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Laura Trapani, Francesca Violo, Valentina Pallottini. Hypercholesterolemia and 3-hydroxy 3-methylglutaryl Coenzyme A reductase regulation in aged female rats. *Experimental Gerontology*, 2010, 45 (2), pp.119. 10.1016/j.exger.2009.10.014 . hal-00559140

HAL Id: hal-00559140

<https://hal.science/hal-00559140>

Submitted on 25 Jan 2011

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Accepted Manuscript

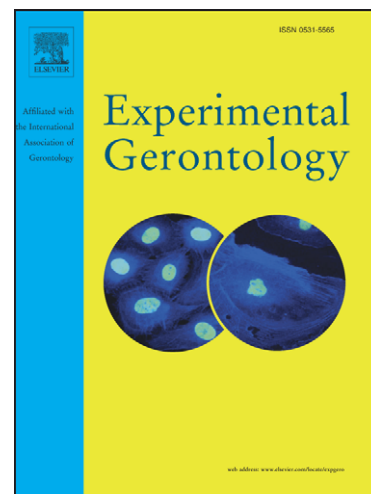
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PII: S0531-5565(09)00230-7
DOI: [10.1016/j.exger.2009.10.014](https://doi.org/10.1016/j.exger.2009.10.014)
Reference: EXG 8666

To appear in: *Experimental Gerontology*

Received Date: 31 August 2009
Revised Date: 12 October 2009
Accepted Date: 29 October 2009



Please cite this article as: Trapani, L., Violo, F., Pallottini, V., Hypercholesterolemia and 3-hydroxy 3-methylglutaryl Coenzyme A reductase regulation in aged female rats, *Experimental Gerontology* (2009), doi: [10.1016/j.exger.2009.10.014](https://doi.org/10.1016/j.exger.2009.10.014)

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**Hypercholesterolemia and 3-hydroxy 3-methylglutaryl Coenzyme A reductase
regulation in aged female rats**

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Running title: HMGR regulation in aged female rats.

Key Words: Ageing, AMPK, estropause, estradiol, hypercholesterolemia, HMGR.

Abstract

Coronary heart disease is less prevalent in pre-menopausal women than in men, but increases at the onset of menopause. This delay is due to estrogen protective effects. The rise of cholesterolemia is one of the main risk factors for coronary disease. Since 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) is the rate-limiting enzyme of the cholesterol biosynthetic pathway, it plays a pivotal role in cholesterol homeostasis maintenance. Aim of this study is to investigate whether HMGR is involved in the cholesterolemia increase that occurs during aging, and to consider its potential role as a target for estrogen protective effects. “In vivo” studies have been performed using the livers of 12-month-old female rats (whose estrogen level decrease is comparable to the one detected at the occurrence of human estropause), 12-month-old female rats treated with 17- β -estradiol, and 3-month-old untreated male and female rats. The results indicated hypercholesterolemic status and a significant increase of HMGR activity according to a reduced activation of AMPK detected in treated rats compared to controls. Furthermore, 17- β estradiol treatment reduced HMGR activity restoring AMPK activation. These findings highlight the correlation between estrogen and HMGR short-term regulation, and suggest the presence of another mechanism underlying the protective role of estrogen in age-related diseases.

Introduction

Coronary heart disease and other vascular diseases account for approximately half of all deaths in women. Although the underlying pathophysiological processes (atherosclerosis and thrombosis) are similar, deaths and other clinical end-points are significantly delayed in women compared to men. Under age 55, the incidence of CVD (Cardiovascular Diseases) in women is one third of that in men; however, at age 75, the incidence is essentially the same in both genders (Skafar et al., 1997). The loss of female sex hormones after menopause contributes to the striking increase in the incidence of cardiovascular morbidity and mortality after menopause. Estrogen replacement therapy improves lipoprotein profiles in postmenopausal women, but this accounts for less than half of the cardioprotective effects of estrogen replacement therapy. The cardioprotective benefits of estrogens include a positive impact on plasma lipids (increased high density lipoprotein (HDL), decreased total cholesterol, triglycerides, low density lipoprotein (LDL), and LDL oxidation) (Skafar et al., 1997, Moorthy et al., 2004), anti-platelet and antioxidant effects and preservation of endothelium-mediated vasodilatation (Maxwell, 1998; Moorthy et al., 2005). These effects may be mediated via genomic and/or nongenomic mechanisms (Skafar et al., 1997). Moreover, studies performed on animal models of estrogen deficiency have indicated a disruption of lipid homeostasis (Heine et al. 2000).

Cholesterol is both synthesized by cells and taken in via food. It plays an essential role in the synthesis of new membranes, the turnover of lipids in existing membranes, and the biosynthesis of products such as steroid hormones and bile acids (Martini and Pallottini, 2007). The liver is the principal site for cholesterol homeostasis maintenance (Dietschy et al., 1993), which involves the regulation of many processes, such as biosynthesis, via 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, E.C. 1.1.1.34) activity, uptake through low-density lipoprotein receptors

(LDLr), lipoprotein release in the blood, storage by esterification, degradation and conversion into bile acids (Weber et al., 2004).

As the central enzyme of the cholesterol biosynthetic pathway, HMGR can catalyze the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate (a four-electron oxidoreduction) (Friesen and Rodwell, 2004), and is tightly regulated (Goldstein and Brown, 1990). The enzyme is subject both to short- and long-term regulations. Short-term regulation of HMGR is achieved phosphorylation and dephosphorylation reactions, both of which can affect enzyme activity. The phosphorylation of residue S872 of HMGR decreases its catalytic activity. Removal of the phosphate by HMGR phosphorylase reactivates the enzyme (Beg et al., 1985; Omkumar et al., 1994). HMGR activation (dephosphorylation) is mediated by protein phosphatase 2A (PP2A), which regulates a significant network of cellular events (Janssens and Goris, 2001). Aside from short-term regulation, HMGR is subject to transcriptional, translational, and post-translational control (Xu et al. 2005). This control can result in changes of over 200-fold in enzyme levels as a function of intracellular sterol levels and cholesterol uptake by LDLr (Goldstein et al., 2006). Both LDLr and HMGR are produced in response to the activation of the Sterol Regulatory Element Binding Proteins (SREBPs) in the liver, particularly SREBP-2 (Brown and Goldstein, 1997; Horton et al., 2002).

The long-term regulation system consists of a pair of membrane-bound proteins, Scap (SREBP cleavage activating protein) and Insig 1 and 2 (Insulin-induced gene), which directly bind sterols and thereby sense their concentrations in the membranes of the endoplasmic reticulum (ER) (Goldstein et al., 2006).

Several hormones act to alter the expression of hepatic HMGR in animals. These include insulin, glucagon, glucocorticoids, thyroid hormone and estrogens (Ness and Chambers, 2000). However, available data regarding to estrogen effects on HMGR regulation remain controversial. Initially, a role for physiologic levels of estrogen as a positive effector of HMGR activity was reported by Carlson and coworkers (Carlson et al. 1980). Other studies show that pharmacological

17- β -estradiol treatment in male rats "in vivo" induces a decline in hepatic HMGR activity after six hours and rapid LDLr induction (Marino et al. 2001). Furthermore, other studies provide evidence that LDLr and HMGR are respectively up- and down-regulated by 17- β -estradiol, which induces growth inhibition or apoptosis in the DLD-1 colon cancer cell line (Messa et al. 2005). Research also reveals that estrogens are able to modulate LDLr transcription through a mechanism mediated by tyrosine kinase signaling in HepG2 cells (Distefano et al. 2002).

Hypercholesterolemia is one of the primary risk factors for CVD, whose incidence is highly related to aging. Almost 25% of men and 42% of women older than 65 years have a serum total cholesterol level greater than 240 mg/dL (Grundy et al., 1999). The mechanisms behind this age-related increase in plasma cholesterol are still incompletely understood. Of particular interest are findings indicating a gradual decline in the fractional clearance of LDL from the circulation with age (Finch and Tanzi, 1997; Holliday R., 2000; Carnes et al., 2003) and evidence of the reduced expression of hepatic LDLr with increasing age in some species (Korpelainen 2000; Gudmundsson et al., 2000). The age-related disruption of cholesterol maintenance could be due to perturbations in homeostasis, which, with progressive declines in physiological capacity and reduced ability to respond adaptively to environmental stresses, are a primary characteristic of elderly patients. In 24-month-old ad libitum-fed male rats, the hepatic HMGR is fully activated and cholesterol synthesis is increased; cholesterol content is unchanged in the liver, but increased in the blood (Marino et al., 2002). In fact, HMGR tends to be completely dephosphorylated and activated (Marino et al., 2002) by the rise of reactive oxygen species (ROS) that occurs during aging (Pallottini et al., 2007).

There is little published research on putative physiological sex-differences in cholesterol homeostasis-related proteins (Abul-Hajj 1978; Carlson et al., 1980; Choi et al. 1988; Hewitt et al. 2003; Kojima & Degawa 2006). Recent data suggest that sex differences in HMGR expression and regulation depend upon variation in regulatory proteins and are related to the presence of estrogen. Some research has shown that HMGR levels and activity are lower in adult female rats than in male

rats. This feature seems to depend on estrogen-dependent Insig 2 levels, which can affect enzyme transcription and degradation (De Marinis et al., 2008). We know of no report to date that analyzes the lack of estradiol during female ageing and putative changes in the protein able to regulate cholesterol homeostasis maintenance, as most existing research has examined males.

This paper evaluates HMGR regulation in estropausal rats to determine if estrogen protective effects on the lipid profile are also mediated via HMGR (estrogen-dependent) regulation.

As an experimental model, 12-month-old female Wistar rats were treated with 17- β estradiol.

At the beginning of estropause (12 months of age), female rats -- like women -- cycle irregularly, but after cycling ceases, 17- β estradiol levels in rats -- unlike those in women and mice -- fall not to zero, but to steady moderate levels (for two months) and then increase again (Berkley et al., 2007). So we can consider 12-month-old female rats to be in a similar condition to that occurring in women at the onset of menopause. Thus, in our experimental model, the putative connection between estrogen decrease, hypercholesterolemia and HMGR activity was investigated.

To this end, the different expression patterns of hepatic proteins involved in HMGR long- and short-term regulation, HMGR activity and ROS production were investigated. The estrogen effects were evaluated treating 12-month-old female rats with pharmacological doses of 17- β estradiol to assess the involvement of HMGR in the protective role played by estrogen.

Materials and Methods

Materials

All materials used were obtained from commercial sources, and were of the highest quality available. All materials with no specified source were obtained from Sigma–Aldrich.

Animals

Three- months-old male and female and 12-month-old female *Rattus norvegicus* Wistar (Morini, Italy) rats were housed under controlled temperature (20 ± 1 °C), humidity ($55\pm10\%$), and light (from 07.00 to 19.00 h). The animals had free access to food and water. Twelve-month-old female rats were injected intraperitoneally with 1 mg/kg of 17- β estradiol 3-benzoate (Sigma Chemical Co.) in vehicle (1 ml/kg dimethyl sulfoxide (DMSO) in saline). Control animals received an equal volume of vehicle. Sampling was then performed at 24 h after the estradiol injection. All female rats were anaesthetized with ether in a fume cupboard. Plasma was obtained from blood collected in EDTA (1 mg/ml blood). Livers were removed and rapidly frozen in liquid nitrogen. Four animals per group were used, and livers were independently analyzed in duplicate. The experiments were performed according to ethical guidelines for the conduct of animal research (Ministero della Salute, Official Italian Regulation N°. 116/92, Communication to Ministero della Salute n° 391/121).

HMGR activity

The assay was carried out via the radioisotopic method, following the production of [^{14}C]-mevalonate from 3- ^{14}C -HMG-CoA (3- ^{14}C -HMG-CoA; specific activity 57.0 mCi/mmol; GE Healthcare, Little Chalfont, UK). Microsomes were prepared in the presence or absence of NaF according to the method of Bruscalupi et al. (1985). NaF was used to block lysosomal phosphatase, thus permitting the analysis of expressed HMGR, while microsomes without NaF were useful in evaluating total HMGR activity. Microsomes (100 μg) were incubated in the presence of cofactors (20 mM glucose-6-phosphate, 20 mM NADP sodium salt, 1 IU glucose-6-phosphate dehydrogenase, and 5 mM dithiothreitol). The assay, in a final volume of 200 μl , was started by the addition of 10 μl (0.088 μCi /11.7 nmol) of 3- ^{14}C - HMG-CoA. The radioactivity of the produced [^{14}C]-mevalonate, isolated by chromatography on AG1-X8 ion exchange resin (Bio-Rad Laboratories), was counted. An internal standard (3- ^3H -mevalonate, specific activity 24.0 mCi/mmol; GE Healthcare) was added to calculate the recovery.

Protein levels analysis

Protein levels were analyzed via Western blotting. An analysis of Insig 1, Insig 2, HMGR and Scap was performed on microsomes prepared as described previously. Total lysates and total membranes were prepared according to Martini et al. (2007). Western blot analysis of LDLr was performed on total membranes while the analysis of SREBP 2 (N-terminus), AMPK, P-AMPK and PP2A was performed on total lysates. Table 1 describes the sources and the antibodies used for the Western blotting assays. Twenty micrograms of protein from solubilized membranes were resolved by 12% (for Insig 1, Insig 2, AMPK, P-AMPK) and 7% (for SREBP 2, LDLr, HMGR and Scap) SDS-PAGE at 100 V for 60 min. The proteins were subsequently transferred electrophoretically onto nitrocellulose for 80 min at 100 V. The nitrocellulose membrane was treated with 3% BSA in 138 mM NaCl, 27 mM KCl, 25 mM Tris-HCl, 0.05% Tween-20 (pH 6.8), and probed at 4°C overnight with primary antibodies, then for 1 h with secondary antibodies. The nitrocellulose membrane was then stripped with Restore Western Blot Stripping Buffer (Pierce Chemical, Rockford, IL, USA) for 10 min at room temperature and reprobed with anti-tubulin antibody (MP Biomedicals, Solon, OH, USA). Bound antibodies were visualized using enhanced chemoluminescence detection (GE Healthcare). All images derived from Western blotting were analyzed with ImageJ (NIH, Bethesda, MD, USA) software for Windows. Each reported value was derived from the ratio between arbitrary units obtained by the protein band and the respective tubulin band (chosen as a housekeeping protein).

Plasma cholesterol analysis

The plasma cholesterol content was assessed through the colorimetric CHOD-POD kit according to manufacturer's instructions (Assel, Rome, Italy). In brief, by the catalysis of cholesterol esterase and cholesterol oxidase, cholesterol ester is catalyzed to yield H₂O₂, which

oxidates 4-Aminoantipyrine with phenol to form a colored dye of quinoneimine. The absorbency increase is directly proportional to the concentration of cholesterol.

Measurement of Reactive Oxygen Species

ROS were detected in liver homogenates by incubating 2 mg protein with 10 μ M dichlorodihydrofluorescein diacetate (DCF; Molecular Probes, Eugene, OR, USA) for 30 min at 37°C in the dark. Samples were then allowed to equilibrate for an additional 60 min. We generally followed the procedures previously published for homogenates and perfused tissues to obtain a overall sense of an increase in oxidative stress (Kehrer and Paraidathathu, 1992; Al-Mehdi et al., 1994). Fluorescence was measured under continuous gentle magnetic stirring at 37°C in a Perkin Elmer LS-50B spectrofluorimeter. Excitation wavelengths were set at 498 nm and emission was set at 530 nm using 5 and 10 nm slits, respectively. A Fenton-type reaction was induced by the addition of 10 μ M FeSO₄ and revealed the capability to continue producing unstable ROS species.

Plasma estrogen analysis

Plasma estrogen content was assessed using the ELISA method in Laboconsult (Rome, Italy).

Statistical analysis

Each mean was derived from four different experiments performed in duplicate. The statistical analysis consisted of ANOVAs followed by the Tukey–Kramer post test. P values under 0.05 were considered statistically significant. All the statistical analyses were performed using GraphPad Instat3 (Graphpad Software Inc., La Jolla, CA, USA) software for Windows.

Results

To analyze the role of HMGR and its regulatory network during estropause, the validity of the experimental model first had to be confirmed, moreover data on 3 month-old male rats to confirm

previous and already published (De Marinis et al., 2008) results obtained on sex-related differences in hepatic regulation of cholesterol homeostasis have been added. Thus, 17- β estradiol levels were analyzed. As shown in Figure 1 (Panel a), the amount of 17- β estradiol decreases significantly in 12-month-old female rats compared to 3-month-old rats. In the attempt to evaluate some of the metabolic parameters of estropausal rats, cholesterol and glucose plasma contents were assessed. As illustrated in Figure 1, a significant increase in cholesterolemia occurred in 12-month-old female rats with respect to 3-month-old rats (Panel b), whereas no differences in glycemia were observed (Panel c).

Because cholesterolemia is dependent upon the presence and functionality of LDLr on hepatocyte membranes, LDLr membrane exposure was evaluated. Figure 2 (Panel a) shows a slight but statistically significant decreased exposure of LDLr on hepatocyte membranes in estropausal rats with respect to 3-month-old female rats. The decreased LDLr exposition, by itself, cannot explain the hypercholesterolemia occurring in 12-month-old female rats, though. So HMGR levels were evaluated to determine whether some variations occurred and combined to induce hypercholesterolemia. Figure 2 (Panel b) illustrates that HMGR protein levels are understood to be higher in 3-month-old males than in female rats, no significant differences were observed between estropausal and 3-month-old female rats, even though a slight increment can be detected.

Although no significant differences in HMGR levels have been found between estropausal and 3-month-old female rats, the increase could suggest the involvement of proteins affecting long-term regulation of HMGR. Thus, the protein levels of Scap (the escort protein for SREBPs), SREBP 2 (the transcription factor able to induce *hmgr* expression), and Insig were evaluated, as illustrated in Figure 3.

The obtained data show no differences in Scap protein levels in 12-month-old rats with respect to the younger controls (Panel a); higher (even though not significant) n-SREBP 2 protein levels in 3-month-old male rats with respect to female counterparts and estropausal rats (Panel b); and no changes in Insig 1 protein levels (Panel c), although lower Insig 2 protein levels were

observed in 3-month-old male rats with respect to their female counterparts and estropausal rats (Panel d).

Taking into account the absence of substantial differences in HMGR protein levels and HMGR regulatory network, it can be gathered that the hypercholesterolemia detected in estropausal rats cannot be attributed to variations in the long-term regulation of HMGR. Thus, our interest has been focused on the short-term regulation of HMGR to determine if hypercholesterolemia in estropausal rats could be due to variations in HMGR activation state. As described above, HMGR activity is regulated by phosphorylation/dephosphorylation: the unphosphorylated enzyme represents the active form of HMGR and the phosphorylated enzyme the inactive one. The enzyme activity was measured in microsomes prepared in the presence or absence of NaF to monitor the total and expressed activity, respectively. The obtained data indicate a higher activation state of HMGR in 12-month-old female rats with respect to younger controls shown both in the table and in Fig. 4, Panel a. Since previous works (Pallottini et al., 2007) have demonstrated that the increased HMGR activity is due to increased ROS content, the reactive oxygen species levels were measured in our experimental rats (data not shown). Although slight variations were observed, none were statistically significant; thus, the increased activation state of HMGR cannot be attributed to ROS content modification.

To determine whether the increased HMGR activity was due to variations in the proteins involved in the rapid phosphorylation/dephosphorylation mechanisms, the AMPK activation state and the PP2A catalytic sub-unit protein levels were measured. AMPK was observed to be lower when phosphorylated and activated in estropausal rats compared with controls (Fig. 4, Panel b), whereas no differences in PP2A were observed (Fig. 4, Panel c).

To determine whether the previously observed variations were ascribable to the estrogen levels falling, 12-month-old female rats were treated with a single pharmacological dose of 17- β estradiol (1 mg/kg). At first, estrogen plasma content was checked in 12-month-old female rats

treated with 17- β estradiol. As shown in Figure 5, estradiol treatment restored the hormone concentrations to the levels observed in 3-month-old female rats.

As such, in the older 17- β estradiol-treated female rats, plasma cholesterol content (Figure 6, Panel a) and LDLr exposure on liver tissue membranes (Figure 6 Panel b) were analyzed. The data indicate that both cholesterolemia and LDLr exposure in treated animals were comparable to the young rats.

Subsequently, the effects of 17- β estradiol treatment on HMGR expressed, total activity and activation state was evaluated (Figure 7). In estradiol-treated estropausal rats, the HMGR activation state results were similar those detected in controls. The 17- β estradiol effects were later evaluated for the proteins involved in short-term regulation of HMGR. As illustrated in Figure 8 (Panel a), treated animals demonstrated a higher phosphorylation and activation state for AMPK compared to 12-month-old female rats, even though AMPK activation was not completely restored (compared to 3-month-old female rats). In addition, 17- β estradiol treatment did not have any observable effect on PP2A protein levels (Figure 8, Panel b).

Discussion

The aim of this work was to evaluate the putative correlation between the variations in the HMGR regulatory network and estrogen declines during the ageing process, at the beginning of female rat estropause, and to determine whether this was related to the occurrence of hypercholesterolemia and the eventual involvement of HMGR in the protective role of estrogen against age-related increases in plasma cholesterol.

We chose, as an experimental model, 12 month-old female rats because only at 12 months is estrogen behavior similar to that occurring in human females at the onset of menopause (Berkley, 2007).

Data obtained from estropausal rats (in which estrogen levels decrease) indicate a higher plasma cholesterol amount and HMGR activation state and a lower level of LDLr membrane

exposure with respect to controls. This event contributes to decreases in cholesterol uptake and a rise in cholesterol synthesis, which supports the correlation between hypercholesterolemia, ageing and estropause. The higher HMGR activation state does not depend on a rise in ROS content, as in aged-match male rats (Straniero et al., 2009), but seems to depend on the lower AMPK activation state observed in 17- β estradiol deficiency (a condition that occurs at the beginning of estropause), thus triggering the HMGR phosphorylation decrease and enzyme activation.

The 17- β estradiol treatment of older female rats restores both proper cholesterolemia and the HMGR activation state, along with LDLr exposure on the plasma membrane. Moreover, AMPK activation results were higher than those in control animals, but activation was not completely restored. However, the increase of the AMPK activation state induced by 17- β estradiol treatment seems to be sufficient to phosphorylate HMGR and to restore its proper activity. The enhancement of estradiol-induced AMPK activation is supported also by other data in the literature (Schulz et al., 2005).

Our results demonstrate that the estradiol decrease occurring at the onset of estropause does not affect the long-term regulation of HMGR, but rather its short-term modulation via AMPK activation-state control. Thus, the existence of a relationship between estrogen changes and cholesterolemia modulation (the latter connected to the HMGR activity) seems to occur in older female rats.

Our work suggests how the protective role played by estrogens on the lipid profile is carried out not only through the increase of HDL and the decrease of LDL plasma content and oxidation -- as previously assessed by numerous papers in the literature (Skafar et al., 1997; Maxwell, 1998, Moorthy et al., 2004)-- but also through the modulation of the AMPK activation state, which, in turn, controls both the normal HMGR activation state and cholesterol synthesis.

Restoration of normal HMGR activation should restore physiological production of cholesterol in cells and, thus, the correct LDLr membrane exposure, depending on cellular sterol content, as described by the classical model generally accepted (Goldstein et al., 2006; Espenshade

and Huges, 2007). In the light of these observations, we can conclude that during the female rat aging process, the diminished AMPK activation state, triggered by estrogen declines at the onset of estropause, can induce hypercholesterolemia. Thus, due to its strict dependence on the AMPK activation state, HMGR can be considered as another mediator of the protective effects of estrogen.

Acknowledgments

The Authors wish to thank past and present members of their laboratories who contributed data and discussions to the ideas presented here. In particular, the invaluable and dedicated work of our mentor Prof. Anna Trentalance is warmly acknowledged.

References

- Abul-Hajj, Y.J., 1978. Stimulation by estradiol benzoate of hepatic beta-hydroxy-beta-methylglutaryl coenzyme A reductase in normal and ovariectomized rats. *Steroids* 31, 841-847.
- Al-Mehdi, A., Shuman, H., Fisher A.B., 1994. Fluorescence microtopography of oxidative stress in lung ischemia-reperfusion. *Lab. Invest.* 70, 579-587.
- Beg, Z.H., Stonik, J.A., Brewer H.B.Jr., 1985. Phosphorylation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase and modulation of its enzymic activity by calcium-activated and phospholipid-dependent protein kinase. *J. Biol. Chem.* 260, 1682-1687.
- Berkley, K.J., McAllister, S.L., Accius, B.E., Winnard K.P., 2007. Endometriosis-induced vaginal hyperalgesia in the rat: effect of estropause, ovariectomy, and estradiol replacement. *Pain* 132, Suppl 1:S150-9.
- Brown, M.S., Goldstein J.L., 1997. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 89, 331-340.
- Bruscalupi, G., Leoni, S., Mangiantini, M.T., Minieri, M., Spagnuolo, S., Trentalance A., 1985. True uncoupling between cholesterol synthesis and 3-hydroxy-3-methylglutaryl-coenzyme A reductase in a early stage of liver regeneration. *Cell. Molec. Biol.*, 31, 365-368.
- Carlson, S.E., Mitchell, A.D., Carter, M.L., Goldfarb, S., 1980. Evidence that physiologic levels of circulating estrogens and neonatal sex-imprinting modify postpubertal hepatic microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *Biochim. Biophys. Acta* 633, 154-158.
- Carnes B.A., Olshansky S.J., Grahn D. (2003) "Biological evidence for limits to the duration of life" *Biogerontology* 4: 31-45
- Choi, Y.S., Goto, S., Ikeda, I., Sugano, M., 1988. Age-related changes in lipid metabolism in rats: the consequence of moderate food restriction. *Biochim Biophys Acta* 963, 237-242.
- De Marinis, E., Martini, C., Trentalance, A., Pallottini, V., 2008. Sex differences in hepatic regulation of cholesterol homeostasis. *J Endocrinol.* 198, 635-643.

- Dietschy, J.M., Turley, S.D., Spady, D.K., 1993. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J. Lipid Research* 34, 1637-1659.
- Distefano, E., Marino, M., Gillette, J.A., Hanstein, B., Pallottini, V., Bruning, J., Krone, W., Trentalance, A., 2002. Role of tyrosine kinase signalling in estrogen-induced LDL receptor gene expression in HepG2 cells. *Biochim. Biophys. Acta* 1580, 145–149.
- Espenshade, P.J., Hughes, A.L., 2007. Regulation of sterol synthesis in eukaryotes. *Annu. Rev. Genet.* 41, 401-427.
- Finch, C.E., Tanzi R.E., 1997. Genetics of aging. *Science* 278, 407-411.
- Friesen, J.A., Rodwell, V.W., 2004. The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductases. *Genome Biology* 5: 248-251.
- Goldstein, J.L., Brown, M.S., 1990. Regulation of the mevalonate pathway. *Nature* 343, 425–430.
- Goldstein, J.L., DeBose-Boyd, R.A., Brown M.S., 2006. Protein sensors for membrane sterols. *Cell* 124, 35–46.
- Grundy, S.M., Cleeman, J.I., Rifkind, B.M., Kuller, L.H., 1999. Cholesterol lowering in the elderly population. Coordinating Committee of the National Cholesterol Education Program. *Arch Intern Med* 159, 1670–1678.
- Gudmundsson, H., Gudbjartsson, D.F., Kong, A.N.T., Gudbjartsson, H., Frigge, M., Gulcher, J.R., Stefansson, K., 2000. Inheritance of human longevity in Iceland. *Eur. J. Hum. Genet.* 8, 743-749.
- Heine, P.A., Taylor, J.A., Iwamoto, G.A., Lubahn, D.B., Cooke, P.S., 2000. Increased adipose tissue in male and female estrogen receptor – a knockout mice. *PNAS* 97, 12729–12734.
- Hewitt, K.N., Boon, W.C., Murata, Y., Jones, M.E., Simpson, E.R., 2003. The aromatase knockout mouse presents with a sexually dimorphic disruption to cholesterol homeostasis. *Endocrinology* 144, 3895-3903.
- Holliday, R., 2000. Ageing research in the next century. *Biogerontology* 1, 97-101.

- Horton, J.D., Goldstein, J.L., Brown, M.S., 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* 109, 1125–1131.
- Janssens, V., Goris, J., 2001. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem. J.* 353, 417–439.
- Kehrer, J.P., Paraidathathu, T., 1992. The use of fluorescent probes to assess oxidative processes in isolated-perfused rat heart tissue. *Free Radic. Res. Commun.* 16, 217–225.
- Kojima, M., Degawa, M., 2006. Gender-related difference in altered gene expression of a sterol regulatory element binding protein, SREBP-2, by lead nitrate in rats: correlation with development of hypercholesterolemia. *J. Appl. Toxicol.* 26, 381–384.
- Korpelainen, H., 2000. Variation in the heritability and evolvability of human lifespan. *Naturwissenschaften* 87, 566–568.
- Marino, M., Distefano, E., Pallottini, V., Caporali, S., Bruscalupi, G., Trentalance, A., 2001. Activation of IP3-protein kinase C- α signal transduction pathway precedes the changes of plasma cholesterol, hepatic lipid metabolism and induction of low-density lipoprotein receptor expression in 17- β -oestradiol-treated rats. *Experimental Physiology* 86, 39–45.
- Marino, M., Pallottini, V., D'Eramo, C., Cavallini, G., Bergamini, E., Trentalance, A., 2002. Age-related changes of cholesterol and dolichol biosynthesis in rat liver. *Mech. Ageing Dev.* 123, 1183–1189.
- Martini, C., Pallottini, V., Cavallini, G., Donati, A., Bergamini, E., Trentalance, A., 2007. Caloric restrictions affect some factors involved in age-related hypercholesterolemia. *J. Cell. Biochem.* 101, 235–243.
- Martini, C., Pallottini V., 2007. Cholesterol: from feeding to gene regulation. *Genes Nutr.* 2, 181–193.
- Maxwell, S.R.J., 1998. Women and heart disease. *Basic Res. Cardiol.* 93, 79–84.

- Messa, C., Notarnicola, M., Russo, F., Cavallini, A., Pallottini V., Trentalance A., Bifulco M., Laezza C., Caruso G.M., 2005. Estrogenic regulation of cholesterol biosynthesis and cell growth in DLD-1 human colon cancer cells. *Scandinavian Journal of Gastroenterology* 40: 1454-1461.
- Moorthy, K., Yadav, U.C., Mantha, A.K., Cowsik, S.M., Sharma, D., Basir, S.F., Baquer, N.Z. 2004. Estradiol and progesterone treatments change the lipid profile in naturally menopausal rats from different age groups. *Biogerontology* 5(6):411-419.
- Moorthy, K., Sharma, D., Basir, S.F., Baquer, N.Z. 2005. Administration of estradiol and progesterone modulate the activities of antioxidant enzyme and aminotransferases in naturally menopausal rats. *Exp Gerontol.* 40(4):295-302.
- Ness, G.C., Chambers, C.M., 2000. Feedback and hormonal regulation of hepatic 3-hydroxyl-3-methylglutaryl Coenzyme A reductase: the concept of cholesterol buffering capacity. *Pro. Soc. Exp. Biol. Med.* 224, 8-19.
- Omkumar, R.V., Darnay, B.G., Rodwell, V.W., 1994. Modulation of Syrian hamster 3-hydroxy-3-methylglutaryl-CoA reductase activity by phosphorylation Role of serine 871. *J. Biol. Chem.* 26, 6810-6814.
- Pallottini, V., Martini, C., Cavallini, G., Bergamini, E., Mustard K.J., Hardie, D.G., Trentalance, A., 2007. Age-related HMG-CoA reductase deregulation depends on ROS-induced p38 activation. *Mech. Ageing Dev.* 128, 688–695.
- Schulz, E., Anter, E., Zou, M.H., Keaney, J.F. Jr., 2005. Estradiol-mediated endothelial nitric oxide synthase association with heat shock protein 90 requires adenosine monophosphate-dependent protein kinase. *Circulation.* 111(25), 473-480.
- Skafar, D.F., Xu, R., Morales, J., Ram, J., Sower J.R., 1997. Female sex hormones and cardiovascular disease in women. *J. Clin. Endocrinol. Metab.* 82, 3913-3918.

- Straniero, S., Cavallini, G., Donati, A., Pallottini, V., Martini, C., Trentalance, A., Bergamini, E., 2009. Stimulation of autophagy by antilipolytic drugs may rescue rodents from age-associated hypercholesterolemia. *Rejuvenation Research*. In press.
- Weber, L.W., Boll, M., Stampfl, A., 2004. Maintaining cholesterol homeostasis: sterol regulatory element-binding proteins. *World Journal of Gastroenterology* 10, 3081-3087.
- Xu, F., Rychnovsky, S.D., Belani, J.D., Hobbs, H.H., Cohen, J.C., Rawson, R.B., 2005. Dual roles for cholesterol in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 14551–14556.
- Yang, T., Espenshade, P.J., Wright, M.E., Yabe, D., Gong, Y., Aebersold, R., Goldstein, J.L., Brown, M.S., 2002. Crucial step in cholesterol homeostasis: Sterols promote binding of SCAP to INSIG 1, a membrane protein that facilitates retention of SREBPs in ER. *Cell* 110, 489–500.

Figure legend

Figure 1. Plasma levels of 17- β estradiol, total cholesterol and glucose in 3-month-old male and in 3- and 12-month-old female rats.

Plasma 17- β estradiol (**a**), cholesterol (**b**) and glucose (**c**) levels measured in 3-month-old male and female rats and in 12-month-old female rats. The data obtained are the means \pm S.D. of four different experiments performed in duplicate. *** = $p < 0.0001$ from ANOVA followed by the Tukey-Kramer test versus 3-month-old female rats.

Figure 2. LDLr and HMGR levels in the livers of 3-month-old male and in 3- and 12-month-old female rats.

The figure illustrates a typical Western blot and densitometric analysis of LDLr (**a**) and HMGR (**b**) protein levels, analyzed in 3-month-old male and 3- and 12-month-old female rat livers. The proteins were resolved via SDS PAGE and levels were normalized to tubulin content (for details see the main text).

*** = $p < 0.0001$ as from ANOVA followed by the Tukey-Kramer test versus 3-month-old female rats.

Figure 3. Scap, SREBP 2, Insig 1, and Insig 2 levels in 3-month-old male and 3- and 12-month-old female rat livers.

The figure shows a typical Western blot and densitometric analysis of Scap (**a**), SREBP 2 (**b**), Insig 1 (**c**), and Insig 2 (**d**) protein levels, analyzed in 3-month-old male and 3- and 12-month-old female rat livers. The protein levels were normalized to tubulin content (for details see the main text). All data presented are the means \pm S.D. of four different experiments, performed in duplicate.

*** = $p < 0.0001$ from ANOVA followed by the Tukey-Kramer test versus 3-month-old female rats.

Figure 4 HMGR, AMPK activation state and PP2A catalytic sub-unit levels in 3-month-old male and 3- and 12-month-old female rat livers.

In Panel **a**, the HMGR activity, table on the top, and activation state are represented. In Panel **b**, the AMPK activation state is expressed as the ratio between the densitometric analysis of P-AMPK and AMPK is shown. Panel **c** illustrates a typical Western blot and densitometric analysis of PP2A protein levels. All experiments were conducted in the livers of 3-month-old male and 3- and 12-month-old female rat. The protein levels were normalized to tubulin content. For details, see the main text. All data presented are the means \pm S.D. of four different experiments performed in duplicate. *** = $p < 0.0001$ from ANOVA followed by the Tukey-Kramer test versus 3-month-old female rats.

Figure 5. Plasma 17- β estradiol levels in 3-month-old, 12 month-old and 17- β estradiol-treated 12-month-old female rats.

The figure illustrates plasma 17- β estradiol levels assayed in 3- and 12-month-old females and on 12-month-old 17- β estradiol-treated female rats. The data presented are the means \pm S.D. of four different experiments performed in duplicate. *** = $p < 0.0001$ from ANOVA followed by the Tukey-Kramer test versus 3-month-old female rats.

Figure 6. Cholesterolemia and LDLr levels in 3-month-old, 12-month-old and in 17- β estradiol-treated 12-month-old female rats.

Panel **a** shows plasma cholesterol levels. Panel **b** illustrates a typical Western blotting and the densitometric analysis of hepatic LDLr protein levels. The experiments were conducted in 3-month-old, 12-month-old and in 17- β estradiol-treated 12-month-old female rats. The data presented are the means \pm S.D. of four different experiments performed in duplicate. *** = $p < 0.0001$ from ANOVA followed by the Tukey-Kramer test versus 3-month-old female rats.

Figure 7. HMGR activity and activation state in the liver of 3-, 12-month-old and 12-month-old 17- β estradiol-treated female rats.

Figure shows the HMGR activity, table on the top, and activation state (ratio between +NaF and -NaF). Bottom is represented a typical Western blotting of HMGR protein levels. The protein levels were normalized to tubulin content. For details, see the main text. All data presented are the means \pm S.D. of four different experiments performed in duplicate.*** = $p < 0.0001$ from ANOVA followed by the Tukey-Kramer test versus 3-month-old female rats.

Figure 8 AMPK activation state and PP2A catalytic sub-unit levels in the liver of 3-, 12-month-old and 12-month-old 17- β estradiol-treated female rats.

Panel **a** represents a typical Western blot and densitometric analysis of the AMPK activation state expressed by the ratio between P-AMPK and AMPK. Panel **b** represents a typical Western blot and densitometric analysis of PP2A catalytic sub-unit levels. Protein levels were analyzed in 3- and 12-month-old females and in 12-month-old 17- β estradiol-treated female rat livers. The protein levels were normalized to tubulin content. For details, see the main text. All the data presented are the means \pm S.D. of four different experiments performed in duplicate.*** = $p < 0.0001$ from ANOVA followed by the Tukey-Kramer test versus 3-month-old female rats. ### = $p < 0.0001$ from ANOVA followed by the Tukey-Kramer test versus 12-month-old female rats.

Fig. 1

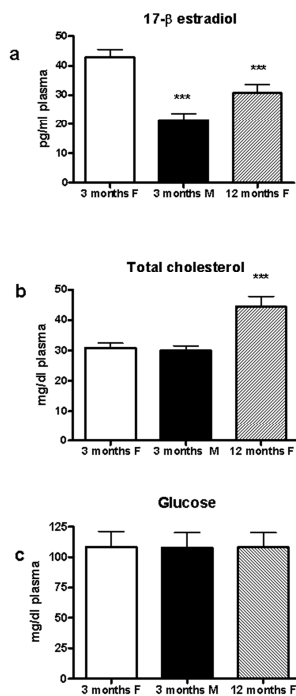
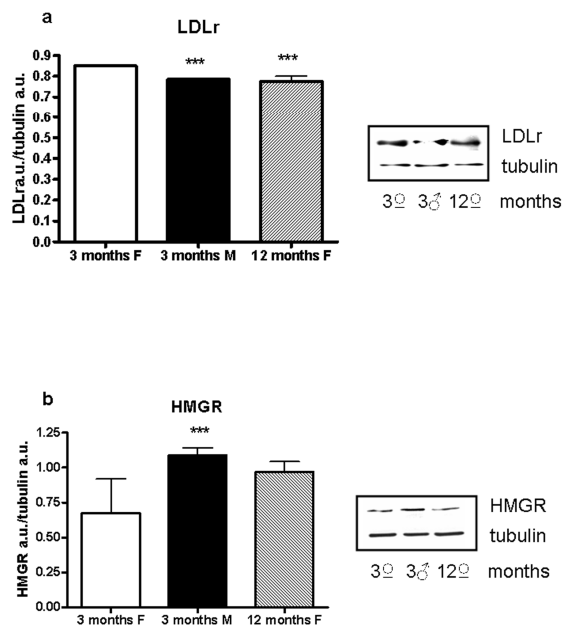


Fig. 2



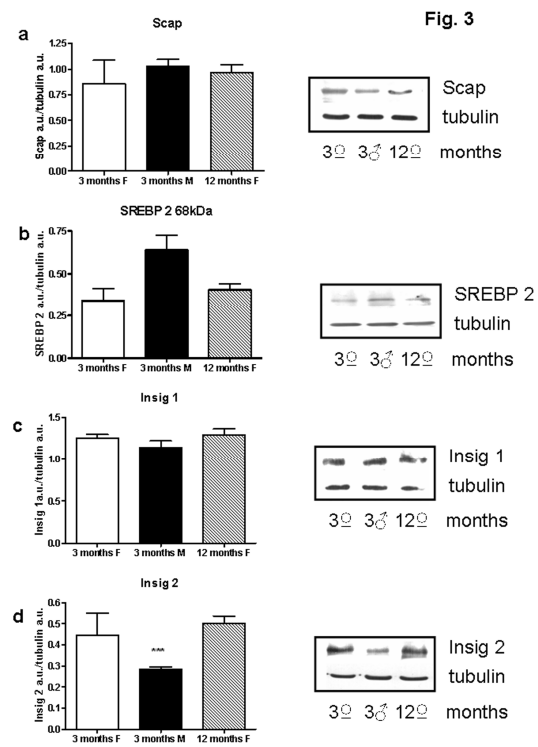


Fig. 4

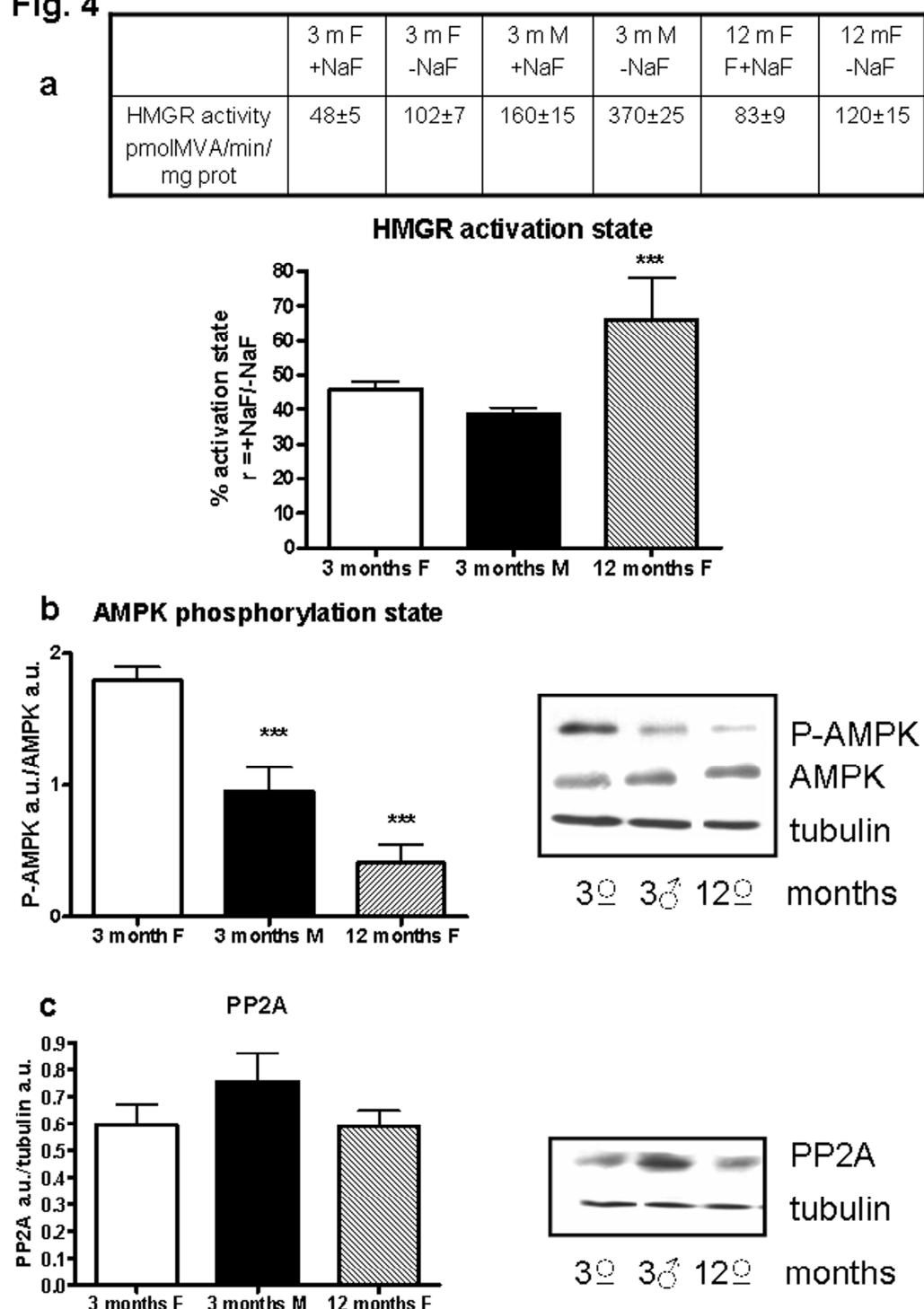


Fig. 5

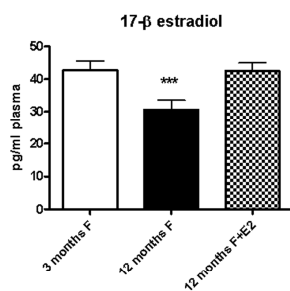


Fig.6

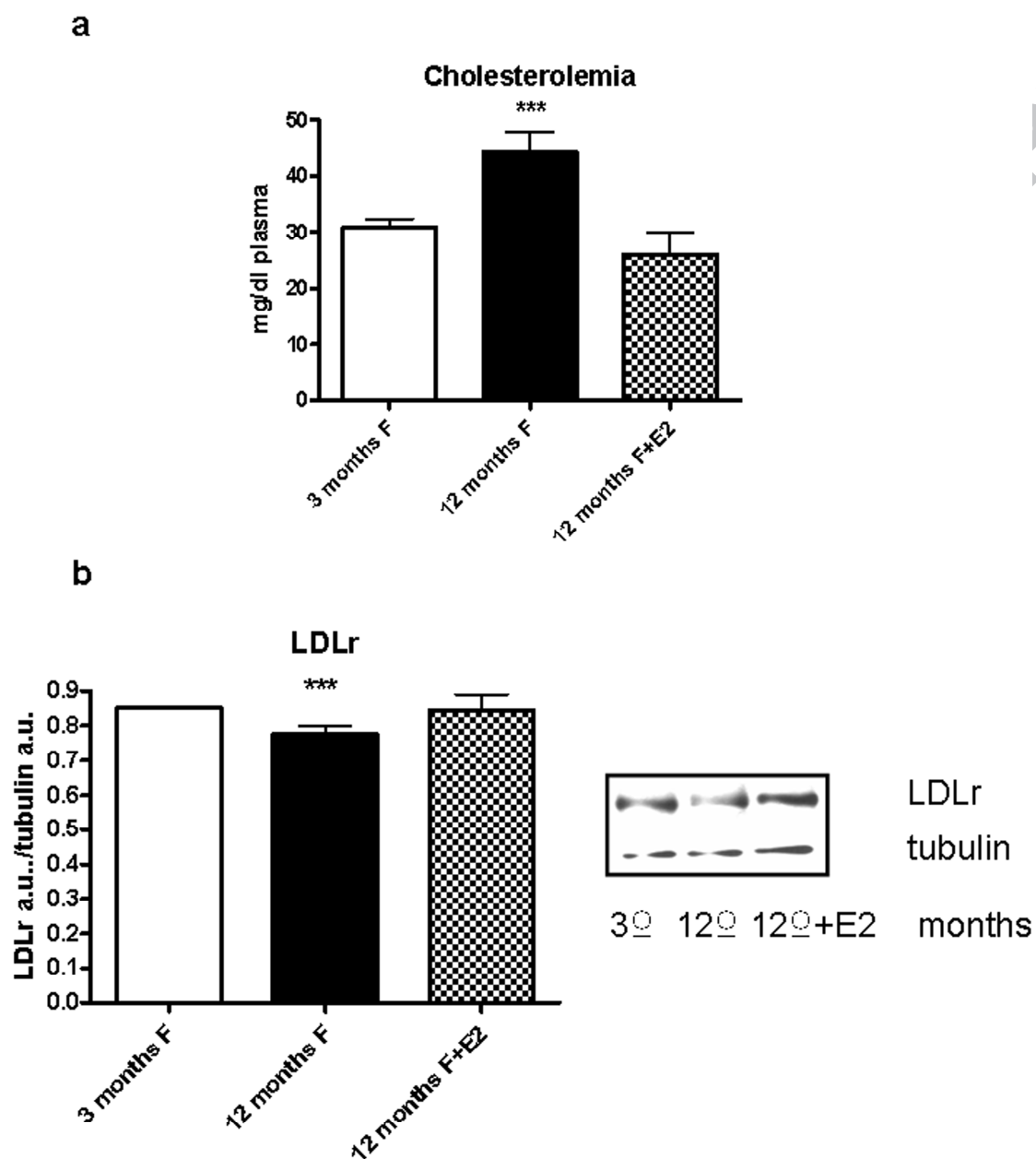


Fig.7

	3 m F +NaF	3 m F -NaF	12 m M +NaF	12 m M -NaF	12 m F+E2 F+NaF	12 m F+E2 -NaF
HMGR activity pmolMVA/min/ mg prot	48±5	102±7	83±9	120±15	50±7	110±9

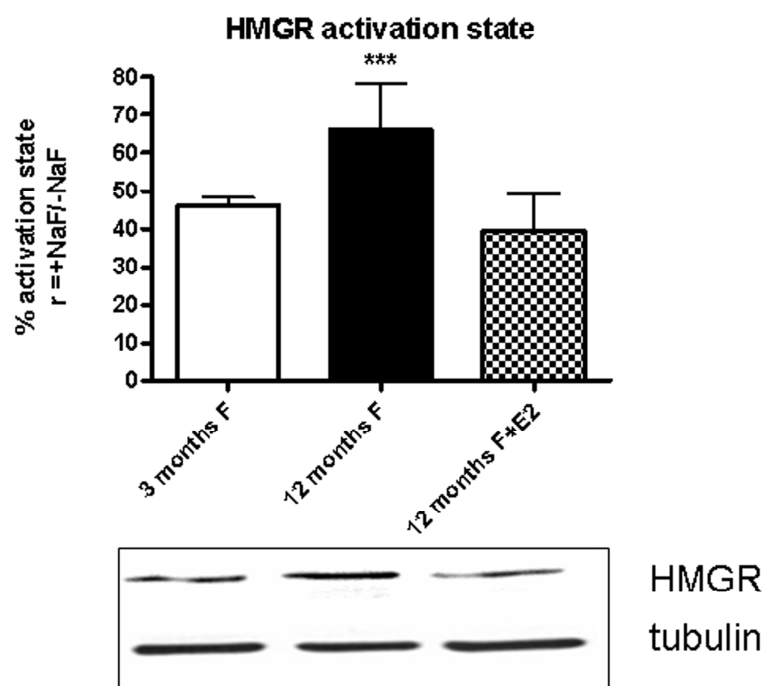


Fig.8

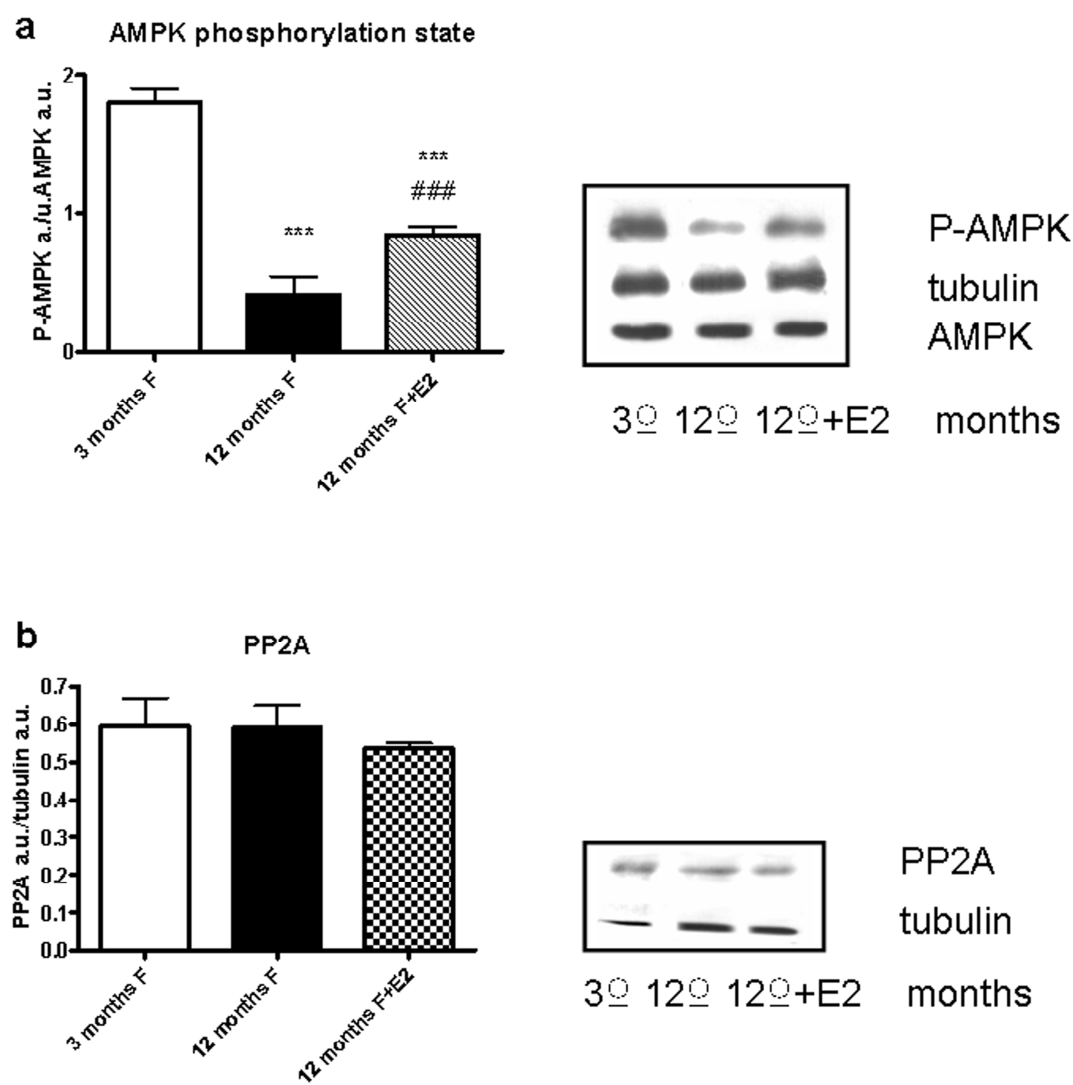


Table 1

List of antibodies used for western blotting assay.

Primary Antibody	Sources	Secondary Antibody	Sources
Anti-HMGR	Upstate, Lake Placid, NY, USA	Goat anti-rabbit conjugated to HRP	UCS Diagnostic
Anti-Insig 1	Novus Biologicals, Littleton, CO, USA	Goat anti-rabbit conjugated to HRP	UCS Diagnostic, Rome, Italy
Anti-Insig 2	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Rabbit anti-goat conjugated to HRP	Chemicon International, Temecula, Canada
Anti-SREBP 2 (N-terminal)	Abcam, Cambridge, UK	Goat anti-rabbit conjugated to HRP	UCS Diagnostic
Anti LDLr	Abcam, Cambridge, UK	Goat anti-rabbit conjugated to HRP	UCS Diagnostic
Anti-tubulin	MP Biomedicals, Solon, OH, USA	Goat anti-mouse conjugated to HRP	UCS Diagnostic
Anti-AMPK α	Cell Signaling technology, Boston, MA USA	Goat anti-rabbit conjugated to HRP	UCS Diagnostic
Anti-P-AMPK α	Cell Signaling technology, Boston, MA USA	Goat anti-rabbit conjugated to HRP	UCS Diagnostic
Anti-Scap	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Rabbit anti-goat conjugated to HRP	UCS Diagnostic
Anti-PP2A	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Goat anti-rabbit conjugated to HRP	UCS Diagnostic