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A NEW MATHEMATICAL MODEL TO STUDY BONE TURNOVER IN GROWING RATS

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A new mathematical model for the study of bone turnover in growing rats was developed. The model predicts a linear relationship between bone mineral content (BMC) and biochemical markers (BMK) of bone turnover assuming that rats are growing, bone turnover is profoundly affected by skeletal maturation, and resorption and formation are physiologically balanced. The model validation was performed by measuring galactosyl-hydroxylysine (GHYL) and hydroxyproline (HYP) in urines. This mathematical evidence supports our proposed use of the specific bone resorption marker GHYL to predict bone mineral content. Further studies on bone turnover will be possible by the application of the same approach.

Galactosyl-hydroxylysine (GHYL) was recently proposed as a suitable marker of bone resorption [1]. Collagen undergoes a glycosylation process and two hydroxylysine glycosides, galactosyl and glucosyl-galactosyl-hydroxylysine (GGHYL), are present in the molecule [2]. Since GHYL is sevenfold more abundant than GGHYL in bone collagen, GHYL is to be considered a bone specific biochemical marker [3].

Since Type I collagen (which represents 90% of the proteins of bone matrix) is directly involved in bone mineralization, it is conceivable that a specific marker of the molecule correlates with the amount of bone mineral. This has been found in human subjects, which show an inverse correlation between bone mineral content, measured by quantitative computed tomography, and GHYL excreted with urines [1].

Thus, it seemed necessary to investigate by a mathematical modelling approach the relationship between growth, bone mineral content and urinary excretion of collagen catabolites.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats, 40 days-old, 6 males and 6 females, were purchased from Charles River (Calco, Italy). Males and females were maintained in separate cages, in groups of three for cage, in a controlled room (light from 8:00 to 20:00; dark from 20:00 to 8:00) and received food (standard diet, Charles River, Italy) and water ad libitum.

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Abbreviations: BMC, bone mineral content; BMK, biochemical markers; GHYL, galactosyl-hydroxylysine; GGHYL, glucosyl-galactosyl-hydroxylysine; HYP, hydroxyproline.

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The experiment lasted 6 months and during this period 24h urine collections and single photon absorptiometry measurements of the proximal third of the hind leg were performed at different times: from 42 to 84 days of age, every week; between 84 and 141 days, every two weeks and the last urine collection and absorptiometry were performed when the rats were 220 days old.

The animals were anesthetized with a combination of xylazine, 200 µg/100g body weight (Rompun, Bayer, Italy), and ketamine, 15 µg/100g body weight (Ketalar, Parke Davis, Italy), to obtain complete relaxation during single photon absorptiometry measurements.

Urine collection

To collect the 24h urines, the animals were housed in horizontal metabolic cages that allow a complete separation of urines from feces and other contaminants. The cages were mounted on a supporting structure that allows the immediate freezing of urine (Tecniplast Gazzada s.r.l., Buguggiate, Italy). To further protect urines from bacterial proliferation, boric acid, 10 mg/tube (C. Erba, Milano, Italy), was added. At the end of the 24 hours, all tubes collected were removed and stored at -20 °C, until used.

Single Photon Absorptiometry

Bone mineral content (BMC, g/cm) was measured by single photon absorptiometry using the Norland Apparatus Model 2780, electronically adapted for studies of bone mineralization in small bones [4], following the method of Sanchez et al. [5], with minor modifications [6]. In order to avoid errors in the longitudinal study of BMC due to the decay of the source strength, all the measurements were performed with high source strength and weekly calibrated instrument [7]. The search threshold was adjusted to 85% and the bone edges were detected automatically. To assure a constant soft tissue equivalent material around the bone, the rat hind limb was clamped in a tissue equivalent bag, filled with saline and held in place by a support bar. The ultraproximal tibio-fibular site was selected for the analysis instead of the femural diaphysis as in the original method, because of its high percentage of trabecular bone. The correct positioning of the scan site over the scanning path was assured by the simple detection of the femur-tibial joint, that was placed just proximal to the edge of the scanning path. The reported BMC values are the mean of five measurements, each after repositioning of the hind limb over the scanning path. Intra-animals and intra-assay coefficients of variation of the measurements were 9% and 8.3% respectively; the origin of such variation was mainly due to the peculiar geometry of the proximal tibia in the rats.

Urine assays

The urinary excretion of galactosyl-hydroxylysine (GHYL), hydroxyproline (HYP) and creatinine, was determined. The amount of GHYL was measured as reported by dc Bernard et al. [8]. Briefly, 100 µl of urine were derivatized with dansyl chloride, according to the method of Gray [9], filtered and then injected in a Model 344 HPLC (Beckman Instruments, Fullerton, CA) connected to a Spectra/Glo fluorometer (Gilson Medical Electronics Inc., Middleton, WI). For the fluorescence measurements, the excitation wavelength was 366 nm and the emission wavelength was 490 nm. The area of the peaks was calculated by an HP 3390 automatic integrator (Hewlett-Packard, Avondale, PA). Hydroxyproline was measured by the procedure of Kivirikko et al. [10], and creatinine by the Jaffe's method, using the kit from Biochemia (Boehringer, Mannheim, Germany). The amounts of GHYL and of HYP were expressed as micromoles/g and millimoles/g creatinine, respectively.

MATHEMATICAL MODELLING APPROACH

Three simple assumptions were made :

a) *The animals weight growth-rate, dw/dt, is proportional to the amount of weight which remains to be reached*, i.e. to the distance from the actual weight w and the maximum obtainable weight w_∞ :

$$dw/dt \div w_\infty - w$$

and by integrating one obtains:

$$w = w_\infty - (w_\infty - w_0) \exp(-\alpha t) \qquad (1)$$

which describes the weight as a function of time, where w_0 represents the initial weight, α is the constant of proportionality between dw/dt and $(w_\infty - w)$ connected to the initial velocity of growth, v_0, by the relation:

$$\alpha = v_0 / (w_\infty - w_0)$$

and where the time parameter t is equal to

$$t = \tau - \tau_0$$

where τ is the animal age and τ_0 is the threshold age for absorptiometry measurements with our experimental apparatus [4].

b) *The bone mineral content, BMC, is proportional to the body weight w* :

$$BMC \div w$$

and, by substituting expression (1), one obtains:

$$BMC = k_1 \{ w_\infty - (w_\infty - w_0) \exp(-\alpha t) \} \qquad (2)$$

which describes BMC as a function of time, where k_1 is the constant of proportionality between BMC and w.

c) *The amount of biochemical markers (BMK) deriving from collagen break-down during bone turnover and excreted with urines is proportional to the growth- rate dw/dt :*

$$BMK \div dw/dt$$

and by substituting the expression of the derivative of w, one obtains:

$$BMK = k_2 \alpha (w_\infty - w_0) \exp(-\alpha t) \qquad (3)$$

which describes BMK as a function of time, where k_2 is the constant of proportionality between BMK and the growth-rate.

Finally, by eliminating the parameter t between expressions (2) and (3) one obtains:

$$BMC = k_1 w_\infty - BMK \; k_1/(k_2 \alpha) \qquad (4)$$

which predicts that, under the hypotheses stated above, the relationship between bone mineral content and the marker excreted with urines is linear, the intercept being independent of the marker employed.

MODEL VALIDATION AND RESULTS

We will proceed step by step showing that each formulated assumption is consistent with experimental data. Regression estimates and related standard errors are reported in figures 1-8 which offer an immediate check of model assumptions.

Step a)

A non-linear regression with expression (1) gives, in females and males respectively, the results illustrated in figures 1, 2 .

Step b)

A linear regression with expression:

$$BMC = k_1 w$$

gives, in females and males respectively, the results illustrated in figures 3, 4 .

Step c)

By substituting the values for w_0 , w_∞ and α , as obtained in step a), in expression (3) and by a linear regression in the single parameter k_2, one obtains, for HYP and GHYL in females and males respectively, the results illustrated in figures 5-8 .

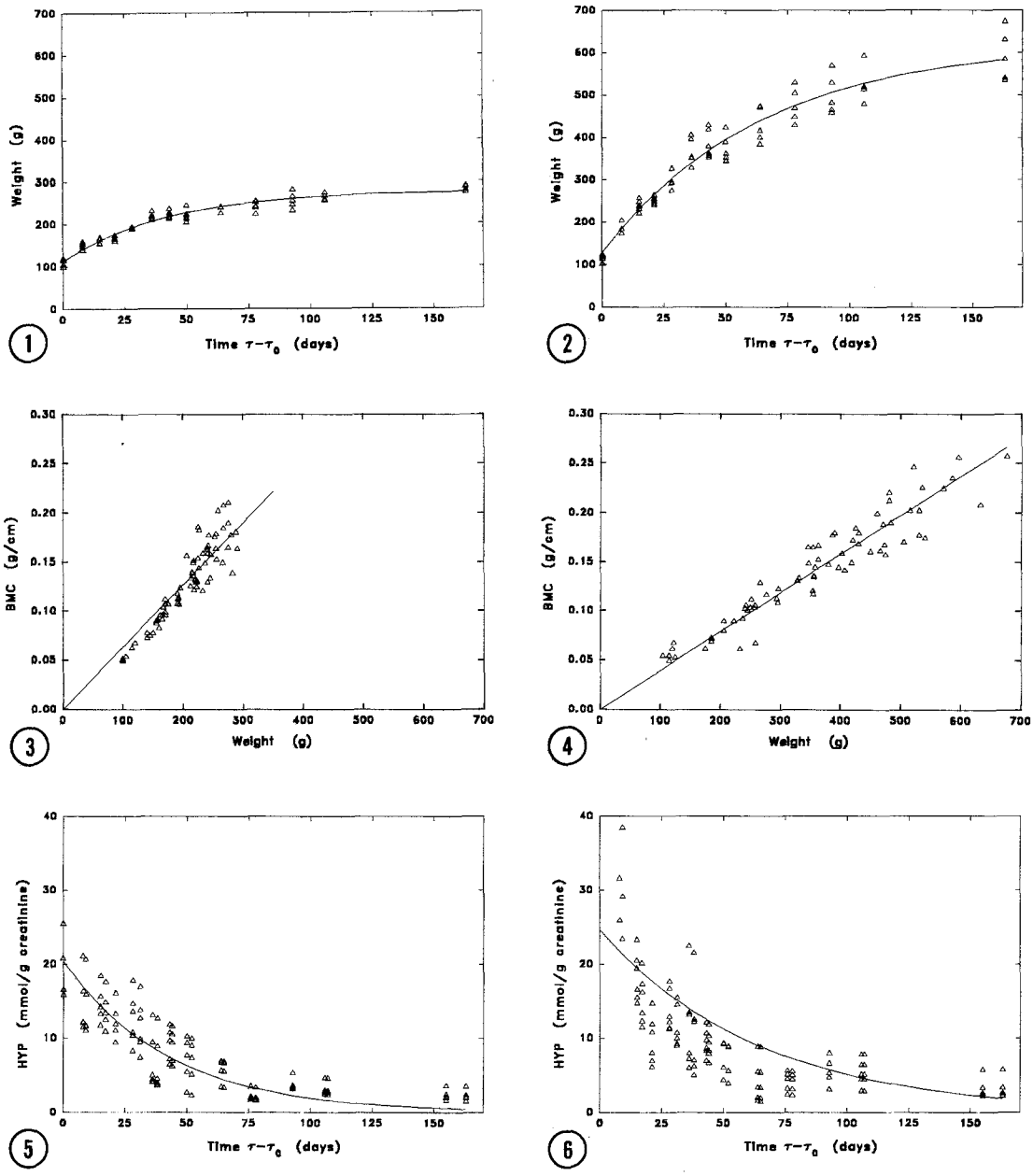

Fig. 1. Weight vs time in females. Regression values are w_0=112.21±3.50; w_∞=278.00±4.36; α=0.0236±0.0017; v_0=3.91.

Fig. 2. Weight vs time in males. Regression values are w_0=126.81±10.00; w_∞=620.96±22.10; α=0.0157±0.0016; v_0=7.76.

Fig. 3. Bone mineral content vs weight in females. Regression values are k_1=0.000634±0.0000091.

Fig. 4. Bone mineral content vs weight in males. Regression values are k_1=0.000395±0.0000053.

Fig. 5. Hydroxyproline vs time in females. Regression values are k_2=5.86±0.40.

Fig. 6. Hydroxyproline vs time in males. Regression values are k_2=3.56±0.37.

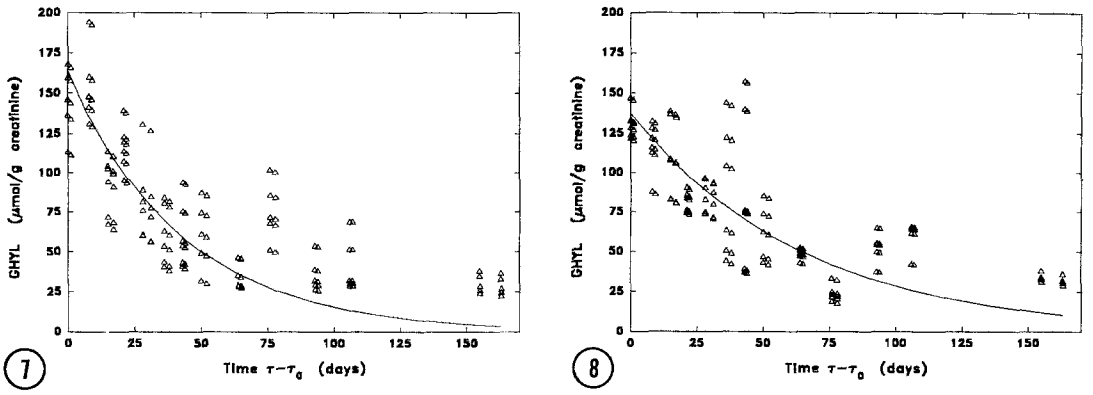

Fig. 7. Galactosyl-hydroxylysine vs time in females. Regression values are k_2=42.04±1.62.

Fig. 8. Galactosyl-hydroxylysine vs time in males. Regression values are k_2=17.79±0.65.

Step d)

The next step to validate the model consists in the calculation of the joint confidence region of intercept, a, and slope, b, of the direct linear regression of BMC versus BMK

$$BMC = a + b\,BMK$$

where a and b represent the observed intercept and slope to be compared with the predicted values $k_1 w_\infty$ and $-k_1/(k_2\,\alpha)$. In the following Tables 1, 2 and related figures 9-12 we report the 99% joint confidence distances, where negative distances mean that the predicted values fall inside the joint confidence region, thus validating the model, whereas positive values indicate a significant lack of fit of experimental data with respect to the model: they are marked with an (*).

DISCUSSION

From the data reported above, it appears that the proposed mathematical model to study bone turnover in growing rats fits completely with the experimental data when the marker employed is GHYL. If the biochemical marker is HYP the accordance is observed only for female animals.

This mathematical model allows to predict BMC from biochemical markers if the following essential assumptions are accepted: rats are growing, bone turnover is profoundly affected by skeletal maturation, and resorption and formation are physiologically balanced [11].

TABLE 1 - Predicted and observed intercept and slope for hydroxyproline in females and males

	Females		Males	
	Predicted	Observed	Predicted	Observed
Intercept	0.18	0.18	0.24	0.18
Slope	-0.0046	-0.0057	-0.0071	-0.0028
99% j.c.d.	-0.026		+2.16(*)	

TABLE 2 - Predicted and observed intercept and slope for galactosyl-hydroxylysine in females and males

	Females		Males	
	Predicted	Observed	Predicted	Observed
Intercept	0.18	0.19	0.24	0.22
Slope	-0.00064	-0.00076	-0.0014	-0.0010
99% j.c.d.	-0.042		-0.0018	

The parameters of the shown linear relationship, BMC vs BMK, can be precalculated by suitable experiments for all animal species and for every marker. In particular, because of the independence of the intercept, $k_1 w_\infty$, from the marker, it is sufficient to calculate only the slope, $-k_1/(k_2 \alpha)$, for different markers.

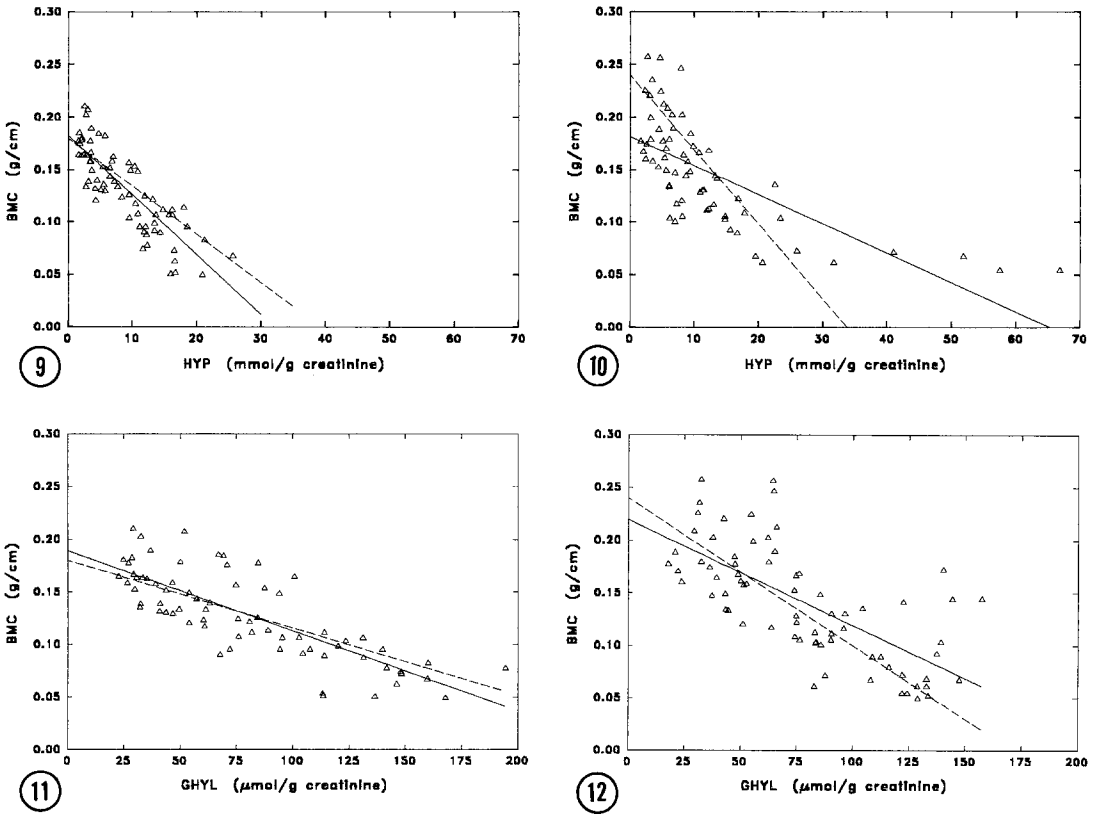

Fig. 9. Bone mineral content vs hydroxyproline in females. --- predicted; ——observed.

Fig. 10. Bone mineral content vs hydroxyproline in males. --- predicted; —— observed.

Fig. 11. Bone mineral content vs galactosyl-hydroxylysine in females. --- predicted; —— observed.

Fig. 12. Bone mineral content vs galactosyl-hydroxylysine in males. --- predicted; —— observed.

When applied to different markers, the mathematical model permits to validate the physiological significance of the specific marker employed. In the case of HYP, the accordance between observed and predicted values, appeared only in female animals, indicates a lower predictive value of HYP in males in comparison with GHYL.

This finding may be explained by the effect produced by androgens on the male growth. Indeed, the difference of predicted vs observed data of urinary excretion of HYP in males becomes significant starting from about 20 days of experimental time (see fig. 6 and fig. 10). By considering that $t = \tau - \tau_0$, this experimental time is characterized by the sexual maturity of the animal and corresponds to about two month of age. Since androgens secretion is known to stimulate liver enzymes [12], higher catabolism of HYP and consequently a lower amount of it in the urine is to be expected (fig. 6). However, the possibility that, at least partially, HYP may undergo a more efficient utilization in the synthesis of new proteins, under the influence of androgens [12], cannot be ruled out.

In conclusion, the proved linear relationship between BMC and urinary biochemical markers of collagen break-down as GHYL, allows the prediction of the former by the measurements of the latter, thus confirming our previous data on 20-80 years old women [1]. This result was obtained on the basis of the model we propose, which not only demonstrates a linear relationship between BMC and BMK, but also provides the physical meaning of the parameters involved. Further studies on bone turnover will be possible using the same mathematical approach.

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