

Osteoporosis in Relation to a Bone-Related Aging Biomarker Derived from the Urinary Proteomic Profile: A Population Study

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Supplementary Data

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Urinary Proteomics

Sample preparation and CE-MS analysis

As described in detail elsewhere [1], urine aliquots were thawed and 700 μL mixed with 700 μL of 2 M urea, 10 mM NH_4OH containing 0.02 % SDS. Subsequently, samples were ultrafiltered using a Centrstat 20 kDa cut-off centrifugal filter device (Satorius, Göttingen, Germany) to eliminate high molecular weight proteins. The obtained filtrate was desalted using a PD 10 gel filtration column (GE Healthcare Bio Sciences, Uppsala, Sweden) to remove urea, electrolytes and salts as well as to enrich the polypeptides. The samples were lyophilized and stored at 4°C before usage. Shortly before CE-MS analysis, the samples were re-suspended in 10 μL HPLC-grade H_2O . Samples were injected into CE-MS with 2 psi for 99 sec, resulting in injection volumes of ~ 280 nL.

A P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA) was coupled with a Micro-TOF MS (Bruker Daltonic, Bremen, Germany). A solution of 20% acetonitrile (Sigma-Aldrich, Taufkirchen, Germany) in HPLC-grade H_2O (Roth, Karlsruhe, Germany) supplemented with 0.94% formic acid (Sigma-Aldrich) was used as running buffer. For CE-MS analysis, the electrospray ionization interface from Agilent Technologies (Palo Alto, CA) was set to a potential of -4.0 to -4.5 kV. Spectra were recorded over an m/z range of 350-3000 and accumulated every 3 s.

CE-MS data processing

After the CE-MS analysis, mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaFinder software [2]. Only signals with $z > 1$ observed in a minimum of 3 consecutive spectra with a signal-to-noise ratio of at least 4 were considered. The resulting peak list characterizes each polypeptide by its mass and migration time. Data were calibrated utilizing 3151 internal standards as reference data points for mass and migration time by applying global and local linear regression, respectively as described previously. Reference signals of 29 abundant peptides were used as internal standards for calibration of signal intensity using local linear regression [3]. This procedure was shown to be an easy and reliable method to address both analytical and dilution variances and differences in glomerular filtration in a single calibration step. This procedure was shown to be highly reproducible and to address both analytical and dilution variances in a single calibration step [3]. The obtained peak list characterizes each polypeptide by its calibrated molecular mass (Da), calibrated CE migration time (min) and normalized signal intensity. All detected peptides were deposited, matched, and annotated in a Microsoft SQL database allowing further statistical analysis.

Sequencing of peptides

Candidate biomarkers were sequenced using CE-MS/MS or LC-MS/MS analysis, as described in detail [4]. MS/MS experiments were using an Ultimate 3000 nano-flow system (Dionex/LC Packings, Thermo Fisher Scientific Inc., Waltham, MA) or a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA), both connected to an LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany) equipped with a nano-electrospray ion source. The mass spectrometer was operated in data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra (from m/z 300–2,000) were acquired in the Orbitrap. Ions were sequentially isolated for fragmentation. Data files were searched against the

UniProt human nonredundant database, using Proteome Discoverer 2.4 and the SEQUEST search engine. Relevant settings were: no fixed modifications, oxidation of methionine and proline as variable modifications. The minimum precursor mass was set to 790 Da, maximum precursor mass to 6000 Da with a minimum peak count of 10. The high-confidence peptides were defined by cross-correlation (Xcorr) >1.9 and rank = 1. Precursor mass tolerance and fragment mass tolerance were 5 ppm and 0.05 Da, respectively. For further validation of obtained peptide sequences, the correlation between peptide charge at the working pH of 2 and CE-migration time was utilized to minimize incorrect sequence assignment [5]. Calculated CE-migration time of the sequence candidate based on its peptide sequence (number of basic amino acids) was compared to the experimental migration time.

References

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Supplementary Table 1. Bone-related function of 19 parental protein identified by 395 sequenced urinary peptides.

Parental proteins identified from the amino-acid sequence			Number of peptides in analysis	Bone-related function
Accession number	Symbol	Name		
P02452	COL1A1	Collagen alpha-1(I) chain	199	Bone trabecula formation, cartilage development involved in endochondral bone morphogenesis, embryonic skeletal system development, endochondral ossification, extracellular matrix structural constituent and organization, ossification, osteoblast differentiation, skeletal system development
P08123	COL1A2	Collagen alpha-2(I) chain	37	Bone mineralization, extracellular matrix structural constituent and organization, skeletal system development
P02458	COL2A1	Collagen alpha-1(II) chain	21	Extracellular matrix structural constituent, cartilage condensation, cartilage development involved in endochondral bone morphogenesis, chondrocyte differentiation, embryonic skeletal joint morphogenesis, endochondral ossification, skeletal system development
P02461	COL3A1	Collagen alpha-1(III) chain	94	Extracellular matrix structural constituent and organization, limb joint morphogenesis
P53420	COL4A4	Collagen alpha-4(IV) chain	3	Extracellular matrix structural constituent, high expression in calvaria
P05997	COL5A2	Collagen alpha-2(V) chain	5	Extracellular matrix organization, ossification, skeletal system development
P12109	COL6A1	Collagen alpha-1(VI) chain	5	Extracellular matrix structural constituent, osteoblast differentiation
Q14055	COL9A2	Collagen alpha-2(IX) chain	2	Extracellular matrix structural constituent and organization, skeletal system development
Q14050	COL9A3	Collagen alpha-3(IX) chain	2	Extracellular matrix structural constituent and organization, involvement in multiple epiphyseal dysplasia
Q03692	COL10A1	Collagen alpha-1(X) chain	1	Extracellular matrix structural constituent and organization, skeletal system development
P12107	COL11A1	Collagen alpha-1(XI) chain	7	Extracellular matrix structural constituent and organization, cartilage condensation, embryonic skeletal system morphogenesis, ossification
P13942	COL11A2	Collagen alpha-2(XI) chain	4	Extracellular matrix structural constituent and organization, cartilage development, skeletal system development
Q5TAT6	COL13A1	Collagen alpha-1(XIII) chain	4	Extracellular matrix structural constituent and organization, endochondral ossification, skeletal system development
P39060	COL18A1	Collagen alpha-1(XVIII) chain	2	Extracellular matrix structural constituent and organization, collagen fibril organization, skeletal system development
Q14993	COL19A1	Collagen alpha-1(XIX) chain	2	Extracellular matrix structural constituent and organization, skeletal system development
Q96P44	COL21A1	Collagen alpha-1(XXI) chain	1	Collagen-containing extracellular matrix, expression in bone marrow
P01344	IGF2	Insulin-like growth factor II	3	Growth factor and hormone activity, osteoblast differentiation
P08493	MGP	Matrix Gla protein	2	Structural constituent of bone, cartilage condensation, ossification, regulation of bone mineralization
Q86Y38	XYLT1	Xylosyltransferase 1	1	Embryonic skeletal system development, ossification involved in bone maturation

For analysis, 395 sequenced urinary peptides derived from 19 parental proteins with a detectable signal in $\geq 70\%$ FLEMENGHO participants were selected (see Figure 1). Accession numbers refer to the Uniprot database (<http://www.uniprot.org/uniprot>), which substantiated the bone-related function of the parental proteins.

Supplementary Table 2. Association of 99 bone-related sequenced urinary peptides with chronological age in the derivation dataset (starts)

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			β (95% confidence interval)	<i>p</i> -value
		Accession number	Symbol	Name		
e99907132	EpGSpGENGAPGmGPR	P02452	COL1A1	Collagen alpha-1(I) chain	0.281 (0.203, 0.359)	9.21E-11
e99910072	GLpGTGGPpGENGKpGEpGPKG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.232 (-0.309, -0.155)	3.29E-08
e99901132	cDDYRLcE	P08493	MGP	Matrix Gla protein	0.230 (0.154, 0.305)	3.85E-08
e99906708	pGpAGEKGSpGADGPAGAP	P02452	COL1A1	Collagen alpha-1(I) chain	0.250 (0.169, 0.332)	5.19E-08
e99904196	PSGDQGASGpAGPSGP	P02458	COL2A1	Collagen alpha-1(II) chain	0.237 (0.156, 0.318)	7.19E-08
e99920065	ARGNDGARGSDGQpGppPPPBTAGFPpGSpGAKGEV GpAGSpGSNGApG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.241 (-0.323, -0.159)	7.27E-08
e99912851	LDGAKGDAGPAGPKGEpGSpGENGApG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.227 (-0.304, -0.151)	1.52E-07
e99917856	ARGNDGARGSDGQPGPpGppGTAGFpGSpGAKGEV GP	P02461	COL3A1	Collagen alpha-1(III) chain	-0.235 (-0.317, -0.153)	1.78E-07
e99919107	DQGPVGRTEVGVAVGpGFAGEKpSGEAGTAGPP GTpGPQG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.218 (-0.297, -0.139)	3.00E-07
e99917922	NTGApGSPGVSGPKGDAGQpGEKGSpGAQpPGAP GPLG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.234 (-0.318, -0.151)	3.04E-07
e99916966	PpGESGREGApGAEGSpGRDGSgAKGDRGETGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.215 (-0.295, -0.135)	3.23E-06
e99906441	TGSpGSpGPDGKTGPpGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.204 (-0.283, -0.126)	7.75E-06
e99912986	ADGQpGAKGEpGDAGAKGDAGPPGPAGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.203 (-0.282, -0.125)	9.27E-06
e99901661	DGpSGAEGpPGp	P02458	COL2A1	Collagen alpha-1(II) chain	0.187 (0.107, 0.267)	2.49E-05
e99918573	DQGPVGRTEVGVAVGpPGFAGEKpSGEAGTAGPP GTpGP	P08123	COL1A2	Collagen alpha-2(I) chain	-0.183 (-0.263, -0.102)	3.26E-05
e99919745	SKGESGNKGEpGSAGPQGpGpSGEEGKRGPNGEA GSAGPPGPpG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.187 (-0.270, -0.105)	3.43E-05
e99905560	GSpGSpGPDGKTGPpGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.191 (-0.269, -0.113)	3.61E-05
e99900340	cDDYRLc	P08493	MGP	Matrix Gla protein	0.189 (0.108, 0.270)	4.30E-05
e99909891	SESPGHpGQPGpPGpPGAAPGP	P02461	COL3A1	Collagen alpha-1(III) chain	-0.189 (-0.271, -0.106)	4.88E-05
e99906709	GSpGSpGPDGKTGPpGPAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.186 (-0.264, -0.108)	5.84E-05
e99906154	EDGHpGKpGRpGERG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.180 (-0.261, -0.098)	6.07E-05
e99912949	LDGAKGDAGPAGpKGEpGSpGENGApG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.191 (-0.271, -0.111)	6.40E-05

Supplementary Table 2 Association of 99 bone-related sequenced urinary peptides with chronological age in the derivation dataset (continued)

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			β (95% confidence interval)	<i>p</i> -value
		Accession number	Symbol	Name		
e99917311	NTGApGSpGVSGpKGDAGQpGEKGSPPGAPGp	P02461	COL3A1	Collagen alpha-1(III) chain	-0.186 (-0.270, -0.102)	9.67E-05
e99915778	ESGREGAPGAEGSPGRDGSpGAKGDRGETGp	P02452	COL1A1	Collagen alpha-1(I) chain	-0.186 (-0.266, -0.106)	0.00011
e99911415	IGPpPAGApGDKGESGPSGAPPTG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.182 (-0.261, -0.104)	0.00012
e99903016	ApGDRGEPGpGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.187 (-0.268, -0.105)	0.00016
e99903372	DGQPQAKGEpGDAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.184 (-0.266, -0.103)	0.00017
e99911777	ANGApGNDGAKGDAGApGApGSQGAPG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.188 (-0.271, -0.104)	0.00019
e99903120	EGSpGRDGSpGAK	P02452	COL1A1	Collagen alpha-1(I) chain	0.188 (0.105, 0.272)	0.00021
e99904945	SpGSPGPDGKTGpGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.186 (-0.270, -0.102)	0.00027
e99906617	MpGSpGGpGSDGKpGpPG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.172 (-0.255, -0.089)	0.00029
e99917288	GpGESGREGAPGAEGSpGRDGSpGAKGDRGETGp	P02452	COL1A1	Collagen alpha-1(I) chain	-0.184 (-0.267, -0.100)	0.00031
e99919885	ERGEQGPAGSpGFQGLpGpAGppGEAGKpGEQGVPGDLGAPGPSG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.179 (-0.261, -0.098)	0.00034
e99918864	GpAGPPGPPPGTSGHPGSPGSPGYQGGPEPGQA GPSGPPG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.164 (-0.246, -0.082)	0.00052
e99904847	GGDkGEDDPGQpGp	P12107	COL11A1	Collagen alpha-1(XI) chain	0.157 (0.074, 0.241)	0.00063
e99917866	NTGApGSpGVSGpKGDAGQpGEKGSPPGAPGPLG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.166 (-0.250, -0.081)	0.00070
e99912508	KGNSGEPGAPGSKGDTGAKGEpGpVG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.176 (-0.260, -0.092)	0.00073
e99920509	GEHGpPGPPGPIGPVQPGAAGADGEPGARGPQGHFGAKGDEGTRGFNGP	P13942	COL11A2	Collagen alpha-2(XI) chain	-0.153 (-0.236, -0.070)	0.00078
e99915442	DVStPPTVLPDNFPRYP	P01344	IGF2	Insulin-like growth factor II	0.148 (0.066, 0.231)	0.00088
e99919425	pGApGVRGFQGGKGSmGDpGLPGPQGLRGDVGDRGPGGAAGP	Q14050	COL9A3	Collagen alpha-3(IX) chain	0.150 (0.066, 0.235)	0.00089
e99917787	GARGLpGpPGSNGNPGPPGPSGPKDGPPGAPNTGAP	P02461	COL3A1	Collagen alpha-1(III) chain	-0.160 (-0.243, -0.076)	0.0011
e99917280	AAGEpGKAGERGVpGpGAVGPAGKDGEAGAQQPGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.169 (-0.253, -0.086)	0.0012
e99913779	DDILASPPRLPEPQPYPGAPHSS	P39060	COL18A1	Collagen alpha-1(XVIII) chain	0.140 (0.059, 0.222)	0.0012

Supplementary Table 2. Association of 99 bone-related sequenced urinary peptides with chronological age in the derivation dataset (continued)

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			β (95% confidence interval)	<i>p</i> -value
		Accession number	Symbol	Name		
e99917212	RTGEVGA VGPpGFAGEKGPSGEAGTAGpPGTpGPQG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.149 (-0.231, -0.067)	0.0012
e99909408	GDDGEAGKpGRPGERGPPGp	P02452	COL1A1	Collagen alpha-1(I) chain	-0.166 (-0.247, -0.084)	0.0013
e99906961	GLpGTGGPpGENGKPGEp	P02461	COL3A1	Collagen alpha-1(III) chain	-0.161 (-0.246, -0.075)	0.0013
e99914645	DVStPPTVLPDNFPRYPVG	P01344	IGF2	Insulin-like growth factor II	0.144 (0.061, 0.226)	0.0014
e99905423	VGpPGPPGpPGPPGPPS	P02452	COL1A1	Collagen alpha-1(I) chain	-0.167 (-0.250, -0.084)	0.0014
e99905074	DGHpGKpGRPGERG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.149 (-0.232, -0.066)	0.0014
e99916419	EAGRDGNpGNDGppGRDQPGHKGERGYpG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.146 (-0.227, -0.064)	0.0016
e99904531	PGTpGSPGPAGASGNPG	P02458	COL2A1	Collagen alpha-1(II) chain	0.147 (0.065, 0.228)	0.0018
e99916785	DPGLMGERGEDGpAGNGTEGFpGFpGYPGNR	P12109	COL6A1	Collagen alpha-1(VI) chain	-0.137 (-0.221, -0.054)	0.0020
e99907513	TGSpGSPGPDGKTGPpGPAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.164 (-0.247, -0.080)	0.0022
e99914815	pGYPGQpGQDGKPGYQGIAGTpGVpGSPG	Q96P44	COL21A1	Collagen alpha-1(XXI) chain	-0.130 (-0.211, -0.049)	0.0023
e99917447	GPpGDpGLMGERGEDGpAGNGTEGFPGFPYPGN	P12109	COL6A1	Collagen alpha-1(VI) chain	-0.134 (-0.219, -0.049)	0.0030
e99911753	GADGQPGAKGEPGDAGAKGDA GppGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.162 (-0.247, -0.077)	0.0031
e99918044	AGPGFPpGApGAKGEAGpTGARGpEGAQGPRGEPGTPGS	P02458	COL2A1	Collagen alpha-1(II) chain	-0.143 (-0.227, -0.058)	0.0039
e99905705	DGQPGAKGEPGDAGAKG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.153 (-0.235, -0.071)	0.0042
e99906176	VGPpGPpGPPGpGPPSA	P02452	COL1A1	Collagen alpha-1(I) chain	-0.157 (-0.241, -0.073)	0.0042
e99915411	PQGPpGPTGpGGDKGDTGPpGPQGLQLpGT	P02461	COL3A1	Collagen alpha-1(III) chain	0.144 (0.060, 0.228)	0.0043
e99918867	QGFQGPAGEPGEPGQTGPAGARGPAGppGKAGEDGHpGKP	P08123	COL1A2	Collagen alpha-2(I) chain	-0.136 (-0.218, -0.053)	0.0043
e99914015	PAGERGEQGAPGPSGFQGLPGpGPPGE	P02458	COL2A1	Collagen alpha-1(II) chain	-0.140 (-0.224, -0.056)	0.0048
e99911879	EPGAGSKGESGNGKGEPSAGpQGp	P08123	COL1A2	Collagen alpha-2(I) chain	-0.138 (-0.222, -0.053)	0.0049
e99917184	GPAGFAGPPGADGQPGAKGEQGEAGQKGDAGAPGPQG	P02458	COL2A1	Collagen alpha-1(II) chain	-0.136 (-0.219, -0.054)	0.0050
e99906686	SpGNIGPAGKEGPVGLpG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.132 (-0.215, -0.049)	0.0065
e99911537	GKNGDDGEAGKpGRpGERGpGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.153 (-0.238, -0.069)	0.0067

Supplementary Table 2. Association of 99 bone-related sequenced urinary peptides with chronological age in the derivation dataset (continued)

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			β (95% confidence interval)	<i>p</i> -value
		Accession number	Symbol	Name		
e99910953	DGQpGAKGepGDAGAKGDAGPpGP	P02452	COL1A1	Collagen alpha-1(I) chain	0.148 (0.066, 0.229)	0.0068
e99901648	GpEGGKGAAGPpG	P02461	COL3A1	Collagen alpha-1(III) chain	0.140 (0.054, 0.225)	0.0078
e99918881	HDGEKGRGKPGDMGPPGQPPGKDGpGVKGE NGHp	Q5TAT6	COL13A1	Collagen alpha-1(XIII) chain	-0.118 (-0.203, -0.033)	0.0085
e99912556	KNGDDGEAGKpGRPGERGpPGPQG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.147 (-0.230, -0.064)	0.0094
e99902032	GGpGSDGKpGpG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.134 (-0.217, -0.050)	0.0095
e99915467	GRDGNpGNDGpGRDQpGHKGERGYpG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.126 (-0.210, -0.043)	0.0098
e99919780	GAPGERGETGpGpAGFAGpPGADGQPGAQKGEQGE AGQKGDAGAPG	P02458	COL2A1	Collagen alpha-1(II) chain	-0.128 (-0.212, -0.045)	0.011
e99915129	ERGEAGIpGVpGAKGEDGKDGSPGEpGANG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.129 (-0.210, -0.047)	0.011
e99906024	SpGSPGPDGKTGpPGPAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.144 (-0.228, -0.061)	0.012
e99913707	GApGQNGEPGGKGERGApGEKGEpGpG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.129 (-0.212, -0.046)	0.013
e99908442	AGEKGPSGEAGTAGPpGTpGP	P08123	COL1A2	Collagen alpha-2(I) chain	0.124 (0.039, 0.208)	0.014
e99916417	RTGEVGAVGpGFAGEKGPSGEAGTAGPpGTpGP	P08123	COL1A2	Collagen alpha-2(I) chain	-0.123 (-0.208, -0.038)	0.015
e99911008	pPGEEGKRGRGDpGTVGpGP	P05997	COL5A2	Collagen alpha-2(V) chain	-0.105 (-0.187, -0.022)	0.016
e99914315	NRGERGSESGPHpGQpGPPGpPGApGP	P02461	COL3A1	Collagen alpha-1(III) chain	-0.127 (-0.210, -0.043)	0.016
e99913816	ERGEAGIpGVpGAKGEDGKDGSpGEpG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.128 (-0.212, -0.044)	0.016
e99901785	DGESGRPGRpG	P02461	COL3A1	Collagen alpha-1(III) chain	0.126 (0.043, 0.210)	0.017
e99905346	GpGKpGDDGEAGKpG	P02458	COL2A1	Collagen alpha-1(II) chain	0.123 (0.039, 0.207)	0.017
e99908598	NSGEPGApGSKGDTGAKGEp	P02452	COL1A1	Collagen alpha-1(I) chain	-0.138 (-0.220, -0.055)	0.018
e99902286	PpGEAGKpGEQG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.139 (-0.224, -0.055)	0.020
e99903284	DGSpGAKGDRGET	P02452	COL1A1	Collagen alpha-1(I) chain	0.138 (0.055, 0.222)	0.021
e99915811	GHPGSPGPPKpGYGSpGLQGEpGLPpGPPGPS	Q03692	COL10A1	Collagen alpha-1(X) chain	-0.099 (-0.183, -0.016)	0.021
e99912156	GPAGpPGEKGEpGDDGPSAEGPPGp	P02458	COL2A1	Collagen alpha-1(II) chain	-0.117 (-0.200, -0.034)	0.025
e99902189	GpPGpGpGpGpG	P53420	COL4A4	Collagen alpha-4(IV) chain	-0.096 (-0.180, -0.012)	0.025

Supplementary Table 2. Association of 99 bone-related sequenced urinary peptides with chronological age in the derivation dataset (ends)

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			β (95% confidence interval)	<i>p</i> -value
		Accession number	Symbol	Name		
e99906650	EGSpGRDGSpGAKGDRG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.129 (-0.209, -0.049)	0.028
e99913065	ADGQpGAKGEpGDAGAKGDAGPpGPAGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.128 (-0.208, -0.048)	0.029
e99917672	GPpGpAGFAGpPGADGQPGAKGEQGEAGQKGDAG APGP	P02458	COL2A1	Collagen alpha-1(II) chain	-0.116 (-0.201, -0.032)	0.029
e99904330	pPGpPGPpGpPGPPS	P02452	COL1A1	Collagen alpha-1(I) chain	-0.132 (-0.216, -0.048)	0.034
e99909989	DGESGRpGRpGERGLpGPPpG	P02461	COL3A1	Collagen alpha-1(III) chain	0.118 (0.034, 0.203)	0.034
e99905551	VGpPpGPPGpGpGPPS	P02452	COL1A1	Collagen alpha-1(I) chain	-0.133 (-0.218, -0.049)	0.035
e99905559	GpAGpRGERGPPGESG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.110 (-0.196, -0.025)	0.0371
e99920326	GSEGARGAPGAPPPGDPLMGERGEDGPAGNGT EGFpGFpGYpGpNR	P12109	COL6A1	Collagen alpha-1(VI) chain	-0.094 (-0.179, -0.010)	0.043
e99918041	NTGAPGSpGVSGpKGDAGQpGEKGSpGAQGppGAP GPLG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.115 (-0.200, -0.030)	0.044
e99906697	VGpPpGpGPPpGPPGPPSAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.129 (-0.213, -0.044)	0.046

FLEMENGHO: Flemish Study on Environment, Genes, and Health Outcomes. The lower-case letters in the amino-acid sequence identify post-translational modifications: c, disulphide bridges; m, oxidized methionine; n, deaminated asparagine; p, hydroxylated proline; k, hydroxylated lysine; q, deaminated glutamine. Accession numbers refer to the Uniprot database (<http://www.uniprot.org/uniprot>). Association sizes (β) between urinary peptides and chronological age were expressed per 10-year increment and were adjusted for the clustering within families, sex, body mass index, the glomerular filtration rate calculated from serum creatinine by the Chronic Kidney Disease Epidemiology Collaboration equation, current smoking, γ -glutamyltransferase, fasting plasma glucose, physical activity, use of diuretics (yes vs no), and socioeconomic status (High or middle vs low). *p*-values were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate for 395 tested associations.

Supplementary Table 3. Association of chronological age with 16 bone-related urinary peptides

Peptide ID number	Parental protein	Derivation dataset (<i>n</i> = 519)		Time-shifted validation dataset (<i>n</i> = 519)		Synchronous validation dataset (<i>n</i> = 187)	
		β (95% CI)	<i>p</i> -value	β (95% CI)	<i>p</i> -value	β (95% CI)	<i>p</i> -value
e99907132	COL1A1	0.281 (0.203, 0.359)	9.21E-11	0.293 (0.205, 0.381)	1.51E-09	0.151 (0.028, 0.274)	0.093
e99910072	COL3A1	-0.232 (-0.309, -0.155)	3.29E-08	-0.171 (-0.260, -0.082)	0.00059	-0.201 (-0.329, -0.073)	0.026
e99901132	MGP	0.230 (0.154, 0.305)	3.85E-08	0.232 (0.146, 0.318)	8.67E-07	0.238 (0.113, 0.363)	0.0007
e99904196	COL2A1	0.237 (0.156, 0.318)	7.19E-08	0.134 (0.042, 0.226)	0.0098	NS	...
e99919107	COL1A2	-0.218 (-0.297, -0.139)	3.00E-07	-0.106 (-0.198, -0.015)	0.034	-0.218 (-0.343, -0.093)	0.0028
e99904847	COL11A1	0.157 (0.074, 0.241)	0.00063	NS	...	NS	...
e99920509	COL11A2	-0.153 (-0.236, -0.070)	0.00078	NS	...	NS	...
e99915442	IGF2	0.148 (0.066, 0.231)	0.00088	0.100 (0.005, 0.194)	0.04	0.160 (0.026, 0.294)	0.028
e99919425	COL9A3	0.150 (0.066, 0.235)	0.00089	0.129 (0.037, 0.221)	0.011	NS	...
e99913779	COL18A1	0.140 (0.059, 0.222)	0.0012	0.113 (0.023, 0.203)	0.018	NS	...
e99916785	COL6A1	-0.137 (-0.221, -0.054)	0.0020	NS	...	-0.140 (-0.276, -0.004)	0.05
e99914815	COL21A1	-0.130 (-0.211, -0.049)	0.0023	-0.100 (-0.189, -0.010)	0.033	NS	...
e99918881	COL13A1	-0.118 (-0.203, -0.033)	0.0085	NS	...	-0.134 (-0.268, -0.001)	0.049
e99911008	COL5A2	-0.105 (-0.187, -0.022)	0.016	NS	...	-0.178 (-0.316, -0.040)	0.019
e99915811	COL10A1	-0.099 (-0.183, -0.016)	0.021	NS	...	-0.179 (-0.312, -0.047)	0.016
e99902189	COL4A4	-0.096 (-0.180, -0.012)	0.025	NS	...	-0.145 (-0.278, -0.012)	0.041

Only urinary peptides with the highest significance per identified protein were selected for this analysis to demonstrate consistency of the associations in 519 participants included in the derivation and the time-shifted validation datasets and in the 187 participants included in the synchronous validation dataset. Association sizes (β) of chronological age with the urinary peptides were expressed per 10-year increment and were adjusted for the clustering within families, sex, body mass index, the glomerular filtration rate calculated from serum creatinine by the Chronic Kidney Disease Epidemiology Collaboration equation, current smoking, γ -glutamyltransferase, fasting plasma glucose, physical activity, use of diuretics (yes *vs* no), and socioeconomic status (high or middle *vs* low). The *p*-values indicate the validation status (*p* < 0.10, given the prior probability established by the significance in the derivation dataset). NS indicates not significant.

Supplementary Table 4. Number of replicated bone-related urinary peptides identifying parental proteins

Peptide ID number	Parental protein	Number of peptide fragments (Percentage replicated compared with derivation dataset)		
		Derivation dataset (<i>n</i> = 519)	Time-shifted validation dataset (<i>n</i> = 519)	Synchronous validation dataset (<i>n</i> = 187)
e99907132	COL1A1	38	16 (42.1%)	19 (50%)
e99910072	COL3A1	21	10 (47.6%)	6 (28.6%)
e99901132	MGP	2	1 (50%)	2 (100%)
e99904196	COL2A1	10	4 (40%)	5 (50%)
e99919107	COL1A2	14	3 (21.4%)	8 (57.1%)
e99904847	COL11A1	1	0 (0%)	0 (0%)
e99920509	COL11A2	1	0 (0%)	0 (0%)
e99915442	IGF2	2	2 (100%)	1 (50%)
e99919425	COL9A3	1	1 (100%)	0 (0%)
e99913779	COL18A1	1	1 (100%)	0 (0%)
e99916785	COL6A1	3	0 (0%)	1 (33.3%)
e99914815	COL21A1	1	1 (100%)	1 (100%)
e99918881	COL13A1	1	0 (0%)	0 (0%)
e99911008	COL5A2	1	0 (0%)	1 (100%)
e99915811	COL10A1	1	0 (0%)	1 (100%)
e99902189	COL4A4	1	0 (0%)	1 (100%)

Replication indicates similar directionality in the association with chronological age with a *p-value* of <0.10 (given the prior probability established by the significance in the derivation dataset).

Supplementary Table 5. Sex difference of bone-related urinary peptides in the derivation dataset (starts)

Peptide ID number	Parental protein	Unadjusted		Adjusted	
		Estimate (95% CI)	<i>p</i> -value	Estimate (95% CI)	<i>p</i> -value
e99918573	COL1A2	-0.551 (-0.716, -0.386)	1.39E-08	-0.490 (-0.663, -0.317)	4.42E-06
e99915129	COL3A1	-0.544 (-0.709, -0.379)	1.25E-08	-0.496 (-0.671, -0.320)	2.49E-06
e99919107	COL1A2	-0.483 (-0.650, -0.316)	7.38E-07	-0.464 (-0.634, -0.295)	3.10E-06
e99915778	COL1A1	-0.440 (-0.608, -0.273)	7.26E-06	-0.324 (-0.497, -0.151)	0.0021
e99909408	COL1A1	-0.426 (-0.594, -0.258)	1.47E-05	-0.357 (-0.533, -0.181)	0.00077
e99917184	COL2A1	-0.424 (-0.592, -0.256)	1.42E-05	-0.375 (-0.553, -0.198)	0.00043
e99913707	COL3A1	-0.419 (-0.587, -0.251)	1.63E-05	-0.403 (-0.582, -0.225)	0.00016
e99915811	COL10A1	-0.418 (-0.586, -0.250)	1.59E-05	-0.435 (-0.614, -0.256)	5.03E-05
e99908598	COL1A1	-0.394 (-0.562, -0.226)	5.59E-05	-0.379 (-0.557, -0.202)	0.00041
e99915411	COL3A1	-0.373 (-0.542, -0.204)	0.00016	-0.321 (-0.501, -0.140)	0.0037
e99903016	COL1A1	-0.355 (-0.524, -0.186)	0.00037	-0.281 (-0.458, -0.105)	0.0091
e99905074	COL1A2	-0.329 (-0.499, -0.160)	0.001	-0.295 (-0.473, -0.117)	0.0068
e99910072	COL3A1	-0.316 (-0.485, -0.146)	0.0017	-0.256 (-0.436, -0.077)	0.019
e99914315	COL3A1	-0.314 (-0.484, -0.145)	0.0017	-0.243 (-0.409, -0.077)	0.016
e99911008	COL5A2	-0.311 (-0.480, -0.141)	0.0019	-0.238 (-0.416, -0.060)	0.03
e99906686	COL1A2	-0.307 (-0.477, -0.138)	0.002	-0.313 (-0.492, -0.133)	0.004
e99913779	COL18A1	-0.291 (-0.461, -0.121)	0.0036	-0.266 (-0.441, -0.091)	0.013
e99912508	COL1A1	-0.287 (-0.457, -0.117)	0.0041	-0.278 (-0.458, -0.097)	0.012
e99916419	COL1A2	-0.284 (-0.454, -0.114)	0.0045	-0.274 (-0.450, -0.099)	0.01
e99906650	COL1A1	-0.271 (-0.441, -0.101)	0.0072	NS	...

Supplementary Table 5. Sex difference of bone-related urinary peptides in the derivation dataset (ends)

Peptide ID number	Parental protein	Unadjusted		Adjusted	
		Estimate (95% CI)	<i>p</i> -value	Estimate (95% CI)	<i>p</i> -value
e99904945	COL1A1	-0.254 (-0.424, -0.084)	0.013	NS	...
e99911777	COL1A1	-0.245 (-0.415, -0.074)	0.016	-0.239 (-0.418, -0.060)	0.031
e99913816	COL3A1	-0.240 (-0.411, -0.069)	0.017	NS	...
e99911753	COL1A1	-0.240 (-0.411, -0.070)	0.018	-0.242 (-0.424, -0.060)	0.03
e99917866	COL3A1	-0.232 (-0.402, -0.061)	0.023	NS	...
e99918041	COL3A1	0.207 (0.036, 0.378)	0.048	NS	...
e99915442	IGF2	0.209 (0.038, 0.380)	0.046	0.261 (0.085, 0.438)	0.016
e99906709	COL1A1	0.243 (0.072, 0.413)	0.017	0.215 (0.047, 0.382)	0.039
e99901661	COL2A1	0.248 (0.078, 0.419)	0.015	0.306 (0.133, 0.478)	0.0034
e99903120	COL1A1	0.250 (0.079, 0.420)	0.014	0.282 (0.102, 0.463)	0.011
e99900340	MGP	0.260 (0.090, 0.430)	0.011	0.308 (0.135, 0.481)	0.0036
e99918044	COL2A1	0.305 (0.135, 0.474)	0.0021	0.260 (0.078, 0.441)	0.019
e99913065	COL1A1	0.311 (0.141, 0.480)	0.0018	0.332 (0.160, 0.505)	0.0015
e99906441	COL1A1	0.323 (0.154, 0.493)	0.0013	0.313 (0.143, 0.482)	0.0024
e99901132	MGP	0.340 (0.170, 0.509)	0.00068	0.372 (0.210, 0.535)	0.00015
e99910953	COL1A1	0.342 (0.173, 0.512)	0.00063	0.285 (0.109, 0.460)	0.0083
e99905560	COL1A1	0.465 (0.298, 0.632)	1.81E-06	0.475 (0.306, 0.643)	1.7E-06

NS: not significant. Estimate was the association size of difference in urinary peptide level in women compared to men (men as reference) derived from the rank-normalized data in the unadjusted and adjusted general linear models. Adjusted models accounted for the clustering within families, age, body mass index, the glomerular filtration rate calculated from serum creatinine by the Chronic Kidney Disease Epidemiology Collaboration equation, current smoking, γ -glutamyltransferase as index of alcohol intake, blood glucose, physical activity, use of diuretics (yes vs. no), and the middle or high socioeconomic status (yes vs. no). The 99 urinary peptides associated with chronological age were included in the analysis in the derivation dataset. *P*-values were corrected for the Benjamini-Hochberg false discovery rate for 99 tested association. 37 (9 parental proteins) was identified in the unadjusted models and 32 urinary peptides (9 parental proteins) in the adjusted models, respectively.

Supplementary Table 6. Weights assigned by elastic net regression to the multidimensional UPP biomarker reflecting aging (starts)

Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			Weight (95% confidence interval)
		Accession number	Symbol	Name	
...	49.577405 (intercept)
e99911879	EPGPAGSKGESGNKGEPSAGpQGp	P08123	COL1A2	Collagen alpha-2(I) chain	-0.006570 (-0.010338, -0.002746)
e99919107	DQGPVGRGTGEVGA VGPpGFAGEKGP SGEAGTAGPPG TpGPQG	P08123	COL1A2	Collagen alpha-2(I) chain	-0.005577 (-0.008878, -0.002536)
e99905705	DGQPGAKGepGDAGAKG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.005512 (-0.013123, 0.001801)
e99902032	GGpGSDGKpGPpG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.005088 (-0.010774, 0.000741)
e99902189	GpPGPpGPpGPpG	P53420	COL4A4	Collagen alpha-4(IV) chain	-0.005073 (-0.008518, -0.001569)
e99916966	PpGESGREGApGAEGSpGRDGSFGAKGDRGETGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.004863 (-0.006168, -0.003606)
e99903372	DGQPGAKGepGDAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.004531 (-0.010864, 0.001061)
e99910072	GLpGTGGPpGENGKpGepGPKG	P02461	COL3A1	Collagen alpha-1(III) chain	-0.004197 (-0.007435, -0.001032)
e99906650	EGSpGRDGSpGAKGDRG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.003875 (-0.009837, 0.002081)
e99906709	GSpGSpGPDGKTGPpGPAG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.003650 (-0.004616, -0.002698)
e99912851	LDGAKGDAGPAGPKGepGSpGENGApG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.003648 (-0.006470, -0.000989)
e99912949	LDGAKGDAGPAGpKGEpGSpGENGApG	P02452	COL1A1	Collagen alpha-1(I) chain	-0.003329 (-0.008020, 0.001176)
e99920509	GEHGpPGPPGPIGPVQPGAAGADGEPGARGPQGHF GAKGDEGTRGFNGP	P13942	COL11A2	Collagen alpha-2(XI) chain	-0.001102 (-0.001807, -0.000415)
e99906441	TGSpGSpGPDGKTGPpGP	P02452	COL1A1	Collagen alpha-1(I) chain	-0.000776 (-0.002173, 0.000571)
e99905560	GSpGSpGPDGKTGPPGp	P02452	COL1A1	Collagen alpha-1(I) chain	-0.000259 (-0.000961, 0.000439)
e99914645	DVStPPTVLPDNFPRYPVG	P01344	IGF2	Insulin-like growth factor II	0.000179 (0.000003, 0.000347)
e99915442	DVStPPTVLPDNFPRYP	P01344	IGF2	Insulin-like growth factor II	0.000350 (-0.000203, 0.000945)

Supplementary Table 6. Weights assigned by elastic net regression to the multidimensional UPP biomarker reflecting aging (ends).

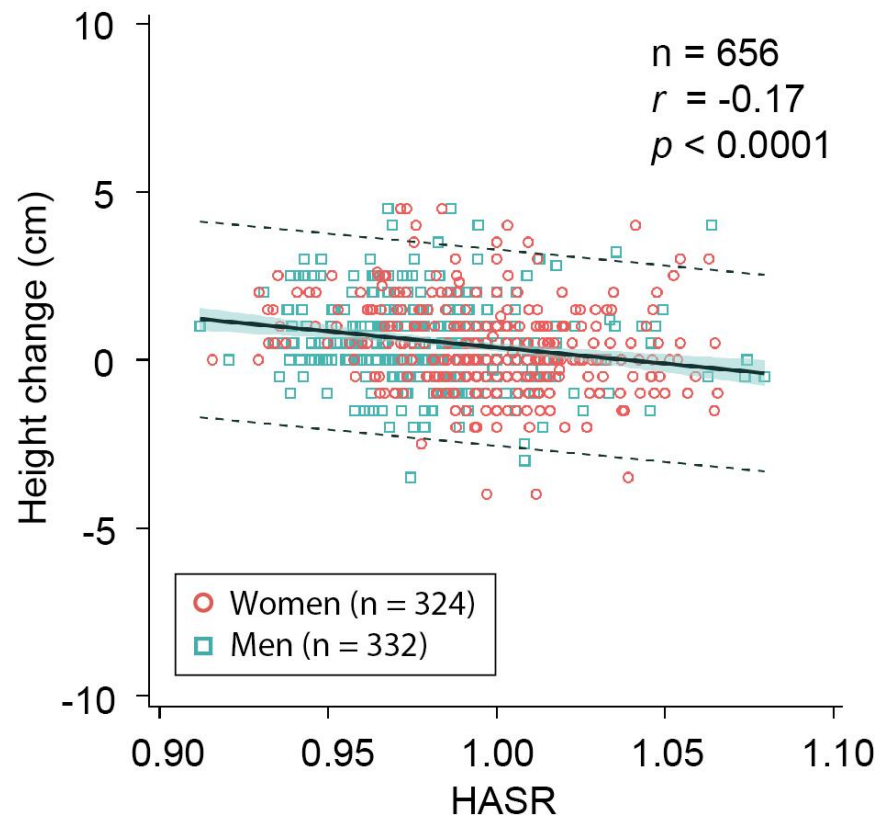
Peptide ID number	Amino-acid sequence	Parental proteins identified from the amino-acid sequence			Weight (95% confidence interval)
		Accession number	Symbol	Name	
e99901132	cDDYRLcE	P08493	MGP	Matrix Gla protein	0.000822 (0.000486, 0.001196)
e99909989	DGESGRpGRpGERGLpGPpG	P02461	COL3A1	Collagen alpha-1(III) chain	0.001280 (0.000333, 0.002376)
e99907132	EpGSpGENGAPGQmGPR	P02452	COL1A1	Collagen alpha-1(I) chain	0.001361 (0.000879, 0.001887)
e99913779	DDILASPPRLPEPQYPGAPHSS	P39060	COL18A1	Collagen alpha-1(XVIII) chain	0.002118 (-0.002625, 0.007012)
e99904196	PSGDQGASGpAGPSGP	P02458	COL2A1	Collagen alpha-1(II) chain	0.002342 (-0.001725, 0.006138)
e99910953	DGQpGAKGEpGDAGAKGDAGPpGP	P02452	COL1A1	Collagen alpha-1(I) chain	0.002584 (0.001634, 0.003548)
e99903284	DGSpGAKGDRGET	P02452	COL1A1	Collagen alpha-1(I) chain	0.002860 (-0.000424, 0.005988)
e99908442	AGEKGPSGEAGTAGPpGTpGP	P08123	COL1A2	Collagen alpha-2(I) chain	0.002947 (0.000610, 0.005217)
e99905346	GPpGKpGDDGEAGKpG	P02458	COL2A1	Collagen alpha-1(II) chain	0.004666 (0.002066, 0.007324)
e99917280	AAGEpGKAGERGVpGPpGAVGPAGKDGEAGAQPpG	P02452	COL1A1	Collagen alpha-1(I) chain	0.005564 (0.003320, 0.007863)
e99916417	RTGEVGAVGPpGFAGEKGPSGEAGTAGPpGTpGP	P08123	COL1A2	Collagen alpha-2(I) chain	0.005706 (0.003379, 0.008284)
e99919425	pGApGVRGFQKQKGSmGDpGLPGPQGLRGDVGDRGP GGAAGP	Q14050	COL9A3	Collagen alpha-3(IX) chain	0.010065 (0.004812, 0.014979)

The derivation dataset included the baseline data of 519 participants in FLEMENGHO. Accession numbers refer to the Uniprot database (<http://www.uniprot.org/uniprot>). Association sizes (β) between chronological age and the levels of the urinary peptides were derived by elastic net regression, which was bootstrapped 1000 times. The regression slopes (β s) reflect the weight of each of the 29 peptide fragments in the construction of the multidimensional UPPost-age biomarker. Of 99 peptides entering the data reduction analysis, 29 entered in at least 60% of models and identified 10 proteins. The 95% confidence intervals of the β s were derived from the bootstrap distribution.

Supplementary Table 7. Risk of having HASD \leq -3 cm in FLEMENGHO participants at baseline (2005-2010).

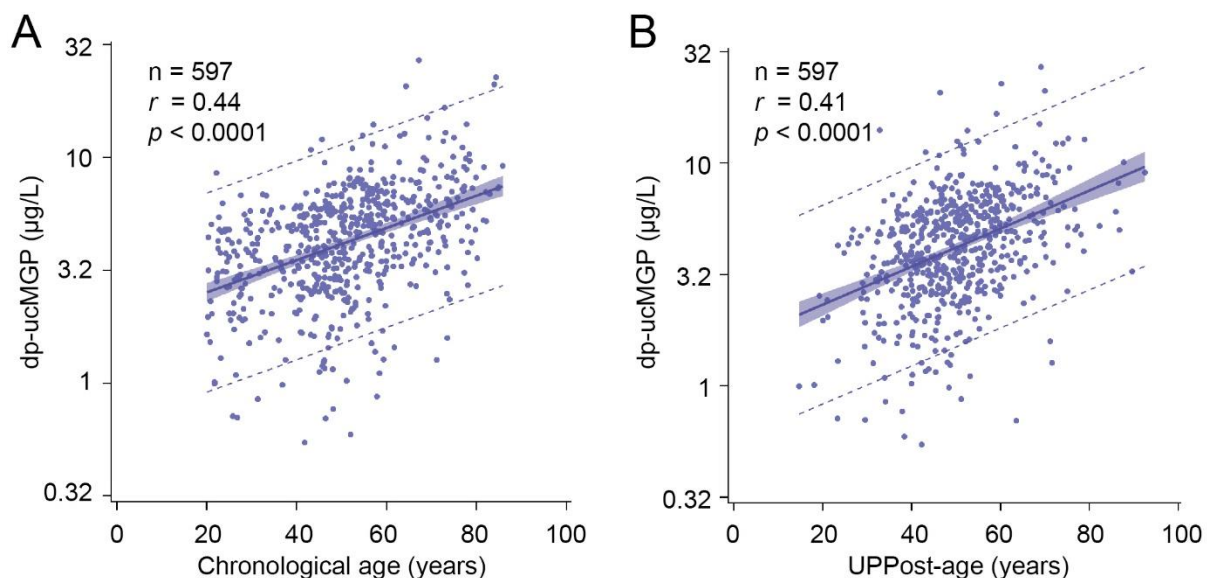
Variable	n/N	Chronological age		UPPost-age	
		OR (95% CI)	<i>p</i> -value	OR (95% CI)	<i>p</i> -value
All participants	278/706	1.43 (1.25, 1.63)	<0.0001	1.31 (1.12, 1.53)	0.0007
Men	188/349	1.40 (1.17, 1.67)	0.0002	1.19 (0.96, 1.48)	0.11
Women	90/357	1.51 (1.21, 1.88)	0.0003	1.43 (1.14, 1.81)	0.0024
Postmenopausal women	62/183	1.57 (1.06, 2.34)	0.025	1.22 (0.88, 1.70)	0.24

Abbreviations: HASD: the difference of body height minus arm span; n/N: number of participants with HASD \leq -3 cm/total number of participants; UPPost-age: age as predicted by the urinary proteomic profile; OR: odds ratio; 95% CI: 95% confidence interval. ORs, given with 95% CI, express the relative risk per 10-year increment in chronological age or UPPost-age with adjustment for clustering within families, sex (in all participants only), current smoking, γ -glutamyltransferase, fasting plasma glucose, physical activity, use of diuretics (yes vs no) and socioeconomic status (high and middle vs low).



Supplementary Figure 1. Correlation between height loss and body-height-to-arm-span ratio in 656 FLEMENGHO participants. HASR: body-height-to-arm-span ratio. r is the Pearson correlation coefficient. Height change was the differences between two body height measurement examined in the interval from 1985 until 2008. During the median time interval of 9.56 years (interquartile range: 6.54, 15.5 years), the mean height change was 0.46 cm (SD: 1.51 cm) in 656 participants (50.6% women; median age at the first measurement: 40.5 year). Height change was significantly correlated with HASR ($r = -0.17$, $p < 0.0001$). The sex-specific correlation coefficients were -0.24 in women (red circles) and -0.095 in men (green squares), respectively ($p = 0.057$ for the sex difference).

SUPPLEMENTARY DATA



Supplementary Figure 2. Regression plot of dp-ucMGP with chronological age (A) and UPPost-age (B) in 597 FLEMENGHO participants. r is the Pearson correlation coefficient. dp-ucMGP denotes desphospho-uncarboxylated matrix Gla protein. UPPost-age is age as predicted by the urinary proteomic profile.

SUPPLEMENTARY DATA