions of various potential factors is a variance-component-pedigree based bi-variate analysis of a pair of traits, such that can subdivide the observed correlation into genetic (genetic correlation, r_G) and environmental (environmental correlation, r_E) components[47]. This approach is very convenient in cases where neither the exact interactions between different physiological/biochemical pathways underlying, for example, the bone remodeling process, nor the chromosomal location of the respective genes have been determined.

Significant genetic correlation may warrant a complex interpretation, but basically it suggests that the variation of the two biochemical factors affected by the same gene product, or the

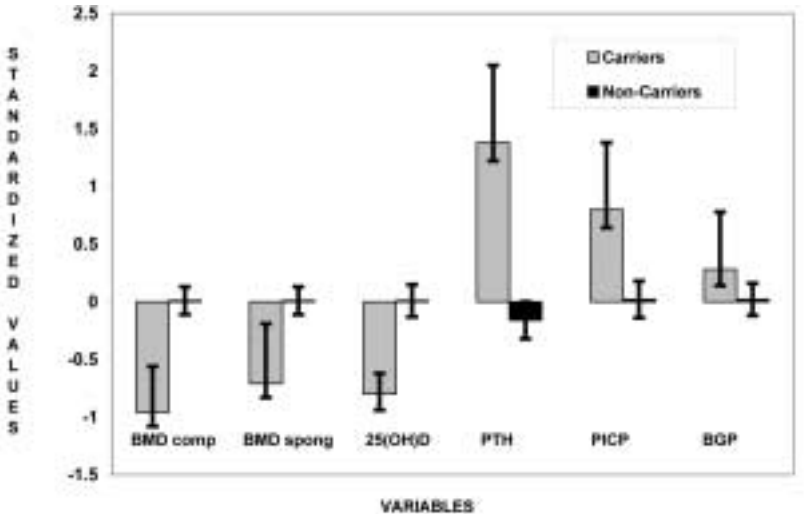

Figure 2. Combined effect of the estrogen receptor-alpha (ERα) and collagen 1 A1 (COLIA1) genes on hand bone mineral density (BMD) and bone turnover in postmenopausal European women. Mean and standard errors for each variable are presented in two groups according to the presence of *Px* haplotype at ERα gene and allele *s* at COLIA1 gene (carriers), vs. absence of both *Px* and *s* (*non-carriers*). Data according to Sapir-Koren et al.[55].

respective genes, are in close linkage disequilibrium. Such information is of importance for the linkage analysis, because the multivariate linkage analysis has more power than does univariate analysis[48,49]. In addition, the nature of the correlation between the different phenotypes may provide clues for deciphering the biochemical pathways underlying the observed correlation. Identification of the genetically correlated traits may thus provide a rational starting point for future identification of the specific genes involved in the determination of quantitative phenotypes of interest. This was the major aim of our studies on the co-variation between several biochemical markers measured in the ethnically homogeneous nuclear families[27,50]. The main findings of these studies are shown in Figure 3.

For all shown pairs of variables, phenotypic correlations were observed that were significant at least at the $p \leq 0.01$ level. The only two exceptions were the correlation between leptin and OPG ($p<0.05$), and that between OPG and bone size. For all the studied genetic and/or environmental correlations, the likelihood ratio tests rejected the null hypotheses of no ($r_G=0$) and complete ($r_G=1.0$) pleiotropy, thus indicating incomplete but significant shared genetic (environmental) effects. It appears that despite the substantial genetic effects on variation of each of the biochemical markers in Figure 3, the pleiotropic genetic (or environmental) effects were rather limited. Yet some of the correlations were of greater interest, namely, those between IGF-I and IGFBP3, between IGFBP3 and IGFBP1, and between IGFBP1 and leptin; because for these three pairs of markers, both genetic and environmental correlations were of relatively substantial magnitude (>0.30) and were reliably significant ($p<0.01$). Their physiological significance is also rather understandable. To begin with, the stability, availability and bioactivity of circulating IGF-I is regulated by its binding proteins (IGFBP 1-6), in particular by IGFBP-3[51]. Secondly, more than 90% of the total IGF-I in serum is complexed with IGFBP-3, which works as a main modulator of IGF-I activity. On the other hand IGFBP-I is likely to regulate bioavailability of IGF-I in response to environmental stimuli such as physical exercise and food intake[52], which is supported by a growing body of evidence that IGF-I is engaged in the regulation of body fat stores, and relates IGF-I and leptin actions. It has been speculated that leptin, which plays an important role in the regulation of food intake and energy expenditure, stimulates the production of positive effectors of IGF-I synthesis, yet at the same time antagonizes IGF-I activity[52,53].

Noteworthy also are two other correlations, namely, the one between M-CSF and BGP, which is not yet clear-cut, and the one between BGP and PICP. Regarding the latter two molecules, they are primary components of bone matrix, but their correlation is likely regulated by some common environmental factors ($p<0.001$) rather than by shared genetic effects ($p>0.05$). In other words, unique genes control the variation of each BGP and PICP. It should be mentioned, however, that significant rE may also be due to non-additive interactions between genes and/or alleles[47]. Clarification of the physiological mechanisms and specific genes involved in the above genetic correlations is an important challenge for future investigations.

Association between circulating levels of biomarkers and their respective structural genes

The aim of this section is to summarize published data on the structural genes of the biomarkers listed in Table 1 and to assess the effect of these genes on the biomarkers variations (Table 2). Chromosomal location of the respective genes is given accord-

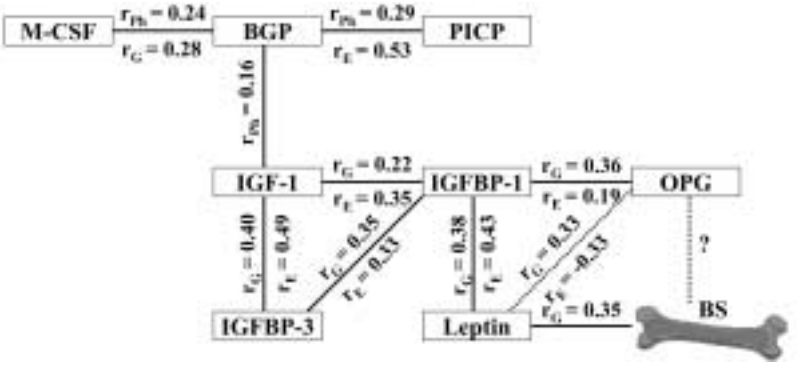

Figure 3. Genetic and familial influences on the correlation between circulating levels of several molecules involved in bone metabolism. rPh, rG and rE are observed phenotypic and estimated genetic and environmental correlations between the variables. Phenotypic correlation presented where genetic and/or environmental correlations have not yet been evaluated.

ing to Online Mendelian Inheritance in Man (OMIM). To date, the effects of most of these genes on circulating levels of the corresponding molecules and on osteoporosis (OP) related phenotypes have been evaluated in numerous studies. Table 2 summarizes only recent reports, and provides results that both confirm and reject the null hypothesis.

Scanning through various relevant publications, it is of interest to note that for some of the listed genes, either no association was found with the circulating levels of the respective molecules (e.g., PTH, OPG) or else association was not consistently observed (e.g., leptin, IGF-1), while for RANKL, MCSF and IGFBP-1 there were no published data at all on this subject. Even so, the data that have appeared in the press with respect to the serum levels of other molecules, such as IL-6, IGFBP-3 and especially TGF-β do consistently suggest significant association of these molecules with their genes. We need to remember that these associations are often evinced while using different methods of analysis, different designs and different samples. Many studies have also examined the association of the above genes with various osteoporosis-related phenotypes, mostly with BMD. For example, Ferrari et al.[54] found that two polymorphisms in the IL-6 promoter were significantly associated with circulating levels of C-reactive protein and markers of bone resorption and weakly with BMD. de Maat et al.[42], reported that 238 G/A polymorphism at the TNF-α gene significantly affected circulating levels of IL-6 and TNF-α. This association was found first using ANOVA of unrelated individuals, but then was confirmed using sib-pair based TDT. As mentioned, such examples are still rare and yet a number of studies have been published during the last few years and some of them are shown in Table 2. Note that although for several of the listed genes (e.g., IGF-1, TGF-β and TNF-α), these results were consistent throughout, they still explained only a small portion (<10%) of the bone trait variation. As for the other genes, their association with bone phenotypes was either not consistent (e.g., PTH, BGP) or was not as yet tested (e.g., M-CSF, IGFBP3, VEGF). Clearly, this area of research must have a significant impact on pharmaco-genomic development, and hence, a further extensive molecular genetic study in this area is both timely and imperative.

Gene expression approaches

Many of the studies in this rapidly developing area focused on various microarrays may pave new ways in our understanding of the specific mechanisms and physiological pathways underlying bone loss and skeletal system degeneration. DNA microarrays, for example, look presently as a promising tool for gene expression studies, especially if they include a thoroughly researched set of relevant, pathway- or disease-focused genes. Because of their focused design, data handling is easier and more straightforward, the new research projects searching for relevant candidate genes can progress more rapidly. Thus, for instance, Oligo GEArrays company suggests TGF-β and BMP signaling pathways that contain 113 genes, which encode members of the TGF-β superfamily and key proteins involved in the corresponding signal transduction pathway. Using such a microarray one may assume with a reasonable degree of confidence that it can be possible to determine specific genes related to the TGF-β/BMP signaling pathway that are differentially expressed among the case/control samples. Similar microarrays exist for a variety of potentially relevant genes including, in particular, numerous genes relevant for skeletal development. The major deficiency of this method is in fact that it requires the introduction of a threshold in phenotype description, like healthy vs. ill in analysis of the data, and currently does not permit to work with quantitative phenotypes. It should be stressed that this relatively new interdisciplinary area that combines bioinformatics, physiology and molecular genetics achievements certainly desires a special review paper that is out of the scope of the present paper. Here, for the aims of illustration, we note just a few relevant recent publications.

One of the most interesting results in this area, in view of this author, was obtained by Onyia et al.[109]. In order to understand potential mechanisms for different skeletal responses to human

PTH treatment, this study utilized DNA microarrays to delineate the genes and pathways that are regulated by intermittent and continuous PTH (1-34) treatment of female rats. The obtained results suggested that 22 genes associated with skeletal development (i.e., collagens, osteocalcin, decorin, and osteonectin) were commonly regulated by both PTH treatment regimens. However, most intriguing was the fact that there were also treatment specific genes. Intermittent PTH regulated 19 additional unique genes while continuous treatment regulated 173 specific genes. This investigation clearly suggests the broad profiling of the gene and pathway changes that occur *in vivo* following treatment of intermittent versus continuous PTH (1-34), and thus indicating very complex relationships between circulating biochemical factors and genes activation. Qin and colleagues[110] using a gene expression technique identified a novel growth factor, amphiregulin, that is PTH-regulated and appears to have an important role in bone metabolism. It is of interest to note here that all EGF-like ligands and their receptors were expressed in osteoblasts, but amphiregulin was the only member that is highly regulated by PTH. Recently, Mohamed et al.[111] applying this methodology examined the role of IL-4 on the intracellular signaling as a potential mechanism for inhibition of osteoclastogenesis. They found evidence that IL-4 may downregulate osteoclastogenesis in part through inhibition of the expression of several transcription factors.

Another interesting example comes from a whole genome scan for 22,000 genes expression in primary osteoblast-like culture from marrow aspirates obtained from three pairs of monozygotic twins[112]. Two pairs were discordant for bone mineral density at the hip by more than one standard deviation, and the third pair was unrelated concordant and used as control. The study revealed that within the twin pair only some 1.5% of genes showed variation in expression as compared to 5% between pairs. Of special significance was the observation that there were several groups of genes showing variations within the discordant pairs and not within the concordant pair. These genes included IL-1β, M-CSF, inhibin βA, and some others, and are known to have potential roles in bone physiology relating to bone density, osteoporosis and osteoarthritis. The authors of this paper conclude that they have shown the potential and cost-effectiveness of further gene expression studies in discordant monozygotic twin pairs. Yet, it is obvious that a replication study for confirmation is essential. Currently, however, there are still very few publications implementing gene expression methods in the analysis of bone physiology and bone loss. It looks therefore as timely and necessary to invest more efforts in these technologies, especially in the situations where phenotypes may clearly be discriminated in two or just a few categories.

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