Racemization and isomerization of type I collagen C-telopeptides in human bone and soft tissues: assessment of tissue turnover

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Urinary excretion of the type I collagen C-telopeptide (CTx) has been shown to be a sensitive index of the rate of bone resorption. The human type I collagen sequence $A^{1209}HDGGR^{1214}$ of CTx can undergo racemization of the aspartic acid residue Asp^{1211} and isomerization of the bond between this residue and Gly^{1212}. These spontaneous non-enzymic chemical reactions takes place *in vivo* in bone, and the degree of racemization and isomerization of CTx molecules may be an index of the biological age and the remodelling of bone. The aim of the present study was to investigate the degree of racemization and isomerization of type I collagen in human connective soft tissues, in order to estimate the rate of collagen turnover in adult tissues and compare it with that of bone. We also performed a systematic evaluation of the pyridinium cross-link content in adult human tissues. Using antibodies raised against the different CTx forms, we found that bone and dermis are the tissues that show most racemization and isomerization. The type I collagen of arteries, lung, intestine, kidney, skeletal muscle and heart shows significantly less racemization and isomerization than that of bone, suggesting that these soft tissues have a faster turnover than bone. We also found that pyridinoline and, to a lesser degree, deoxypyridinoline are distributed throughout the different tissues investigated. Because bone type I collagen is characterized by a high degree of both racemization/isomerization and deoxypyridinoline cross-linking, the concomitant assessment of these two post-translational modifications is likely to result in a highly specific marker of bone resorption.

Key words: aging, aspartic acid, pyridinium cross-links.

INTRODUCTION

Type I collagen, the major constituent of the extracellular matrix, is widely distributed in many tissues and organs, including bone, tendon, ligament and skin [1–4]. It is a heterotrimer $[(\alpha1)_2\alpha2]$ composed of a helicoidal domain with C- and N-telopeptides at either end. Recently it has been shown that the $A^{1209}HDGGR^{1214}$ sequence of the C-telopeptide of the $\alpha1$ chain of type I collagen can undergo isomerization and racemization [5,6]. These two processes, which are known to occur in several proteins *in vivo*, are believed to derive from a spontaneous non-enzymic chemical reaction [7], and their end-products could represent a potential biochronological tool for investigating bone turnover *in vivo*. For example, we showed previously that, in Paget's disease of bone (characterized by a marked increase in bone turnover), the degree of isomerization is much lower than in adult normal bone [8]. However, at the present time it is not known if these processes are specific for bone tissue or can also occur in the extracellular matrix of soft tissues.

The C-telopeptide of the $\alpha1$ chain of type I collagen also contains a lysine residue which is involved in the formation of pyridinium cross-links in collagen. Pyridinoline (Pyr) and deoxypyridinoline (DPyr) are trifunctional cross-links which stabilize the structure of mature collagen fibres within the extracellular matrix. Pyr is mainly distributed in bone and cartilage, and to a lesser extent in collagen of other connective tissues, whereas DPyr is present mainly in bone and dentin [9–12]. Pyr and DPyr are virtually absent from skin. Urinary excretion of Pyr, DPyr and associated collagen type I C-telopeptide (CTx) or N-telopeptide fragments are sensitive indices of the rate of bone resorption [13]. Some studies have investigated soft tissue collagen turnover in animals by the incorporation of radiolabelled proline into collagen and by measurement of the specific radioactivity of proline and hydroxyproline in body tissues [14,15]. Because these methods are invasive, few data are available in humans [3,16–18].

The purpose of the present study was to investigate the degree of isomerization and racemization of type I collagen in human soft tissue, in order to determine the rate of soft tissue turnover in adults. In addition, we performed a systematic evaluation of Pyr and DPyr content in adult human tissues.

EXPERIMENTAL

Tissue source

Human tissue samples, including bone, dermis, tendon, ligament, skeletal muscle, intestine, heart, arteries, lung, liver and kidney, were obtained from 10 apparently normal subjects (five women and five men; age 48–90 years) during post-mortem autopsy. The samples were cleaned, cut into small pieces with a scalpel, finely ground in a Spex Freezer-Mill (Spex Industries), washed extensively at 4 °C with ice-cold water, defatted with ice-cold acetone and freeze-dried.

Aliquots of powdered tissue (20 mg wet weight/ml) were

Abbreviations used: CTx, fragment derived from the C-telopeptide of the $\alpha1$ chain of type I collagen containing the sequence AHDGGR; α_L, native peptide; β_L, isomerized peptide containing a β-Asp bond; α_D, native peptide containing a D-Asp residue; β_D, isomerized peptide containing a D-Asp residue; Pyr, pyridinoline; DPyr, deoxypyridinoline.

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Table 1 Content of collagen, Pyr and DPyr in human tissues

Collagen, Pyr and DPyr contents are expressed in nmol/g of wet tissue. Pyr and DPyr contents are also expressed in mmol/mol of collagen.

	Content (nmol/g wet tissue)			Content (mmol/mol of collagen)		
Tissue	Collagen	Pyr	DPyr	Pyr	DPyr	Pyr/ DPyr ratio
Bone	307 ± 71	60 ± 21	17 ± 6	197 ± 67	55 ± 19	4 ± 1
Dermis	335 ± 64	5 ± 4	1 ± 1	16 ± 12	3 ± 3	6 ± 2
Tendon	677 ± 57	370 ± 124	13 ± 2	547 ± 183	19 ± 3	23 ± 9
Ligament	510 ± 84	208 ± 60	11 ± 4	408 ± 117	21 ± 8	20 ± 6
Liver	39 ± 18	16 ± 4	1.7 ± 0.4	405 ± 89	44 ± 10	9 ± 1
Lung	108 ± 25	22 ± 4	3 ± 1	202 ± 34	31 ± 7	7 ± 1
Intestine	161 ± 27	64 ± 15	9 ± 1	396 ± 94	54 ± 8	7 ± 1
Skeletal muscle	59 ± 17	22 ± 5	3 ± 1	376 ± 91	49 ± 15	8 ± 2
Arteries (aorta)	295 ± 92	59 ± 10	13 ± 4	201 ± 34	46 ± 13	4 ± 1
Heart	25 ± 5	10 ± 5	1.2 ± 0.4	410 ± 209	49 ± 15	8 ± 2
Kidney	87 ± 30	26 ± 7	4 ± 2	302 ± 82	42 ± 24	8 ± 2

hydrolysed with 6 M HCl at 110 °C for 24 h, and tissue hydrolysates were assayed for hydroxyproline content by colorimetric assay [19]. Collagen content was calculated from the hydroxyproline concentration, using the following equation:

Collagen (mol) = [hydroxyproline (g) × 7.5]/300000) [9].

Determination of tissue pyridinium cross-links

Aliquots of soft-tissue powders were washed three times with 2 M NaCl (6 ml/100 mg), and extracted with 4 M guanidine hydrochloride/0.05 M Tris, pH 7.4, for 4 days with a change of buffer every day. Bone was demineralized in 0.5 M EDTA/0.05 M Tris/1 % (w/v) NaN_3, pH 7.5, for 24 h and extracted with 4 M guanidine hydrochloride/0.05 M Tris, pH 7.4, for 3 days. Samples were centrifuged at 3500 ***g*** for 30 min. The pellets containing the collagenous proteins were washed in ice-cold water and freeze-dried.

Extracted collagen (10 mg dry wet weight/ml) was hydrolysed in 6 M HCl at 110 °C for 24 h in sealed glass tubes. Pyridinium cross-links were extracted from collagen hydrolysates using cellulose CF1 partition column chromatography. Samples were dissolved in 1 % (v/v) heptafluorobutyric acid and analysed by reverse-phase HPLC on a Beckman Ultrasphere ODS column (5 μm particle size; 25 cm × 4.6 mm) protected by a Brownlee RP-18 Guard cartridge (7 μm; 15 mm × 3 mm) at a flow rate of 1 ml/min with an isocratic gradient of 12 % (v/v) acetonitrile in 0.15 % (v/v) heptafluorobutyric acid. The effluent was monitored for fluorescence at an emission wavelength of 395 nm and an excitation wavelength of 297 nm using a highly sensitive fluorescence detector (Jasco FP-920).

Determination of CTx ratios in human bone and soft tissues

Aliquots of 20 mg of extracted tissue collagen were reconstituted in 2 ml of 100 mM NaH_2PO_4, pH 7.6, and digested overnight at 37 °C with 10:1 (w/w) trypsin XIII (EC 3.4.21.4; tosylphenylalanylchloromethane ('TPCK')-treated from bovine pancreas; Sigma, St. Louis, MO, U.S.A.). After centrifugation for 30 min at 3000 ***g***, supernatants were analysed for the native peptide form of CTx (αL) and an isomerized form containing a β-Asp bond (βL) using the α-CrossLaps® RIA and CrossLaps® ELISA respectively. A racemized form of CTx containing a D-Asp residue (αD) and an isomerized/racemized form (βD) were measured as described in the previous paper [6].

To compare directly results obtained with αL, βL, αD and βD, immunoassays were calibrated using known amounts of purified synthetic peptides. Under these conditions, the values for αL shown in this paper are the raw values given by the α-CrossLaps® RIA multiplied by a factor of 0.0205. For βL, values were obtained by multiplying the raw data from the CrossLaps® ELISA by a factor of 0.0365 [8].

Wilcoxon paired tests were used to compare the αL/βL, αL/αD and αL/βD CTx ratios in the human tissue specimens.

RESULTS

Collagen and pyridinium cross-link content in human bone and soft tissues

As shown in Table 1, tendon and ligament are the tissues with the highest collagen content, whereas heart, liver and skeletal muscle contained relatively low amounts of collagen. Bone, dermis and arteries displayed similar collagen contents.

All connective soft tissues, except for skin, contain significant amounts of pyridinium cross-links. When the results are expressed in mmol of cross-links per mol of collagen (Table 1), the Pyr content of all of the soft tissues is higher than that of bone. Although the DPyr content expressed in mmol per molecule of collagen was highest for bone, the DPyr content of most soft tissues was high and similar to that of bone, with the exception of tendon and ligament. Consequently the Pyr/DPyr ratio is higher for soft tissues than bone, especially in tendon and ligament, but the values for bone and arteries are virtually identical. When results are expressed per g of wet tissue (Table 1), tendon and ligament have the highest Pyr content (respectively 6 and 3.5 times higher than that of bone). The amounts of Pyr in intestine and arteries were similar to that of bone. Skeletal muscle, lung, liver and heart have a low Pyr content. When the DPyr results are expressed in nmol per g of wet tissue, bone is the tissue with the highest DPyr content. Tendon, ligament and arteries contain significant amounts of DPyr, and the remaining soft tissues analysed have DPyr contents 2–20 times lower than that of bone.

CTx ratios (αL/βL, αL/αD and αL/βD)

The relative amounts of αL, βL, αD and βD CTx fragments were determined in the trypsin digests of bone and soft tissues, and the

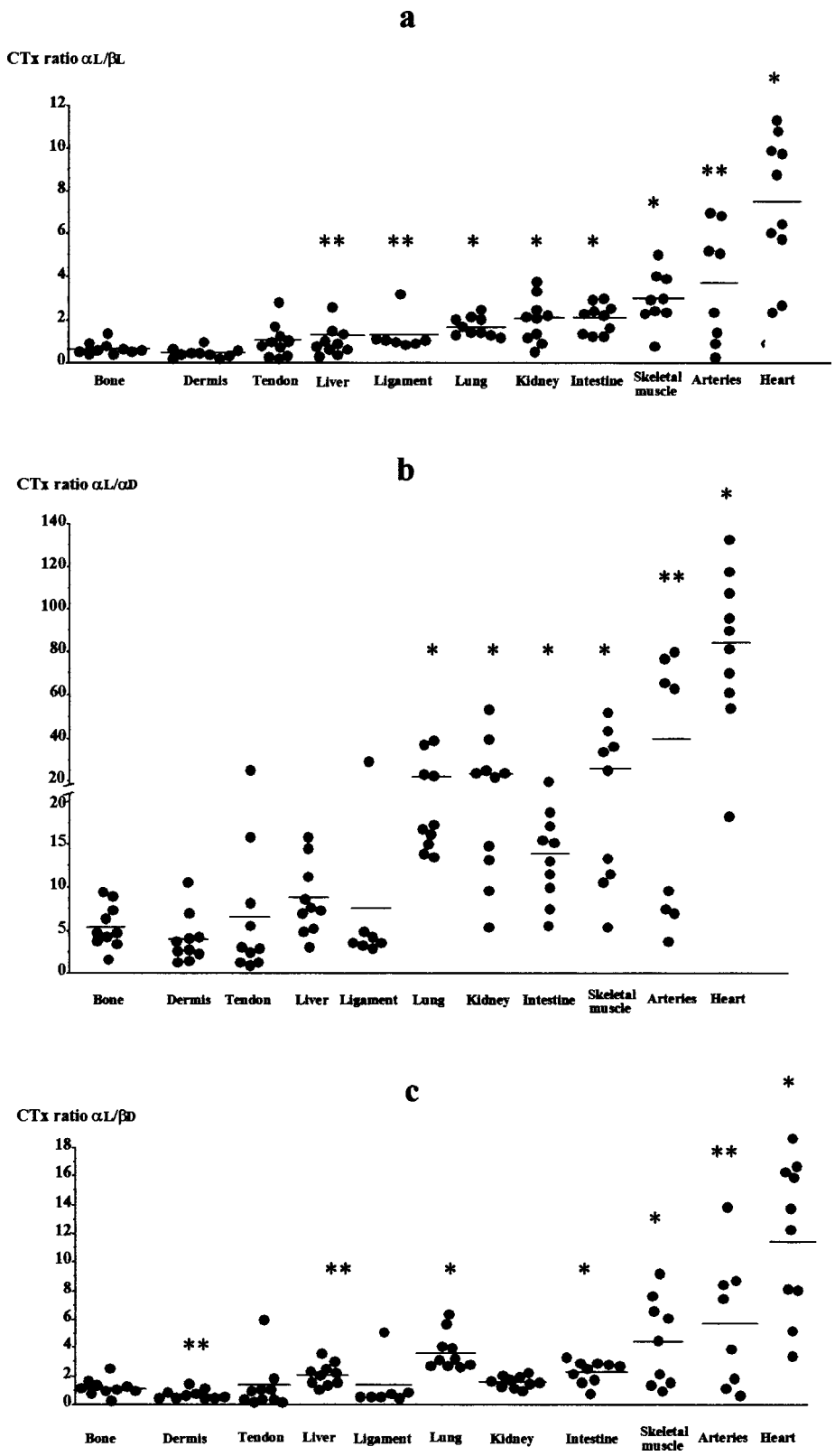

Figure 1 Isomerization (a), racemization (b) and isomerization/ racemization (c) of type I collagen in human bone and soft tissues

A higher $\alpha L/\beta L$ ratio (**a**) is an indicator of a lower degree of isomerization of type I collagen molecules, a higher $\alpha L/\alpha D$ ratio (**b**) is indicator of a lower degree of racemization of type I collagen molecules, and a higher $\alpha L/\beta D$ ratio (**c**) is indicator of a lower degree of isomerization and racemization of type I collagen molecules. Each symbol (●) represents an individual measurement, and horizontal bars (—) represent the averages of measurements. Significance levels, as determined by Wilcoxon paired tests, are given: $^{*}P < 0.01$, $^{**}P < 0.04$–0.01 compared with bone.

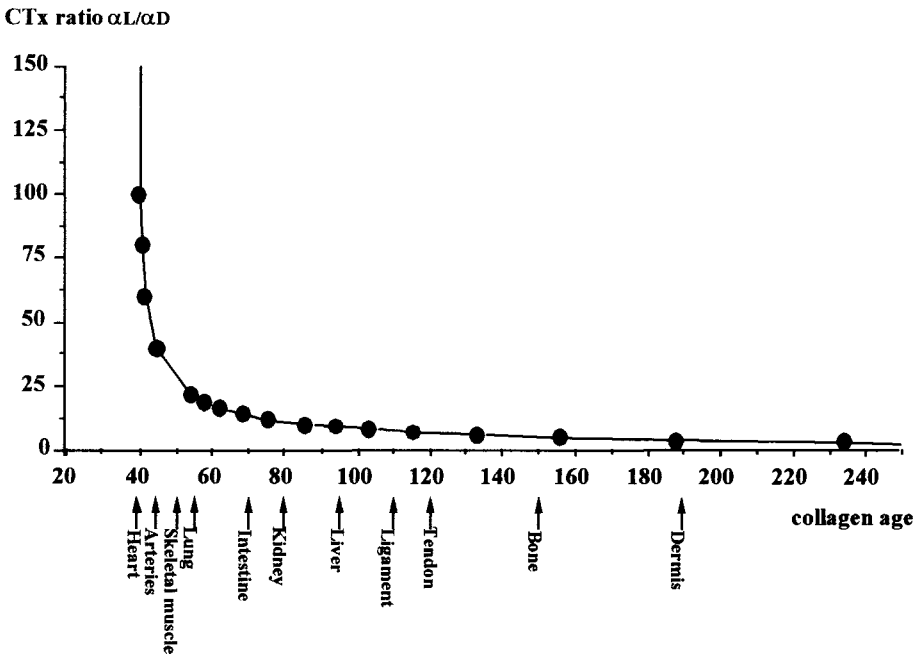

Figure 2 Racemization kinetic curve and estimated age of human tissue collagen

The time-dependent variation in the $\alpha L/\alpha D$ CTx ratio at 37 °C was calculated from *in vitro* kinetic analysis of a synthetic peptide, as described by Cloos and Fledelius [6] (preceding paper). The estimated age of collagen in the different human tissues is indicated on the curve, as the average $\alpha L/\alpha D$ ratio determined (see Figure 1b).

$\alpha L/\beta L$ (isomerization), $\alpha L/\alpha D$ (racemization) and $\alpha L/\beta D$ (isomerization/racemization) CTx ratios were determined (Figures 1a, 1b and 1c respectively). Bone and skin were the tissues with the lowest ratios. Arteries and heart were the soft tissues with the highest ratios. In ligament and liver the $\alpha L/\beta L$ ratio, but not the $\alpha L/\alpha D$ ratio, was significantly increased compared with bone. In dermis the $\alpha L/\beta D$ ratio was significantly decreased compared with bone. For skeletal muscle, arteries and heart, the mean ratios were higher than those of bone and dermis, but it should be noted that there was great variability between individuals for these tissues. Very high levels for the $\alpha L/\beta L$ ratio were found in foetal dermis (5.95 ± 0.98; $n = 3$) and foetal bone (18.8; $n = 1$).

Figure 2 shows the time-dependent variation in the $\alpha L/\alpha D$ CTx ratio at 37 °C, calculated from *in vitro* kinetic analysis of the synthetic peptide [6]. The estimated average ages for collagen type I for each tissue are indicated on the curve. Arteries and heart have the lowest collagen ages, estimated to be less than 50 days. Skeletal muscle, liver, kidney, intestine and lung have an estimated collagen age of between 50 and 100 days. Tendon and ligament have an estimated age of more than 100 days. Bone and dermis have the greatest collagen age, estimated at around 150 and 190 days respectively.

DISCUSSION

To our knowledge, this is the first study to analyse post-translational modifications of collagen type I in several human soft tissues and to derive an index of the rate of turnover of type I collagen in these tissues. Our data suggest that type I collagen in soft tissues – with the possible exception of dermis – is turned over at least as fast as that in bone. We also report an extensive determination of pyridinium cross-links in these tissues.

Isomerization and racemization of type I collagen C-telopeptides take place *in vivo*. It has been shown that the accumulation of the altered molecular species (βL, αD and βD forms) in tissues depends on the rate constants for the inversion of the different isomers, and also on protein turnover [6]. Interestingly, the kinetics of racemization and isomerization assayed *in vitro* using the AHDGGR synthetic hexapeptide were similar to those in bone collagen under physiological conditions [6]. Thus it is likely that the values for racemization and isomerization that we measured in the soft tissues can be directly compared and used as an index of collagen turnover.

In all soft tissues analysed, except dermis, we found a significantly lower content of racemized and/or isomerized type I collagen molecules than in bone, suggesting a higher rate of turnover in these tissues compared with bone. This is especially true for skeletal muscle, heart and arteries. The assessment of tissue turnover has previously been measured in animals mainly using radiolabelled proline/hydroxyproline. These invasive methods are technically difficult to perform and require rather complex mathematical analysis. Because the variety of animal models comprise different ages, a comparison of the studies is difficult. In the rat, the rate of turnover of collagen is 9 %/day in the heart, 4 %/day in the lung and less than 1 %/day in the dermis [14]. Collagen synthesis in rabbits occurs at a rate of 10 %/day in the lung, 3 %/day in skeletal muscle, 4 %/day in skin and 5 %/day in heart [15]. In humans, high turnover in arteries and heart has been reported in patients with hypertension and myocardial disease [3,16,17]. Conversely, dermal collagen appears to turn over slowly [18]. Histomorphometric studies have estimated that, in adults, the period between two bone resorption events is 2–5 years [20]. Thus, although the available comparative data are limited, they appear to be in agreement with the rates of tissue turnover that we have determined from assessment of the CTx isomerization/racemization processes.

We have also confirmed, using a large number of tissue types and individuals, that pyridinium cross-links – both Pyr and DPyr – are not bone-specific, but are widely distributed (especially Pyr). The contribution of soft tissues to the global pool of serum and urine pyridinium cross-links is likely to depend on several parameters, including cross-link content per amount of tissue, tissue mass, its rate of turnover and the clearance process. Thus, although the concentrations of Pyr and DPyr in tendon, ligament and arteries are similar to or even higher than that in bone, in addition to a higher turnover rate, their contribution to the pool of pyridinium cross-links is likely to be small under normal conditions due to the small mass of these tissues compared with that of bone. Conversely, the amount of type I collagen in the skin is important (about 4–5 kg for an adult), and our results suggest that its rate of turnover is similar to that of bone. However, as the pyridinium cross-link content (especially DPyr) is very low, skin pyridinium cross-links probably represent only a small fraction of the global pool. One tissue which should be taken into consideration when evaluating pyridinium cross-links is skeletal muscle. Indeed, the amount of skeletal tissue is high, representing about 40 % of whole-body mass. The Pyr and DPyr content in this tissue is only 3 and 6 times lower respectively than that in bone, and skeletal muscle is characterized by a turnover rate higher than that of bone, as deduced by the racemization/isomerization process. Thus, at least theorically, a significant contribution of muscle Pyr to the global pool could be expected.

It is of interest to note that, in patients with hyperthyroidism, a condition characterized by a general increase in tissue turnover (including bone and skeletal muscle), a very large increase in free Pyr was reported, even greater than that of cross-linked peptides [21]. The increase in type I collagen cross-linked peptides in patients treated with thyroxine was blunted by bisphosphonate, a specific inhibitor of bone resorption, whereas that of free cross-links was not, suggesting a contribution of non-bone Pyr to the global pool of free cross-links [22]. Our combined data for isomerization/racemization and pyridinium cross-link analysis suggest that one of the most promising strategies for increasing the specificity of bone-resorption surrogate markers would be to use an assay that recognizes isomerized/racemized type I collagen peptide cross-linked by DPyr, because both of these post-translational modifications are greatest in bone.

Our study has some limitations. The age range of the individuals was very narrow, thus precluding any analysis of changes in tissue turnover with chronological age. However, our preliminary data from a human foetus indicate much higher turnover in bone and skin compared with adults, as expected. Although the number of individuals analysed was limited, we found significant differences between paired tissues, suggesting that the comparison between tissues is reliable. The cause of death of the individuals was not available for ethical reasons, and we cannot exclude the possibililty that the results for some tissues may reflect not only physiological turnover, but also the potential effect of underlying diseases.

In conclusion, although isomerization and racemization are not restricted to bone CTx peptides, these post-translational modifications are most prominent in this tissue. Using these modifications as an index of biological age, we found that most soft tissues turn over more rapidly than bone. As the degree of isomerization/racemization and the DPyr content is highest in bone, assessment of the urinary excretion of isomerized/racemized type I collagen peptides cross-linked by DPyr could be a valuable strategy to obtain a highly specific marker of bone resorption.

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