

Section 1: Materials and Methods

Section 2: Figures and figure captions

Section 3: Tables

Section 1: Materials and Methods

DXA: Lunar PIXImus densitometer (software version 1.45) was calibrated using a hydroxyapatite phantom provided by the manufacturer (Lunar Corporation, Madison, WI). After calibration, mice were anesthetized with 2% isoflurane, and whole-body scans except the head were conducted to obtain bone area (cm²), total tissue mass (TTM, g), percent fat tissue (%Fat, %). Derived parameters were calculated as follows: Bone mineral density (BMD, g/cm²) = BMC / bone area. Fat mass (g) = %Fat * TTM, Lean mass (g) = TTM – Fat mass

μCT using Scanco system: After DXA measurement, mice were scanned by μCT (Scanco Medical) at high resolution (10.5 μm isotropic voxel size) for quantitative determinations and integrated into 3D voxel images at the right tibia. Scan settings included X-ray tube potential (70 kVp), X-ray density (114 μA), and integration time (250ms). At a threshold of 260 mg/cm³, and a Gaussian filter (sigma = 0.8, support = 1), cortical volume of interest (VOI) was determined using 58 slices (0.61 mm) at the tibial mid-diaphysis. Trabecular VOI was made on 97 slices (1.02 mm) away from the growth plate, to avoid the primary spongiosa, and evaluated at a threshold of 240 mg/cm³, and a Gaussian filter (sigma = 1.2, support = 2). Scanco Eval Program v6.6 was used for measuring bone volume.

Bone histomorphometry

The structural indices were measured in the trabecular bone of the secondary spongiosa, using a semi-automatic image analysis system (Sony DXC 950P Camera; DMRB microscope, Leica Microsystems, Germany; ImaScope Bones X, version 10.80, La Rochelle, France) at a magnification x 10 to obtain bone volume fraction (BV/TV, %), and trabecular thickness (Tb.Th, mm). The cellular parameters were measured at a magnification x 20 (Zeiss Axio Scope A1, Germany; morphométrie osseuse animale, version 2.13) including Osteoclast Number per Bone Area (N.Oc/B.Ar, mm²), Osteoclast Number per Bone perimeter (N.Oc/B.Pm, c/mm), Osteoclast Surface per Bone Surface (Oc.S/BS, %), Osteoclast Length (Oc.Le, μm). The dynamic indices of bone formation included single-labelled surface per bone surface (sLS/BS, %), and double-labelled surface per bone surface (dLS/BS, %). Mineral Apposition Rate (MAR, μm/day) was calculated from the mean distance between the labels divided by the number of days between labelings under magnification x40. Mineralizing Surface per Bone Surface (MS/BS, %) was calculated as ½ sLS/BS + dLS/BS. Bone - Formation Rate per Bone Surface (BFR/BS, μm³/μm²/day) was calculated by multiplying MS/BS by MAR.

Section 2: Figures and figure captions

Fig. S1. Experimental timeline. Female mice were fed regular diets (RD) adapted to their growth stage since weaning. Experimental diets were done at the beginning of 16 months old for 90 days. Data and samples were collected at the times indicated.

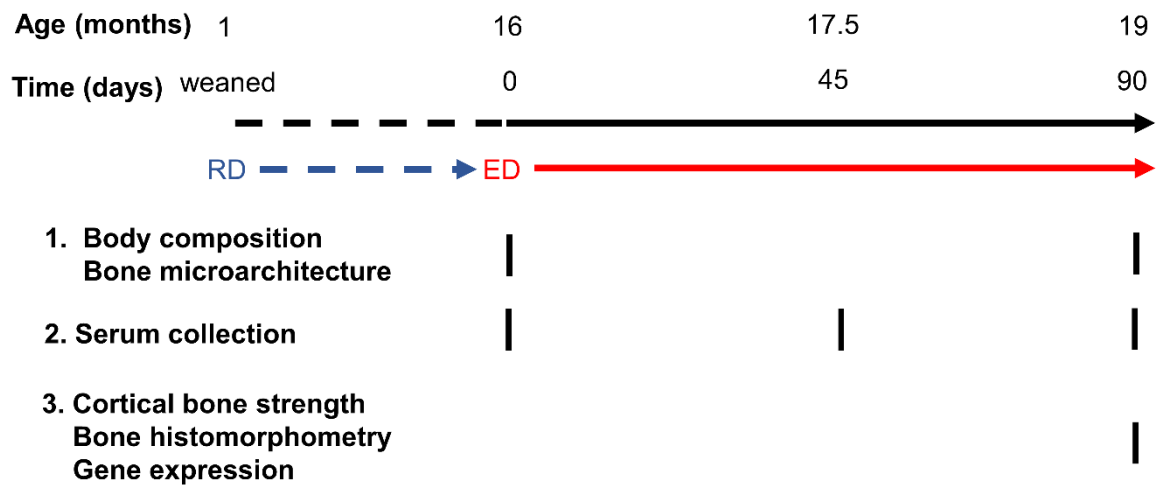


Fig. S2. Changes in body weight in mice. Absolute changes of body weight in (A) all mice included in this study (n=17 per Standard, n=21 per CaCO₃, n=18 per Nacre); and (C) the subset of mice on which body composition was assessed with DXA (Standard: n=6, CaCO₃: n=7, Nacre: n=6). Data are represented mean ± standard error. ^a vs. Standard, ^a p ≤ 0.05, ^{aa} p ≤ 0.01. (B) Representative image of an animal per group under anesthesia at 2.5% isoflurane. (D, E) The changes of lean mass and fat mass from baseline (day 0) to follow-up (day 90) at the whole body in the longitudinal study by DXA (PIXImus). The raw data show the mean, and 95% CIs. p-values in green: vs. Standard, in pink: vs. CaCO₃. * vs. Day 0, *p≤0.05.

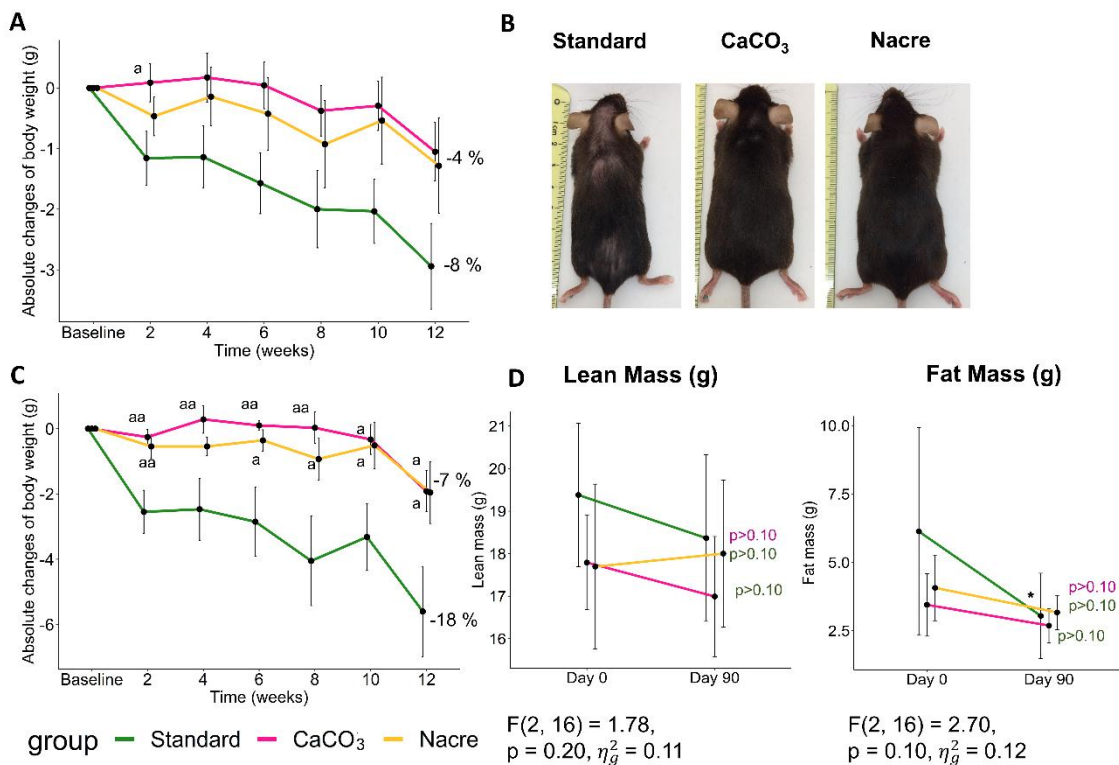


Fig. S3. Comparisons of linear regressions between (A) Stiffness and body weight and (B) Fmax and body weight for Standard versus Nacre and CaCO₃ versus Nacre.

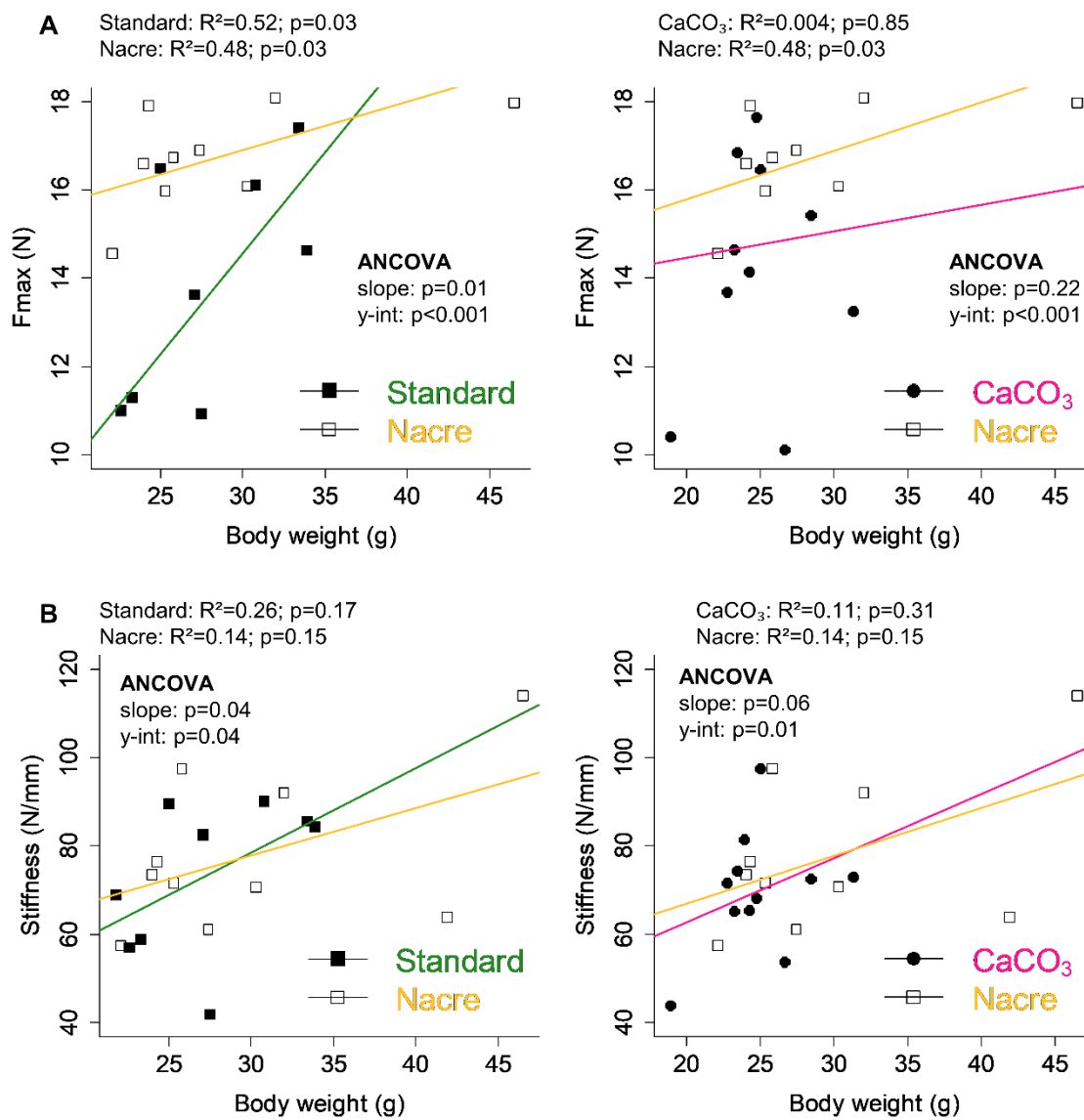
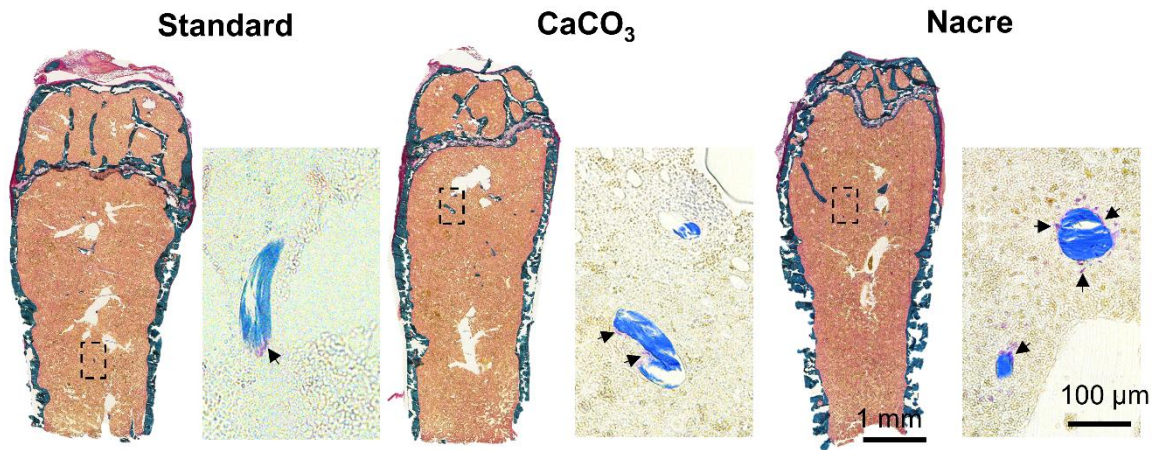


Fig S4. Representative images of Goldner's trichrome (left panel, x10), in which the dashed squares depict the corresponding zone stained by tartrate – resistant acid phosphate (TRAP) staining (right panel, x40; arrows indicate red-stained osteoclasts).



Section 3: Tables

Table S1. Ingredient composition of the diets fed to mice

	Standard	Standard + 0.25% CaCO ₃	Standard + 0.25% Nacre
Acid amin Mix (g/kg) ^a	21.5	21.5	>21.5*
Fat acid Mix (g/kg) ^b	19.8	19.8	>19.8*
Vitamin Mix (g/kg) ^c	1.7	1.7	1.7
Soya trace	free	free	free
Mineral Mix (g/kg) ^d	27.8	28.8	28.8
Calcium content (g/kg)	8.5	9.5	9.5

Note: **Pinctata maxima*'s nacre contains 2.7% organic matrix (Bourrat X et al., *CrysEngComm*, 2007 (Bourrat et al., 2007)) ; 0.068 g nacre organic content per 1kg food.

^a acid amin Mix – Arginine : 6.5, Cysteine : 2, Lysine : 4.4, Methionine : 1.8, Tryptophane : 1.5, Glycine : 5.3

^b Fat acid Mix – Palmitic acid : 2.2, Stearic acid : 0.4, Oleic acid : 6, Linoleic acid : 11.2, Linolenic acid : 0.4

^c Vitamine Mix – Vit A : 5, Vit D3 : 9, Vit B1 : 0.005, vit B2 : 0.006, Vit B5 : 0.01, vit B6 : 0.002, vit B12 : 2.10⁻⁵, vit E : 0.025, vit K3 : 0.0025, Niacine : 0.07, acid Folic : 0.0005, Biotine : 0.00004, Choline : 1.6

^d Mineral Mix – Phosphate : 5, Sodium : 2.2, Potassium : 6.3, Magnesium : 1.9, Maganese : 0.09, Fer : 0.27, Copper : 0.016, Zinc : 0.060, Chlorure : 3.5

Table S2. Primer sequences used in this study

Genes (protein encoded)	Sense (5'-3')	Antisense (5'-3')
<i>Hprt1</i>	tgttgatacaggccagact	caacttgcgctcatcttagg
<i>Runx2</i> (RUNX2)	gacactgccacctctgactt	gcaggtacgtgtggtagtga
<i>Spp1</i> (Osteopontin)	cccggtgaaagtgactgattc	atggcttcattggaattgc
<i>Sparc</i> (Osteonectin)	aggtatgcagcaatgacaac	attcagtcagctcggaatcc
<i>Bmp2</i> (BMP-2)	gatctgtaccgcaggcactc	gttctccacggcttcttcg
<i>Acp5</i> (TRAP)	ggcaacgtctctgcacagat	ttgagccaggacagctgagt
<i>Cathepsin K</i> (Cathepsin K)	tgaccactgcctccaatac	tgccgtggcgttatacatac
<i>Sost</i> (Sclerostin)	tcctctgagaacaaccagac	tgtcaggaagcgggtgtagtg

Table S3. Densitometry parameters measured by PIXImus at Baseline and Follow-up in the whole body except the head of mice in the *in vivo* longitudinal study

Characteristic (unit)	Estimate	Group (No. of mice)		
		Standard (6)	CaCO ₃ (7)	Nacre (7)
BMD (mg/cm ²)	Baseline	0.05 (0.002)	0.05 (0.001)	0.05 (0.002)
	Follow-up	0.05 (0.003)	0.05 (0.002)	0.05 (0.003)
	Adjusted follow-up	0.05	0.05	0.05
BMC (g)	Baseline	0.46 (0.05)	0.46 (0.03)	0.44 (0.03)
	Follow-up	0.50 (0.05)	0.49 (0.05)	0.46 (0.02)
	Adjusted follow-up	0.05	0.05	0.05
Fat mass (g)	Baseline	6.1 (3.6)	3.5 (1.2)	4.0 (1.1)
	Follow-up	3.1 (1.5)*	2.7 (0.7)	3.1 (0.6)
	Adjusted follow-up	2.5	3.0	3.3
Lean mass (g)	Baseline	19.4 (1.6)	17.8 (1.2)	17.6 (1.7)
	Follow-up	18.4 (1.9)	16.9 (1.5)	18.0 (1.5)
	Adjusted follow-up	17.5	17.3	18.5

Values are mean (standard deviation). Standard: n=6, CaCO₃ or Nacre: n=7.

For comparisons between groups, analysis of covariance generalized linear model using baseline values as covariable followed by Tukey's HSD post-hoc test

For comparisons between baseline and follow-up values in each group, Student's t paired test followed by Benjamini-Hochberg adjustment

*p<0.05, *vs. baseline

Table S4. μ CT (Scanco) parameters of trabecular and cortical bone at Baseline and Follow-up at the proximal tibiae and midshaft of mice in Standard, CaCO₃, and Nacre groups (*in vivo* longitudinal study)

Characteristic (unit)	Estimate	Group (No. of mice)		
		Standard (10)	CaCO ₃ (10)	Nacre (6)
(A) Trabecular parameters (proximal tibia)				
BV/TV (%)	Baseline	1.7 (0.6)	2.2 (0.7)	1.9 (0.6)
	Follow-up	1.8 (1.2)	2.4 (1.7)	3.2 (1.2)
	Adjusted follow-up	2.1	2.1	3.2
Conn.D (1/mm ³)	Baseline	2.9 (2.4)	4.7 (4.4)	4.9 (1.7)
	Follow-up	3.8 (4.8)	4.0 (5.2)	18.2 (20.2)*
	Adjusted follow-up	5.2	3.3	17.2^b
Tb.N (1/mm)	Baseline	2.0 (0.8)	1.7 (0.2)	1.6 (0.3)
	Follow-up	1.9 (0.8)	1.6 (0.2)*	1.6 (0.1)
	Adjusted follow-up	1.7	1.7	1.8
Tb.Th (mm)	Baseline	0.05 (0.01)	0.05 (0.01)	0.05 (0.01)
	Follow-up	0.05 (0.01)	0.06 (0.01)*	0.05 (0.01)
	Adjusted follow-up	0.05	0.06	0.05
Tb.Sp (mm)	Baseline	0.6 (0.1)	0.6 (0.1)	0.6 (0.1)
	Follow-up	0.6 (0.2)	0.7 (0.1)	0.6 (0.1)
	Adjusted follow-up	0.6	0.7	0.6
SMI [†]	Baseline	2.2 (0.4)	2.2 (0.4)	1.9 (0.4)
	Follow-up	2.2 (0.3)	2.2 (0.6)	1.8 (0.4)
	Adjusted follow-up	2.2	2.2	1.8
BMD (mg/cm ³)	Baseline	1010.2 (36.4)	1018.3 (34.9)	1005.9 (14.8)
	Follow-up	1006.3 (47.1)	1009.4 (31.9)	991.5 (28.4)
	Adjusted follow-up	1008	1006	996
(B) Cortical parameters (midshaft tibia)				
Ct.Ar (mm ²)	Baseline	0.62 (0.07)	0.62 (0.08)	0.70 (0.06)
	Follow-up	0.65 (0.08)	0.63 (0.04)	0.69 (0.07)
	Adjusted follow-up	0.66	0.64	0.67
Ct.Th (mm)	Baseline	0.14 (0.008)	0.14 (0.01)	0.16 (0.02)
	Follow-up	0.14 (0.01)	0.14 (0.01)	0.16 (0.03)
	Adjusted follow-up	0.14	0.14	0.15
Ma.Ar (mm ²)	Baseline	0.67 (0.09)	0.68 (0.06)	0.69 (0.13)
	Follow-up	0.73 (0.07)*	0.70 (0.08)	0.65 (0.11)
	Adjusted follow-up	0.73	0.70	0.65^a
TMD (mg/cm ³)	Baseline	1270 (45)	1260 (38.5)	1260 (26.5)
	Follow-up	1240 (48.8)	1240 (30.7)	1290 (23.7)
	Adjusted follow-up	1239	1243	1285
Ct.Po (%)	Baseline	10.2 (5.8)	9.1 (3.2)	7.4 (1.2)
	Follow-up	11.3 (4.0)	8.6 (1.9)	7.5 (2.1)
	Adjusted follow-up	10.8	8.6	8.5

Values are mean (standard deviation). Standard or CaCO₃, n=10, Nacre: n=6.

For comparisons between groups, analysis of covariance generalized linear model using baseline values as covariable followed by Tukey's HSD post-hoc test

For comparisons between baseline and follow-up values in each group, Student's t paired test followed by Benjamini-Hochberg adjustment

† For the structure model index, 0 indicates parallel plates, 3 cylindrical rods, and 4 spheres

*p<0.05, *vs. baseline

^a p<0.05; ^a vs Standard, ^b vs. CaCO₃

References

BOURRAT, X., FRANCKE, L., LOPEZ, E., ROUSSEAU, M., STEMPFLÉ, P., ANGELLIER, M. & ALBÉRIC, P.
2007. Nacre biocrystal thermal behaviour. *CrystEngComm*, 9, 1205-1208.