# Retinal Tissue Develops an Inflammatory Reaction to Tobacco Smoke and Electronic Cigarette Vapor in Mice

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# Abstract

Cigarette smoke has been identified as a major risk factor for the development of age-related macular degeneration (AMD). As an alternative of conventional cigarette (C-cigarette), electronic cigarette (E-cigarette) has been rapidly promoted and used globally. The increasing usage of E-cigarette raises concerns with regard to long-term consequences related to retinal tissue. In the present study, a controlled study in mice models was conducted to probe the comprehensive effects of E-cigarette on retina, RPE and choroid tissues by (1) comparing the effect of C-cigarette smoke and E-cigarette smoke on retina; (2) determining the effects of E-cigarette vapor on the RPE and analyzing the changes with regard to inflammatory and angiogenic mediators in retina/RPE/choroid. The data showed that C-cigarette smoke exposure promoted an inflammatory reaction in the retina in vivo. Mice exposed to E-cigarette (nicotine-free) vapor developed inflammatory and angiogenic reactions more pronounced in RPE and choroid, while nicotine-containing E-cigarette vapor caused even a more serious reaction. Both, inflammatory and pro-angiogenic reactions increased with the extension of exposure time. These results demonstrate that exposure to C-cigarette smoke is harmful to the retina. Likewise, the exposure to E-cigarette vapor (with or without nicotine) increases the occurrence and progression of inflammatory and angiogenic stimuli in the retina, which might be similar effects causing the onset of wet AMD in humans.


# Key Messages

- C-cigarette smoke exposure promotes an inflammatory reaction in the retina in vivo
- Mice exposed to E-cigarette (nicotine-free) vapor develop inflammatory and angiogenic reactions more pronounced in RPE and choroid, while nicotine-containing E-cigarette vapor causes even a more serious reaction
- Both inflammatory and pro-angiogenic reactions increase with the extension of E-cigarette vapor exposure time

# Introduction

Age-related macular degeneration (AMD) is one of the leading causes of severe vision impairment among the global population [1]. According to the pathological characteristics, AMD is divided into dry and wet, two types based on the absence or presence of choroidal neovascularization (CNV), respectively [2]. The inflammation and the immune dysregulation play crucial roles in the pathogenesis of AMD. Previous studies have demonstrated that pro-inflammatory cytokines interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) can promote angiogenesis in choroidal neovascular membranes [3,4], and are able to disrupt the structure and function of the outer and inner blood-retinal barrier (BRB) [5-7], leading to the progression of wet AMD. Moreover, inflammatory enzymes, inducible nitric oxide synthase (iNOS), which can produce large amounts of nitric oxide (NO) enduringly, is upregulated in some pathological conditions such as inflammation or in the presence of certain cytokines (TNF, IL etc.); the excessive NO not only causes oxidative stress, but also reacts with superoxide anion radicals forming peroxynitrite,

witch further contributes to vascular damage and promotes the development of AMD or other retinopathies [8]. Additionally, pro-angiogenic cytokine, vascular endothelial growth factor (VEGF), participates in the complex regulation of angiogenesis and vascular permeability, and is the crucial promoter for CNV. However, the impaired VEGF signaling would result in a dysfunction of RPE/Bruch's membrane (BrMb), which is presumably involved in the pathogenesis of dry AMD [9]. In contrast, pigment epithelium-derived factor (PEDF) is an anti-angiogenic and neuroprotective factor. It affects the proliferation and the oxidative stress state of choroidal endothelial cells [10]. A balance between VEGF and PEDF has been demonstrated in the RPE and choroid, and a disruption of this balance would result in pathological angiogenesis [11,12].

Among numerous risk factors of AMD, cigarette smoking is the largest single preventable factor [13]. The epidemiological evidences highly supported the causal association between cigarette smoking and progression of AMD [14]. However, E-cigarette, as an alternative of C-cigarette, its vapor's effects on fundus haven't been reported yet. E-cigarettes are battery-powered devices delivering vapor to the user by heating e-liquid that normally contains solvents, flavoring agents, with or without nicotine [15]. As advertised as "less dangerous" alternatives, the E-cigarettes are supposed to generate less toxicants. Although some aerosol studies revealed that the particles generated from E-cigarette have a significantly lower biological activity than C-cigarette smoke [16,17], the increasing evidences suggest that these ultrafine particles still can induce inflammatory reactions, and the usage of E-cigarette would generate toxicity and induce the release of inflammatory mediators [18-20]. Animal studies also showed that nicotine-containing E-cigarette vapor exposure could increase the pulmonary inflammation and oxidative stress in mice [21,22]. To date, the scientific evidence regarding the health effects of E-cigarette vapor on the retina or fundus is still limited, thus posing a question whether E-cigarette has any adverse effects as similar with C-cigarette. Herein, we assess the effect of C-cigarette smoke and E-cigarette vapor on the retina by molecular investigation on smoke mice model. Furthermore, the effects of E-cigarette vapor on RPE, choroid were determined and the changes with regard to inflammatory and angiogenic mediators in retina/RPE/choroid were analyzed, to evaluate the probable relationship of E-cigarette vapor exposure and the induction of inflammatory angiogenic effects in mice, which might be similar effects leading to the onset of AMD in humans.

## Materials And Methods

The data presented in this paper are part of a large multidisciplinary experimental setup targeted at describing the effect of smoke and vapor exposure on different tissues of the body, in particular the lung. Mice used in the experiments were C57BL/6J from our own colony or directly purchased from Charles River Deutschland, Sulzfeld, Germany. Animals were housed under a 12:12 h, light-dark cycle and food and water supply ad libitum during experiment. Overview of the experimental groups is displayed in Fig. 1.

## Experimental Animals and Smoke Exposure

Retina tissue was harvested in the C-cigarette exposure groups and E-cigarette short exposure subgroup. In E-cigarette medium and long-term exposure subgroups, all the retina, RPE and choroid were harvested. In each group, retinae were pooled in order to perform the different assays presented in this paper. In the E-cigarette setup, mice were exposed to the vapor of nicotine-free liquid or liquid containing 18 mg/ml nicotine. The only exception is the long-term exposure time-point, where mice were exposed only to vapor from nicotine containing liquid. Age-matched mice that were kept under identical conditions without cigarette-smoke or -vapor exposure were used as corresponding controls.

# C-cigarette smoke exposure

Male C57BL/6J mice (12 ± 4 weeks old) were divided into two subgroups with different exposure time (Fig. 1a). Whole body exposure to the mainstream smoke of 3R4F cigarettes (Kentucky Tobacco Research and Development Centre, USA, containing 0.7 mg nicotine per cigarette) generated by a smoke generator (Burkhart, Wedel, Germany) was done as previously described [23,24]. The particle concentration was adjusted to 200 mg/$m^3$ and mice were exposed for 6 hours per day, 5 days per week for a period of 3 (medium-term) or 8–12 (long-term) months [25]. Mice in the control group remained in the room air environment, while the other conditions were similar to the corresponding experimental group.

# E-cigarette vapor exposure

Male C57BL/6J mice (12 ± 4 weeks old) were exposed in a whole-body manner to E-cigarette vapor for 6 hours per day, 5 days per week, using inExpose inhalation exposure system (SCIREQ, Montreal, Canada). The E-cigarette liquid is vaporized at 2 puffs per minute (2 sec evaporation per puff) and hence 720 puffs in total for one day (6 hours). There are three different subgroups in the E-cigarette vaping setup (Fig. 1b) with different exposure time of 2 weeks, 3 months, 8–12 months as short, medium and long-term subgroups respectively. In order to evaluate the effect of nicotine on retina/RPE/choroid, both the short and medium subgroups were further divided into two independent vaping subgroups using different kind of E-cigarette liquids (nicotine-free liquid and liquid containing 18 mg/ml nicotine) without flavoring reagents (Avoria GmbH, Nuremberg, Germany).

# Retina, RPE, choroid/scleral dissection

Following euthanasia, the eyes were enucleated and placed in 1×PBS buffer on ice immediately. The retina was removed carefully by cutting the optic nerve. Dissection and isolation of RPE and choroid/scleral were done according to Wei et al. [26]. Tissue was harvested and immediately stored at –80 °C until further use. All retinae were pooled per subgroup in order to obtain sufficient material to perform all assays (see below).

# Protein extraction and enzyme-linked immunosorbent assay (ELISA)

Proteins were isolated using AllPrep RNA/Protein Kit (80404, QIAGEN, Germany) and used to quantify the levels of cytokine IL-1β and VEGF, which were analyzed by mouse IL-1beta/IL-1F2 DuoSet and mouse VEGF DuoSet ELISA kits (R&D Systems), and levels of PEDF, iNOS, and TNF-α proteins by using Mouse Pigment epithelium-derived factor (PEDF) ELISA Kit (KTE70449, Abbkine, China), Inducible Nitric Oxide Synthase (iNOS), ELISA Kit (MBS030771, My BioSource, USA) and mouse TNF-α DuoSet ELISA kits (R&D Systems) respectively. All assays were performed according to related manufacturer's protocol.

# Data statistics

Values were expressed as mean ± SD (standard deviation). One or two-way analysis of variance (1 or 2 way-ANOVA) or multiple t tests were used to determine statistical significance. All statistics were carried out using GraphPad Prism 7 (GraphPad Software Inc, SanDiego, CA, USA), P value less than 0.05 was defined as statistically significant.

## Results

# The effect of C-cigarette smoke or E-cigarette vapor exposure on the retina

In medium-term C-cigarette smoke exposure subgroup, the level of pro-inflammatory cytokines TNF-α (Fig. 2c) was reduced in retina. The level of anti-angiogenic cytokines PEDF (Fig. 2e) slightly increased, and the Ratio of VEGF *vs.* PEDF (Fig. 2f) decreased by about 40% compared to control mice. However, after long-term exposure, only the level of IL-1β (Fig. 2a) is higher in C-cigarette smoke exposure mice than control mice (about 1.8 folds).

In short-term E-cigarette vapor exposure groups, the expression of iNOS (Fig. 2h) in nicotine-free subgroup was increased, while the levels of TNF-α (Fig. 2i) and Ratio of VEGF *vs.* PEDF (Fig. 2l) decreased significantly. Results from nicotine-containing E-cigarette subgroup showed a decresed level of TNF-α (Fig. 2i) and down-regulated VEGF over PEDF (Fig. 2l) in vapor exposure mice than the control mice.

In medium-term exposure groups, the level of iNOS (Fig. 2h,) and Ratio of VEGF *vs.* PEDF (Fig. 2l) are increased significantly in nicotine-free E-cigarette subgroup. In contrast, the data from nicotine-containing subgroup indicated that the expression of IL-1β (Fig. 2g) decreased, but TNF-α (Fig. 2i) increased significantly. There is no significant difference between nicotine-containing subgroup and control mice for the Ratio of VEGF *vs.* PEDF, though with a higher level of PEDF in vapor exposure subgroup.

Interestingly, long-term nicotine-containing E-cigarette vapor exposure reduced the expression of TNF-α (Fig. 2i) in retina, and down-regulated the Ratio of VEGF *vs.* PEDF (Fig. 2l) as well.

# Comparing the effect of C-cigarette smoke with E-cigarette vapor (nicotine-free or nicotine-containing) on retinal tissue

In medium-term exposure groups, the expression of pro-inflammatory mediator TNF-α (Fig. 3c) proteins in retinal tissues from mice exposed to C-cigarette smoke was decreased by 27%, along with a decreased ratio of VEGF *vs.* PEDF (Fig. 3f) by 38%. Surprisingly, most of the investigated factors in the nicotine free E-cigarette subgroup changed in an opposite trend with C-cigarette subgroup, except of TNF-α (Fig. 3c). In the nicotine-containing E-cigarette subgroup, the expression level of IL-1β (Fig. 3a) decreased by 44% after exposure, while TNF-α (Fig. 3c) increased by 103%. Besides, the regulation trends of iNOS, VEGF and PEDF are similar to the C-cigarette subgroup.

In long-term exposure group, the changes trend in C-cigarette exposure subgroup are similar to the medium-term group. The most significant change is the upregulated IL-1β protein by 81% (Fig. 3a), while the change of the Ratio of VEGF *vs.* PEDF (Fig. 3f) is less. In nicotine-containing E-cigarette group, IL-1β and TNF-α were both down-regulated by 10% and 30% respectively, while the iNOS was up-regulated by 17%. In addition, the expression levels of VEGF (Fig. 4d) and PEDF (Fig. 3e) were increased simultaneously, giving the decreased Ratio of VEGF *vs.* PEDF (Fig. 3f) by 17%.

# Comparing the different effects of medium- and long-term exposure of E-cigarette vapor on RPE and choroid.

In RPE, after medium-term nicotine-free E-cigarette vapor exposure, the protein levels of IL-1β (Fig. 4a), iNOS (Fig. 4b), and VEGF (Fig. 4d) were up-regulated, and the Ratio of VEGF *vs.* PEDF (Fig. 4f) increased significantly as well. Simultaneously, the data from nicotine-containing subgroup indicated that the levels of IL-1β, TNF-α (Fig. 4c) increased significantly. The increasement of the Ratio of VEGF *vs.* PEDF is more significant than nicotine-free subgroup. In long-term nicotine-containing exposure subgroup, the changes of protein levels of pro-inflammation mediators are similar but less significant than that from medium-term exposure subgroup, and the Ratio of VEGF *vs.* PEDF was not significant anymore.

In choroid, after medium-term nicotine-free E-cigarette vapor exposure, the levels of IL-1β (Fig. 4a), TNF-α (Fig. 4c), and VEGF (Fig. 4d) all increased significantly. The data from nicotine-containing subgroup showed a slightly increase of IL-1β, and a significantly decreased Ratio of VEGF *vs.* PEDF (Fig. 4f). In addition, long-term exposure of nicotine-containing E-cigarette vapor resulted in higher levels of IL-1β, iNOS and Ratio of VEGF *vs.* PEDF in choroid.

# The comprehensive effects of long-term exposure of E-cigarette (nicotine-free vs nicotine-containing) vapor on retina, RPE and choroid.

Mice that were exposed to nicotine-free E-cigarette for medium-term, only the expression of iNOS (Fig. 5b) increased obviously in retina. In RPE tissue, the Ratio of VEGF *vs.* PEDF (Fig. 5f) increased by 219% surprisingly. However, the regulation trends of mediators in choroidal tissue are different from retina and RPE, in which, the levels of IL-1β (Fig. 5a) and TNF-α (Fig. 5c) were increased by 298% and 398% dramatically.

In nicotine-containing E-cigarette subgroup of medium-term exposure, the level of TNF-α (Fig. 5c) increased by 103% in retina. In RPE tissue, the levels of IL-β (Fig. 5a) and TNF-α (Fig. 5c) dramatically increased by 421% and 113% respectively. Besides, the Ratio of VEGF *vs.* PEDF (Fig. 5f) significantly increased by 445%. However, the change of cytokines levels in choroid was not as obvious, wherein, only IL-β (Fig. 5a) was upregulated by 130%.

In long-term nicotine containing E-cigarette vapor exposure subgroups, the change of cytokines levels was not significant in both retina and RPE. However, in choroid, the level of VEGF (Fig. 5j) was up-regulated by 128%, accompanied with the down-regulated level of PEDF (Fig. 5k) by 29%, resulting in a dramatically increased Ratio of VEGF *vs.* PEDF (Fig. 5l) by 221%.

## Discussion

Most of the reported studies about the association of smoking with AMD are mainly focused on the effect of smoking on RPE and scarcely on the retina. In the present study, the effect of smoking towards the retina, RPE and choroid tissue was investigated by studying the data for different cytokines after medium- and long-term exposure to C-cigarette smoke, as well as short-, medium-, and long-term exposure to nicotine containing or nicotine-free E-cigarette vapor. With regard to E-cigarette vapor, the medium and long-term exposure were conducted to simulate the human's vaping habits and to investigate the association of E-cigarette vapor with increased inflammation and angiogenesis. The short-term exposure was performed as well to assess the immediate adverse effects of E-cigarette vapor on retina.

Our results indicate that after short-term exposure to E-cigarette vapor (both nicotine-free and nicotine-containing), in retina tissue, the anti-angiogenic pathway is activated and no significant change occurred with regard to the expression of pro-inflammatory mediators. This suggests that short-term exposure to E-cigarette vapor only has limited pro-inflammatory effect on retinal tissue.

The pathogenesis of the two types of AMD are not alike. Dry AMD is assumed to begin with RPE dysfunction followed by the dysfunction/loss of photoreceptors and choriocapillaris. In wet AMD, it probably starts with the dysfunction/loss of choroidal vasculature alone or with RPE layer together,

followed by the accumulation of pro-inflammatory mediators in choriocapillaris, and the subsequent production of excessive angiogenic substances by RPE because of hypoxia, which will result in angiogenesis from the choroidal vessels into the retina (CNV), and photoreceptor loss [27,28].

In the present study, the comprehensive results from retina, RPE and choroid after medium-term exposure to E-cigarette vapor revealed that both nicotine-containing and nicotine-free E-cigarette vapor could stimulate the expression of pro-inflammatory and angiogenic mediators and accumulate in the RPE and choroidal tissues. This is likely the cause that leads to an inflammatory response in these tissues and induces CNV. Interestingly, such results are in line with the pathogenesis of wet AMD as mentioned above, which suggests that even without nicotine or flavoring agents, E-cigarette vapor only derived from basic solvents (propylene glycol, vegetable glycerin) still can promote the occurrence and progression of wet AMD. In addition, the adverse effects, which are stimulated by nicotine-containing E-cigarette vapor on the RPE are stronger than that by nicotine-free one. As the only difference between these two E-cigarette vapors is the nicotine component, it further corroborates the notion that the nicotine component enhances the harmful effect of basic solvents on RPE and choroid. As shown in Fig. 5f, the relative change of the Ratio of VEGF *vs.* PEDF in the nicotine-containing subgroup (445%) is about 2 times higher than the value in the nicotine-free subgroup (219%). This result is consistent with the experimental study by Pons and colleagues, wherein they confirmed that nicotine could increase the Ratio of VEGF *vs.* PEDF by combining with nicotinic acetylcholine receptors in RPE, which is critical in the development of wet AMD for second-hand smokers [29]. Previous studies have demonstrated that nicotine is an agent with pro-angiogenic effect and can stimulate the proliferation of CNV [30], and the impact of nicotine on the expression of pro-angiogenic and inflammatory mediators has also been reported [31]. All these reported findings could rationalize our results of nicotine-containing E-cigarette vapor exposure, that even the e-liquid vapor which only contains the basic solvents can stimulate the expression of pro-inflammatory and angiogenic mediators in RPE, as well as pro-inflammatory mediators in choroid. The study by Bekki and colleagues showed that heating the basic solvents could generate some carbonyl compounds such as formaldehyde, acetaldehyde, acetone, acrolein and so on [32]. Other studies suggested that acrolein also exists in cigarette smoke, which could potentially induce inflammatory reaction from macrophages and epithelial cells [33,34]. Based on these reports, we speculate that the acrolein or other relative carbonyl compounds in the vapor from solvents induced the expression of pro-inflammatory mediators from RPE cells and the choroidal capillary endothelial cells or some unique inflammatory cells. Nevertheless, further longitudinal studies are needed to unveil how the nicotine free E-cigarette vapor causes angiogenesis.

By comparing the data from medium-term to long-term E-cigarette vapor exposure, we observed that the predominantly impaired tissues changed from RPE and choroid to choroid alone. Moreover, the levels of VEGF and PEDF decreased dramatically in RPE, and turned even lower than in related control mice. According to the pathological progression of wet AMD, it is assumed that with the extension of exposure period, the damage of RPE might become more serious, and the RPE layer becomes dysfunctional or even apoptotic/necrotic, leading to a decreased ability to produce pro-inflammatory and angiogenic factors. On the other hand, due to the increasing loss of choroidal blood vessels, the hypoxia of choroidal capillaries becomes more serious [35], which would stimulate the choroidal capillary endothelial cells to

produce more angiogenic substances like VEGF, and hence the expression of antiangiogenic mediator PEDF decreases, promoting the generation of CNV in lesions at the RPE/retina interface, which is a hallmark sign in the development of AMD in humans.

To date, the exact mechanism of cigarette smoking on retina/RPE/choroid is still unknown. Nevertheless, some reviews have summarized that cigarette smoking can increase the oxidative stress burden and hence induce the inflammation response on RPE and choroid, causing the damage to retina/RPE. Furthermore, impairing the choroidal blood flow and decreasing the perfusion pressure could result in hypoxia and promote angiogenesis, and eventually cause the development of AMD [36-38]. Another review elaborated that E-cigarette vapor exposure not only disrupt pulmonary homeostasis but also can increase inflammatory response and oxidative stress via airway [39]. Although there are no specialized studies regarding the mechanism of E-cigarette vapor on the retina, based on the respiratory system studies, we assumed that the vapor could also cause an inflammatory response and oxidative stress to the fundus similar to C-cigarette smoke.

With regard to limitations of our study, it should be considered that whole-body exposure was performed in this mouse model. The anterior tissue of the ocular surface such as cornea as well as skin surrounding the eye have been exposed to the smoke or vapor, which might have an impact on our results. However, anterior/posterior diffusion of molecules in the eye is not easily possible due to the presence of physiological barriers. Therefore, since this exposure is also similar to human smoke/vapor exposure, it should not interfere significantly with the data presented here. A second limitation is the relatively low number of eyes analyzed in some of the subgroups (E-cigarette, long-term exposure). This renders the interpretation of the respective data less. However, these initial data on a small number of animals provide a first view on these yet under-investigated mechanisms and pave the way for further experiments with an increased number of animals.

In conclusion, by providing molecular experimental evidences, our study demonstrated for the first time that exposure to E-cigarette vapor (with or without nicotine) induces the occurrence and progression of inflammatory and angiogenic effects in the retina. The nicotine component in vapor likely enhances the harmful effect of the basic humectants. In addition, with the extension of exposure period, nicotine-containing E-cigarette vapor further increases the likelihood of the generation of pathologic effects similar to those of wet AMD.

# Declarations

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**Conflicts of interest** The authors declare no conflict of interest.

**Availability of data and material** The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Code availability** Not applicable.

**Authors' contributions** Conceptualization, K.S. and N.W.; methodology, F.W., S.H., E.T.; B.F., A.J., T.W.; validation, K.S., N.S., S.H.; formal analysis, K.S., F.W., B.L., S.L; data curation, F.W.; writing-original draft preparation, F.W., K.S., S.H.; writing-review and editing, K.S., B.L., S.L., N.W..; visualization, F.W.; All authors have read and agreed to the published version of the manuscript.

**Ethics approval** All animal experiments were approved by the local ethics committee for animal welfare (Regierungspräsidium, Giessen, Germany, GI 74/2016) and performed in accordance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

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# Figures

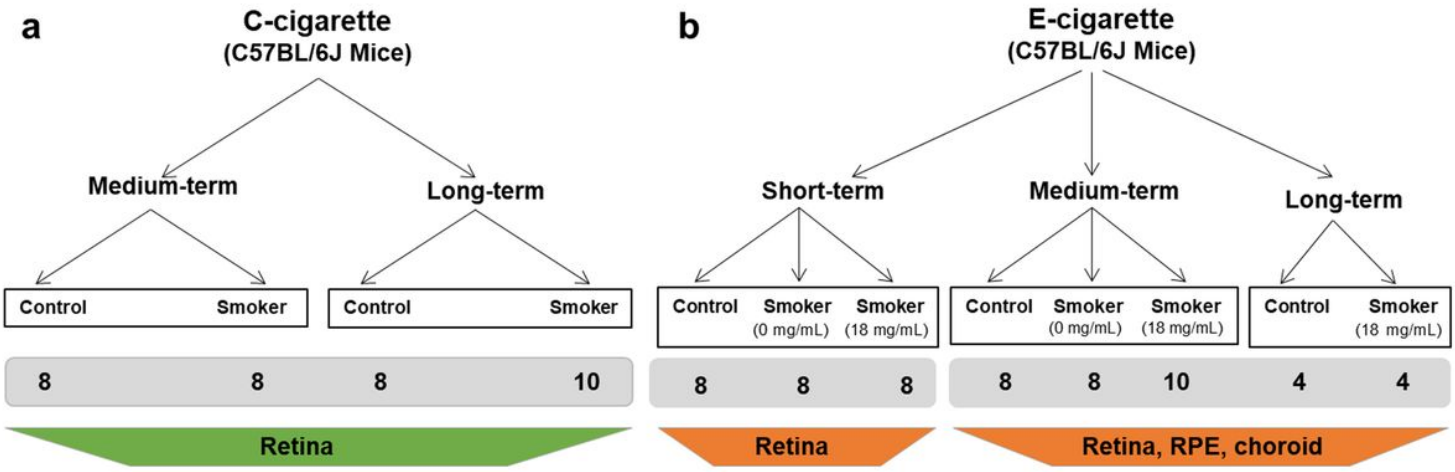
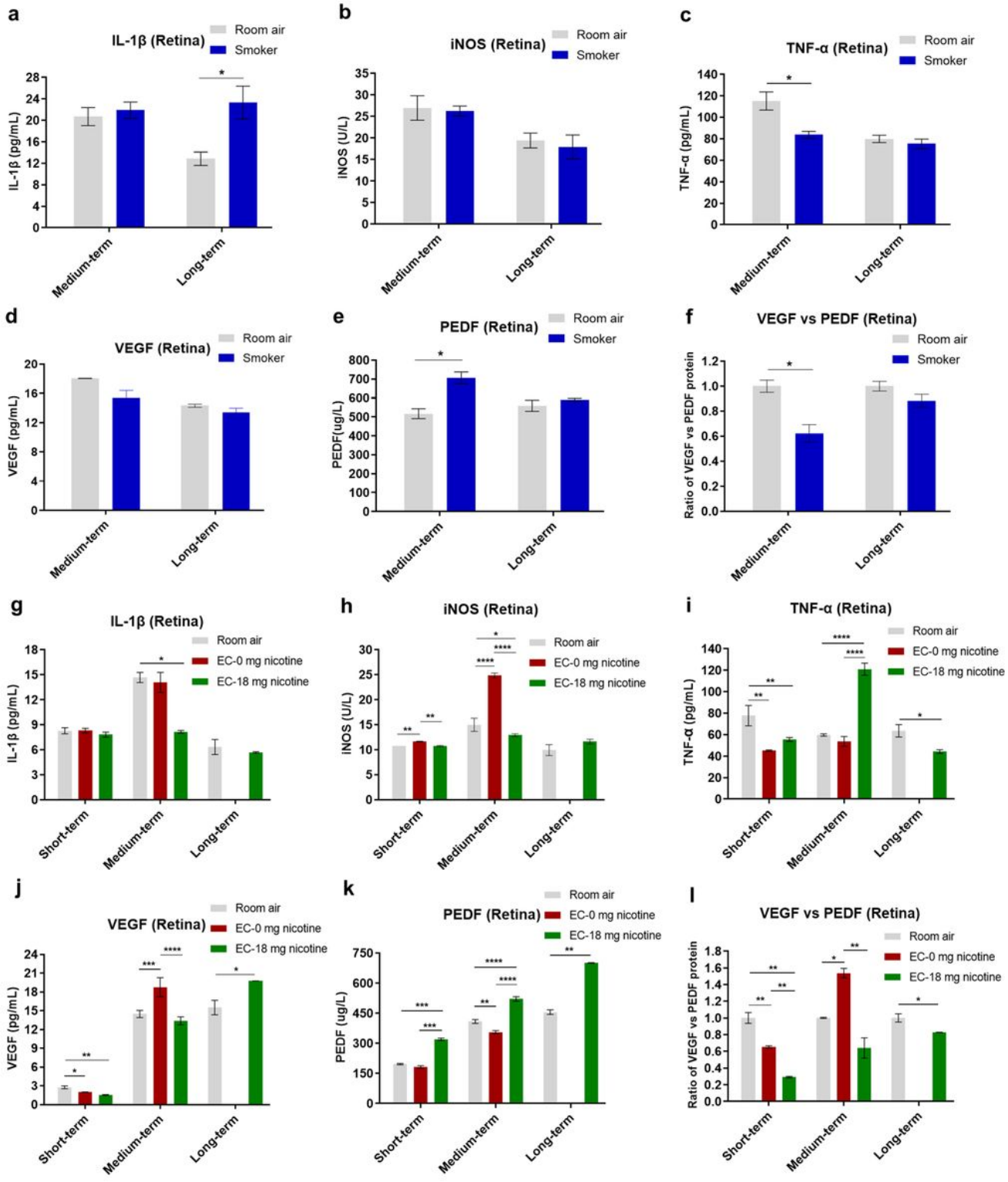


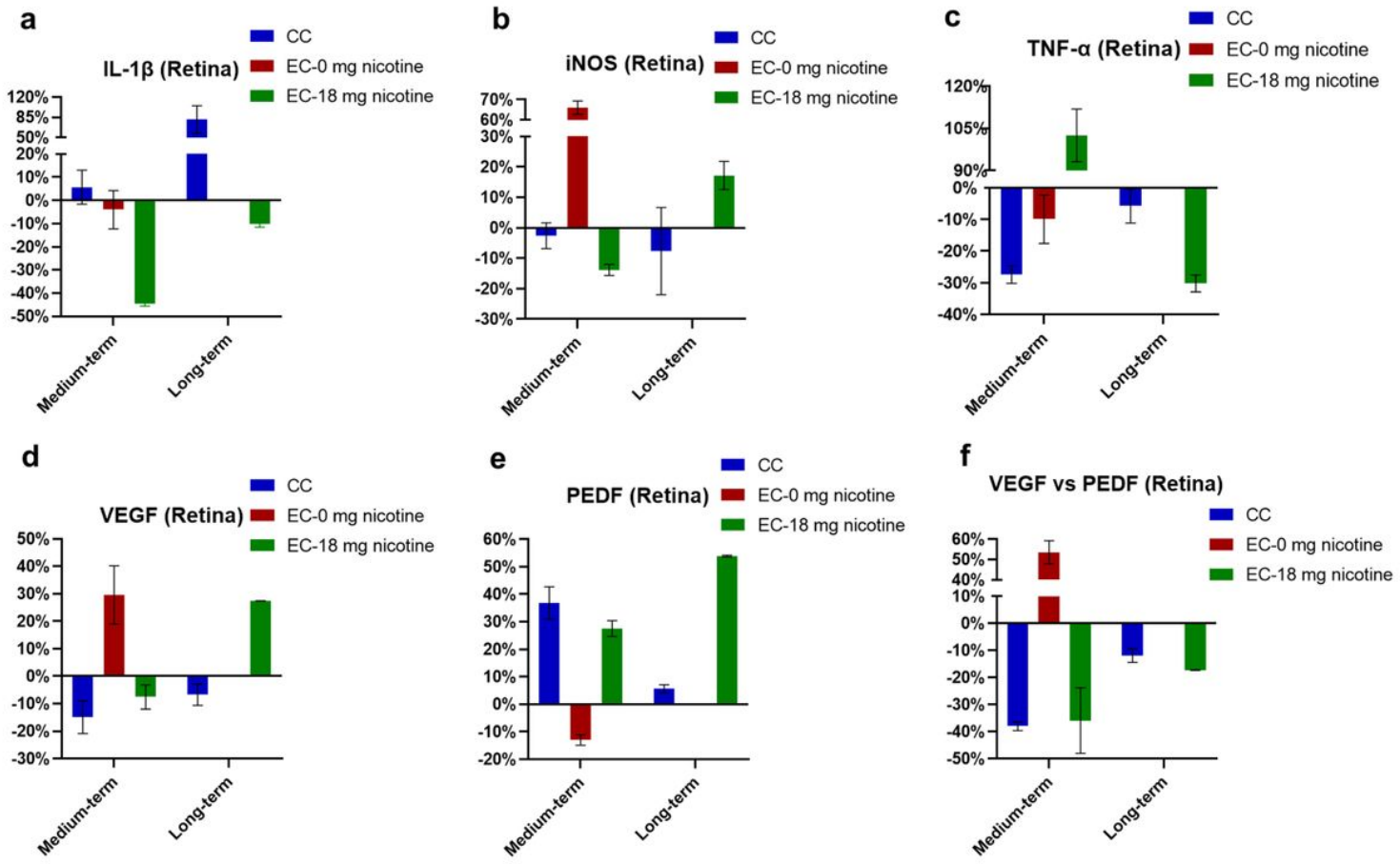

**Figure 1**

Overview of the experimental groups. (a) C-cigarette exposure groups contain medium- and long-term subgroups and the corresponding control mice (b) E-cigarette exposure groups contain short-, medium- and long-term subgroups wherein nicotine-free E-cigarette and nicotine-containing E-cigarette were used with the exception of the long-term subgroup, in which only nicotine-containing E-cigarettes was used. Number of eyes per group are listed in the grey box below each group.



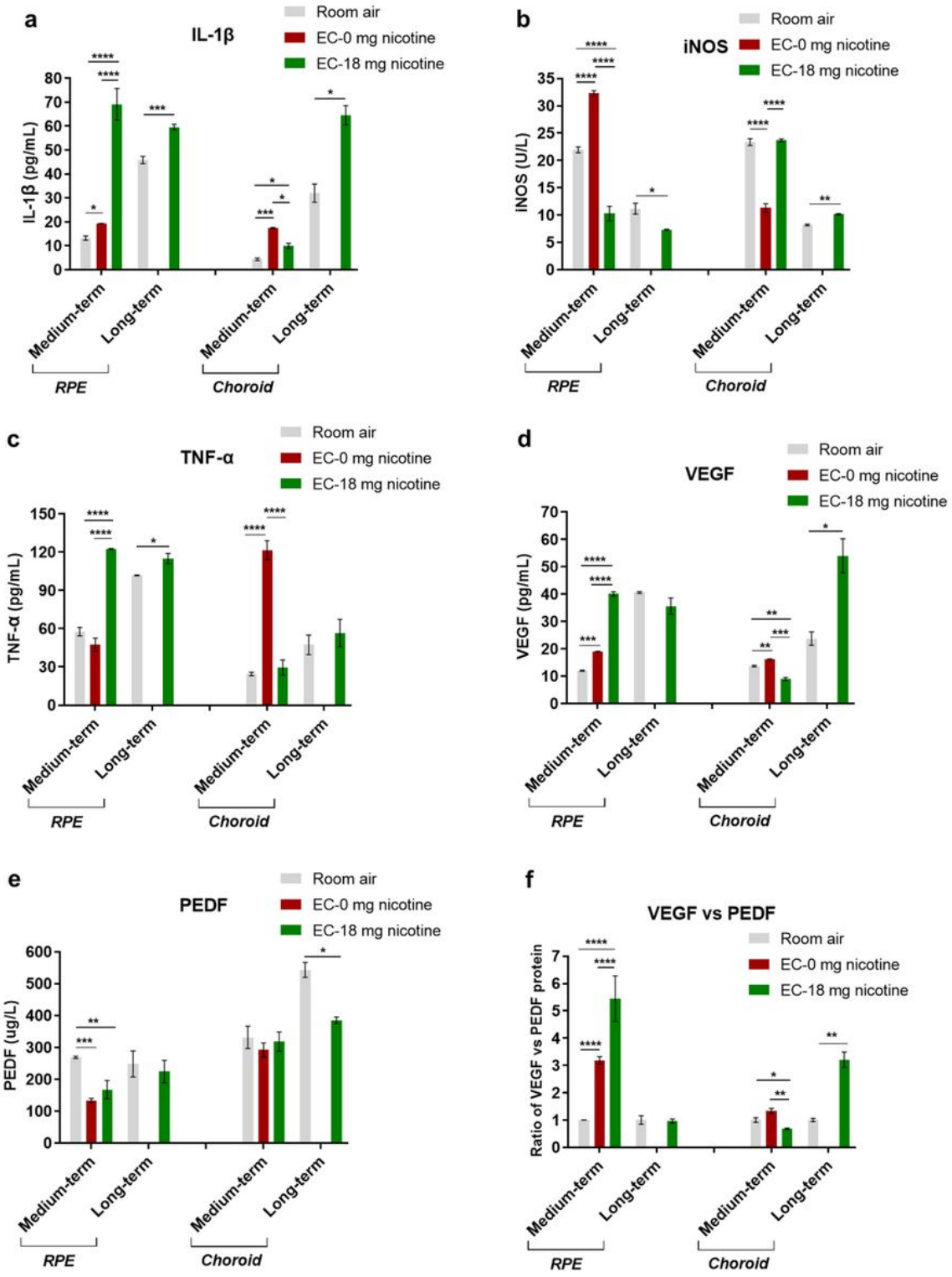

## Figure 2

Protein concentrations in the retina of animals from the C-cigarette smoke or E-cigarette vapor exposure subgroups. Data presented are from factors IL-1β (a, g), iNOS (b, h), TNF-α (c, i) and VEGF (d, j), PEDF (e, k). The Ratio of VEGF vs. PEDF proteins (f, l) reflect the comprehensive effect of pro-angiogenic. Histograms a-f were from C-cigarette smoke exposure groups; histograms g-l were from E-cigarette vapor exposure groups. Data are presented as mean ± SD. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.




## Figure 3

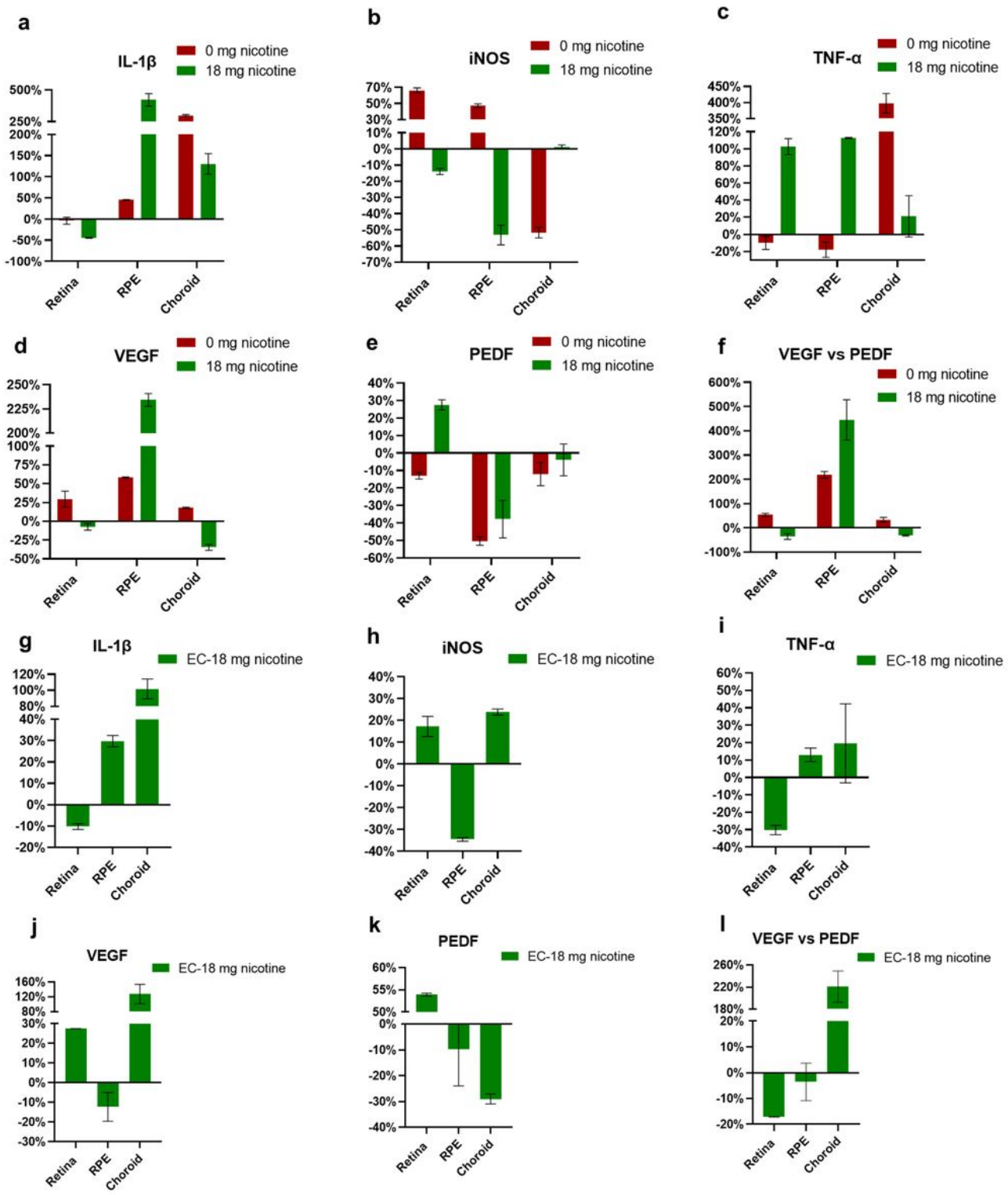
Comparison of the percentage changes in protein concentration between C- and E- cigarette exposure subgroups. Presented is the percentage change in protein concentration for cytokines IL-1β (a), iNOS (b), TNF-α (c), VEGF (d), PEDF (e), and the Ratio of VEGF vs. PEDF (f) in retinal tissue of each experimental subgroup after C-cigarette smoke or E-cigarette (nicotine free and nicotine-containing) vapor exposure. Ratio of VEGF vs. PEDF reflects the changes to the equilibrium of both factors at the RPE/retina interface. CC: C-cigarette smoke; EC-0 mg nicotine: E-cigarette (nicotine free) vapor; EC-18 mg nicotine: E-cigarette (nicotine containing) vapor.




## Figure 4

Protein concentrations in RPE and Choroid of animals from the E-cigarette exposure subgroups. Data presented are from factors IL-1β (a), iNOS (b), TNF-α (c), VEGF (d), and PEDF (e) protein secreted by RPE and choroid in mice. The Ratio of VEGF vs. PEDF (f) protein of each control was set as 1.0, this ratio reflects the changes to the equilibrium of both factors at the RPE/retina interface. The long-term E-

cigarette exposure subgroup was exposed to nicotine-containing E-cigarettes only. All the data are presented as mean ± SD. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.




## Figure 5

Comparison of percentage changes in protein concentration in retina, RPE, and choroid in the E-cigarette medium-term or long-term exposure subgroups. Data sets are presented for factors IL-1β (a, g), iNOS (b, h), TNF-α (c, i), VEGF (d, j), PEDF (e, k), and the Ratio of VEGF vs. PEDF (f, l). Ratio of VEGF vs. PEDF

reflects the changes to the equilibrium of both factors at the RPE/retina interface. Histograms a-f were from medium-term exposure groups; histograms g-l were from long-term exposure groups.