Methods

Materials
All the powdered reference compounds (i.e. ACP, OCP, DCPD, HA, CHA, and Ca acetate) were purchased from Sigma-Aldrich (USA) and directly used without further modification.

Mice
Both male and female Mgp⁻/⁻ mice are in C57Bl/6 background. All the knockout mice were whole-body knockouts. Mgp⁻/⁻ mice lack exon 1 to 3 and part of exon 4 of the MGP gene (1). Mice were maintained in a pathogen-free standard animal facility. The animal use protocol and all procedures were reviewed and approved by the Shriners Hospitals for Children Animal Care Committee and the McGill Institutional Animal Care and Use Committee. Shriners Hospitals for Children - Canada and McGill University are accredited and followed the guidelines of the Canadian Council on Animal Care. Genotypes were determined by PCR on genomic DNAs isolated from tail biopsies. The nucleotide sequences of the primers used for genotyping are available upon request.

Tissue preparation
Thoracic and abdominal aorta segments were isolated from 1 week, 2 week, 3 week, 4 week, and 5 week old Mgp⁻/⁻ mice and from 3 week and 5 week old wild-type (WT) mice. For whole tissue analyses, aortas were fixed in 4% buffered paraformaldehyde overnight, washed in phosphate buffered saline (PBS), transferred to 70% ethanol, and dried under vacuum overnight before analysis. For cross section analyses, aortas were fixed overnight in freshly prepared 4% paraformaldehyde, washed in PBS, transferred to 70% ethanol, embedded in methyl methacrylate, sectioned (7-μm-thick), and deplastified with 2-methoxyethyl acetate before analysis.

Bones were isolated from 5 week old WT mice. For the analyses, bones were fixed in 10% formalin overnight, washed in PBS, kept into 70% ethanol and then dried under vacuum overnight.

Histological analysis
Mouse aortas were fixed overnight in 4% buffered paraformaldehyde, embedded in methyl methacrylate, sectioned (7 μm), and stained by von Kossa and van Gieson (VKVG). All histological images were captured using a digital camera (DP72; Olympus Canada Inc), acquired with DP2-BSW software (XV3.0; Olympus Canada Inc) and processed using PhotoShop software (Adobe).

Alizarin red staining
For alizarin red staining of aortas, thoracic cages were fixed overnight in 95% ethanol, stained in 0.015% Alcian Blue dye (Sigma-Aldrich) in a 1:4 solution of glacial acetic acid and absolute ethanol for 24 hours. Tissues were then treated with 2% potassium hydroxide for another 24 hours (or until the soft tissues were dissolved) and then stained by 0.005% Alizarin Red (Sigma-Aldrich) in a 1% potassium hydroxide solution. Finally, the stained skeletal tissues were clarified in 1% potassium hydroxide/20% glycerol for 2 days.

**Fourier Transform infrared (FTIR) spectroscopy**
FTIR spectra of powdered aortas mixed with an appropriate 50% weight/weight ratio of KBr were collected on a Bruker Tensor 27 spectrometer equipped with a diffuse reflectance (DRIFT) accessory and a DTGS detector. The spectra were collected from 400 to 4000 cm⁻¹ with 128 scans at 4 cm⁻¹ resolution. The reflected signals were converted and reported as absorbance in the figures shown in this study. FTIR measurements were performed on three points per sample and three samples per condition were analyzed. The data was analyzed using OPUS software (OPUS 7.0.0, Bruker, Karlsruhe, Germany).

**Raman spectroscopy**
Whole aortas were analyzed by Raman spectroscopy using a Bruker Senterra confocal Raman spectrophotometer equipped with a 785nm diode laser coupled with an Olympus optical microscope, using a 40x objective. Spectra were collected at 100 mW laser power and spectral resolution of 3.5 cm⁻¹, between 400 and 1800 cm⁻¹, with an integration time of 60 seconds and 4 co-additions. In total, 3 thoracic and 3 abdominal samples were analyzed for each mouse age, and 20 points per sample were analyzed. The data was analyzed using OPUS software (OPUS 7.0.0, Bruker, Karlsruhe, Germany).

**X-ray diffraction (XRD)**
XRD data of grounded aortas were obtained with a Bruker D5000 diffractometer using a Cu Kα radiation source (Kα1 0.17 Å), and a monochromator operated at 40 kV and 40 mA within the 10° to 80° range in 2θ. Three samples per condition were analyzed. The FWHM were analyzed using GraphPad software, Inc. (2016)

**X-ray photoelectron spectroscopy (XPS)**
The atomic composition of aorta cross sections was characterized using a Thermo Scientific Kα spectrometer, equipped with an Al Kα X-ray source (1486.6 eV, 0.843 nm), a micro-focused monochromator, and an ultrahigh vacuum chamber (10⁻⁹ Torr). Survey scans and high resolution spectra were acquired with an X-ray spot with a diameter of 50 μm. At least three survey scans were collected between 0 and 1200 eV with a step size of 1 eV for each sample. Elemental high resolution scans were acquired with a step size of 0.1 eV. Samples were hit with a flood gun shooting low energy electrons during the measurement to prevent charging. Ten points were randomly selected along the cross section of each aorta and three samples were analyzed per condition. Peak fitting and quantitative analysis of the survey spectra were performed using the Thermo Advantage software (version 4.60).
Ca K-edge Near Edge X-ray absorption fine structure (Ca K-edge NEXAFS) spectroscopy

Ca K-edge NEXAFS spectra on whole aortas were collected at the bulk-XAFS end-station of the Soft X-ray Microcharacterization beamline (SXRMB) at the Canadian Light Source (CLS), Saskatoon, Saskatchewan. Powdered reference compounds of known chemical compositions were analyzed, including ACP, OCP, DCPD, hydroxyapatite (HA), CHA, and Ca acetate (Ca(CH$_3$COO)$_2$). Spectra were collected with energies between 1.7 and 10 keV in fluorescence mode and with a photon beam spot size of 2 mm x 6 mm. The spectra were calibrated, aligned, and normalized using Athena software (Demeter 0.9.20). Linear combination fitting (LCF) was performed on the NEXAFS regions of the aorta sample spectra using all the possible combinations of the reference samples. The quality of the fits was evaluated by the R-factor value: values below 0.02 indicate that the fitting results are satisfactory (37). For each spectrum, the best LCF combination (i.e. the combination giving the lowest R-factor) was retained. LCF was performed using Athena software.

Micro-X-ray fluorescence (μ-XRF) and μ-NEXAFS spectroscopy

μ-XRF Ca and P maps and μ-XAFS spectra were collected on whole aortas at the microprobe end-station of the Soft X-ray Microcharacterization beamline (SXRMB) at the Canadian Light Source (CLS), Saskatoon, Saskatchewan. μ-XRF maps were obtained by scanning the sample under a monochromatic beam with a spot size of 10 x 10 μm$^2$ at an X-ray energy of 4100 eV. μ-NEXAFS spectra were collected on hot spots, i.e. spots that contain the highest amounts of Ca with energies between 1.7 and 10 keV in fluorescence mode and with a photon beam spot size of 10 x 10 μm$^2$. μ-XRF maps were created and analyzed using the SMAK software program and μ-NEXAFS spectra were analyzed using Athena software.

Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS)

Aorta cross sections were characterized using a combination of SEM and EDS. Samples were secured to an aluminium sample holder with carbon tape, and then coated with 8.5 nm carbon (carbon sputter coater, EMS150R ES, Electron Microscopy Sciences (EMS)). The samples were then imaged using an Inspect-50 field emission SEM (FEI, Japan), at 10 kV operating voltage under high vacuum. EDS spectra and elemental maps were obtained in the regions of interest using an EDX spectrometer (EDX, Thermo Scientific, USA).

Statistical analysis
Statistical data analysis was performed using GraphPad software, Inc. (2016). Mean values were expressed as average ± standard deviation. Statistical analysis on mean values were performed by Student’s t test or one-way ANOVA test followed by Tukey’s test correction for multiple comparisons, and p< 0.05 was considered significant. In the Raman experiments, median values instead of means were used because the data was not normally distributed. In this case, median values were expressed as median ± standard deviation; the statistical analysis was performed using Mood’s median test followed by Tukey’s test correction for multiple comparisons, and p< 0.05 was considered significant.
Reference