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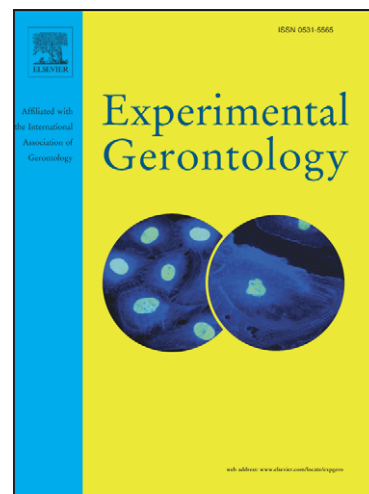
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## **Apolipoprotein E and its role in aging and survival**

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**Short title:** Apolipoprotein E and aging

**Abstract**

The study of biological aging has seen spectacular progress in the last decade and markers are increasingly employed for understanding physiological processes that change with age. Recently, it has been demonstrated that apolipoprotein E (apoE) has a major impact on longevity, but its mechanisms are still not fully understood. ApoE-deficient ( $E^0$ ) mice have proved to be a very popular model for studying spontaneous hypercholesterolemia and the subsequent development of atherosclerotic lesions, but only limited data are available with regard to aging and aging changes. We used this murine model to better characterize the involvement of apoE in aging and to evaluate its role in the maintenance of normal organ morphology. Our results show that  $E^0$  mice at different ages (6, 12, 20 weeks old) developed age-dependent morphological and biochemical alterations, including fibrosis (newly formed collagen), pro-inflammatory cytokine (IL-6 and iNOS), lipofuscin accumulation, and decrease of antioxidant enzymes (superoxide dismutase and catalase) in several organs (kidney, liver and heart). It is significant that the observed degenerative findings in  $E^0$  mice at different ages (6, 12, 20 weeks old) were not identified in control mice (C57BL), at 6, 12 and 20 weeks of age. Consequently, since these mice showed enzymatic and structural alterations, normally linked to the age, such as increase of lipofuscin, pro-inflammatory cytokines and decrease of antioxidant enzymes, we can conclude that apoE is a useful player in studies of longevity and age-related diseases, such as inflammatory status and atherosclerosis that are known risk factors for functional decline and early mortality. Moreover, it is possible that apoE may also play a role in other pathological conditions including, for example, cancer, rheumatoid arthritis and macular degeneration.

KEYWORDS: Apolipoprotein E, aging, liver, kidney, heart

## Introduction

Aging is a multifactorial process and attempts at understanding the fundamental causes of aging are limited by the complexity of the problem (Kirkwood, 2005; Rajawat et al., 2009). However, studying aging processes at the cellular and molecular levels is complicated and subjected to confounding experimental and environmental factors (Dice, 1993). The lack of precise, well defined and reliable cellular and biochemical markers of aging has hindered efforts to identify the primary mechanisms and separate them from secondary effects. Many theories have been proposed about the causes of aging (Weinert and Timiras, 2003) but broadly these can be divided into two main categories (Troen, 2003): the stochastic theories and the genetic theories. The fundamental concept behind the stochastic theories is the progressive accumulation of “damage” that occurs throughout the entire lifespan of cells and tissue (Rattan, 2008). Such damage may result from toxic by-products of routine metabolism or inefficient repair and defensive systems. It is apparent that in long-lived post-mitotic cells, such as neurons and cardiomyocytes, accumulation of lesions may prove more critical and detrimental. The genetic theories consider aging as part of the genetically programmed and controlled events during the lifespan that includes periods of development, maturation, senescence and death (Finch and Tanzi, 1997). The genetic and stochastic theories, however, are not mutually exclusive. Since the aim of this current study has been to consider the contribution of stochastic events in the aging process, further discussion of the genetic theories is not considered.

The stochastic theories speculate that cumulative damage induced by random environmental factors or insults at various levels of an organism represent the main causes of aging. These include damage to DNA or inability to repair, damage to cell organelles and tissues by free radicals of oxygen, protein cross-linking and the limited ability of the cellular turnover mechanisms to remove such damaged structures and molecules. The progressive accumulation of such damage at a level that inhibits or disrupts normal physiological function may lead to progressive aging changes and senescence (Rajawat et al., 2009). Moreover, it has been demonstrated that free radicals or reactive

oxygen species (ROS) accumulate over time causing progressive deterioration in the function of cells, tissues and organ systems, which may ultimately cause the death of cells and damage to a system. An excessive increase in ROS production has been implicated in the pathogenesis of many disorders including: atherosclerosis, cardiovascular diseases, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative and immuno-inflammatory diseases (Roberts and Sindhu, 2009). Moreover, ROS are highly reactive and may modify and inactivate proteins, lipids, DNA, and RNA and induce cellular dysfunctions. To prevent free radical-induced cellular damage, the organism has developed a defense mechanism, the antioxidative system. This system includes antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Melov, 2002).

In order to detect the age-related changes in the biochemical composition of tissues, many markers of aging have been described, for example an increased accumulation of lipofuscin (age pigment) (Florini et al., 1981), increased cross-linking in molecules of the extracellular matrix such as collagen (Bjorksten, 1974) and alteration in pro-inflammatory cytokines (Chung et al., 2002). Accumulated data strongly suggest that continuous (chronic) upregulation of pro-inflammatory mediators (e.g. IL-6, iNOS) are induced during the aging process due to an age-related redox imbalance that activates many pro-inflammatory signaling pathways, including the NF- $\kappa$ B signaling pathway (Chung et al., 2009). Other notable age-related changes involve alterations in the rate of transcription of specific genes, the rate of protein synthesis and turnover and numerous post-translational protein modifications, such as glycation and oxidation (Schneider and Rowe, 1996). These alterations with aging presumably lead to a diminished ability to maintain homeostasis and a subsequent reduced ability to adaptively respond to environmental stimuli or insults (Adelman et al., 1978).

Biomarkers are increasingly employed in empirical studies of human populations to understand physiological processes that change with age, including diseases whose onset appears linked to age, and the aging process itself (Crimmins et al., 2008). Recently, it has been demonstrated that apolipoprotein E (apoE) has a large impact on longevity (Smith, 2002; Ang et al., 2008) even if the

mechanisms involved in aging are not fully understood. In addition, apoE is well known to play a major role in lipid homeostasis, particularly in determination of the levels of low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol, which are directly and inversely correlated with risk for cardiovascular disease, respectively (Smith, 2002).

Numerous murine models are available for studying the aging process. Most of these models are based on known mutations that cause progeroid disease in humans or are involved in DNA repair and cell senescence (Ang et al., 2008). In particular, apoE-deficient ( $E^0$ ) mice, since their introduction in the early 1990s, have proved to be a very popular model for studying spontaneous hypercholesterolemia and the subsequent development of atherosclerotic lesions (Coleman et al., 2006), however, only limited data are reported in regard to successful aging. Moghadasian et al. (2001) observed that 35% of  $E^0$  mice died suddenly after 18 months, whereas Ang et al. (2008) showed that on a high fat diet, these mice, even after 7 months of age, exhibited major pathological disorders including: cachexia, severe cutaneous xanthomas, dramatic loss of subcutaneous fat and low body mass, kyphosis and hypokinesia.

On the basis of the above premises, and in order to evaluate the role of apoE in aging processes, we performed a morphological and biological study on apolipoprotein E knock-out mice at different ages aged 6 to 20 weeks at sacrifice to characterize better the spontaneous changes in key organs such as kidney, liver and heart of  $E^0$  mice. So, we decided to use hematoxylin–eosin and periodic acid Schiff staining for morphological study and biological markers, such as IL-6, iNOS, SOD and catalase since, as above reported, are a useful tool for aging damage.

## **Materials and methods**

### **Apolipoprotein E-deficient ( $E^0$ ) mice**

$E^0$  mice are widely used as animal models for atherosclerosis, since they develop severe hypercholesterolemia on a regular chow diet and the histopathology of their lesions closely resemble those in humans (Plump et al., 1992; Meir and Leitersdorf, 2004). The mice stock

(provided courtesy of Prof. Jan Breslow, Rockefeller University, NY) were bred and housed in the Animal Care Facility of the Rappaport Faculty of Medicine, and the protocols for the study were approved by the Committee for Supervision of Animal Experiments of the Technion-Israel Institute of Technology. Female E<sup>o</sup> mice were divided into six groups: group I (control, 6 weeks old at sacrifice); group II (control, 12 weeks old); group III (control, 20 weeks old); group IV (E<sup>o</sup> mice, 6 weeks old); group V (E<sup>o</sup> mice, 12 weeks old); group VI (E<sup>o</sup> mice, 20 weeks old). The age-matched control mice were C57BL6 and not deficient in apolipoprotein E. Mice were anesthetized with ether using a nasal cone and hearts, kidney, and liver were rapidly dissected, washed briefly in phosphate buffer (0.1M, pH 7.4) and immersion-fixed in 10% neutral buffered formalin, prior to embedding in paraffin wax using routine procedures. 5µm thick serial sections were cut using a microtome. For histopathological evaluation, sections were stained with hematoxylin-eosin, picosirius red and periodic acid Schiff staining. These latters have been made according to Junqueira et al. (1979) and Stacchiotti et al. (2006) respectively. Regarding picosirius red staining, under polarized light microscopy, the type III collagen (constitutive) appeared green, while type I collagen (newly formed) yellow/red (Rizzoni et al., 2006).

### **Immunohistochemical localization**

For the immunohistochemical study, sections were deparaffinized and rehydrated by routine protocol and immersed in 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in methanol for 30 min to quench endogenous peroxidase. Sections obtained from all groups were incubated for 1 h, side by side, at room temperature, in normal serum (diluted 1:5 Dako Cytomation, Glostrup, Denmark). The samples were then incubated with primary antibodies directed against the specific antigens (4 and IL-6, SOD, CAT and iNOS, Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:100 in Tris buffer (0.1M, pH7.4; TBS) overnight. Sections were washed in TBS, then incubated with a biotinylated secondary antibody diluted 1:50 for 1h, and then with the avidin-biotin horseradish peroxidase complex (ABC Kit; Dakopatts, Milan, Italy), prepared according to manufacturer's



instructions for a further 1hr. Sections were immersed in a solution of 0.05% 3,3-diamino-benzidine tetrahydrochloride (DAB) and 0.03% H<sub>2</sub>O<sub>2</sub> for 10 min, then counterstained with hematoxylin, dehydrated, cleared and mounted according to routine protocols.

Control reactions were performed in the absence of primary antibody and in the presence of isotype-matched IgGs. For quantitative analysis, all samples were evaluated at 400 x magnification using an Olympus BX50 optical microscope, (Olympus, Hamburg, Germany), equipped with an image analysis program (Image-Pro Plus<sup>TM</sup>, Media Cybernetics, Silver Spring, MD). The integrated optical density (IOD), resulting from DAB staining intensity, was calculated for arbitrary zones in 10 fields of standard area (0.04 mm<sup>2</sup>/ field) per slide. The DAB staining intensity was calculated using monochromatic (grey) light and the controls were subtracted. Five sections were analyzed for each animal. The data obtained from the different experimental groups were analyzed and scored blindly. Statistical significance of differences between the experimental groups was estimated using ANOVA and Bonferroni tests with  $p < 0.05$  considered significant.

### **Measurement of morphological damage**

For quantitative analysis of morphological damage, visualized by hematoxylin-eosin, the percentage of glomerulosclerosis, tubular number and percentage of polyploid hepatocytes were calculated in 10 fields for each sample by an independent observer, using an optical polarized light microscope at a final magnification of 200 x . Statistical significance of differences between the experimental groups was estimated using ANOVA and Bonferroni testes with  $p < 0.05$  considered significant.

For quantitative analysis of morphological damage, visualized by picosirius red and periodic acid Schiff staining, digital pictures at 40X were taken using an optical microscope (Olympus, Germany) and then analyzed using an image program (Image-Pro Plus<sup>TM</sup> 4.5.1, Milan, Italy) in order to calculate the percentage of stained area. The yellow/red and green fibers, for picosirius staining, and the pink lipofuscins, for periodic acid Schiff, were measured counting ten randomly collected

fields of identical area ( $0.86 \text{ mm}^2$ ) for each section. The data were pooled to represent a mean value and a statistical analysis. Statistical significance of differences between the experimental groups was estimated using ANOVA and Bonferroni tests with  $p < 0.05$  considered significant.

## Results

### Histology and immunohistochemistry in kidney, liver and heart

#### *Kidney*

Kidneys stained by the picrosirius Red method, both cortex and medulla showed very little collagen, distributed throughout anatomical structures. Under a polarized light microscope, these collagen fibers appeared as a green colour indicating the presence of constitutive collagen (Fig.1A). Hematoxylin-eosin staining from control animals (6, 12 and 20 weeks old) showed a similar morphology with normal cytoarchitecture (Fig.1A, inset).

On the contrary,  $E^o$  (6, 12, 20 week old) mice showed interstitial fibrosis, which was more evident in the 20 week old mice (Fig.1B) and glomerulosclerosis, tubular atrophy (Fig.1B, inset). Marked interstitial fibrosis was seen both around tubules and glomeruli in the cortex and around vasa recta in the medulla. The extracellular matrix deposition around glomeruli, tubular structures, and vasa recta were observed under polarized light microscope; this fibrosis showed mainly newly formed collagen indicated by yellow/red fibers. The age-dependent quantitative data are reported in Fig.1C. The tubule number decreases with age (Fig.2A) whereas tubular atrophy increases. The renal morphology is showed in the insets of Figs.2A and 2B. In addition, the percentage of glomeruli showing global glomerulosclerosis increases in  $E^o$  mice from 6 to 20 weeks and it was higher in the subcapsular cortex compared to juxtamedullary zone (Fig.2B). In detail, the cortical changes consisted in tubular and glomerular injury with dilation of the lumen and Bowman's capsule, respectively. Alterations of the medulla showed vasa recta disruption.

IL-6 and iNOS immunostaining showed a similar positivity in the renal cortex and medulla. In detail, IL-6 immunostaining displayed very weak positivity in control animals at different ages

(Fig.3A) and moderate staining in E<sup>o</sup> mice aged 6 weeks. With aging the positivity increased for both the proteins and in E<sup>o</sup> mice (20 weeks old), it become strong (Fig. 3B). In particular, IL-6 and iNOS immunostaining was localized in epithelial cells of tubules.

No iNOS increase and no antioxidant enzyme decrease were found in control animals at different age (Figs.4A, 4B). As regards the antioxidant enzyme (SOD and catalase) analysis, we observed a decrease of their positivity already in 6 week old E<sup>o</sup> animals; the positivity ranged from strong to moderate in animals 20 weeks old (Figs. 4B), both in the cortex and medulla.

### *Liver*

The control animals at different age showed normal cytoarchitecture of hepatic parenchyma. On the contrary, with aging in E<sup>o</sup> mice, the liver showed evident morphological alterations, including disorganization of hepatic parenchyma with widespread cell swelling and congestion of sinusoids, with a significant increase of fatty deposits in hepatocytes. We observed an accumulation of lipofuscin (red stained with periodic acid Schiff method according to Brunk and Terman, 2002) within the hepatocytes linked to age in comparison with age-matched control mice (Fig.5A, 5B). This accumulation was very high in 20 week old E<sup>o</sup> mice. The quantitative data regarding lipofuscin accumulation are reported in Fig.5C. 20 week old E<sup>o</sup> mice liver also showed polyploid hepatocytes and the percentage of these increased by 40% when compared to the control animals (Fig.6).

Picrosirius red staining demonstrated limited perisinusoidal fibrosis in 6 week old E<sup>o</sup> mice, whereas such fibrosis was well evident in 20 week old E<sup>o</sup> mice. Polarized microscopy showed few perisinusoidal yellow/red fibers of collagen in young animals (E<sup>o</sup> mice), whereas more fibers of this collagen were evident in 20 week old mice (data not shown). No fibrosis was evident in control animals at different age (data not shown).

IL-6 and iNOS immunostaining, evident in cytoplasm of the hepatocytes, were scarce in liver of control animals at different age, whereas it increased with age in E<sup>o</sup> mice (Figs.7A, 7B). As

depicted in Figs.4A, 4B, the obtained results for antioxidant enzymes were similar to those observed in the kidney.

### *Heart*

The connective tissue in heart of control animals at different age was thin, well highlighted and green stained under light polarized microscope after Picrosirius Red method (Fig.8A). The parenchyma had a normal morphology with abundant cardiomyocytes and scarce connective tissue (Fig.8A, inset). Degenerative changes were evident in E<sup>o</sup> mice in a time-dependent manner. The connective tissue was clearly increased and the fibrosis, under light polarized microscopy after Picrosirius Red method, was evident around these cardiomyocytes (Fig.8B).The fibers still had abundant cytoplasm as in the control mice (Fig.8B, inset). The age-dependent quantitative data are shown in Fig.8C. Moreover, the fibrosis increase was associated with a decrease in the number of nuclei per myocytes and with accumulation of lipofuscin pigment (data not shown).

In 6 week old E<sup>o</sup> mice, cardiomyocytes showed a weak positivity for iNOS and IL-6. This immunohistochemical pattern changed with ageing showing an increase in immunostaining of these proteins with a strong positivity at 20 weeks of age, very different from control animals as shown in Figs.4A, 9A, 9B. Both of the proteins were immunolocalized in the sarcoplasm of cardiomyocytes. As depicted in Fig.4B, the results for the antioxidant enzymes were similar to those observed in the kidney. No variation in the studied markers was found in control animals at different ages.

### **Discussion**

Our results showed that E<sup>o</sup> mice at different ages had age-dependent morphological and biochemical alterations, such as fibrosis, pro-inflammatory cytokine increase, lipofuscin accumulation and a decrease of antioxidant enzymes in different organs (kidney, liver and heart). It is significant that the observed observations in E<sup>o</sup> mice at different age (6, 12, 20 weeks old) were not identified in age-matched control animals.

The generation of E<sup>o</sup> mice has been one of the most critical advances in the elucidation of factors affecting atherogenesis (Coleman et al., 2006; Dursun et al., 2009). These mice have marked hypercholesterolemia because of deficiency of apolipoprotein E, develop spontaneous atherosclerotic plaques with many features similar to human lesions and allow investigation of atherosclerosis from its earliest stages (Rosenblat et al., 2006). It is currently the most popular murine model in cardiovascular research and has revealed important insights into atherosclerosis (Aviram et al., 2008). This mouse model provides the opportunity to analyze protein changes during various stages of atherogenesis under well-defined laboratory conditions and in animals with identical genetic background, facilitating proteomic comparisons by limiting biological variation (Rodella et al., 2007). Whereas the important role of apoE protein in atherosclerosis has been well characterized, its involvement in longevity and age-related diseases is still not resolved. Although many potential mechanisms, mainly in recent years, have been discussed, much more progress needs to be made, and researchers in this field are still debating whether increasing or decreasing apoE expression would be beneficial in aging (Smith, 2002).

As far as we are aware, there are no morphological data regarding the cytoarchitecture of organs of E<sup>o</sup> mice and few biochemical and molecular findings showing changes in apoE expression linked to age. Our results showing damage in several organs (kidney, liver and heart) could be a useful tool for studying a progressive decline of different cellular functions related to aging.

Regarding renal structural changes that we have found, there are many papers reporting similar age-related alterations. Zhou et al., (2008) showed that renal aging is associated with alterations in renal morphology and a decline in renal function, which is accelerated and/or accentuated by diseases such as diabetes mellitus and hypertension. The histological changes indicate glomerulosclerosis, tubular atrophy and interstitial fibrosis. These features are soundly correlated with aging-related intimal fibrosis of the interlobular arteries, a change that may be accelerated by hypertension and diabetes mellitus (Zhou et al., 2007). In addition, the same authors, in agreement with other researchers (Martin and Sheaff , 2007), showed that, in parallel with a reduction in the number of

viable glomeruli, there is an increase in the number and percentage of glomeruli showing age-related sclerosis. Subcapsular cortical glomeruli are particularly susceptible to sclerosis in comparison to juxtamedullary region; this condition is generally thought to be a non-specific end-stage morphological change resulting from a large range of insults, including ischemia and a wide range of immunological disorders; however, different types of segmental sclerosing lesions have been clearly shown to result from different stimuli. These changes may be interpreted as an attempt to protect against adverse processes influencing the safety of the kidney and reducing its capacity to respond to later insults.

With regard to liver and heart morphology we found an accumulation of lipofuscin and this is a recognized hallmark of aging occurring with a rate inversely related to longevity (Schmucker, 2001; Terman and Brunk, 2005). Lipofuscin is not an inert material but may have a chemically very reactive surface that can disturb cellular metabolism. Lipofuscin inherently causes toxic effects, in part because of its ability to bind metals, such as iron, copper, zinc, manganese, and calcium, in a concentration up to 2%. One of the dramatic effects of lipofuscin is its ability to decrease proteasomal and lysosomal degradation. In using lysosomal enzyme capacity and lysosomal space, it reduces lysosomal degradation capacity (Massey et al., 2006). On the other hand, the proteasome is inhibited by lipofuscin, perhaps as a result of binding proteasomal complexes in unsuccessful attempts of degradation. Moreover, it has been shown by different authors that the lipofuscin-occupied volume reaching maximal values of about 40% in the cytoplasm of aged animal cells and up to 75% in the motor neurons of human centenarians (Szweda et al., 2003). Comparing tissue samples from several organs (heart, liver, skeletal muscle, lung, kidney, cerebellum, and testis) of rats of different ages (2, 11, 29.5 months) showed a low amount of lipofuscin in the youngest animals and significant increases, up to 28-fold, in the oldest ones (29.5 months) (Jung et al., 2007).

It is important also remember that another marker linked to age is the increase, in Apo-E mice respect to controls, of polyploidy hepatocytes. This is in agreement with other results reported

from Sanjeev Gupta (2000) indicating that hepatic polyploidy is an advancement toward terminal differentiation and cellular senescence with greater probabilities of apoptosis.

Another important point of this study has been the evaluation of pro-inflammatory cytokines (IL-6 and iNOS). Their immunolocalization increases in E<sup>0</sup> mice with age, whereas it was very low in control animals and in young (6 week old) E<sup>0</sup> mice. There is accumulating evidence that aging is associated with a pro-inflammatory shift in vascular phenotype (Csizar et al., 2007), kidney (Rodwell et al., 2004), liver (Coban et al., 2008) and heart (Brinkley et al., 2009). We must comment that the presence of IL-6 in epithelial cells could be odd, but this result is in agreement with other data suggesting its expression in progressive renal disease (Zhang et al., 2009). In fact, these Authors proposed that ROS are likely involved in the expression of inflammatory cytokines such as MCP-1 and IL-6 production in renal tubular epithelial cells. Moreover, our results showing the increase of IL-6 expression in cardiomyocytes are consistent with the experiments carried out from Kurdi et al. (2009). Kurdi et al. reported that the mechanical stretch of cardiomyocytes presented an increased expression of IL-6. Increased production of ROS, normally due to a decrease of antioxidant enzymes (i.e. catalase, SOD), as we showed in all studied organs, is thought to be implicated in the inflammatory process by acting as second messenger; the pro-inflammatory cytokines are able to induce an increased degree of interstitial fibrosis and an influx of infiltrating mononuclear cells, suggesting that inflammation plays an important role in age-related pathological alterations in the kidney (Csizar et al., 2007). We showed increased fibrosis, not only in the kidney, but also in the liver and heart probably linked to the age and we propose that these organs show similar age-dependent metabolic changes. In particular, the age-dependent pro-inflammatory cytokines could induce an increased number of fibroblasts that produce extracellular matrix which *inter alia* contains collagen and elastin, important proteins for the structural integrity of the organs. It is possible that with age, the relative amounts of the different proteins produced are changed; the ratio of newly formed to constitutive collagen is shifted towards the fibrotic one and there are also structural changes in elastin resulting in reduced elastic recoil.

In conclusion, our data indicate several important roles of apoE and it is possible to consider this protein very important in the pathology of atherosclerosis, as previously reported, and also as a useful player in longevity and age-related diseases, i.e. inflammatory status that is a risk factor for mortality and functional decline. It is possible that the data presented may not be a direct result of apolipoprotein E deficiency, but rather that of apolipoprotein E induced oxidative stress, with changes in paraoxonases (PONs) and/or the development of atherosclerosis, though further studies would be needed to resolve this point. We consider that further studies of apoE protein could be applied in other areas, included its role, for example, in cancer, rheumatoid arthritis and macular degeneration.

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**Legends to figures**

**Figure 1.** Photomicrographs showing kidney of control (A) and 20 weeks old E° (B) mice. Sirius Red staining and polarization optics (A and B), Hematoxylin-eosin staining (insets). Bar: 10µm. (C) Graph showing the quantitative analysis of collagen content. \*P<0.05 vs. Control. \*\*P<0.05 vs. 6 weeks old E° and control mice.

**Figure 2.** Histograms showing the tubular number (A) and the percentage of glomerulosclerosis (B) in kidney of control and of E° mice 6 and 20 weeks old. \*P<0.05 vs. Control. \*\*P<0.05 vs. 6 weeks old E° and control mice.

**Figure 3.** Photomicrographs showing IL-6 immunostaining in the kidney of control (A) and of E° mice 20 week old (B). Bar: 10µm.

**Figure 4.** Histograms showing quantitative analysis (IOD) of iNOS (A) and endogenous antioxidant enzymes (B) in kidney, liver and heart of control and of E° mice 6 and 20 weeks old. \*P<0.05 vs. Control. \*\*P<0.05 vs. 6 week old E° and control mice.

**Figure 5.** Photomicrographs showing liver of control (A) and 20 weeks old E° (B) mice. PAS staining. Bar: 10µm. (C) Histogram showing the quantitative analysis of lipofuscin content. \*P<0.05 vs. Control. \*\*P<0.05 vs. 6 weeks old E° and control mice.

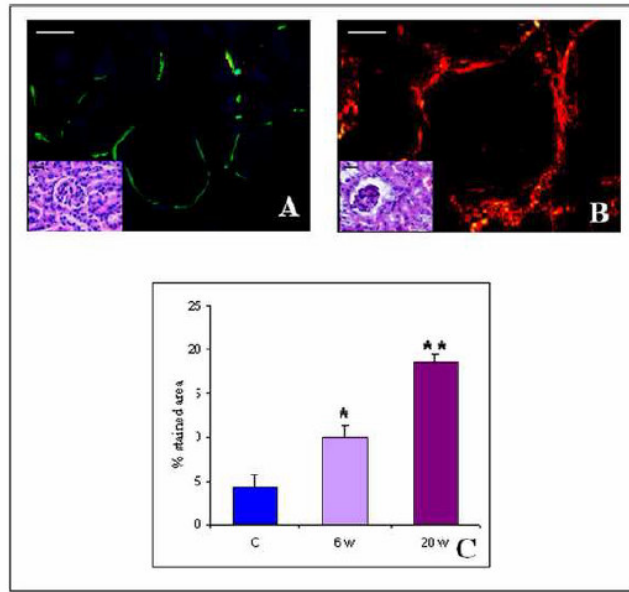
**Figure 6.** Histogram showing percentage of polyploid hepatocytes in liver of control and of E° mice 6 and 20 weeks old. \*P<0.05 vs. Control. Hematoxylin-eosin staining.

**Figure 7.** Photomicrographs showing IL-6 immunostaining in the liver of control (A) and of E° mice 20 weeks old (B). Bar: 10µm.

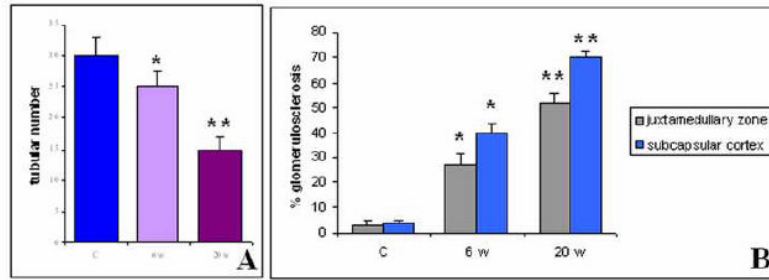
**Figure 8.** Photomicrographs showing heart of control (A) and E° (B) mice 20 weeks old. Sirius Red staining with polarization optics (A and B), hematoxylin-eosin staining (insets). Bar: 10µm. (C) Histogram showing the quantitative analysis of collagen content. \*P<0.05 vs. Control. \*\*P<0.05 vs. 6 week old E° and control mice.

**Figure 9.** Photomicrographs showing IL-6 immunostaining in the heart of control (A) and of E<sup>o</sup> mice (B) 20 weeks old. Bar: 10µm.

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