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α-Linolenic Acid-Enriched Diet Prevents Myocardial Damage and Expands Longevity in Cardiomyopathic Hamsters

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Randomized clinical trials have demonstrated that the increased intake of ω-3 polyunsaturated fatty acids significantly reduces the risk of ischemic cardiovascular disease, but no investigations have been performed in hereditary cardiomyopathies with diffusely damaged myocardium. In the present study, δ-sarcoglycan-null cardiomyopathic hamsters were fed from weaning to death with an α-linolenic acid (ALA)-enriched versus standard diet. Results demonstrated a great accumulation of ALA and eicosapentaenoic acid and an increased eicosapentaenoic/arachidonic acid ratio in cardiomyopathic hamster hearts, correlating with the preservation of myocardial structure and function. In fact, ALA administration preserved plasmalemma and mitochondrial membrane integrity, thus maintaining proper cell/extracellular matrix contacts and signaling, as well as a normal gene expression profile (myosin heavy chain isoforms, atrial natriuretic peptide, transforming growth factor-β1) and a limited extension of fibrotic areas within ALA-fed cardiomyopathic hearts. Consequently, hemodynamic indexes were safeguarded, and more than 60% of ALA-fed animals were still alive (mean survival time, 293 ± 141.8 days) when all those fed with standard diet were deceased (mean survival time, 175.9 ± 56 days). Therefore, the clinically evident beneficial effects of ω-3 polyunsaturated fatty acids are mainly related to preservation of myocardium structure and function and the attenuation of myocardial fibrosis. ***(Am J Pathol 2006, 169:1913–1924; DOI: 10.2353/ajpath.2006.051320)***

A direct relationship between increased intake of ω-3 polyunsaturated fatty acids (ω-3 PUFAs), either from dietary sources or as pharmacological supplementation, and beneficial effects on the cardiovascular system has become evident throughout the years. Dietary sources of ω-3 PUFAs include mainly fish oils rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid and vegetable (eg, soybean, canola, walnut, and flaxseed) oils rich in α-linolenic acid (ALA).[1]

Randomized secondary prevention clinical trials with either EPA and docosahexaenoic acid[2,3] or ALA[4,5] demonstrated a strong association between the intake of these ω-3 PUFAs and significant reductions in cardiovascular risk and compared favorably with landmark secondary prevention trials with lipid-lowering drugs.[6] ω-3 PUFAs exhibit positive effects on hemostatic factors, thrombogenesis, blood pressure, plasma lipids, and heart susceptibility to ventricular arrhythmias.[7,8] Their administration in the form of dietary fish or fish oil capsules has been shown to cause a 30% reduction in the mortality of infarcted patients compared with untreated controls[9] and to induce relevant protective effects in primary prevention of cardiovascular disease in animal models including monkeys.[7] Accordingly, long-chain ω-3 fatty acid consumption has been promoted for all individuals, es-

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pecially those at risk of developing cardiovascular diseases.[7,9] However, the major studies on ω-3 PUFAs' beneficial effects have been performed in patients or experimental models suffering from cardiac ischemic disease, whereas no epidemiological or experimental studies have investigated their effects in hereditary cardiomyopathies. In addition, most investigations have been performed using marine-derived ω-3 PUFAs,[9] and only a few epidemiological studies have evaluated ALA's potential against cardiovascular diseases. Furthermore, although the cardioprotective ability of ω-3 PUFAs has been mainly related to anti-arrhythmic and anti-fibrillatory effects,[10] and among others, recent studies indicate that they could act by altering lipid composition[11] and plasma membrane structure to regulate intracellular signaling[12] and metabolism,[13] further in-depth research is needed to establish the amount of dietary ω-3 PUFAs that maximally affects the greatest number of cardiovascular risk factors and to determine the exact cellular and molecular mechanisms through which ω-3 PUFAs elicit their beneficial effects on the cardiovascular system.

The present study was designed to test the hypothesis that ω-3 PUFAs could beneficially affect the pathophysiological mechanisms of hereditary cardiac hypertrophy. The UM-X7.1 hamster strain was used as the experimental model because this strain displays several pathological characteristics, among which are an abnormal accumulation of ω-6 fatty acids in the heart[14,15] and severely damaged cardiac mitochondrial[16] and cellular membranes.[17] These features are part of a more complex pathophysiological pattern. In fact, UM-X7.1 hamsters exhibit a cardiomyopathic phenotype associated with the deletion of the δ-sarcoglycan (δ-SG) gene,[18] representing a unique model for investigating well-defined patterns of myocardial degeneration that ultimately result in heart failure.[19–21] The ablation of δ-SG, a structural glycoprotein of skeletal and cardiac muscle cell membranes,[22] causes diffuse alterations of cell/cell and cell/extracellular matrix (ECM) contacts, detachment of the basal membrane, and an aberrant intracellular signaling pattern.[17,23] Therefore, the aim of this study was to modulate the lipid composition of the hearts of cardiomyopathic hamsters (CMPHs) by administering an ALA-enriched diet, in an attempt to attenuate cardiomyopathic structural and functional damages. ALA was chosen because the hamster's capability to uptake, transport, and store this specific PUFA has been extensively investigated.[24]

Materials and Methods

Animal Model and Dietary Treatment

In the present study, CMPHs (strain UM-X7.1), affected by δ-SG gene deletion, were used as the experimental model and were compared with healthy Golden Syrian hamsters (GSHs) bred under the same conditions. Three different groups of hamsters were considered: CMPHs and GSHs fed with a standard pellet chow diet (PT) (Rieper, Bolzano, Italy) and CMPHs fed with an ALA-enriched diet (flaxseeds, apples, and carrots) (FS). A fourth group of FS-fed GSHs was initially considered; however, because after 250 days their survival curve was overlapping with that of the GSH-PT, and no significant signs were detectable when myocardial samples were analyzed with light microscopy, only the GSH-PT group was considered as healthy control. Animals were allowed to consume each diet component *ad libitum* from weaning to death. In the FS diet, fresh fruit and vegetables supplied carbohydrates and vitamins, whereas flaxseeds were the only source of fats, with ALA representing 52% of total lipids. Flaxseeds were selected assuming that they would be more palatable for hamsters. Every 24 hours, at the end of the dark period, food remnants were carefully harvested and weighed. The total amount of food consumed by each animal was not significantly different between healthy and cardiomyopathic individuals (CMPH/FS, 6.9 ± 0.7 g/day/100 g body weight; CMPH/PT, 6.5 ± 1.1 g/day/100 g body weight; GSH/PT, 7.1 ± 1.3 g/day/100 g body weight; $n = 15$ per group). In addition, the amount of each component ingested by every animal per day was estimated to be 50% apple, 20% carrot, and 30% flaxseeds. Assuming this composition as the closest to the diet actually consumed, two independent nutritional analyses of the PT and FS diets were performed on the basis of international standard procedures; one was performed by the Laboratory for Food Control of the Italian National Institute of Health (ISS) and another by a private organization (Biodigit srl, Campobasso, Italy) involved in nutritional studies. The analyses indicated that all macro- and micronutrients needed for animal health maintenance were in due proportion in both dietary regimens. In 100 g of fresh PT or FS diet, the caloric power was 222.5 and 202.8 kcal, respectively, whereas major components were differently represented, as shown in Table 1. However, every 7 days, animal weights were recorded to exclude possible decrements attributable to calorie restriction.

Table 1. Major Components of Estimated Experimental Diets

	PT	FS
Kcal/100 g	222.5 ± 48.1	202.8 ± 44.7*
Moisture (%)	37.4 ± 5.2	53.8 ± 4.1
Ash (%)	4.6 ± 0.7	1.7 ± 0.6*
Crude carbohydrate (%)	37.9 ± 4.2	9.9 ± 3.6*
Crude fat (%)	2.9 ± 0.2	14.4 ± 0.5*
Total ω-3 fatty acids (%)	0.13 ± 0.03	2.58 ± 0.05†
Total ω-6 fatty acids (%)	1.11 ± 0.08	0.64 ± 0.05†

Values are means ± SD of eight different diet lots shared at random between laboratories in charge for nutritional analyses. PT, standard diet; FS, flaxseed diet (apple 50%, carrot 20%, flaxseeds 30%).
*$P < 0.01$ and †$P < 0.005$ versus PT.

Harvesting of Ventricular Tissue

Animals were anesthetized with urethane (400 mg/kg i.p.) and sacrificed. Ventricles were rapidly excised, washed in ice-cold 1× phosphate-buffered saline (PBS), pH 7.4, weighed, frozen in liquid nitrogen, and stored at −80°C until use. Alternatively, ventricles were fixed with 4% formaldehyde and embedded in paraffin for light micros-

copy or with 2.5% glutaraldehyde in PBS for electron microscopy. All animal handling procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the institutional Animal Care and Use Committee of the University of Rome Tor Vergata.

Heart and Plasma Fatty Acid Composition

To analyze fatty acid composition, plasma and heart samples were obtained from seven animals per group. Heart tissues were homogenized in H_2O, at 48°C, using an Ultra-Turrax T25 homogenizer (6 × 15 seconds, setting 5; Janke & Kunkel, Staufen, Germany). Lipids were extracted from aliquots of plasma or tissue homogenates according to Folch and colleagues.[25] Fatty acid methyl esters were obtained by transesterification with 3% sulfuric acid in methanol under nitrogen (1 hour, 75°C). Gas chromatography of fatty acid methyl esters was performed with a HRGC 5300 gas chromatograph (Carlo Erba Instruments, Milan, Italy) equipped with a SP-2330 capillary column (30 cm × 0.25 mm, Supelco; Sigma-Aldrich srl, Milan, Italy) and flame ionization detector.

Morphological Analysis of Hamster Hearts

Serial paraffin-embedded ventricular sections (4 μm) were stained with hematoxylin and eosin (H&E). Morphometric analysis was performed using the DS Software (Delta Sistemi, Rome, Italy), as previously described.[19] The extent of myocardial fibrosis was quantified after Masson trichrome staining (Bio-Optica, Milan, Italy); the green area corresponding to fibrosis was quantified (DS Software) from eight visual fields selected at random from 11 animals per group (objective, ×40; Leica DMRB microscope; Wetzlar, Germany). Ultrastructural analysis was performed on myocardial fragments processed for transmission electron microscopy (Philips CM10; Milan, Italy), as previously described.[26] Two independent observers evaluated at least five different hearts per each hamster group (CMPH/FS, CMPH/PT, and GSH/PT). Heart serial sections (4 μm) of at least eight animals per group were analyzed by immunofluorescence (Leica DMRB microscope; objective, ×20): sections were incubated with a primary anti-α-dystroglycan antibody (1:200) (Novocastra, Newcastle, UK) and a secondary fluorescein isothiocyanate-labeled antibody (1:300) (Vector Laboratories Inc., Burlingame, CA). Anti-collagen type I (1:200; Santa Cruz Biotechnologies, Santa Cruz, CA) and anti-laminin α-2 (1:200; Santa Cruz Biotechnologies) were used to evaluate the expression of the corresponding genes, which was assessed by peroxidase immunostaining (Vector Laboratories Inc.).

Western Blot Analysis of Hamster Myocardial Extracts

Myofibrillar extracts were prepared using the Caforio's procedure, as previously described.[27] Briefly, tissue samples were homogenized in a low-salt buffer solution (20 mmol/L KCl, 2 mmol/L K_2HPO_4, and 1 mmol/L EGTA, pH 6.8); after centrifugation at 2000 × *g*, pellets were resuspended in high-salt solution (40 mmol/L $Na_4P_2O_7$, 1 mmol/L $MgCl_2$, and 1 mmol/L EGTA, pH 9.5) and centrifuged at 10,000 × *g*. The protein content of the clarified supernatant samples was evaluated by the Bradford method (Amresco, Solon, OH), and equivalent protein amounts were separated onto 8 to 22% gradient polyacrylamide gels and blotted to polyvinylidene difluoride membranes. Specific antibodies were against α and β myosin heavy chain (α-MHC and β-MHC) (Novocastra) and myosin light chain 1 (MLC1) (BiosPacific, Emeryville, CA) and 2 (MLC2) (Santa Cruz Biotechnologies). Secondary antibodies were peroxidase-linked (Vector Laboratories Inc.) and revealed with enhanced chemiluminescence (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Western blot bands were quantified by the DS Software. Band intensities were expressed as fold increase versus band of the same protein from age-matched GSH/PT. Protein loading was checked by probing for β-actin expression.

In Vitro *Motility Assay*

This assay measures the sliding rate of rhodamine-phalloidin-labeled actin filaments translocated by myosin monomers bound to a nitrocellulose-coated surface. The assay was performed by essentially following the Cuda et al procedure, as previously described.[28]

Northern Blot Analysis

Northern blot analysis of atrial natriuretic peptide (ANP) and transforming growth factor (TGF)-β1 was performed essentially as previously described.[19] In brief, total RNA, extracted from hamster hearts, was electrophoretically separated under denaturing conditions and transferred to nylon membranes. After hybridization with ^{32}P-labeled cDNA probes, nylon membranes were subjected to autoradiography. cDNA of the GAPDH housekeeping gene was used as probe to check the amount of loaded RNAs; bands were quantified by DS Software.

Hemodynamic Assessment

Aortic and intraventricular pressures were measured in 150-day-old CMPH/PT, CMPH/FS, and GSH/PT anesthetized with urethane (400 mg/kg i.p.), as previously described.[19] In brief, a polyethylene catheter (PE50), connected to a Statham P23Gb transducer (Gould Instruments, Cleveland, OH), was introduced into the left ventricular cavity via the right carotid artery; intra-arterial and intraventricular pressures were recorded by a multichannel polygraph (Gould Instruments) after a 20-minute stabilization.

Hamster Survival Analysis

Three hamster groups were considered: GSH/PT (n = 46), as healthy controls, and two CMPH groups,

Table 2. Molar Percent Composition of Individual Fatty Acids from 150-Day-Old CMPH and GSH Ventricles and Plasma

	LV			RV		
	GSH/PT	CMPH/PT	CMPH/FS	GSH/PT	CMPH/PT	CMPH/FS
16:0	16.64 ± 0.89	16.72 ± 1.40	10.34 ± 1.22†	16.91 ± 0.58	21.30 ± 1.86	11.95 ± 1.22†
18:0	12.69 ± 0.42	15.72 ± 0.72	19.70 ± 0.94†	11.76 ± 0.27	16.61 ± 1.35	19.77 ± 0.75†
18:1	20.31 ± 3.22	12.72 ± 0.85	14.24 ± 1.11	18.91 ± 2.86	14.44 ± 1.13	12.37 ± 0.44
18:2	32.29 ± 2.51	31.93 ± 0.45	32.59 ± 0.67	34.91 ± 2.35	26.35 ± 2.28	30.62 ± 0.91†
18:3 ω-6	0.14 ± 0.11	0.43 ± 0.12	ND	0.28 ± 0.11	0.23 ± 0.15	ND
18:3 ω-3	0.59 ± 0.12	0.37 ± 0.11	7.3 ± 1.23*	0.65 ± 0.15	0.45 ± 0.11	7.56 ± 0.89*
20:4 ω-6	6.63 ± 0.68	10.12 ± 1.34	7.6 ± 0.72*	6.88 ± 0.35	9.35 ± 0.42	7.18 ± 0.48*
20:5 ω-3	ND	ND	1.04 ± 0.28*	ND	ND	1.64 ± 0.12*
22:5 ω-6	0.38 ± 0.13	0.53 ± 0.11	ND	0.43 ± 0.11	0.73 ± 0.12	ND
22:5 ω-3	0.59 ± 0.09	0.92 ± 0.24	0.93 ± 0.15	0.62 ± 0.12	0.85 ± 0.15	1.13 ± 0.09
22:6 ω-3	6.35 ± 0.53	9.31 ± 0.61	5.77 ± 0.49†	6.19 ± 0.49	6.92 ± 0.44	7.6 ± 0.17

Values are mean ± SD of seven animals per group. LV, left ventricle; RV, right ventricle; SP, interventricular septum; PT, standard diet; FS, ω-3 PUFA-enriched diet; ND, not detectable.
*$P < 0.005$ and †$P < 0.01$ versus age-matched CMPH/PT.

(table continues)

CMPH/PT ($n = 47$) and CMPH/FS ($n = 47$), of which CMPH was randomly divided at the beginning of the study. The survival study was performed from weaning (30 days of age) up to 450 days. Data analysis was performed with SPSS for Windows (version 11.5; SPSS Inc., Chicago, IL). Hamster survival curves were built by the Kaplan-Meier analysis. Differences between survival mean ages were analyzed by one-way analysis of variance and were considered statistically significant when P was < 0.05. The significance of differences between curves was verified by log-rank test; differences was considered statistically significant when $P < 0.05$.

Statistical Analysis

Results are presented as mean ± SD. Unless stated otherwise, the comparison between FS and PT data was performed by the unpaired *t*-test.

Results

ALA-Enriched Diet Modulates Heart and Plasma Fatty Acid Composition

To evaluate the capability of an ALA-enriched diet to target the myocardium, the molar percent presence of each fatty acid species, obtained by *trans*-esterification of total lipids from hearts and plasma of 150- and 90-day-old CMPH/PT, CMPH/FS, and GSH/PT, was assessed (Table 2). At 150 days of age, CMPH/FS exhibited up to 20- and 7-fold increases in the molar percentage of ALA (18:3 ω-3) in all of the ventricular regions (left ventricular free wall, right ventricular free wall, interventricular septum) and plasma, respectively, compared with CMPH/PT. Moreover, in these animals, EPA (20:5 ω-3), which was not detectable in either plasma or myocardial tissue or plasma of GSH/PT or CMPH/PT, was present (up to 1.7 molar percent) in the different heart regions of CMPH/FS but remained absent from the FS-fed animal plasma, suggesting that its synthesis was myocardium-specific. By contrast, there was a 30% decrease in arachidonic acid (20:4 ω-6, AA) molar percent, and a consequent higher EPA/AA ratio, in all ventricular regions considered and in plasma of 150-day-old CMPH/FS compared with age-matched CMPH/PT. Furthermore, concerning saturated fatty acids, plasma and cardiac lipids from CPMH/FS hearts were enriched in stearic acid (18:0) and depleted in palmitic acid (16:0), compared with those from CMPH/PT. Comparable effects of the FS diet were attained for all ventricular regions and plasma of 90-day-old CMPH groups (data not shown). The effects of the FS diet on the levels of docosahexaenoic acid (22:6 ω-3) in all heart and plasma samples were not uniform regardless of age.

ALA-Enriched Diet Preserves Cell Membrane Integrity and Myocardial Morphology

The hamster ventricular weight to body weight ratio was assessed throughout the entire study period. This ratio was slightly, but not significantly smaller in CMPH fed with FS (3.46 ± 0.21 mg/g body weight) versus PT (3.84 ± 0.29 mg/g body weight) diet, whereas in healthy controls (GSH/PT) the ratio was 3.02 ± 0.23 mg/g body weight ($n = 10$ animals per group). Morphometric analysis showed that the total lumen area of ventricles from 150-day-old CMPH/FS was smaller than that of age-matched CMPH/PT ventricles (18.5 ± 1.2 versus 21.9 ± 1.5 mm^2; $P < 0.05$) but similar to that of GSH/PT controls (16.5 ± 1.7 mm^2) and although ventricular muscle area proportion of CMPH/FS (64.5 ± 1.8%) was significantly smaller than that of GSH/PT (71.3 ± 1.8%; $P < 0.01$), it was significantly greater than that of CMPH/PT (43.9 ± 1.9%; $P < 0.01$). Moreover, H&E staining of ventricular sections demonstrated a closer to physiological tissue organization in CMPH/FS ventricles (Figure 1c) compared with CMPH/PT ventricles (Figure 1b). Although CMPH/PT hearts were marked by the presence of enlarged cardiomyocytes displaying myofibril disorganization surrounded by copious fibrosis (Figure 1e), hearts from CMPH/FS showed partial myofibril organization and scant ECM deposition (Figure 1f), closely resembling the structure of normal GSH/PT hearts (Figure 1d). Also, in CMPH/PT hearts, infiltration by macrophages and gran-

Table 2. Continued

SP			Plasma		
GSH/PT	CMPH/PT	CMPH/FS	GSH/PT	CMPH/PT	CMPH/FS
17.11 ± 1.16	18.57 ± 1.35	11.78 ± 0.89†	25.15 ± 1.12	23.85 ± 1.13	19.01 ± 0.93†
12.62 ± 1.72	17.61 ± 1.77	19.49 ± 1.01†	8.8 ± 0.72	10.56 ± 1.1	15.12 ± 2.97†
20.36 ± 1.42	13.25 ± 3.77	12.61 ± 1.15	17.59 ± 1.53	16.77 ± 2.16	15.07 ± 2.84
32.84 ± 4.82	29.46 ± 5.29	30.36 ± 1.24	34.84 ± 1.75	33.07 ± 2.52	33.35 ± 2.91
0.16 ± 0.06	0.18 ± 0.15	ND	ND	ND	ND
0.57 ± 0.09	0.38 ± 0.18	7.36 ± 1.57*	1.29 ± 0.36	2.07 ± 0.84	15.03 ± 3.91*
6.17 ± 0.37	9.53 ± 1.1	7.17 ± 0.55*	4.57 ± 0.23	7.21 ± 2.46	4.37 ± 0.94*
ND	ND	1.72 ± 0.43*	ND	ND	ND
0.31 ± 0.05	0.65 ± 0.13	ND	0.17 ± 0.03	0.18 ± 0.05	ND
0.77 ± 0.20	0.81 ± 0.07	1.45 ± 0.25	0.36 ± 0.07	0.53 ± 0.21	1.56 ± 0.19
6.94 ± 0.45	7.05 ± 0.76	8.05 ± 0.23	4.72 ± 0.19	3.16 ± 0.57	2.77 ± 0.91

ulocytes, although rare, was greater than that in GSH/PT controls; in CMPH/FS hearts, the presence of inflammatory cells was comparable with that of healthy controls (data not shown). In addition, although mitochondria in CMPH/PT cardiomyocytes were irregular in size and often exhibited damaged outer membranes and altered cristae organization (Figure 1h), those in CMPH/FS cardiomyocytes (Figure 1i), although dispersedly packaged within myofibrils, displayed normal structure. Mitochondria in GSH/PT cardiomyocytes (Figure 1g) were distributed in an orderly manner along myofibrils and were regular in size. Furthermore, although plasmalemma was often interrupted in CMPH/PT cardiomyocytes (Figure 1h), its integrity was apparently preserved in CMPH/FS cardiomyocytes (Figure 1i). The above observations suggested evaluating the integrity of the dystrophin glycoprotein complex, a large multicomponent complex endowed with both mechanical stabilizing and signaling roles in mediating interactions among cytoskeleton, cell membranes, and ECM.[29] Immunofluorescence analysis indicated that, although α-dystroglycan (α-DG) expression was not evenly distributed in CMPH/PT cardiac tissue (Figure 1n), its expression in CMPH/FS (Figure 1o) was similar to that of healthy GSH/PT controls (Figure 1m). It is noteworthy that the localization of α-DG expression in CMPH/PT versus controls showed asymmetrical variations even among littermates; by contrast, the protein's distribution in CMPH/FS was homogeneous. In CMPH/PT myocardia (Figure 1k), the laminin signal was markedly faint in the basal membrane and was arranged in bundles surrounding cardiomyocytes. In CMPH/FS myocardia (Figure 1l), the localization of laminin at the basal membrane was comparable with that observed in GSH/PT myocardia (Figure 1j). Controls with nonimmune serum did not show any specific signal (data not shown).

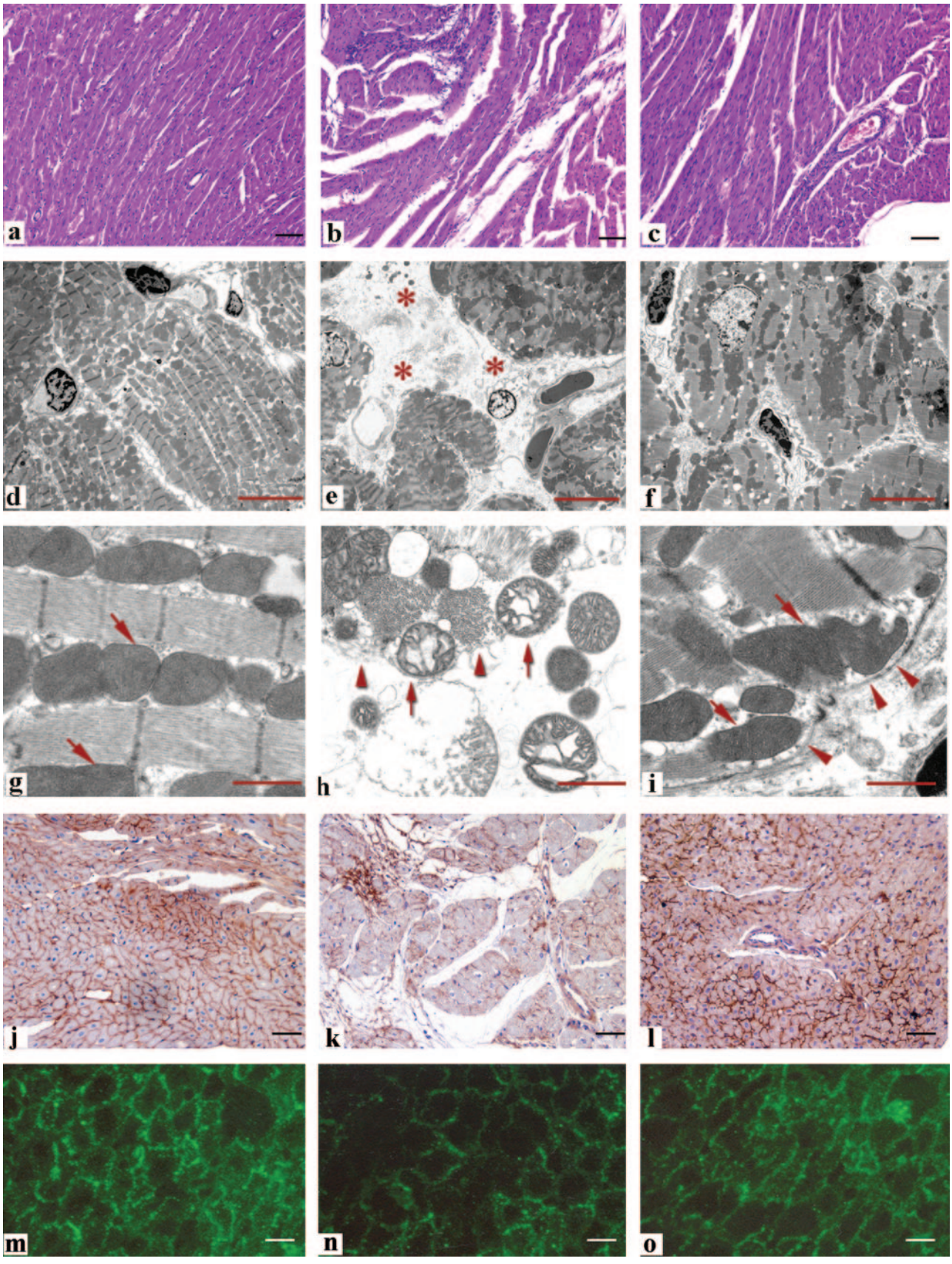
ALA-Enriched Diet Prevents Myocardial Fibrosis

In light of the above-mentioned effects, the extent of myocardial fibrosis in hamsters feeding on different diets was evaluated. Transmural samples were obtained from the areas near the apex and the base of the right ventricle, the septal region, and the left ventricle of hearts from 150-day-old hamsters, and samples were analyzed after Masson trichrome staining. Fibrotic areas represented 37 and 11% of the myocardium in CMPH/PT (Figure 2b) and CMPH/FS (Figure 2c), respectively. To confirm the antifibrotic action of the FS diet, the expression of collagen type I was qualitatively and semiquantitatively investigated via immunohistochemical analysis. Hearts from GSH/PT displayed weak expression of collagen type I, predominantly localized in the stroma around blood vessels (Figure 2d). Conversely, CMPH/PT hearts (Figure 2e) showed a high expression level of collagen type I that paralleled the increased fibrotic process and that was mostly surrounding cardiomyocytes. By comparison, hearts from CMPH/FS (Figure 2f) displayed moderate expression of collagen type I with localization comparable with that observed in normal hearts (Figure 2d). Similar distribution and expression patterns were observed in all ventricular regions. Controls processed with nonimmune serum did not show any specific signal (data not shown).

Because TGF-β1 and ANP are well-known markers of myocardial hypertrophy, and TGF-β1 stimulates fibrosis through the accumulation of ECM,[19,20,30] the expression of these two genes in 150-day-old hamster ventricles was assessed by Northern blot analysis. Compared with CMPH/PT, CMPH/FS myocardium displayed lower ANP and TGF-β1 mRNA levels (Figure 3) that were consistent with satisfactory cardiac cell and tissue organization and meager myocardial fibrosis.

ALA-Enriched Diet Secures CMPH Cardiac Contractility

The remarkably well-preserved myofibrillar organization observed in CMPH/FS cardiomyocytes instigated the investigation of a possible concomitant beneficial effect on myocardial contractile function. Using an *in vitro* motility assay that measures the sliding rate of actin translocated by myosin molecules, it was found that the actin-sliding velocity in 150-day-old CMPH/FS ventricles was greater than that in ventricles of age-matched CMPH/PT (3.76 ± 0.22 μm/second and 2.73 ± 0.27 μm/second, respec-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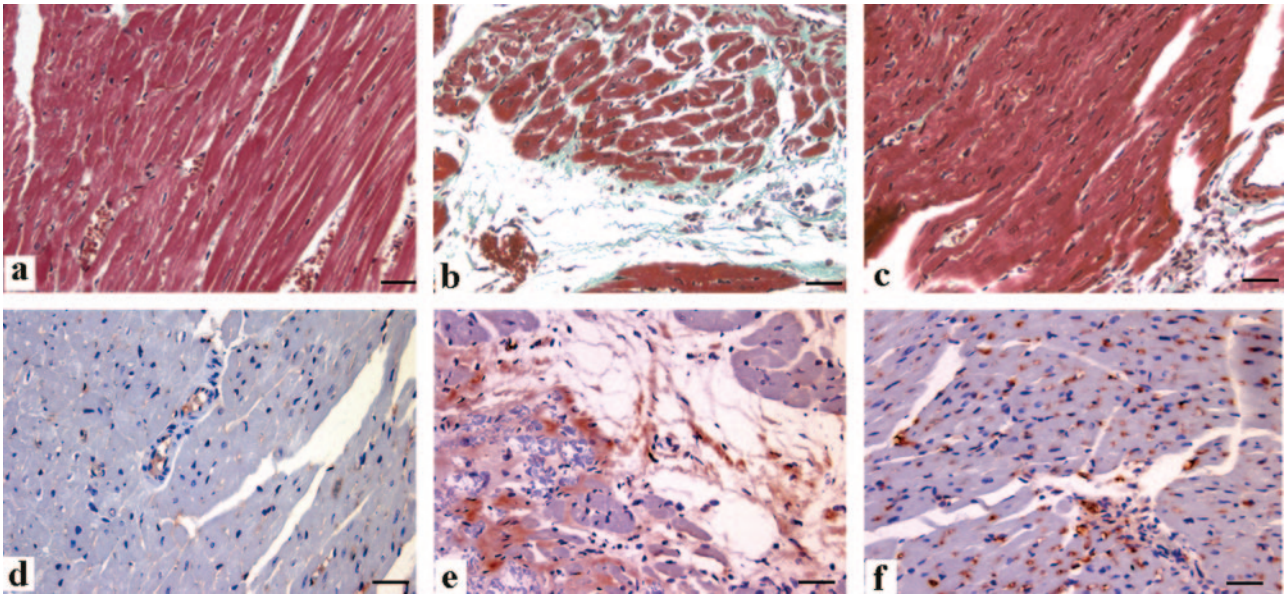

Figure 2. Myocardial fibrosis in ventricles from hamsters fed with different diets. Left ventricular fibrosis visualized by Masson-trichrome dye staining: GSH/PT showing normal morphology (**a**); large fibrosis areas (green) in CMPH/PT ventricular myocardium (**b**); and minute fibrosis areas (green) in CMPH/FS ventricular myocardium (**c**). Representative patterns of collagen type I expression in left ventricular sections: GSH/PT left ventricle showing weak expression of type I collagen that is predominantly localized in the perivasal stroma (**d**); CMPH/PT left ventricle with increased stromal expression of type I collagen, and its deposition surrounding cardiomyocytes (**e**); and CMPH/FS left ventricle with moderate type I collagen expression and localization in the perivasal stroma (**f**). Immunoperoxidase staining and counterstaining with hematoxylin. Similar results were obtained from the right ventricle and interventricular septum. All micrographs were from 150-day-old hamsters (11 animals per group). In each animal, at least eight visual fields/Masson-trichrome dye and type I collagen immunohistochemically stained sections were assessed by image analysis, as described in Materials and Methods. PT, standard diet; FS, ω-3 PUFA-enriched diet. Scale bars = 50 μm (**a–c**); 25 μm (**d–f**).

tively) (Figure 4a). Because fiber velocity depends on MHC isoform composition,[31] the effects of the FS diet on MHC expression in 90-, 150-, and 420-day-old hamster myocardia were assessed by Western blot analysis. In healthy rodent ventricles, α-MHC is the predominant isoform; age and disease, such as cardiomyopathy,[19] determine a progressive decrease in α-MHC content with a parallel increase in β-MHC. A 2.7- and 4.7-fold higher α-MHC expression was detected in ventricles of CMPH/FS aged 90 and 150 days, respectively, compared with that in ventricles of age-matched CMPH/PT controls. Conversely, 77 and 64% lower β-MHC expression was detected in 90- and 150-day-old CMPH/FS hearts, respectively (Figure 4b), compared with hearts from age-matched CMPH/PT controls. These data indicate that the FS diet maintains the α/β MHC ratio close to healthy values up to at least 150 days of age. On the other hand, by the time CMPH/FS and GSH/PT were 420 days of age, and at which time all members of the CMPH/PT group had already perished, there was a twofold higher α-MHC expression and a concomitant 80% lower β-MHC expression in ventricles of FS-fed animals versus those of age-matched GSH/PT healthy controls (Figure 4b). No relevant effects of the dietary regimen on the expression of MLC1 and MLC2 in CMPH ventricles were observed (data not shown).

ALA-Enriched Diet Maintains Adequate Hemodynamic Parameters in Cardiomyopathic Hamsters

Consistently, and along with the above-mentioned beneficial effects on myocardial structure, a significant effect on the CMPH hemodynamic status was observed in 150-day-old CMPH fed with FS versus PT diet. In particular, FS diet preserves a lower, and closer to normal, left ventricular end diastolic pressure as well as other hemodynamic values that tend to match those of healthy controls (Table 3).

Figure 1. Microscopy analysis of ventricular tissue from hamsters fed with different diets. Morphological analysis of H&E-stained sections: GSH/PT left ventricular sections displaying normal morphology (**a**); large areas of myofibril loss in CMPH/PT myocardium (**b**); and myofibril loss areas are almost completely absent in CMPH/FS myocardium (**c**). Analysis of myofibrillar organization and ECM deposition by EM: GSH/PT ventricular sections with normal morphology (**d**); myofibrillar disorganization and extensive ECM deposition (**asterisks**) in CMPH/PT ventricles (**e**); and partial myofibrillar organization and scarce ECM deposition in CMPH/FS ventricular sections (**f**). Analysis of mitochondrial and plasmalemmal morphology by EM: GSH/PT mitochondria (**arrows**) with normal morphology regularly located along myofibrils (**g**); CMPH/PT plasmalemma ruptures (**arrowheads**), mitochondrial membrane damage, and alteration in cristae organization (**arrows**) (**h**); and normal mitochondria dispersedly packaged among myofibrils (**arrows**) and preserved plasmalemma (**arrowheads**) in CMPH/FS ventricles (**i**). Immunohistochemical analysis of laminin expression and localization: basal membrane localization of laminin in healthy GSH/PT left ventricle (**j**); CMPH/PT ventricles with severely reduced expression of laminin (**k**); and CMPH/FS ventricles with laminin localization at basal membrane level (**l**). Immunoperoxidase staining with hematoxylin counterstaining. Analysis of α-DG expression and localization by immunofluorescence staining: GSH/PT left ventricle with α-DG localization within the sarcolemma (**m**); CMPH/PT left ventricles with severe reduction in α-DG expression (**n**); and CMPH/FS left ventricular tissue with α-DG localization at the sarcolemmal level (**o**). Similar results were obtained from the right ventricle and interventricular septum. All micrographs are from 150-day-old hamsters. PT, standard diet; FS, ω-3 PUFA-enriched diet. Scale bars = 50 μm (**a–c**, **j–l**); 5 μm (**d–f**); 0.5 μm (**g–i**); 25 μm (**m–o**).

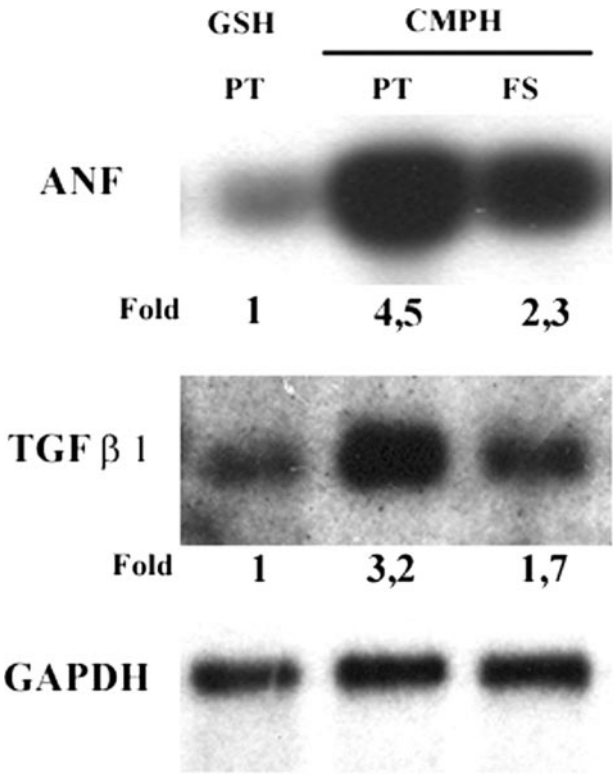

Figure 3. Northern blot analysis of atrial natriuretic factor (ANP) and TGF-β1 mRNA in 150-day-old CMPH ventricles fed with ALA-enriched diet. Similar results were obtained from three independent experiments. After normalization to the housekeeping internal control (GAPDH), the intensity of each band was expressed as fold increase versus age-matched GSH/PT expression of the same gene. PT, standard diet; FS, ω-3 PUFA-enriched diet.

CMPH Fed with ALA Exhibit Remarkable Extension of Longevity

To verify whether beneficial effects induced on myocardial structure and function could be relevant for CMPH survival, the effects of FS versus PT diet on hamster longevity were investigated. The first death was observed at 240 days of age in GSH/PT and at 70 and 77 days in CMPH/PT and CMPH/FS, respectively. The mean survival time of CMPH/FS (293 $\pm$ 141.8 days) was significantly longer ($P < 0.005$; one-way analysis of variance) than that of CMPH/PT (175.9 $\pm$ 56 days). The progression of the survival curves of each of the CMPH groups was significantly different from that of the GSH group (log-rank test: CMPH/PT versus GSH, $P < 0.01$; CMPH/FS versus GSH, $P < 0.05$). At 320 days, when all CMPH/PT had perished, survival rates were 67% (31 of 47) and 98% (45 of 46) for CMPH/FS and healthy controls, respectively (Figure 5). At 450 days (ie, 130 days beyond the death of the last CMPH/PT), the final survival rates were 61% (28 of 47) and 92% (42 of 46) for CMPH/FS and healthy controls, respectively (Figure 5). CMPH curve shapes started diverging at 110 days of age, and the divergence increased at 150 days. After this age, whereas CMPH/PT showed a negative exponential shape, CMPH/FS displayed a more linear progression and a less pronounced slope. Congestive heart failure caused the death of all CMPH/PT and CMPH/FS.

Discussion

Epidemiological studies have reported that the consumption of ω-3 PUFAs from both marine and plant sources positively correlates with reduced mortality from all causes[32] and, above all, from coronary heart disease.[7] Nevertheless, only preliminary information is available concerning the biological mechanisms of ω-3 PUFAs' beneficial effects on the ischemic myocardium, and there are no published reports regarding their effects on the diffuse myocardial damage caused by hereditary cardiomyopathies.

The present study is the first to demonstrate that dietary ω-3 PUFAs (particularly ALA) determine a remarkable elongation of the survival of hamsters suffering from

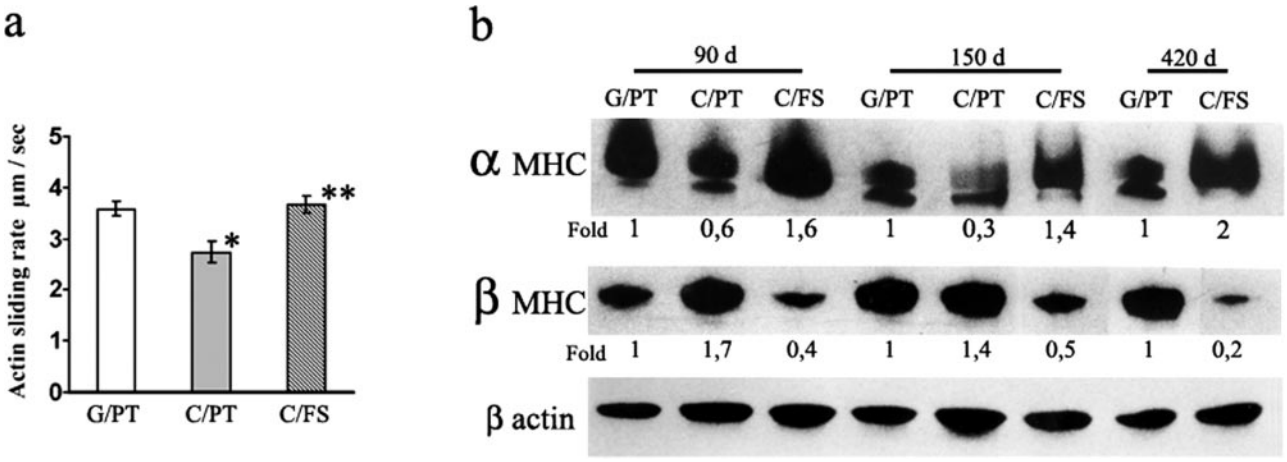

Figure 4. Heart contractile function in CMPH fed with ALA-enriched diet. Actin sliding rate in 150-day-old hamster left ventricle as evaluated by *in vitro* motility assay: values are expressed as mean $\pm$ SD of 50 to 100 filament determinations for each animal group (seven animals per group). $^{*}P < 0.001$ versus age-matched GSH healthy controls; $^{**}P < 0.005$ versus CMPH/PT (**a**). Expression of MHC isoforms (α and β) from left hamster ventricle myofibrillar extracts by Western blot analysis: G/PT, healthy golden Syrian hamsters fed with standard diet; C/PT, cardiomyopathic hamsters fed with standard diet; C/FS, cardiomyopathic hamsters fed with ω-3 PUFA-enriched diet; d, days. Protein loading was checked by β-actin expression. Bands were quantified as reported in Materials and Methods, and their intensities were expressed as fold increase versus band of the same protein from age-matched GSH/PT (**b**).

Table 3. Hemodynamic Parameters in 150-Day-Old GSH/PT, CMPH/PT, and CMPH/FS

	GSH/PT	CMPH/PT	CMPH/FS
HR (beats/minute)	353 ± 22	312 ± 39	337 ± 12*
SAP (mm Hg)	125.3 ± 12.3	92.4 ± 9.5	118 ± 9.7*
LVSP (mm Hg)	130.6 ± 9.2	101.2 ± 9.8	123 ± 8.7*
LVEDP (mm Hg)	7.9 ± 1.0	17.2 ± 3.9	11.3 ± 3.5†
+dP/dt (mm Hg/second)	5667 ± 621	3751 ± 328	4769 ± 458†
−dP/dt (mm Hg/second)	5335 ± 422	3372 ± 383	4620 ± 486†

Values are mean ± SD of 12 animals per group. HR, heart rate; SAP, systolic arterial pressure; LVSP, left ventricular peak systolic pressure; LVEDP, left ventricular end-diastolic pressure; +dP/dt, peak rate of left ventricular pressure development; −dP/dt, peak rate of left ventricular pressure decrease; PT, standard diet; FS, ω-3 PUFA-enriched diet.

*$P < 0.005$ versus age-matched CMPH/PT.

†$P < 0.001$ versus age-matched CMPH/PT.

hereditary cardiomyopathy and that this extended longevity is sustained by ω-3 PUFAs' protective effects on cardiac cells and on cardiac tissue structure and function against damage induced by δ-SG ablation[18] and mitochondrial gene mutation.[16] In this study, such protection was achieved by administering ALA to CMPH from weaning to death, ie, animals, throughout their entire life, never consumed standard diet.

Because significant extension of longevity in rodents could be achieved by reduced calorie intake,[33] great attention was paid to ensuring that the animals used in the present study did not suffer from this limitation. In fact, the extended longevity of CMPH/FS is directly related to the increased intake of ALA, which targeted the myocardium, as demonstrated by its massive presence in plasma and heart. In addition, although heart lipid composition was not substantially different in CMPH/PT versus GSH/PT hearts before weaning,[15] adult CMPH/PT cardiac tissue lipids contained a lower percentage of palmitoleic and oleic acid and were enriched in linoleic and arachidonic acid, compared with age-matched GSH/PT.[15] It is conceivable that the milk of CMPH/PT mothers (before weaning) and the ALA-enriched diet (after weaning) slow the effects of lipogenic enzyme dysregulation, thus preserving normal cardiac lipid composition.[34] The increased presence of ALA could concur to maintain the plasmalemma's fluidity[35] closer to physiological levels and to protect its structural and functional integrity against stress-induced damage.[36]

The identification of lipogenic enzymes potentially involved in the dysregulation of lipid metabolism in CMPH hearts is beyond the scope of the present study; however, previous investigations performed in the same animal model have demonstrated a generalized disturbance of lipid metabolism sustained by an altered expression of liver lipogenic enzymes, such as fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1), and S14.[15] It is worthy to note that enhanced stearic acid accumulation was observed in the plasma and heart lipids of ALA-conditioned hamsters, presumably because of reduced SCD1 activity. However, because the ALA-enriched diet did not restore δ-SG expression in CMPH cardiomyocytes (data not shown), it can be assumed that at least two major players, whose interrelationship remains to be elucidated, are associated in the pathogenesis of the hamster cardiomyopathy: lipid enzyme dysregulation and δ-SG gene deletion, from which other relevant pathogenic mechanisms (eg, altered plasmalemma and mitochondrial membranes and function, and so forth) could stem. This working hypothesis was indirectly corroborated by the observation that an enduring lower survival rate affected ALA-fed CMPH versus GSH/PT, a difference very likely sustained by the continued δ-SG deficiency in the former.

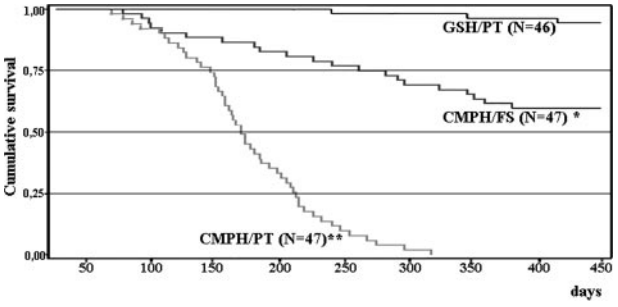

Figure 5. Survival rates of CMPH and GSH: Kaplan-Meier survival curves for CMPH/PT and CMPH/FS, compared with GSH/PT healthy controls. Statistically significant differences are observed between GSH/PT healthy controls and each of CMPH/FS (*$P < 0.05$ by log-rank test) and CMPH/PT (**$P < 0.01$ by log-rank test). PT, standard diet; FS, ω-3 PUFA-enriched diet.

Sarcoglycans are a family of transmembrane-spanning glycoproteins organized in a complex that combines with the dystrophin glycoprotein complex[37] and participates in a composite network that bridges the basal lamina and the cytoskeletal array fastening myofibrils.[22,29] The presence of the sarcoglycan complex is crucial in stabilizing the transmembrane bridge, and some isoforms are suspected to play a signaling role.[37] The δ-SG ablation has been associated with a dystrophic phenotype that is characterized by diffuse alterations of cell/cell and cell/ECM contacts, the detachment of the basal membrane, an aberrant intracellular signaling pattern, and a warped gene expression profile.[17,19,23,38] Consistently, amelioration of striated muscle injury and significant lifespan elongation were achieved in δ-SG-null TO2 hamsters with dilatative cardiomyopathy by δ-SG re-expression, confirming this gene's role in the pathophysiology of the disease.[39] In CMPH suffering from hypertrophic cardiomyopathy caused by the same δ-SG mutation, comparable protective effects on injured striated muscle and on lifespan duration have been achieved by increasing the myocardial content of ω-3 PUFAs, in the absence of δ-SG re-expression. This suggests that, in CMPH/FS cardiomyocytes, maintaining an adequate ω-3/ω-6 balance is de-

cisive in preserving the plasmalemma's mechanical features, as demonstrated by the expression and proper alignment of membrane proteins involved in cell contacts and signaling. Among others, α-DG and laminin expression very likely sustained the capability of the CMPH/FS plasmalemma to establish suitable mechanical links with neighboring structures and to signal correctly to the cardiomyocyte nucleus. α-DG is a component of the dystrophin glycoprotein complex that binds to the ECM through $\alpha 2$ chain of laminin 2 and contributes to cell signaling and cytoskeleton reorganization.[40] The observation that cardiomyocyte lipid composition is important in maintaining CMPH sarcoglycan, and above all, dystrophin glycoprotein complex function implies that the role of δ-SG *in vivo* may not be as crucial as predicted *in vitro*[37] and that, in the absence of δ-SG, other contact and signaling proteins could preserve their orientation and function within the ω-3-protected plasmalemma: the maintenance of myofibrillar orientation observed in CMPH/FS could mark this process. However, additional potentially beneficial mechanisms could be activated in CMPH/FS hearts by the massive presence of ALA and its metabolite EPA and the relatively low AA content. AA is the precursor of the ω-6-derived prostaglandins (PGE_2), leukotrienes, and thromboxanes (LTB_4). Its metabolism can be competitively inhibited by EPA, with the consequent suppression of ω-6, and the increase in ω-3 (PGE_3 and LTB_5) eicosanoids' synthesis, which display a reduced inflammatory potential.[41,42] In addition, EPA induces the suppression of tumor necrosis factor-α and interleukin-1β production,[43] the inhibition of platelet aggregation,[44] and the generation of ω-3-derived mediators (resolvins and protectins) that could provide novel mechanisms of tissue damage repair.[12,45] Furthermore, EPA also modulates gap junction[10] and cell signaling by influencing intracellular trafficking and the localization of membrane proteins. In CMPH/FS hearts, the tissue-specific synthesis of EPA (not detectable in CMPH/FS plasma nor in GSH/PT and CMPH/PT plasma or cardiac tissue) and the remarkable consensual decrease in AA boosted the EPA/AA ratio; this could reduce the myocardial risk of inflammatory injuries and of further structural and functional damages.

ω-3 PUFAs' observed beneficial effects were not confined to plasmalemma structure and intracellular compartments. In the CMPH/FS myocardium, ω-3 PUFAs also preserved a proper ECM, as evidenced by the increased expression and localization of laminin within the basal membrane, and the reduced extension of fibrotic areas and of collagen type I expression, compared with CMPH/PT hearts. Collectively, the ALA-enriched diet remarkably counteracted the CMPH's potential derangement of cardiac tissue texture and function. Consistently, CMPH/FS hearts exhibited preserved myofibrillar spatial organization and intracardiomyocyte signaling, as witnessed by a closer to physiological expression of MHC isoforms with a proper α/β MHC ratio, and a consequent MHC functional efficiency similar to that of healthy controls, as measured by an *in vitro* actin motility assay. A further sign of the physiological signaling capability of CMPH/FS versus CMPH/PT cardiomyocytes is the decreased expression of ANP and TGF-β1 genes, indicating the absence of aberrant signaling cascades that lead to myocardial hypertrophy and fibrosis, respectively.[20]

Ultimately, CMPH/FS displayed hemodynamic indices similar to those of GSH/PT, very likely owing to the suitable ventricular muscle area proportion, the meager fibrosis, and the high cardiomyocyte mechanical efficiency and energy availability. The latter is evidenced by ultrastructural analysis showing a much improved morphology of CMPH/FS versus CMPH/PT mitochondrial membranes and is a consequence of the preservation of the mitochondrial membrane's structural and functional integrity by ω-3 fatty acid embedding, an effect previously demonstrated in age-associated or ischemic damage of myocardial mitochondria.[46,47]

These multifaceted effects suggested testing whether common unifying controller factors that orchestrate the mechanisms activated by ALA could be identified. Possible candidates, as transcriptional controllers of genes involved in cardiac fatty acid uptake and oxidation and in the prostaglandins/leukotrienes cascade, are the nuclear peroxisome proliferator-activated receptors (PPARs).[48] PPARs, which are activated by long-chain fatty acids and a variety of related compounds, heterodimerize with the 9-*cis*-retinoic acid receptor RXR and bind cognate DNA regulatory elements in target genes,[49] attenuating, among others, myocardial fibrosis and inflammation,[50] and preserving the integrity of mitochondrial membranes.[51] Nonetheless, the issue of PPARs' role in the pathophysiology of the heart is controversial.[52] The possibility that the aberrant regulation of PPAR expression and function could be associated to δ-SG ablation in determining the cardiomyopathic phenotype, and the potential ability of dietary ω-3 PUFAs to correct this aberration are very intriguing and warrant further investigations.

However, it still remains that the mechanisms underlying the effects of dietary ω-3 PUFAs on cardiomyopathic hamsters culminated in a postponement of the onset of cardiac failure[19] and an impressive consequent extension of their longevity: by the time all CMPH/PT perished, more than 60% of CMPH fed with flaxseeds were still alive and apparently in good health. Previous investigations ascribed ω-3 PUFAs' potential to counteract postischemic mortality to their anti-arrhythmic and anti-fibrillatory effects.[8,13,53,54] In our view, ω-3 PUFAs' cardiac beneficial effects involve cellular and tissue mechanisms wider and more complex than previously hypothesized, and their anti-arrhythmic potential is only part of this scenario and corollary of the preservation of a suitable cardiomyocyte plasmalemma and intracellular environment. Furthermore, all previous studies investigated ω-3 PUFAs' effects in experimental and/or human heart ischemic disease.[8] This investigation is the first to demonstrate that ω-3 PUFAs can be used to successfully prevent the cardiac damage of hereditary diseases and that this preventive treatment is very efficient, safe, and economically advantageous, compared with drugs presently in use to reduce myocardial fibrosis[30] or to improve cardiac function,[55] while waiting for a more effective gene[56] and/or cell therapy.[57]

In conclusion, the present study sheds light on the pathogenic mechanism of hamster hereditary cardiomyopathy, suggesting, for the first time, the possibility that some of the hereditary muscular diseases could have a multifactorial origin. In cardiomyopathic hamsters, the pathogenesis of the disease seems to result from the complex interplay between genetic and environmental factors. Among the latter, and as the present study unequivocally indicates, an ω-3 PUFA-enriched diet delays fibrosis, preserves heart performance, and induces a marked prolongation of the cardiomyopathic hamster's longevity. Although the ω-3-enriched dietary regimen administered to CMPH was extreme and far from the standard pellet chow, its effects on the hamster's cardiomyopathy and survival were clear and consistent. A number of international organizations have now made recommendations relating to the intake of long-chain ω-3 PUFAs[7] for all individuals, especially those at risk of developing cardiovascular disease, mainly to ameliorate cardiac arrhythmias[53,54] or plasma triacylglycerol levels.[58] This study, although experimental, indicates that the intake of long-chain ω-3 PUFAs has profound and unexpected protective effects on the pathogenesis of cardiac diseases. Therefore, it seems plausible to consider the administration of plant-originated ω-3 PUFAs as a safe adjuvant strategy for attenuating major damages associated with hereditary cardiomyopathy.

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