

## TCDD aggravates the formation of the atherosclerotic plaque in ApoE KO mice with a sexual dimorphic pattern

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2	sexual dimorphic pattern				
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11	Keywords: atherosclerosis, AhR, TCDD, sexual dimorphism				
12					
13	Abbreviations				
14	AhR: Aryl hydrocarbon receptor				
15	ApoE KO: Apolipoprotein E Knock-Out				
16	bHLH/PAS: basic Helix Loop Helix/Per ARNT Sim				
17	CD68: <i>Cluster</i> of Differentiation 68				
18	ICAM1: InterCellular Adhesion Molecule 1				
19	MCP1: Monocyte Chemoattractant Protein 1				
20	SEM: standard error of the mean				
21	$\alpha$ -SMA: <i>a</i> -smooth muscle actin				
22	TCDD: 2,3,7,8-Tetrachlorodibenzo-para-dioxin				
23	VCAM1: Vascular Cell Adhesion Molecule 1				
24	VSMCs: vascular smooth muscle cells				

- VSMCs: vascular smooth muscle cells

#### 27 Abstract:

Aryl hydrocarbon receptor (AhR) ligands are recognized as aggravating factors in 28 29 cardiovascular diseases but little is known about the role of the AhR in atherosclerosis considering the effects of age and gender. We exposed male and female ApoE knock-out 30 31 mice, a model to study the pathogenesis of atherosclerosis, to a potent AhR ligand, 2,3,7,8tetrachlorodibenzo-p-dioxin (TCDD) by an intraperitoneal injection of 1 µg/kg/week for 8 32 weeks. Atherosclerotic lesions, histological parameters and critical atherosclerotic markers in 33 34 aorta were analysed. TCDD increased atherogenic lesions in 35-week old female mice, leading to a switch of vascular smooth muscle cells (VSMCs) from a contractile to a pro-35 atherogenic phenotype and increased expression for VCAM1. AhR activation accelerates the 36 formation of atherosclerotic plaques with sex and age differences due to the phenotypical 37 switch of VSMCs. 38

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#### 40 **1. Introduction**

The AhR is a bHLH/PAS transcriptional factor and a xenobiotic receptor. It is of interest in 41 the fields of toxicology because of its exogenous agonists such as 2,3,7,8-tetrachlorodibenzo-42 *p*-dioxin (TCDD), a potent carcinogen. In addition to the environmental chemicals, dietary 43 and endogenous AhR ligands have been identified including tryptophan metabolites and 44 flavonoids [1]. After ligand binding, AhR transactivates the expression of xenobiotic 45 metabolizing enzymes and many other genes involved in cell proliferation and inflammatory 46 responses. Endogenous ligands are inferred to be involved in the regulation of cardiac 47 48 functions since vascular abnormalities are among the most common phenotypes observed in AhR knock-out (KO) mice [2-4]. Epidemiological studies suggest the role of AhR in 49 dyslipidemia, hypertension, carotid atherosclerosis and myocardial ischemia [5,6] and mouse 50 studies have reported a role for AhR in the increased expression of inflammatory genes in the 51 aorta [7,8]. One of the mechnisms by which AhR could play a role in atherosclerosis is the 52 modulation of inflammatory cytokines, observed in multiple models [9–12]. 53

Atherosclerosis results from the accumulation of lipids in the arterial wall with the 54 55 occurrence of atheromatous plaques which disrupt the permeability of the vessel wall leading to infiltration of low-density lipoproteins whose oxidized forms are specifically captured by 56 57 infiltrated macrophages. This last process is stimulated by the expression of biomarkers 58 including VCAM1 and ICAM1, two adhesion proteins which attract monocytes which then differentiate into macrophages. Macrophages and vascular smooth muscle cells (VSMCs) 59 differentiate into CD68-expressing foam cells following the internalization of oxidized LDLs 60 [13,14]. Normally present in the medial layer of blood vessels, VSMCs switch from a 61 contractile phenotype to a migratory/secretory phenotype with an increased extracellular 62 63 matrix production. In ApoE KO mice, the progression of atherosclerosis is associated with a 64 decreased expression of  $\alpha$ -SMA and an increased expression of cell adhesion molecules (ICAM1 and VCAM1) on VSMCs [15]. 65

Although the role of AhR has been studied in the early stages of atherosclerotic development [7], few studies have focussed on its role in the progression of the pathology. Here, we studied the effects of chronic TCDD treatment on the progression of atheroslerosis in ApoE KO mice that are in advanced phases of atherosclerosis. We tested the hypothesis that chronic stimulation of the AhR aggravates the development of atherosclerosis and assessed the influence of sex and age as they are important biological variables in atherosclerosis [16–20].

#### 72 **2. Materials and Methods**

#### 73 **2.1. Animals**

ApoE KO mice purchased from Charles River were bred in our facility on a 12-hour lightdark cycle and a standard chow diet (temperature 22°C) in accordance with EU directive 2010/63/EU for animal experiments. 20- and 27-week-old male and female ApoE mice (n = 10 per group) were included in the study. They received an intraperitoneal (ip) injection of corn-oil or 1  $\mu$ g/kg of TCDD (LGC Standards) in corn-oil / week for 8 weeks (table 1). This dose was chosen as the highest dose with no observed effect in a previous study [21]. They were sacrified one week after the last injection by intraperitoneal injection of ketamine-HCl

81 100 mg/kg and xylazine 20 mg/kg.

	Number of mice	
	Corn-oil	TCDD
28-week old females	5	5
35-week old females	5	5
28-week old males	4	6
35-week old males	6	4

#### 82

 Table 1: Composition of the groups and number of ApoE KO mice studied

#### 83

#### 84 **2.2.** Quantification of atherosclerotic plaques (Oil red O staining)

The sizes of atherosclerotic lesions were measured in oil red O-stained frozen transverse 85 sections of aortas. The root of the aorta was dissected under a macroscope and frozen in OCT 86 embedding medium at -80°C. Five sections of 10 µm (at 200, 400, 600 and 800 µm from the 87 cusps) were harvested. The sections were counterstained with hematoxylin. The oil red-O 88 stained slides were observed with AxioObserver Z1 (Zeiss) at a magnification of 5 and 89 90 analysed by Leica Qwin software (Leica) to quantify the surface area of the lesion and of the vessel. The density of the lesion was calculated by dividing the surface area of the lesion by 91 92 the surface area of the vessel.

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#### 94 2.3. Immunohistochemistry

Ethanol-fixed cryosections of aorta were blocked in 5% BSA and 0,1 % fish gelatin and then
processed for immunostaining with CD68 (rat, Serotec), aSMA (rabbit, Abcam) and VCAM1

(goat, R&D) antibodies in 1X PBS – 1% BSA and the anti-rat alexa fluor 647, anti-rabbit
rhodamine and anti-goat FITC secondary antibodies (Jackson ImmunoReasearch). The nuclei
were visualized with Hoechst (Molecular Probes).

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#### 101 **2.4. Statistical analysis**

The statistical analyses were performed using GraphPad Prism. All data were expressed as
the mean +/- SEM. The control and TCDD-treated groups were compared using either
Kruskal Wallis followed by Mann-Whitney test or directly by Mann-Whitney test. A p-value
of < 0.05 was considered statistically significant.</li>

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#### 107 **3. Results**

# 3.1. Exposure to TCDD aggravates the development of atherogenic lesions specifically in ApoE KO female mice

Twenty- and 27-week-old ApoE KO animals were injected with 1 µg of TCDD/kg/week for 8 weeks and sacrifed one week after the last injection. At the end of the study, they were respectively 28 and 35 weeks old.

The atherosclerotic plaque surface was quantified to determine the lesion density, obtained by dividing the surface area of the lesion by the surface area of the vessel (Figure 1A-D). This ratio avoids the overestimation of the surface area of lesions and facilitates the comparison between animals. Both of them were increased by TCDD specifically in 35-week-old ApoE KO female mice (Figure 1B, E-H), whereas they were not affected in corresponding male mice. The stage of atherosclerosis development also seems be a differentiating factor: in 28week old mice, TCDD had no effect on the lesion density (Figure 1A, C).





35 week-old TCDD-exposed female mice

121 Figure 1. Lesion density in the aortas of 28- and 35 week-old ApoE KO female (A-B) and male (C-D) mice. It is calculated as the ratio, lesion area / aorta area x100, and is expressed 122 as mean ± SEM. \* : p<0.05. Oil red-O staining of aortas from 35 week-old female mice 123 exposed or not to TCDD. Two representative aortas are displayed per group (control mice: E-124 125 F and TCDD-exposed mice: G-H).

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#### **3.2.** Exposure to TCDD leads to a migratory phenotype of VSMCs in the atherosclerotic 127 plaque of ApoE KO female mice. 128

We focused on 35 week-old female mice to investigate the effect of TCDD exposure in the 129 130 aorta on the expression of markers: 1) CD68, a marker of foam cells, inflammatory cells and of SMCs with a migratory phenotype, 2) VCAM1, expressed on activated endothelial cells 131 and on VSMCs from the atherosclerotic plaque, 3)  $\alpha$ -SMA, a marker of VSMCs with a 132 contratile phenotype, which normally decreases in the atherosclerotic plaque. The increase in 133 the number of CD68+ cells was not significant in the TCDD treated group (Figure 2A-E). 134 However, we observed that  $\alpha$ -SMA+ cells were significantly decreased (Figure 2A-D, 2F) 135 while a significant increase of VCAM1+ cells was found (2A-D, 2G). The increased 136 development of atherosclerotic lesions observed in TCDD-treated mice can be explained by 137 138 the switch of VSMCs from a contractile phenotype to a migratory and secretory phenotype which promotes atherosclerotic plaque development along with expression of pro-139 atherosclerotic markers such as VCAM1. 140



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Figure 2: Immunostaining and pourcentage of CD68, α-SMA, VCAM1+ cells in the aortic root of 35 weeks-old control or TCDD-exposed female mice. Aortas from 35 weekold control or TCDD-exposed female mice were stained for CD68 (pink), α-SMA (red) and VCAM1 (green) and the nuclei were visualized with Hoechst. Two representative aorta are displayed per group (control: A-B or TCDD-exposed mice: C-D). The number of positive cells was normalised by the total cell number and represented as the mean  $\pm$  SEM for each group (\* : p<0.05, E-G).

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#### 150 **4. Discussion**

In this study, we aim to better understand the role of the AhR in the development of atherosclerosis. In fact, AhR has been suggested to be involved in the growth of atherosclerotic plaques in ApoE-KO mice and the expression of the AhR gene is increased in the atherosclerotic mouse aorta and in human carotid artery disease tissue [9]. In the early steps of atherosclerotic development, AhR activation has been shown to aggravate lesions
through an inflammatory response [7]. This is coherent with the many studies which directly
link the AhR pathway to the regulation of pro-inflammatory cytokines [9–12].

In this manuscript, we describe for the first time, a gender difference in effects of TCDD on the progression of atherosclerotic plaques. We exposed 20 and 27 week-old female and male mice to 8-weeks of ip injections of TCDD, a AhR ligand. Increased lesion densities were present in the older group of ApoE female mice exposed to TCDD as compared to the same aged male mice.

We then focused on the phenotypic switching of VSMCs. VSMCs play a crucial remodeling role in the vessel wall potentially recruiting monocyte/macrophages into the plaque [14]. AhR activation leads to a significantly decreased expression of  $\alpha$ -SMA due to a decreased number of contractile VSMCs and an increased expression of VCAM1. Our data are in agreement with those of Guo et al. [22] who reported that TCDD promotes proliferation and migration of human VSMCs with a decreased expression of  $\alpha$ -SMA.

The influence of gender and age in the the development of atherosclerosis has been documented. The atheroprotective effects of estrogen have been reported in ApoE KO mice on high cholesterol diets [18] but other studies showed larger surface lesions in female ApoE KO mice as compared to male mice [17,19,20]. It also can be interesting to explore if the sexual dimorphism is due to a differential response to AhR activation in aortic cells between males and females as it was already observed in the liver of C57BL/6 mice [23].

Our results suggest that the development of AhR antagonists may be useful therapeutically, 175 with age and gender as important parameters to be considered. The plasticity of the AhR 176 which binds a variety of ligands (aromatic hydrocarbons, tryptophan metabolites, indoles 177 178 from the diet...) will be also a key factor [1]. Interstingly, these ligands are reported to have differential or even opposite effects on Th17 differentiation [24,25]. The competition 179 between the different types of ligands for the AhR could influence the development of the 180 atherogenic plaques over time and it might be interesting to consider, as a preventive 181 pharmacological approach, antagonizing the effect of aromatic hydrocarbons. 182

#### **183** Author contributions

- 184 Conception of the work: XC, MJK; planning of experiments, collection & analysis of data:
- 185 LB, MJK; writing of manuscript: XC, MJK. All authors have read and approved the final
- 186 manuscript.
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- 190 **Conflicts of Interest:** The authors declare no conflict of interest.
- 191

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