SUPPLEMENTARY INFORMATION

DIETARY PHOSPHATE RESTRICTION PREVENTS THE APPEARANCE OF SARCOPENIA SIGNS IN OLD MICE

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SUPPLEMENTARY METHODS

Arterial blood pressure measurements

Arterial blood pressure was measured one week before the sacrifice, in conscious animals by means of a tail-cuff sphygmomanometer (LE 5001 Pressure Meter, Letica Scientific Instruments, Hospitalet, Spain), as described previously [1]. To prevent stress and minimize the variability, animals were trained for 3 days before starting the measurement, and they were prewarmed at 30°C with a heater (LE5660/6, Letica Scientific Instruments). Data of basal blood pressure was recorded in 2 consecutive days at the same times (11:00-12:00 am), with at least 20 determinations by day.

Nuclear magnetic resonance imaging data acquisition

T2-weighted (T2-W) spin-echo images were acquired with a rapid acquisition with relaxation enhancement (RARE) sequence in axial and coronal orientations with the following parameters: TR = 8000 ms, TE = 35 ms, RARE factor = 8, Av = 8, axial and coronal FOV = 2.5cm/5cm respectively, acquisition matrix = 256 × 256, slice thickness = 1.00 mm without gap and number of slices = 20 and 14, axial and coronal respectively. Diffusion tensor data were acquired with a spin echo single shot echo planar imaging (EPI) pulse sequence using the following parameters: TR/TE 3500/40ms; a signal average of 4, 7 noncollinear diffusion gradient scheme with a diffusion weighting b = 100 and 1400s/mm2, slices thickness 1 mm without a gap, field of view 35x35mm and acquisition matrix = 128x128. The diffusion gradient timing parameters δ (duration) and Δ (separation from onset to onset) were 3,5 and 20 milliseconds, respectively. Magnetization transfer (MT) maps were acquired with a multi-slice multi-echo sequence in axial orientation and the following parameters: TR = 2500 ms, TE = 9.8 ms, Av = 1, a field of view = 3 cm, acquisition matrix = 128 × 128, with and without magnetization transfer. Sequences of T2 mapping were acquired in the axial plane with the following parameters: 50 spin echo images with 50 different echo times with a constant repetition time of 12 ms (12 ms….600 ms), matrix size 128x128, 10 slices with 2mm thickness, a field of view 3 cm. Mean diffusivity (MD) and MT and T2 maps were calculated with a homemade software application written in Matlab (R2007a).

Hematoxylin & eosin (H&E) staining

For histological analysis, 10 µm serial transverse sections of frozen gastrocnemii were cut at -20ºC by cryostat (Leica CM3000 Cryostat, Leica Biosystems, Nussloch, Germany), mounted on glass slides and stored at -80ºC until assayed. Tissue sections were hydrated in two 10-min-washes with distilled water before haematoxylin staining. Samples were incubated with Harris' haematoxylin solution (10-2331; Casa Álvarez, Madrid, Spain) for 5 min and then rinsed twice in distilled water for 2 min. Next, sections were stained with 0.5% eosin in ethanol (10-3001; Casa Álvarez, Madrid, Spain) for 15 seconds and dehydrated in increasing solutions of ethanol (80-96-100%), for 2 min each. Finally, samples were immersed in xylene for 2 min and mounted using DPX mounting medium fast (PanReac AppliChem, Barcelona, Spain) for viewing under a microscope. Nuclei were stained in purple and cytoplasmic components of muscle fibres in pink. At least 40% of the muscle fibre cross-sectional-area (FCSA) per microscopic field were quantified using ImageJ software 2.6 (http://rsbweb.nih.gov/ ij/) and classified into each size range.

Succinate Dehydrogenase assay

The succinate dehydrogenase (SDH) activity of the fibres, which reflects their oxidative metabolism, was evaluated histochemically in frozen gastrocnemius sections. In brief, tissue sections were fixed in 4% paraformaldehyde for 5 min and rinsed three times in phosphate buffered saline (PBS) for 5 min each. Then, tissue sections were incubated for 1 h at 37°C in the staining solution containing (0.2 M succinate and 0.5 mM Nitro Blue tetrazolium (N5514; Sigma Aldrich, St Louis, MO, USA) in 0.1 M phosphate buffer, pH 7.2). For specificity controls, the endogenous SDH activity was measured by incubating a sample in a solution lacking succinate. Samples were washed in PBS for 15 min, dehydrated in increasing concentrations of ethanol for 2 min each, placed for 10 min in xylene, and finally, mounted in DPX mounting medium fast for viewing by microscope. The percentage of oxidative fibres was calculated using ImageJ software 2.6 (http://rsbweb.nih.gov/ ij/). Analysis of images and data was done blindly by two different researchers.

Fast and slow muscle fibers immunostaining

For fast or slow myosin heavy chain (MHC) immunostaining, 10 µm sections of frozen gastrocnemii were pre-washed in PBS for 10 min and immersed in the blocking solution (0.2% TRITON X-100 and 2% donkey serum (C05SB; Bionova, Madrid, Spain) in PBS) for 1 hour in a humid chamber. Then, tissue sections were incubated with primary antibodies Slow MHC (1:2000; ab11083; abcam, Cambridge, UK) and Fast MHC (1:2000; ab91506; abcam, Cambridge, UK) in blocking solution overnight in a humid chamber at 4°C. Next day, samples were rinsed in three 10-min washes in PBS and incubated with the secondary antibodies Cy™2 Donkey anti-mouse (1:1000; 711-165-15; Jackson ImmunoResearch, Cambridge, UK) and Cy™3 Donkey anti-rabbit (1:200; 715-225-15; Jackson ImmunoResearch, Cambridge, UK) in blocking solution for 1 hour in a humid chamber at room temperature. Next, sections were incubated with DAPI for 10 min in a humid chamber. Finally, three 10-min washes in PBS were performed and tissue sections were mounted with VectaMount™ Permanent Mounting Medium (Vector Laboratories Inc., CA, USA). Photographs were obtained using an Olympus DP 71 camera and Cell R software.

Sirius red staining

The amount of connective tissue in the gastrocnemius muscle was analysed using the Picro-Sirius Red Stain Kit (Abcam, Cambridge, UK). 10 µm tissue sections were hydrated in distilled water and immersed in Sirius Red Solution for 20 min. Then, samples were rinsed in acetic acid solution and absolute ethanol. Finally, tissue sections were mounted in DPX mounting medium fast for viewing under a microscope. Sirius red intensity was measured on 20x magnification photographs using Image Pro Plus software (http://www.mediacy.com/imageproplus).

Supplementary methods reference

1. Martínez-Miguel P, Valdivielso JM, Medrano-Andrés D, Roman-García P, Cano-Peñalver JL, Rodríguez-Puyol M, et al. The active form of vitamin D, calcitriol, induces a complex dual

upregulation of endothelin and nitric oxide in cultured endothelial cells. *Am J Physiol Endocrinol Metab.* 2014; **307:**E1085–E1096.

Supplementary table 1. Diets composition.

	STANDARD DIET	LOW PHOSPHATE DIET
Macronutrients		
Protein (%)	14.5	17.7
Fat (%)	4	5.1
Fiber (%)	4.5	5
Ash (%)	4.7	5.1
Carbohydrate (%)	48	47.3
Minerals		
Calcium (%)	0.7	0.9
Phosphorus (%)	0.6	0.2
Ratio Ca:P	1.17	4.5
Magnesium (%)	0.2	0.2
Potassium (%)	0.89	0.89
Metabolizable energy	12.1 KJ/g	15.5 KJ/g
Commercial Name	Teklad Global Rodent Diets 2014 ENVIGO, IN, USA	EF Low Phosphate S9723-E020 Ssniff, Spezialdiäten GmbH

Supplementary table 2. Intraclass correlation coefficients (ICC) values of the results depicted in box plots.

Figures 1, 2, 3, 4 and 5

	ICC
Gastrocnemius mass	0.1412532
Tibialis mass	0.1241644
Stride length	0.1247825
Hind paw base width	0.1083836
Orientation time	0.2834108
Transition time	0.551654

Figure 7

	ICC
Serum P levels	0.5496269
Grip strength	0.231092

Supplementary figure 1. Muscle strength measured by a grip test from fourteen 5-month-old animals (5m), ten 9-month-old animals (9m), ten 12-month-old animals (12m), fourteen 18-month-old animals (18m), and thirteen 24-month-old animals (24m). $^{*}p<0.05$ vs 5m.