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

3

4 **Laetitia Bey¹, Xavier Coumoul¹, Min Ji Kim²**

5

6 **Institutions :**

7 ¹ Université de Paris, INSERM UMR-S1124, Paris, France

8 ² Université Sorbonne Paris Nord, Bobigny, INSERM UMR-S1124, Paris, France

9 **Correspondance: Min Ji Kim** email: min-ji.kim@inserm.fr

10

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: *Cluster* of Differentiation 68

18 ICAM1: InterCellular Adhesion Molecule 1

19 MCP1: Monocyte Chemoattractant Protein 1

20 SEM: standard error of the mean

21 α -SMA: α -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

25

26

27 **Abstract:**

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

39

40 **1. Introduction**

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

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

66 Although the role of AhR has been studied in the early stages of atherosclerotic development
67 [7], few studies have focussed on its role in the progression of the pathology. Here, we
68 studied the effects of chronic TCDD treatment on the progression of atherosclerosis in ApoE
69 KO mice that are in advanced phases of atherosclerosis. We tested the hypothesis that chronic
70 stimulation of the AhR aggravates the development of atherosclerosis and assessed the
71 influence of sex and age as they are important biological variables in atherosclerosis [16–20].

72 2. Materials and Methods

73 2.1. Animals

74 ApoE KO mice purchased from Charles River were bred in our facility on a 12-hour light-
75 dark cycle and a standard chow diet (temperature 22°C) in accordance with EU directive
76 2010/63/EU for animal experiments. 20- and 27-week-old male and female ApoE mice (n =
77 10 per group) were included in the study. They received an intraperitoneal (ip) injection of
78 corn-oil or 1 µg/kg of TCDD (LGC Standards) in corn-oil / week for 8 weeks (table 1). **This**
79 **dose was chosen as the highest dose with no observed effect in a previous study [21].** They
80 were sacrificed 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)

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

93

94 2.3. Immunohistochemistry

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

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

100

101 **2.4. Statistical analysis**

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

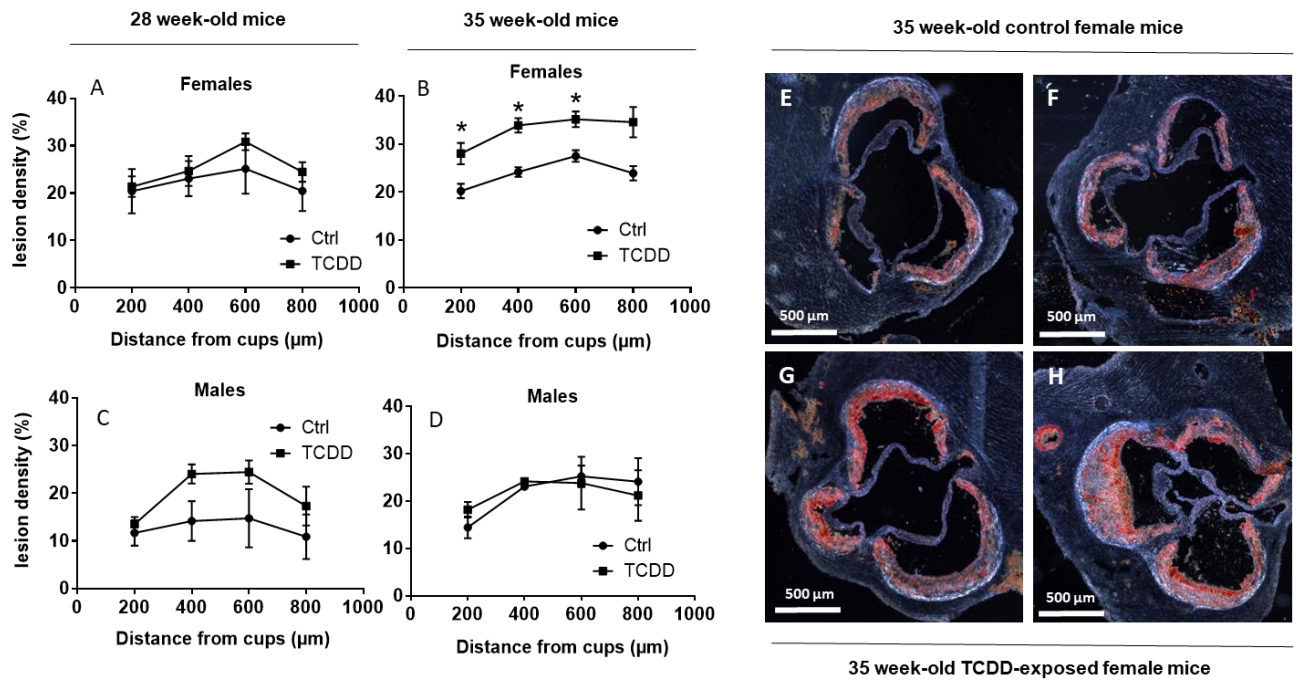
106

107 **3. Results**

108 **3.1. Exposure to TCDD aggravates the development of atherogenic lesions specifically in** 109 **ApoE KO female mice**

110 Twenty- and 27-week-old ApoE KO animals were injected with 1 µg of TCDD/kg/week for
111 8 weeks and sacrificed one week after the last injection. At the end of the study, they were
112 respectively 28 and 35 weeks old.

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

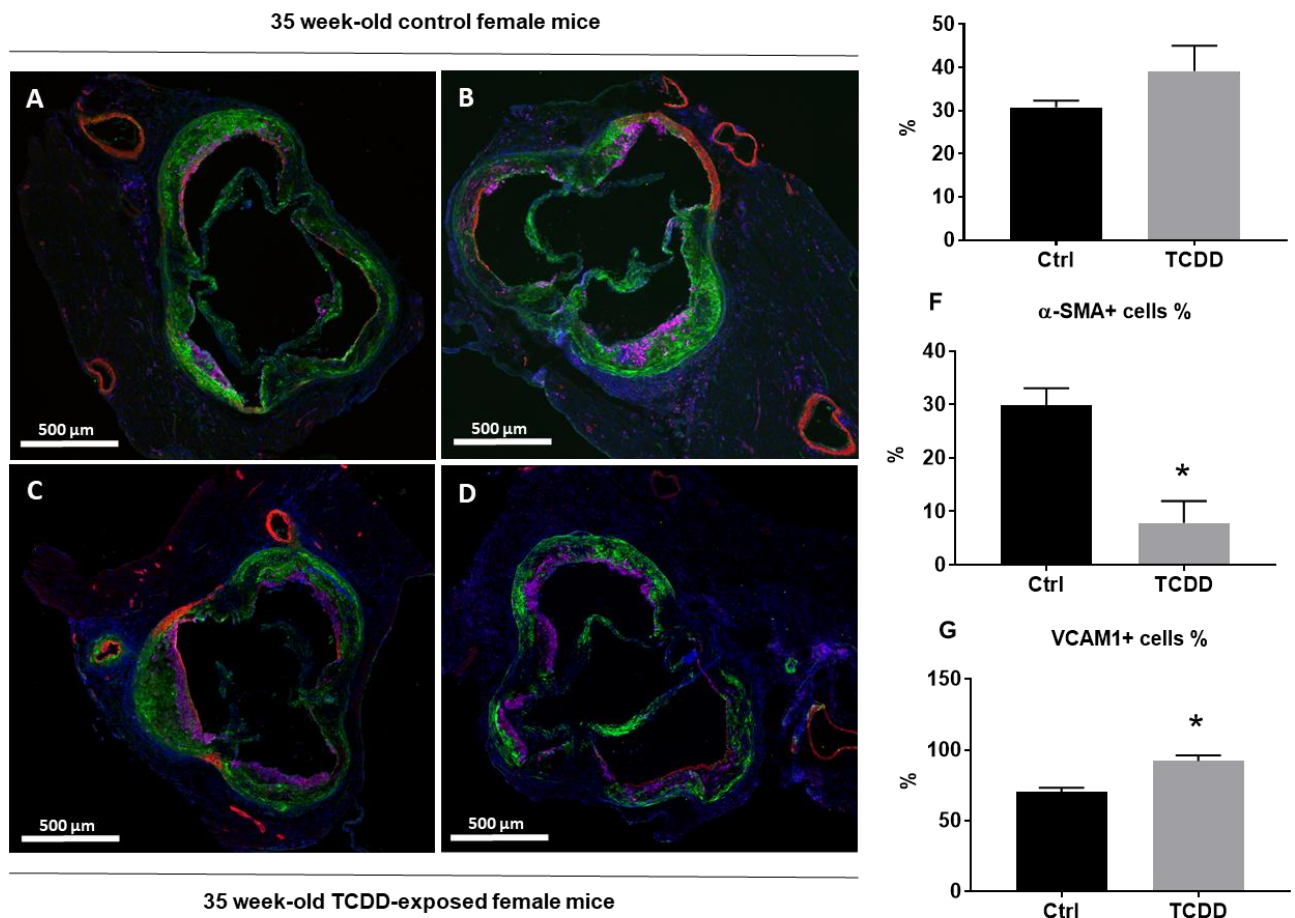


120
 121 **Figure 1.** Lesion density in the aortas of 28- and 35 week-old ApoE KO female (A-B) and
 122 male (C-D) mice. It is calculated as the ratio, lesion area / aorta area x100, and is expressed
 123 as mean \pm SEM. * : $p < 0.05$. Oil red-O staining of aortas from 35 week-old female mice
 124 exposed or not to TCDD. Two representative aortas are displayed per group (control mice: E-
 125 F and TCDD-exposed mice: G-H).

126

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

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



141

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

149

150 4. Discussion

151 In this study, we aim to better understand the role of the AhR in the development of
 152 atherosclerosis. In fact, AhR has been suggested to be involved in the growth of
 153 atherosclerotic plaques in ApoE-KO mice and the expression of the AhR gene is increased in
 154 the atherosclerotic mouse aorta and in human carotid artery disease tissue [9]. In the early

155 steps of atherosclerotic development, AhR activation has been shown to aggravate lesions
156 through an inflammatory response [7]. This is coherent with the many studies which directly
157 link the AhR pathway to the regulation of pro-inflammatory cytokines [9–12].

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

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

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

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

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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