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ORIGINAL RESEARCH

Development of a New Immunoassay for Human Cathepsin K-Generated Periostin Fragments as a Serum Biomarker for Cortical Bone

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Abstract Periostin is a matricellular protein mainly expressed by periosteal cells and osteocytes in bone, but is also present in several other tissues. Available immunoassays use antibodies of unclear specificity. The aim of the study was to develop a bone-specific periostin ELISA based on the detection of fragments generated by the osteoclastic and osteocytic protease cathepsin K. In vitro digestion of human recombinant intact periostin by cathepsin K leads to the generation of multiple fragments. Using LS–MS/MS, it was found that the GSLQPIIK peptide was the most efficiently and abundantly generated periostin fragment. A rabbit polyclonal antibody directed against the synthetic GSLQPIIK sequence was produced. Immunohistochemistry experiments of the tibia showed that the GSLQPIIK fragments localized at the periosteal surface and within the osteocytes. Using the same antibody, we developed an ELISA for the measurement of GSLQPIIK in the serum. This ELISA demonstrated intra- and interassay variability below 14% with a sensitivity allowing accurate determinations in the serum of healthy individuals. Serum GSLQPIIK was measured in 160 healthy postmenopausal women (mean age 65 year) participating in the Geneva Retiree Cohort. Serum GSLQPIIK levels did not correlate with total periostin, hip BMD, and the bone markers PINP and CTX. However, GSLQPIIK was negatively correlated (p values ranging from 0.007 to 0.03) with Hr-pQCT measures of tibia and radius cortical bone, but not with trabecular parameters. We have developed the first assay for the detection of periostin fragments generated by cathepsin K. Because serum levels of this new marker significantly correlated with cortical bone measurements in postmenopausal women, it may prove to be useful for the clinical investigation of patients with osteoporosis.

Keywords Periostin · Osteoporosis · Periosteum · Bone marker

Introduction

The periosteum covers long bones and although in adults its metabolism is considered to be low, it plays an important role for controlling the diameter of bones and thus bone strength [1]. Currently however, there are no available non-invasive biological tools allowing the assessment of periosteal metabolism as current bone markers reflect mainly endosteal bone remodeling, and not periosteal metabolism [2]. The concept of developing biological markers that reflect the remodeling of a particular bone compartment has been suggested for some proteins including osteocalcin which is more concentrated in the cortical than trabecular bone [3], although clinical data with serum measurements were not conclusive. Moreover, despite several markers of osteoblast and osteoclast activities are available, there is only one serum marker potentially reflecting osteocytes' number and/or activity, i.e., sclerostin, but its clinical utility remains unclear.

Periostin is mainly expressed by periosteal osteoblasts and osteocytes, although osteoclasts may also express low levels [4–6]. Periostin is an 811-amino acid protein composed of a signal sequence, followed by an Emilin (EMI) domain rich in cysteine, four repeated and conserved Fasciclin-1 (FAS-1) domains, and a C-terminal hydrophilic

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and variable domain. Alternative splicing of the C-terminal domain gives rise to seven different human isoforms. This protein belongs to the matricellular protein family because it contains binding sites for extracellular matrix proteins, such as type I and type V collagens, and the cell surface receptor integrins [4, 5]. In adults, periostin has been shown to be overexpressed at the periosteal surface, but also in other collagen-rich tissues subjected to mechanical strain such as periodontal ligaments, heart valves, and tendons [4, 5].

In bone biology, mice studies have shown that periostin is an important regulator of the osteoblastic activity, bone formation, and regulation of bone strength by altering collagen crosslinking, particularly at cortical sites and also osteoclastic activity by increasing osteoprotegerin [7–10]. Periostin is also an important mediator of the effects of mechanical factors and parathyroid hormone on cortical bone mineral density (BMD) and bone strength by modulating the canonical Wnt signaling pathway with a down regulation of sclerostin expression [9].

Because periostin is a secreted protein, it can be detected in peripheral blood and thus periostin immunoassays have been developed for rodents [11] and humans [12]. Three ELISAs from three different diagnostic companies (USCN, Adipogen, Biomedica) for human serum periostin are commercially available and use antibodies raised against intact human recombinant periostin or FAS-1 domains [12, 13]. However, the exact epitope of these antibodies on the POSTN sequence is undetermined and it is unclear which molecular entities—in addition to intact form— they are detecting in the blood. Because intact periostin is secreted by several tissues, it is unlikely that these assays reflect specifically bone metabolism. Despite these limitations, we previously found a weak but positive association between total circulating periostin levels (USCN) and cortical structure parameters [14]. Higher total periostin levels were also reported to be associated with fracture risk, particularly with non-vertebral fractures in postmenopausal women [15, 16].

Cathepsin K is a lysosomal cysteine protease mainly secreted by osteoclasts, but also by osteocytes in certain conditions including mechanical loading. It is the most efficient proteases to solubilize bone collagen [17], but can also degrade non-collagenous bone proteins such as osteonectin [18]. It is thus critical in osteoclast-mediated bone resorption, as in osteoporosis and other metabolic bone diseases, as well as in osteocytic osteolysis as seen during lactation. We recently showed that mouse recombinant periostin was degraded by recombinant cathepsin K, generating different yet uncharacterized fragments [19].

In this study, we hypothesize that human periostin is also a substrate for cathepsin K and that the detection of cathepsin K-generated periostin fragments could serve as a novel marker of bone metabolism, particularly at cortical surfaces.

Subjects and Methods

Healthy Postmenopausal Women

The healthy subjects were randomly selected from the Geneva Retiree Cohort (GERICO) which has been described in details elsewhere [20, 21]. This study comprises more than 1000 subjects from the general population 63- to 67-year-old. In this study, we analyzed 160 women randomly selected from the GERICO cohort with a mean age of 65.0 ± 1.40 years. At baseline, BMD at the femoral neck and bone microstructure at the tibia and radius were assessed.

BMD was determined by dual-energy X-ray absorptiometry using Hologic QDR Discovery instrument. Trabecular and cortical bone microstructure at the non-dominant distal tibia and radius were measured by high-resolution peripheral quantitative computed tomography (Hr-pQCT) as described in details elsewhere [21]. Hr-pQCT measurements were performed using a XtremeCT instrument (Scanco Medical AG, Basserdorf Switzerland).

Fasting serum samples were also collected for the measurement of POSTN levels and conventional bone markers. All samples were stored at −80 °C until analysis and were not freeze/thaw more than twice.

Digestion of Human Periostin by Cathepsin K and Identification of Periostin Fragments by LC–MS/MS

Human recombinant periostin (R&D system, UK) was incubated with active human recombinant cathepsin K [18] at 37 °C for various times at a periostin/cathepsin K ratio of 50/1. The inhibitor E64 (Sigma-Aldrich, France) (250 μM) was added to stop the digestion at the end of incubation. The digests were then analyzed by SDS-PAGE electrophoresis. Various periostin fragments bands were excised from the SDS-PAGE gel and digested with trypsin. The tryptic peptides were concentrated and separated by reverse phase chromatography on a PepMap100, C18, 5 μm, 100 Å, 300 μm × 25 mm column from Dionex (ThermoScientific) using a gradient of 5–40% acetonitrile, 0.1% formic acid in 60 min at 300 nL/min. Furthermore, to maximize the sequence coverage, the analysis was conducted on two mass spectrometers, LTQ Velos Mass Spectrometer (ThermoScientific) and Qstar XL Mass Spectrometer (Applied Biosystems).

Production of Anti-periostin Fragments Antibodies

Synthetic peptides, including biotinylated and keyhole limpet hemocyanin (KLH)-coupled peptides, were synthesized to 85% purity using standard 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis [22].

Rabbits were injected intraperitoneally with 1 mL (0.5 mg/mL of conjugated peptide) of the synthetic ^{688}GSLQPIIK^{695} peptide (K-Postn) derived from the sequence of human periostin conjugated to KLH using glutaraldehyde [23] emulsified in equal volume with Freund's complete adjuvant. Immunizations were repeated 3 times every month for 3 months using the same immunogen but emulsified in Freund's incomplete adjuvant as previously described [23]. At each bleeding, antiserum was screened by titration for the presence of anti-K-Postn antibodies. Titration was performed by investigating the binding of subsequent dilutions of the antiserum on microtiter plates coated with biotinylated K-Postn peptide (see below). The titer was defined as the dilution of the antiserum giving 50% of the absorbance of the undiluted antiserum. The antiserum with the highest titer was selected for the immunohistochemistry experiments and the development of the ELISA for serum measurements (see below).

Immunolocalization of POSTN-CatK Peptide in Mouse Bone Tissue

The right and left tibiae from mice were excised and subsequently fixed in 4% paraformaldehyde overnight at 4 °C. They were then decalcified in 19% EDTA and 4% phosphate-buffered formalin for 3 weeks. The tibiae were then dehydrated in an ascending series of ethanol, cleared in Propar (Anatech LTD, Battle Creek, MI), and embedded in paraffin blocks. 8-µm-thick sections were cut from the blocks at the tibia mid-shaft level using a RM2155 microtome (Leica, Germany) and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburg, PA). Sections were air-dried overnight at room temperature. Prior to staining, they were incubated at 60 °C for 1 h, deparaffinized in xylene, and rehydrated in a descending series of ethanol. Deparaffinized slides were pre-treated in 3% hydrogen peroxide in methanol to quench endogenous peroxidase and rinsed in tap water followed by non-specific avidin/biotin blocking (Vector Laboratories, Burlingame, CA) according to the manufacturer's directions. All incubations took place in a humidified chamber. Additional protein blocking was accomplished with Protein Block-Serum Free (DAKO, Carpinteria, CA). Using the Vectastain Elite ABC (Rabbit IgG) Kit (Vector Laboratories, Burlingame, CA), the slides were incubated in 1.5% normal goat serum for 30 min at room temperature. The primary antibody (Rabbit anti-K-Postn) was diluted in Antibody Diluent (DAKO, Carpinteria, CA) to a final concentration of 1:6000 and incubated at 4 °C overnight. The following day, slides were rinsed in Wash Buffer (DAKO, Carpinteria, CA) for 15 min on a rocker at room temperature and incubated in biotinylated goat anti-rabbit (Vectastain Kit) secondary antibody diluted 1:1000 for 30 min at room temperature, followed by another rinse in Wash Buffer for 15 min on a rocker at room temperature. The ABC reagent from the Vector Kit was prepared according to the manufacturer's directions at a dilution of 1:250 and the slides were incubated in it for 30 min at room temperature and rinsed, as above, in Wash Buffer. All incubation steps were performed at room temperature and all rinse steps employed the DAKO Wash Buffer at room temperature on a rocker. The following protocol was used: incubation in streptavidin-HRP diluted at 1:100 for 30 min, and washed for 15 min. Slides were developed in a working solution of 3,3′-diaminobenzidine (DAB Substrate Kit for Peroxidase Kit, Vector Laboratories, Burlingame, CA) prepared according to manufacturer's directions for 10 min at room temperature. Following a final rinse in deionized water, the slides were mounted in Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI). For the negative control, primary antibody incubation was replaced by TRIS 0.1 M buffer. Digital images were obtained using a microscope with a camera AxioCam MRc5 controlled by Axiovision AC software (Carl Zeiss MicroImaging GmbH, Germany).

ELISA for the GSLQPIIK Fragment of Periostin Generated by Cathepsin K (K-Postn)

Biotinylated GSLQPIIK peptide (100 µL) diluted in TRIS 10 mM/$CaCl_2$ 10 mM/NaCl 150 mM/0.5% BSA PH 7.4/ buffer (assay buffer) was pipetted into each well of an streptavidin-coated plate (Thermo Fisher, France). The plate was incubated for 1 h at room temperature. The plate was then washed 3 times with PBS/BSA-0.5%/Tween 20–0.005% (washing buffer) and 50 µL of calibrator (free synthetic GSLQPIIK peptide), control, or unknown samples was pipetted into each well.

Fifty microliters of primary antibody (polyclonal antibody against K-Postn peptide) diluted at the titer in assay buffer was pipetted into each well. After incubation for 20 h at 4 °C, the plate was washed 5 times with washing buffer, and 100 µL of a solution of peroxidase-conjugated goat anti-rabbit antibody (The Jackson Laboratory, Bar Harbor, ME) diluted in the assay buffer was pipetted into each well. The plate was incubated for 1 h at room temperature. After incubation, the wells were washed 4 times and 100 µL H_2O_2/tetramethylbenzidine substrate indicator solution (Sigma) was added. After incubation at room temperature for 30 min, the colorimetric reaction was

stopped by the addition of 100 µL 100 H_2SO_4 0.2 M, and the optical density at 450 nm was recorded. All samples were measured in duplicate.

Specificity of the ELISA for K-Postn

The specificity of the antibody used in the K-Postn ELISA was investigated by competitive inhibition with the GSLQPIIK peptide (standard of the assay), intact human recombinant periostin, intact POSTN degraded with cathepsin K, and GSLQPIIK peptide extended by 1 amino (GSLQPIIKT) or shortened by 1 amino acid (GSLQPII) at the C-terminal end.

Total Periostin and Bone Marker Measurements

Serum total periostin was measured by ELISA using the assay from USCN (China) previously described and used in different population studies [13, 15]. This is a sandwich ELISA using a polyclonal antibody raised against the FAS-1 domain common to all periostin isoforms. Intra- and interassay precision errors were below 10 and 15%, respectively [13]. The procollagen type I *N*-propeptide (PINP) and crosslinked C-telopeptide of type I collagen (CTX), which are the bone markers recommended by the IOF-IFCC [24], were measured by the automated assay (Elecsys, RocheDiagnostics, Basel, Switzerland) with intra- and interassay precision below 5%.

Statistical Analyses

Statistical analyses were performed using MedCalc Statistical Software version 13.1.2 (MedCalc Software bvba, Ostend, Belgium). All data were reported as mean ± SD. Normal distribution was evaluated by d'Agostino–Pearson test. To take into account that not all variables were normally distributed, the differences between groups were assessed by Mann–Whitney *U* test. Correlations of K-Postn with BMD, bone structure parameters, and bone markers were analyzed Spearman Rank correlation.

Results

Cathepsin K Efficiently Degrades Human Periostin In Vitro and the ^{688}GSLQPIIK^{695} Peptide is the Main Proteolytic Fragment

Silver staining of the SDS-PAGE analysis showed that when intact periostin was incubated with active cathepsin K, there was a time-dependent disappearance of the electrophoretic band corresponding to intact periostin at 95 Kd and the simultaneous appearance of smaller bands. After 3 h of incubation, two major bands were detected at 35 and 7 Kd, respectively (Fig. 1). LS–MS/MS of cathepsin K digest of human periostin showed that several fragments were obtained. The sequence of 17 of them was determined by targeted LC–MS/MS. The abundance was highest for the ^{688}GSLQPIIK^{695} sequence (Fig. 2). By varying the periostin/cathepsin K ratio, it was found that the amount of peptides increased with the increasing amounts of cathepsin K saturating at a ratio of 5:1 (data not shown). The appearance of 17 different periostin fragments was monitored during cathepsin K-dependent periostin digestion lasting from 15 min to 2 h, at a periostin/cathepsin K ratio of 50:1 (Fig. 2). The fragment generated the earliest was again GSLQPIIK appearing within 15 min from reaction starts, followed by several other fragments. Most of the periostin digestion ended within 60 min from the start of reaction (Fig. 2).

The Cathepsin K-Generated Periostin Fragment GSLQPIIK is Present in Cortical Bone

To investigate whether the cathepsin K-digested periostin fragment GSLQPIIK is also present in vivo, immunohistochemistry of mouse cortical bone was performed using the specific polyclonal antibody that was generated. As shown in Fig. 3, GSLQPIIK was detected at mouse periosteum surface of cortical bone region, specifically in osteocytes and the lacuno canalicular system as well as in the bone matrix.

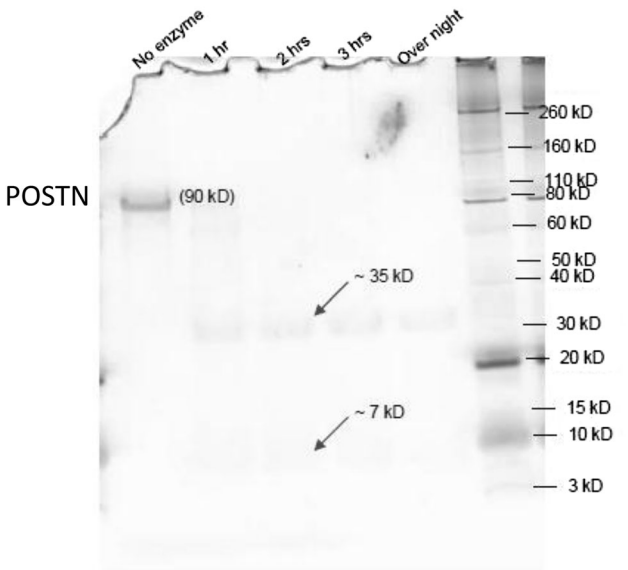

Fig. 1 SDS-PAGE gel of cathepsin K-digested human periostin. Digestion lasting for 1, 2, 3 h, or overnight with a constant periostin/cathepsin K ratio of 50:1. POSTN fragments were visualized by silver staining; dominant band at around 90 kDa corresponds to intact periostin

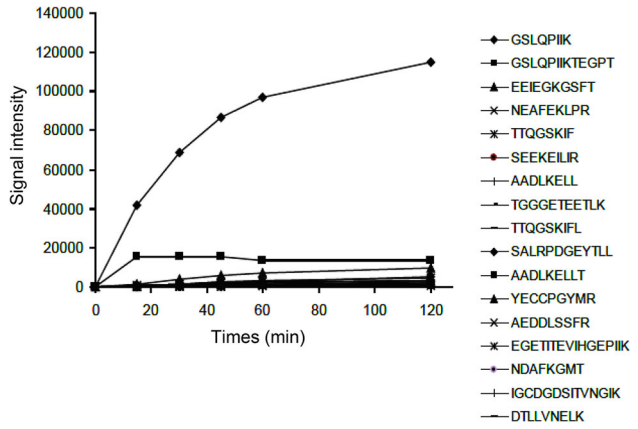

Fig. 2 Time course appearance of the 17 most abundant peptides generated during degradation of recombinant intact periostin with active cathepsin K

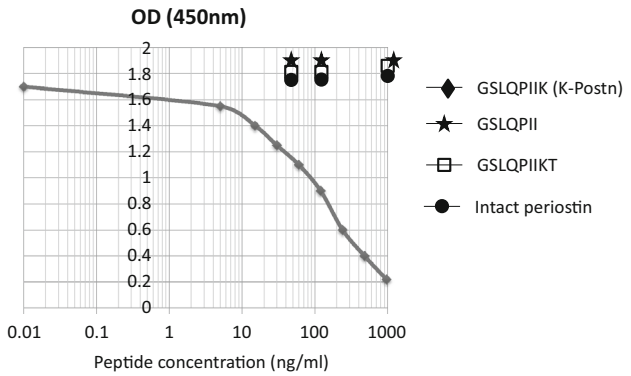

Fig. 4 Typical standard curve and specificity of the enzyme-linked immunosorbent assay (ELISA) using serum levels of GSLQPIIK fragment. The *graph* shows the competitive inhibition of ELISA with GSLQPIIK peptide used as standard, GSLQPIIK peptide shortened by 1 amino acid (GSLQPII) or extended by 1 amino acid (GSLQPIIKT) at the C-terminal end and intact human recombinant periostin. The *y*-axis shows the optical density value for the binding of K-Postn antibody on GSLQPIIK-coated microtiter plates at different concentrations of each peptide. The *x*-axis shows the concentration of each peptide for purposes of comparison

Analytical Performances of the K-Postn ELISA

We developed a competitive ELISA using a polyclonal antibody raised against the GSLQPIIK sequence. A typical standard curve is shown in Fig. 4. The lower limit of detection, defined as the concentration corresponding to 3SD above the mean of 15 determinations of the zero calibrator, was determined to be 7 ng/mL. The lower limit of quantification, defined as the concentration of GSLQPIIK in serum samples that can be measured with a coefficient of variation (CV) below 20%, was estimated at 12 ng/mL. The intraassay CVs assessed by 10 measurements of four different serum samples (mean levels of 32.7, 50.8, 132.9, and 201 ng/mL) in the same run ranged from 8 to 12.8%. The interassay CVs determined by the measurements of three different serum samples (mean levels of 7.5, 27.2 and 40.8 ng/mL) in 10 different runs were 17.2, 13.8, and 10.8%, respectively. The median dilution recovery of serum was 108.5% ranging from 99 to 124% (Table 1).

The specificity of the antibody was investigated by experiments involving competitive inhibition between GSLQPIIK and synthetic peptides, intact periostin, or cathepsin K digest of intact periostin. As shown in Fig. 4, there was no significant crossreactivity of the antibody with the GSLQPIIK peptide that was shortened by 1 amino acid at the C-terminal end or with the GSLQPIIKT peptide (which corresponds to the K-Postn sequence extended by 1 amino acid) up to a concentration of 1000 ng/mL. Similar experiments showed that the antibody used in the ELISA did not demonstrate significant immunoreactivity with human intact periostin at a concentration of up to 1000 ng/mL (Fig. 4). Conversely, when intact periostin was digested by recombinant cathepsin K for 4 h at a molar ratio of 1/10, 29 ng/mL of GSLQPIIK was measured in the ELISA.

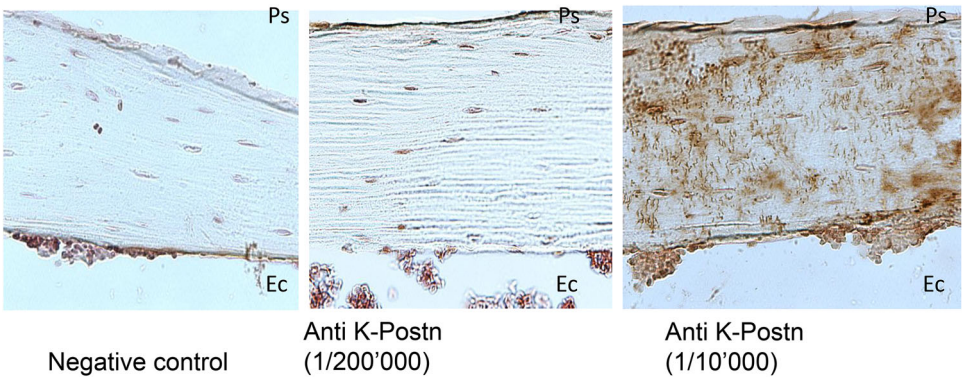

Fig. 3 Immunolocalization of the periostin fragment GSLQPIIK generated by cathepsin K in mouse tibia cortical bone. *Ps* periosteum, *Ec* endocortical surface

Table 1 Dilution of serum samples in the K-Postn ELISA

Serum ID	Dilution	K-Postn fragment (ng/mL)	% recovery
A	Neat (endogenous concentration)	201	
	1/2	104	103
	1/4	55.1	110
	1/8	31.1	124
B	Neat	314	
	1/2	187	119
	1/4	84	107
	1/8	39	99

The GSLQPIIK Fragment Circulates in Blood and is Associated with Bone Cortical Structure in Healthy Postmenopausal Women

To investigate whether the GSLQPIIK fragment can be detected in circulating blood samples and may serve as a clinical biomarker, we measured GSLQPIIK with ELISA in 160 healthy women of the GERICO cohort. GSLQPIIK values ranged from 10.9 to 170.3 ng/mL (median: 38.8 ng/mL). GSLQPIIK levels did not correlate with age in this population within a narrow age range (data not shown). Serum GSLQPIIK was not correlated with serum total periostin, PINP, or CTX (Table 2). GSLQPIIK was also not correlated with hip BMD by DXA or BV/TV (bone volume on total volume) nor with trabecular volume as assessed by HR-pQCT of the tibia and radius. In contrast, GSLQPIIK significantly and negatively correlated with cortical area ($p = 0.02$), cortical thickness ($p = 0.03$), and cortical perimeter ($p = 0.009$ and 0.007 at the tibia and radius, respectively) (Table 2) at the tibia and radius. When postmenopausal women were categorized in tertiles of GSLQPIIK, we found that those with levels in the highest tertile had significantly lower levels of cortical area than the other subjects (Fig. 5). The ratio of serum GSLQPIIK/serum CTX as a putative index of cortical resorption was only modestly correlated with cortical thickness ($r = 0.13$, $p = 0.04$) and trabecular bone volume ($r = 0.16$, $p = 0.01$) at the radius.

By comparison, serum PINP and CTX moderately correlated with femoral neck BMD and both trabecular and cortical bone parameters (r values ranging from -0.17 to -0.19, $p < 0.01$–0.001). Serum total periostin was also modestly associated—but positively—with cortical thickness both at the tibia and radius ($r = 0.21$ and $r = 0.20$, respectively, all $p < 0.05$) and trabecular bone volume ($r = 0.16$ and $r = 0.20$, respectively, all $p < 0.05$).

Table 2 Correlation of serum K-Postn in 160 healthy women with serum bone markers, femoral neck BMD, and structural parameters of distal tibia and radius

Variables	*R* value	*p* value
Serum total POSTN	0.07	0.33
Serum PINP	−0.08	0.26
Serum CTX	−0.09	0.20
Femoral neck BMD	−0.02	0.78
BV/TV tibia	−0.08	0.30
BV/TV radius	−0.06	0.44
Tb. vBMD (mgHA/cm^3) tibia	−0.08	0.30
Tb. vBMD (mgHA/cm^3) radius	−0.07	0.41
Ct. area tibia (mm^2)	−0.17	0.02
Ct. area radius (mm^2)	−0.14	0.10
Ct. Th tibia (mm)	−0.16	0.03
Ct. Th radius (mm)	−0.16	0.05
Ct. Pm tibia (mm)	−0.17	0.009
Ct. Pm radius (mm)	−0.17	0.007

Tb. vBMD trabecular bone volume, *Ct. area* cortical area, *Ct. Th* cortical thickness, *Ct. Pm* cortical perimeter

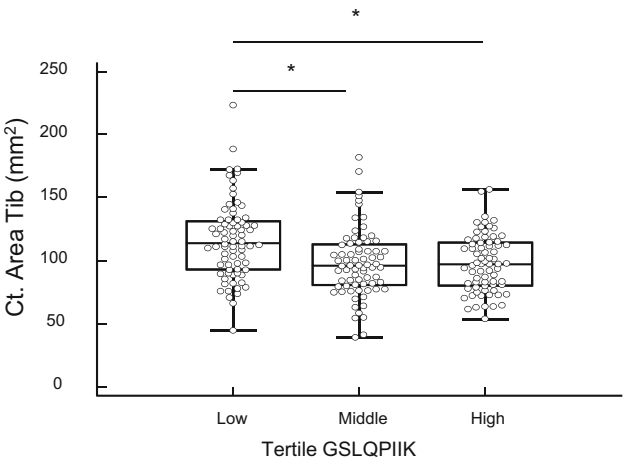

Fig. 5 Cortical area of the tibia by Hr-pQCT in 160 healthy postmenopausal women categorized in tertile of serum K-Postn levels. *$p < 0.05$

Discussion

In this study, we report the development of a new marker reflecting the cathepsin K-mediated degradation of periostin (GSLQPIIK). We demonstrate that the identified fragment is present at both tissue level in mice and in the peripheral blood of healthy individuals. The serum levels of GSLQPIIK did not correlate with serum total periostin nor with the conventional markers of bone formation and resorption, indicating that this new biomarker reflects biological processes which are largely independent of the total level of substrate detected in the circulation (from various tissues) and from the level of bone turnover as assessed by standard biochemical markers. In healthy women, serum GSLQPIIK levels were negatively associated with cortical, but not trabecular bone structure, further suggesting selectivity for cortical bone metabolism. By comparison, the association of total periostin with cortical bone parameters was weaker and positive. These data suggest that increased levels of serum GSLQPIIK may selectively reflect cathepsin K activity in cortical bone, particularly periosteal metabolism—where periostin is mainly expressed—and thereby cortical bone strength.

Current immunoassays for serum periostin use antibody raised against intact periostin or a large portion of it, such as the FAS-1 domain. However, the exact epitope of these antibodies on the periostin sequence is undermined and thus it is unclear which immunoreactive forms they are detecting. The lack of information on the specificity of these antibodies makes the interpretation of the clinical data generated with these tests difficult. To overcome these limitations, we used a rationalized process based on the following steps. In order to obtain a marker which is more bone specific than total periostin, we identified the proteolytic cleavage of periostin by cathepsin K in vitro, because cathepsin K is active mainly in bone osteoclasts and osteocytes. This strategy has proved to be successful for the development of the cathepsin K-mediated degradation of type I collagen to develop the established CTX and NTX bone resorption markers [13]. We then performed immunohistochemistry of bone tissue to verify that the fragment is also generated in vivo. We developed a sensitive and precise ELISA for accurate determination in circulating blood and finally measured serum levels in postmenopausal women, a target population for such biomarker.

The analysis of cathepsin K digest of human intact periostin by LC–MS/MS showed that cathepsin K is able to degrade efficiently periostin and generate several proteolytic fragments. The sequences of the fragments generated suggest that amino acids lysine, threonine, glutamic acid, arginine, and leucine are preferred cleavage sites. These are consistent with the structure of some of fluorogenic peptide substrates for cathepsin K which have been reported during the initial characterization of this enzyme [18]. Time course experiments and dose response in periostin/cathepsin K ratio showed that the fragment with the GSLQPIIK sequence was the faster to be released and highest in relative amount among the different peptides. This peptide which is localized in the C-terminal domain of periostin presents with 100% homology with the corresponding sequence in mouse, dog, and rabbits, indicating that it may also be used in animal studies.

The polyclonal antibody we developed against this GSLQPIIK fragment was highly specific for the C-terminal end of this peptide. Competitive experiments showed no immunoreactivity with intact periostin and with the GSLQPIIK shortened or elongated by one amino acid at the C-terminal end, but the antibody detected intact periostin cleaved by cathepsin K. It remains to be investigated whether other cysteine proteases such as cathepsin B or L can also cleave periostin at the same site. Using this antibody, immunohistochemistry studies indicated that the GSLQPIIK fragment localized in the mouse cortical bone, particularly at the periosteal site and within osteocytes, i.e., where the substrate, periostin, has previously shown to be mainly expressed [7, 25]. We cannot preclude, however, that this fragment could also be present at low levels in other tissues expressing both periostin and cathepsin K such as the lung and the skin [26, 27].

Our findings indicating tissue expression of the GSLQPIIK fragment in cortical bone prompted us to develop an ELISA for use as a circulating biomarker. The ELISA demonstrated adequate precision for such a manual competitive ELISA and was sensitive enough to measure accurately serum levels of the GSLQPIIK fragment in all the healthy postmenopausal women we investigated. In these women, we found that GSLQPIIK levels were not associated with conventional markers of bone turnover and hip BMD, data which are consistent with those previously reported for total periostin levels in another population of healthy postmenopausal women [15]. This finding indicates that serum GSLQPIIK and bone markers reflect different biological processes. May be more unexpectedly, there was no significant association between GSLQPIIK levels and total periostin values, further indicating that total periostin as evaluated by most commercial assays so far is not bone specific and/or suggesting that the rate of synthesis of periostin is unrelated from its cathepsin K-mediated degradation.

When we investigated the association of this new biomarker with bone structure parameters assessed by Hr-pQCT of the tibia and radius, we found that GSLQPIIK correlated negatively with cortical but not trabecular

measurements. These data are consistent with the preferential localization of this fragment in cortical bone. Animal studies have indeed showed that periostin deficiency is associated with decreased bone formation and bone strength [7]. The association is modest, possibly because we cannot measure accurately periosteal bone volume/size with current imaging tools. A similar weak association has been reported between bone turnover markers and BMD.

In summary, we have developed the first fully characterized biomarker of cathepsin K-mediated degradation of periostin. The ELISA for GSLQPIIK is precise and sensitive and serum levels were associated with structural features of cortical bone in postmenopausal women. Provided that these data are confirmed in larger independent clinical studies, GSLQPIIK may be useful for the clinical investigation of patients with osteoporosis and other metabolic bone diseases. In this respect, we recently showed that serum GSLQPIIK measured by the assay described in this report—but not total periostin—predicted the incidence of low trauma fracture independently of BMD and FRAX in a cohort of 695 postmenopausal women [28]. Whether serum GSLQPIIK is associated with changes in cortical bone turnover induced by treatments such as PTH remains to be investigated.

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Compliance with Ethical Standards

Conflict of interest P. Garnero and N. Bonnet have no conflict of interest to disclose. S. Ferrari received speaker fees and research grant from Merck.

Human and Animal Rights and Informed Consent Human and animal studies were approved by local ethical committees and informed consent was obtained from all participants.

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