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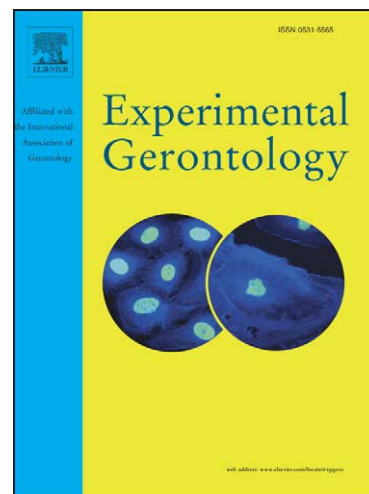
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**EFFECT OF A CALORIC RESTRICTION REGIMEN ON THE ANGIOGENIC CAPACITY  
OF AORTA AND ON THE EXPRESSION OF ENDOTHELIN-1 DURING AGEING.**

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**Abstract**

Ageing is accompanied by impaired angiogenesis, as well as by a deficient expression of several angiogenic growth factors and the alteration of endothelial functions. Caloric restriction (CR) is the only intervention that can extend lifespan and retard age-related-decline functions in mammals by reducing the rate of ageing and the progression of the associated diseases. Herein, we have investigated the effects of ageing and of a caloric restriction regimen (mild or severe) on the angiogenic response and on the expression of endothelin-1 (ET-1) in the aorta of male 3-, 12- or 24-month-old Sprague Dawley rats fed *ad libitum* (AL), fed *ad libitum* and fasted 1 day a week (mild CR) or fasted every other in alternate days (severe CR).

Our findings, using the rat aorta ring assay, show that the angiogenic capacity of aorta decreases with ageing in the oldest rats only. Furthermore, caloric restriction counteracts the age-related changes caloric restrictions actually give raise to a similar recovery. Interestingly, the mRNA ET-1 levels as well as ET-1 expression in aorta sprouting decreases both in middle and in aged animals. Mild and severe caloric restriction regimens prevents ET-1 changes.

## 1. Introduction

Angiogenesis, that is the formation of new vessels from existing vasculature, is a complex process that includes activation, migration, and proliferation of endothelium. The neovascular process is regulated by a wide range of locally released or circulating soluble mediators (D'Amore and Thompson, 1987).

Aging is associated with a progressive deterioration of physiological function that impairs the ability of an organism to maintain homeostasis and, consequently, increases the organism's susceptibility to disease and death (Harman, 2001). Angiogenesis is both delayed and altered with age (Rivard et al., 1999). Diseases such as diabetes, atherosclerosis and cancer can affect vascular function (Williams, 1998; Edelberg and Reed, 2003). In this connection, animal models offer identical genetic backgrounds, controlled environmental exposures and thus a suitable way to define the role of ageing in vascular impairment (Edelberg and Reed, 2003).

Caloric restriction (CR) is an important model to investigate mechanisms of biological ageing. Researches spanning for more than 70 years have shown that a 40% caloric restriction consistently extends the median and maximum lifespan and health span in rodents and perhaps in primates and humans (Roth, 1999; Masoro, 2002; Mattinson et al., 2003) and may counteract age related changes in tissue function (Payne et al., 2003). A milder (10%) regimen of food restriction had significant beneficial effects on animal's longevity, decreased the incidence of neoplastic and non-neoplastic lesions, counteracted the aging-related changes in cell maintenance (autophagy) and biomarker accumulation (dolichol) (Duffy et al., 2004a,b; Parentini et al., 2005). Publications on the effects of a 40% severe CR regimen on angiogenesis are conflicting: CR was reported to increase microvascular density and cerebral blood flow in aged rats (Lynch et al., 1999); to reduce vascular density in peritumoral (but not intratumoral) tissue (Thompson et al., 2004); to have significant anti-angiogenic effects in three distinct brain tumor models (Mukherjee et al., 2004). The effects of milder CR regimens on angiogenesis were not explored.

In the present study we have tested the effects of mild and severe CR regimens on age-related decline in the angiogenic capacity of rat aorta using an *ex vivo* model (Nicosia et al., 1994) that approximates many of the physiologic aorta properties (Nicosia et al., 1994) and it is suitable to quantify spontaneous aorta angiogenesis (Blacher et al., 2001). We also focused on a factor playing a pivotal role on the physiological aorta functions such as endothelin-1 (ET-1) (Marasciulo et al., 2006). ET-1 is in fact the most abundant isoform of a family of small, structurally-related, vasoactive peptides

which is produced by vascular endothelial cells (Yanagisawa et al., 1988),

Taken together our data show that the angiogenic capacity of aorta using an *ex-vivo* model as well as ET-1 levels were significantly reduced in the aorta of 12 and 24 months old rats in comparison to 3.5 months old rats. Furthermore, mild or severe caloric restriction (CR) treatments recover such a capability modulating ET-1 levels in aged animals.

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## 2. Materials and methods

### 2.1. Animals

Male rats of the Sprague-Dawley strain were divided into three groups, 3.5, 12 and 24 month old respectively, which were maintained (a) on standard laboratory food (Teklad, Harlan Italy, S. Pietro in Natisone, Italy) ad libitum (AL); (b) fed ad libitum and fasted 1 day a week (FW); (c) on an every other day (EOD) ad libitum feeding regimen. All rats had free access to water. Food was withdrawn 16 h before experimentation. Rats on the EOD restricted diet were sacrificed on the day of fasting. At the given age, rats were anaesthetized by the intraperitoneal injection of pentobarbital (50 mg/kg body weight) and aorta was quickly excised. In Table 1 is reported the body weight, the food consumption and the survival of young and aged rats (3.5, 6, 12, 18 and 24-month old rats) submitted to AL, FW or EOD regimen.

### 2.2. Rat aorta ring assay

Angiogenesis was studied by culturing aortic explants in three-dimensional matrix gels according to a previous paper (Nicosia and Ottinetti, 1990). Thoracic aortas were removed and immediately transferred to a culture dish containing cold serum-free Dulbecco modified Eagle medium (DMEM, Sigma Aldrich). The periaortic fibroadipose tissue was carefully removed with fine microdissecting forceps and iridectomy scissors paying special attention not to damage the aortic wall. Aortic rings were obtained by cross-sectioning at 1 mm intervals the thoracic aorta (approximately 15 per aorta) and extensively rinsed in 5 consecutive washes of DMEM. The proximal and distal 1 mm of the aorta, which were used to hold the explants with the forceps during the dissection, were discarded. Before embedding the aortic rings in collagen gel, the bottom of each well was coated with 40  $\mu$ l of collagen solution (2mg/ml collagen stock solution extract from tail of rat). The rings were then placed individually on the bottom of 1.2 cm<sup>2</sup> wells (8-well NUNC dishes) with the luminal axis of each ring lying parallel to the bottom of the culture dish. After the collagen solution was allowed to gel for 5 min at 37 °C, 0.3 ml serum-free Endothelial Cell Basal Medium (Clonetics) was added to each culture. The aortic ring/collagen gel preparations were kept in a humidified CO<sub>2</sub> incubator at 37 °C. The medium was changed three times a week starting from day 3. The angiogenic response of aortic cultures was measured in the live cultures by counting the number of neovessels over time. The following criteria were used for counting microvessels: (a) microvascular sprouts were distinguished from fibroblasts

based on their unique morphology; (b) one sprouts generated two new sprouts; (c) each loop was counted as two sprouts since it was usually formed by a process of anastomosis between two converging microvessels.

Collagen was prepared as here described: isolated rat tail tendons was dissolved in 0.5 M acetic acid, shacked for 48 h at 4 °C, dialyzed in 0.1X DMEM (pH 4.0), and stored at 4 °C. The final collagen solution was prepared by mixing on ice 8 volumes of 2 mg/ml collagen, 1 volume of 23.4 mg/ml sodium bicarbonate, and 1 volume of 10X DMEM.

### 2.3. RT-PCR analysis

Total RNA was extracted from aorta using the Rneasy Mini Kit (Qiagen) according to specialized protocol for tissue rich of contractile proteins, connective tissue and collagen. The RNA concentration was determined spectrophotometrically at 260nm. Total RNA (1-5 µg) was reverse transcribed into complementary DNA (cDNA) using eAMV-RT Kit (Sigma); PCR reactions were performed in 50 µl using 200 µM dNTPs (Sigma), 1.5 U of RED-Taq polymerase (Sigma), 1X RED buffer (Sigma), 0.3 µM of specific oligonucleotide primers, and 2 µl of cDNA was amplified. The sequence of the primers, the lengths of PCR products and the annealing temperatures are listed in Table 2. After an initial denaturation at 95°C for 15 minutes, PCR was for 40 cycles with denaturation at 95°C for 30 seconds, annealing at the appropriate temperature for 15 seconds, and extension at 72°C for 30 seconds. Following the final cycle of amplification, 10-minutes incubation at 72°C was performed to ensure that all products were full length. Ten microliters of the RT-PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide, visualized with an ultraviolet transilluminator, and photographed. The expression of beta-actin mRNA was used as housekeeping gene. Finally, to check the amplification of the correct band, all the bands were purified and sequenced.

### 2.4. Immunohistochemistry

Rat aorta were fixed in 10% neutral buffered formalin for 72h and then embedded in paraffin. Consecutive four-micrometer sections were analysed by immunohistochemistry. Briefly, sections were deparaffinized in xylene, rehydrated through graded concentrations of ethanol, and then incubated with hydrogen peroxide to block endogenous peroxidase activity and finally washed in Tris-buffered saline solution. Then, they were heated for 5 min in citrate buffer pH 6.0 (microwaves 700W) and incubated for 18h at 4°C with the primary antibody mouse anti- rat ET-1 (1:200, Affinity Bioreagent). Negative controls were obtained omitting the primary antibody. Sections were then incubated with a biotinylated



anti-mouse secondary antibody (Vector) for 30min at room temperature. Reactions were developed using DAB as chromogene and counterstained with Mayer' hematoxylin. All experimental and control slides were prepared in parallel to avoid technical variability.

### 2.5. Statistical analysis

The ANOVA one-way test was used to determine statistical significance. For all statistical analyses the level of significance was set at  $p < 0.05$ .

GENE	PRIMERS	FRAGMENT SIZE bp	TEMPERATURE OF ANNEALING (Celsius degree)
$\beta$ -ACTIN	Sense 5'-TCCTAGCACCATGAAGATC Antisense 5'-AAACGCAGCTCAGTAACAG	190	47
ET-1	Sense 5'- TTGCTCCTGCTCCTCCTTGAT Antisense 5'-TAGACCTAGAAGGGCTTCCTAGT	119	60

Table 2: primer sequences, cDNA fragment lengths and annealing temperatures used for RT-PCR (for ET-1: Iemitsu et al, 2002).

### 3. Results

Figure 1 (panel A) shows that the capacity of angiogenesis decreased in 24 months old rats (90%,  $p < 0.001$  vs 3.5-months-old rats), but was not modified in 12 months old rats with respect to young ones (Fig.1, A). Panel B of the same figure shows that both EOD and FW feeding enhanced angiogenic capacity in aged animals (45%,  $p < 0.05$  and 63%,  $p < 0.05$  vs untreated animals for EOD and FW, respectively), and caused partial recovery.

Furthermore, Table 3 shows that ET-1 levels in rat aorta decreased significantly both in 12-month (-29%) and 24-month (-41%) aged animals with respect to young once ( $p < 0.001$ ). In contrast, both mild and severe caloric restriction fully prevented the decline in ET-1 aorta mRNA expression in middle and aged animals (Table 3). Accordingly, immunohistochemistry analysis confirmed a decreased level of expression of ET-1 in aged animals (12-month old animals data not shown) with respect to 3-month old rats (Fig.2, panel A). The caloric restriction regimen prevented, in addition, such a decrease (Fig. 2, panel B). On the other hand, no specific correlation between vessel aorta sprouts and ET-1 expression was observed in young animals.

## Discussion

### *Effect of ageing and caloric restriction regimen on rat aorta sprout capacity*

The process of angiogenesis, during which new blood vessels are formed, is impaired during ageing (Edelberg and Reed, 2003). Ageing is a universal process affecting most cells, and concurrent studies *in vitro* have confirmed that the age-related delay in angiogenesis is associated with several deficits in cell functions. The latter include slowed migration of aged microvascular endothelial cells (Reed et al., 2000) and fibroblasts (Reed et al., 2001; Mogford et al., 2002); lower nitric oxide (NO) production and increased sensitivity of endothelial cells to apoptotic stimuli (Vasa et al., 2000; Hoffmann et al., 2001; Chavakis and Dimmeler, 2002); a defective response in aged cells to the mitogenic effects of vascular endothelium growth factor (VEGF) (Rivard et al. 1999) and the anti-proliferative effects of transforming growth factor (TGF)- $\beta$  (McCaffrey and Falcone, 1993).

In the present paper we have investigated the effect of age and anti-ageing CR regimens (mild or severe) on the unstimulated angiogenic capacity of rat aortal. The rat aortic ring model has gained broad acceptance as an angiogenic assay (Zhu and Nicosia, 2002). Our data show that unstimulated angiogenesis is retained by aorta explants from middle-aged rats, and is lost by aortic rings from older rats almost completely.

With regard to the effects of caloric restriction on angiogenesis, no improvement in wound repair, in reduction in wound area over tissue, and in the rate of proliferative fibroblasts and endothelial cells at the wound edge was reported by (Reed et al. 1996) in old 40% CR mice (but beneficial effects were seen in CR and re-fed mice). To our knowledge, the effects of a mild antiageing CR regimen were not studied. Here we show that antiageing CR had a significant beneficial effect on unstimulated angiogenesis in the aorta model, which was quite small, however, unlike most other antiageing effects of CR (e.g. Masoro, 2002), including the effects on ET-1 expression in this paper. The effects of a mild CR on unstimulated angiogenesis were not smaller than those of a severely restricted regimen. This finding should not be surprising, since very low levels of CR were shown to have a significant effect on survival, and the incidence of neoplastic lesions is known to be lower in CR than in AL rats but very similar in 10% and 40% CR rats (Duffy et al., 2004a). On the other hand, severity of chronic heart and kidney diseases was reduced in all CR groups with a significant CR-dependent linear trend (Duffy et al., 2004b). With regard to the effects at the cellular and molecular level, a mild CR regimen had sub maximal beneficial antiageing effects on age-changes in liver autophagy and on tissue accumulation of a biomarker of ageing like dolichol, while severe CR fully prevented the age-related impairment of both parameters (Parentini et al., 2005).

*ET-1 level during ageing and caloric restriction regimen*

In order to gain insight into the molecular mechanism on endothelial cells triggered by ageing and antiageing CR, we have investigated effects on the level of expression of ET-1 under the same conditions. Recently, a decrease in the expression of ET-1 mRNA was found in the aortas of a sedentary aged group compared with the sedentary young group, and change was prevented by exercise training (Maeda et al., 2002). Our data show that the levels of ET-1 decrease during ageing, and may support the conclusions by Maeda et al. (2002). Very interesting, CR, the most effective antiageing intervention, restored the expression of ET-1 to levels detected in young animals (3.5 month old) both in middle aged and in aged rats. CR was shown to reduce several age-dependent degenerative alterations in rat aorta and an antiatherosclerotic action was suggested (Fornieri et al., 1999). To our knowledge this is the first study demonstrating an anti-aging effect of CR on ET-1 expression.

Taken together, our findings show that ageing impairs the angiogenic capacity of rat aorta and ET-1 expression with a different time-lag that CR may counteract change in part and that mild and severe CR regimens may have similar beneficial effects. Loss of endothelial function associated with advancing age may have important clinical implication for the pathogenesis of cardiovascular disease.

In conclusion, our observation that low level CR may exert a significant beneficial effect on aortic endothelium, invites to investigate on an effective mild CR regimen as anti-ageing intervention in spite of an intensive regimen (like EOD).

**Legends to figure**

Figure 1. Panel A. The angiogenic capacity of rat aortic rings. Thoracic aorta was removed by young (3.5-months-old rats), middle (12-months-old) and aged (24-months-aged) untreated animals (AL). Bars showed the number of microvessels  $\pm$  SD counted at 8<sup>th</sup> day in four independent rings obtained by each rat analysed (3 rats for each point).

Panel B. The angiogenic capacity of rat aorta rings isolated by 24-months-old rats untreated (AL) or treated (EOD or FW). The angiogenic response was measured as described in panel A.

Figure 2. Expression of ET-1 in aorta of 3.5- and 24-month old rats (panel A) and in animals submitted to EOD or FW regimen (panel B) by means of immunohistochemistry.

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**Table 1** – Body weight, food consumption and survival data.

	AL	FW	EOD
<b>Body weight (g)</b>			
6 months	529 ± 37.4	514 ± 37.0	451 ± 32.3*
12 months	627 ± 48.4	599 ± 48.2	497 ± 42.5*
18 months	673 ± 45.3	644 ± 38.9	507 ± 47.7*
24 months	633 ± 58.4	621 ± 49.6	508 ± 58.4*
<b>Food intake (g/day)</b>			
6 months	21.6 ± 0.73	20.9 ± 1.28	16.8 ± 0.30*
12 months	23.6 ± 0.54	21.4 ± 0.84	17.6 ± 0.48*
18 months	24.2 ± 1.51	21.1 ± 0.64	16.5 ± 0.45*
24 months	22.3 ± 1.18	19.4 ± 0.49*	16.5 ± 0.94*
<b>Percent survival</b>			
6 months	100%	100%	100%
12 months	100%	100%	100%
18 months	90%	95%	95%
24 months	60%	78%	95%

**Body weight and food intake:** values represent the mean ± SD (four rats for each point); \*p<0.05 with respect to AL group.

Survival was determined analysing four rats for each point.

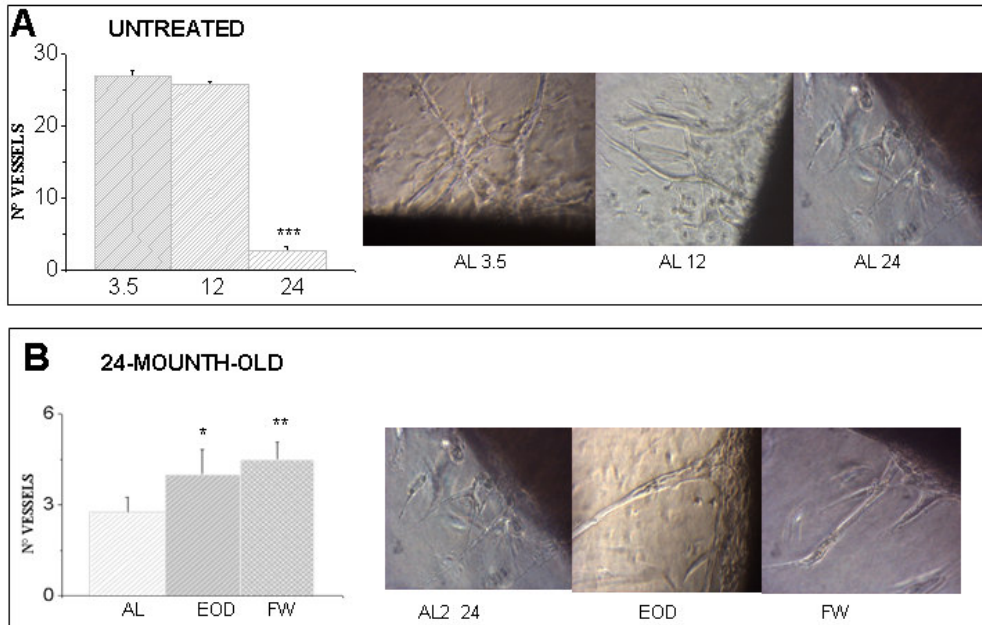
Table 3. Levels of ET-1 mRNA in the aorta 3.5, 12 and 24 month old rats and in immunohistochemistry to EOD or FW regimen.

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	AL	EOD	FW
3.5 month old	0.90±0.04		
12month old	0.65±0.03	1.07±0.02	1.07±0.03
24 month old	0.53±0.02	1.13±0.03	1.08±0.07

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The values are the ratio between the means  $\pm$  S.D. of densitometric value of ET-1 with respect to the densitometric value of beta actin (three independent rats, two samples for each rat).



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Figure 1

A

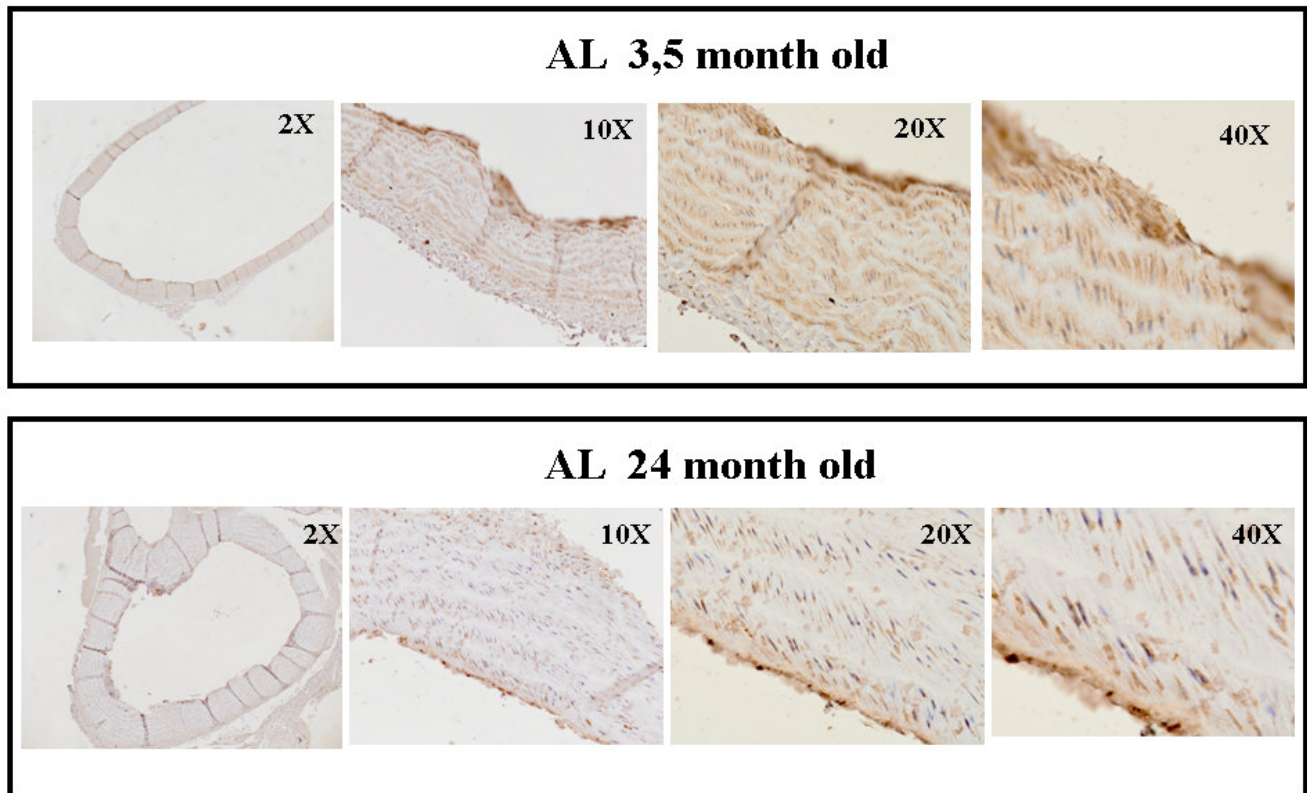
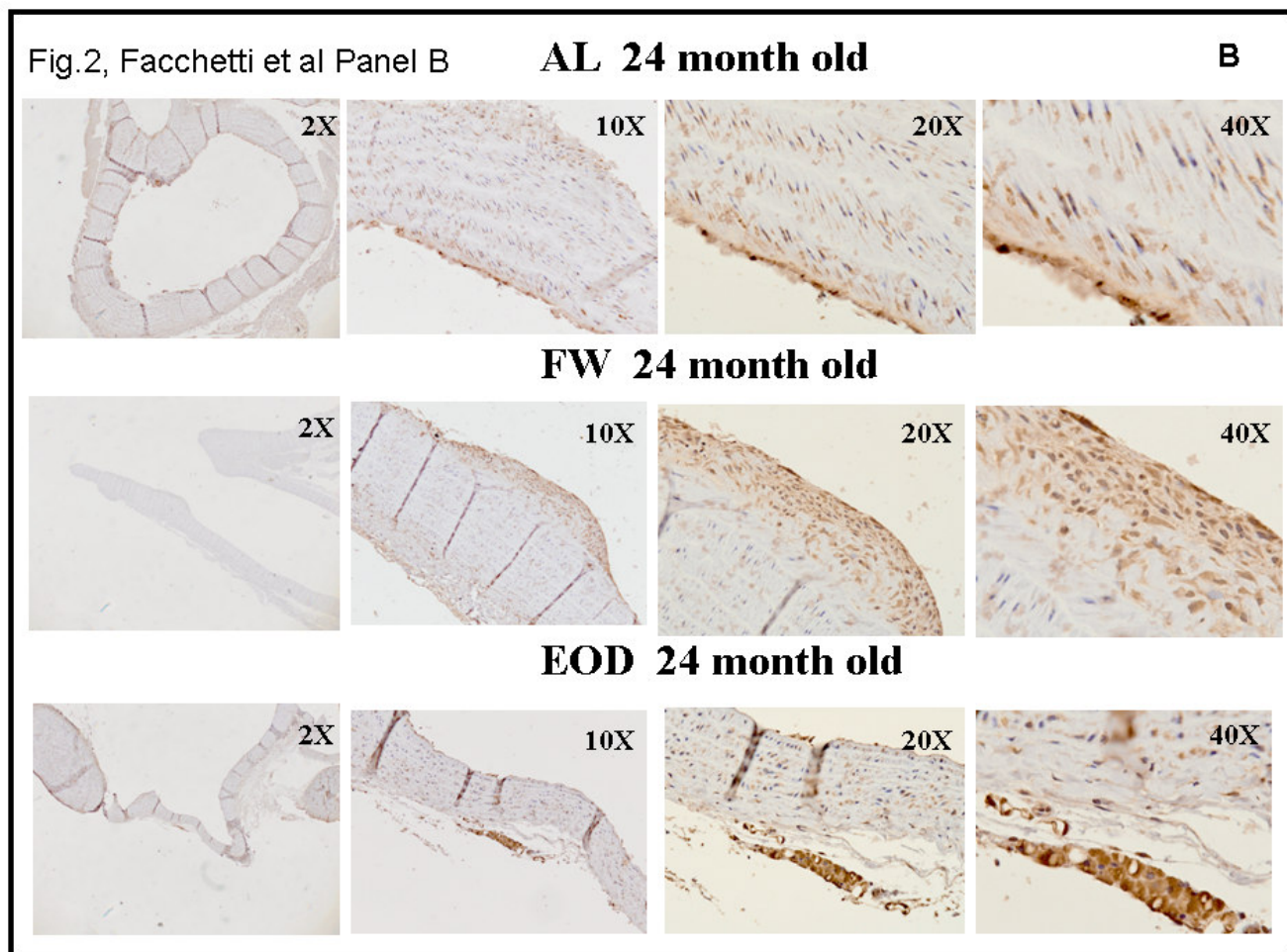


Figure 2 Facchetti et al. Panel A

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