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Inflammation contributes to the atherogenic role of intermittent hypoxia in apolipoprotein-E knock out mice

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ABSTRACT

Rationale. Obstructive sleep apnea results in nocturnal intermittent hypoxia (IH) as a main trigger for cardiovascular morbidity, including atherosclerosis. IH induces hemodynamic, hormono-metabolic, oxidative and immuno-inflammatory alterations that could differentially contribute to atherosclerosis. Our study aimed at examining the respective contribution of these consequences to the proatherogenic role of IH in atherosclerosis-prone mice.

Methods. Fifteen-week-old male apolipoprotein E-deficient (ApoE^{-/-}) mice fed on a high-cholesterol diet (HCD) for 6 weeks and exposed for the last 14 days to IH (21-5% FiO₂, 60s cycle, 8h/day) or air, were investigated for aortic atherosclerosis and lipid alterations. Then IH proatherogenicity was assessed in 15 and 20-week-old ApoE^{-/-} mice fed on a standart-chow diet (SCD) exposed to IH or air for 14 days and assessed for atherosclerosis, lipid, hemodynamic and inflammation alterations.

Results. IH aggravated atherosclerosis in HCD-fed mice, whereas the extremely high cholesterol levels due to HCD were not different between normoxic and hypoxic animals. In SCD-fed mice, IH also aggravated atherosclerosis, more severely in 20 compared to 15-week-old animals. However, total and LDL-cholesterols, which increased with IH, were not different in the two SCD-fed groups. The 20-week-old animals had higher plasma triglycerides and arterial blood pressure. Moreover IH induced systemic and prominent vascular inflammation, including increased splenocyte proliferation with decreased IL-10 secretion, and increased T-lymphocytes within atherosclerotic plaques.

Conclusions. A short IH exposure without HCD has proatherogenic effects through inflammatory, dyslipidemic and hemodynamic alterations. Inflammation, which appeared as a prominent component in our experimental model, should be considered in sleep apnea patients.

Key words: sleep apnea, intermittent hypoxia, atherosclerosis, dyslipidemia, inflammation,
mice

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INTRODUCTION

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2 Obstructive sleep apnea (OSA) syndrome is a highly prevalent disease, affecting up to 10% of
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4 middle aged men in the general population¹, and is recognized as an independent
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6 cardiovascular risk factor². The Wisconsin sleep cohort showed that untreated severe sleep
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8 apnea patients are five times more likely to die from cardiovascular causes¹. However, the
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10 relationship between OSA and mortality appears to be found only before 70 years old³. OSA-
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12 patients present early signs of atherosclerosis (e.g. carotid artery intima-media thickening,
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14 carotid plaques and arterial stiffness) that are independent of other cardiovascular or
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16 metabolic risk factors^{4, 5}, and are reversible in some patients by continuous positive airway
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18 pressure (CPAP) therapy⁶. OSA is a clinical situation with dyslipidemia⁷, hypertension⁸ and
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20 systemic inflammation⁹ which are in part imputable to the main component of OSA, i.e.
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22 intermittent hypoxia (IH). These several OSA-consequences could promote cardiac and
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24 vascular disease such as atherosclerosis^{2, 8}. However, their respective contribution is
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26 unknown. Moreover, current pharmacologic therapies in apneic patients only aim at
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28 normalizing blood pressure and metabolic alterations.
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36 Recent studies showed that long term IH exposure (up to 12 weeks) with high-fat high-
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38 cholesterol diet caused atherosclerosis and dyslipidemia in C57BL6 mice, or aggravated both
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40 atherosclerotic plaque progression and dyslipidemia in atherosclerosis-prone mice¹⁰⁻¹². In
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42 these studies, the metabolic disorder (dyslipidemia and lipid peroxydation) appeared as the
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44 main factor linking atherosclerosis to IH. However, it is well established that atherosclerosis
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46 is a chronic inflammatory disease¹³, and there is growing evidence that OSA is a chronic low
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48 grade inflammation. Therefore, IH-induced inflammation could be an additional link between
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50 OSA and atherosclerosis⁹, without the use of a dyslipidemic diet and even after a short-term
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52 of IH exposure, as we previously showed that 14 days of IH were sufficient to induce vascular
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54 alterations^{14, 15}.
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1 We exposed atherosclerosis-prone mice (apolipoprotein E knock-out (ApoE^{-/-}) mice) to 14
2 days of IH and assessed atherosclerotic plaque progression and inflammatory alterations. The
3
4 genesis of atherosclerosis in ApoE^{-/-} mice is well documented. Mice fed on standard chow
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6 diet develop foam cell lesions as early as 8 weeks, whereas intermediate lesions containing
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8 foam cells and smooth muscle cells appear at 15 weeks, and fibrous plaques at 20 weeks of
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10 age¹⁶. Western dyslipidemic diet accelerates this process and exacerbates plasma cholesterol
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12 levels. Therefore, we evaluated the effect of IH on different stages of atherosclerosis
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14 development, using 15 and 20-week-old ApoE^{-/-} mice fed either on a standard-chow diet or a
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16 high-cholesterol diet.
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METHODS

Animals

We first assessed the proatherogenic effect of IH in 15-week-old male ApoE^{-/-} mice (C57BL6 background) fed on a high-cholesterol diet (HCD) (17.3% fat, 1.30% cholesterol; Safe) for 6 weeks. During the last 14 days, mice were exposed to IH or air (see below), then assessed for atherosclerotic lesions and lipid alterations. Since the animals developed extremely severe dyslipidemia, which is an unusual situation in sleep apnea patients, the other experiments were conducted in mice fed on a standard-chow diet (SCD). Fifteen and 20-week-old male ApoE^{-/-} SCD-fed mice were exposed to IH or air for 14 days, then investigated for atherosclerotic lesions, lipid, blood pressure, and inflammation alterations. Animals were weighed before and at the end of each week of exposure. All the experiments were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Council of Europe, European Treaties ETS 123, Strasbourg, 18 March 1986), and to the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996).

Intermittent hypoxia

IH was performed in experimental cages accommodating 10 mice each, as previously described¹⁵. The animals were exposed to IH (cyclic 21-5% FiO₂, 60s cycle, both normoxic and hypoxic phases for 30s, 8h/day during daytime). FiO₂ was measured with a gas analyzer (ML206, ADInstruments) throughout the experiment. In similar experimental cages, normoxic mice (N) were exposed to air at similar flows than the IH stimulus, thereby reproducing equivalent levels of noise and turbulences related to gas circulation. Ambient temperature was maintained at 20-22°C. The day following the last exposure period, animals were anesthetized under a mixture of ketamine-xylazine (100mg/kg-10mg/kg, intraperitoneal

1 injection). Hemodynamic parameters were recorded, blood was collected from cardiac
2 puncture on EDTA tubes, and tissues were harvested and either frozen in liquid nitrogen then
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4 stored at -80°C until analysis, or processed for splenocyte proliferation and cytokine assays.
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8 9 **Atherosclerotic lesion size**

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11 Atherosclerotic lesions of the thoraco-abdominal aorta and aortic roots were analyzed by Oil-
12 red-O staining. For each aortic root, we quantified lipid deposition from 5 sections (8 µm
13 thickness), separated by 80 µm from each other, using computer image analysis (MetaMorph6
14 software, Zeiss microscope)¹⁷.
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24 **Lipid measurements**

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26 Total cholesterol was measured in plasma collected after the 14-day period of exposure, using
27 both the Infinity kit (Thermo Electron Corporation) according to the manufacturer's
28 guidelines, and the Modular P® device (Roche Diagnostics). HDL-cholesterol and
29 triglycerides (TG) were quantified by a colorimetric enzymatic reaction using the Modular
30 P® device (Roche Diagnostics). LDL-cholesterol was calculated using the Friedewald
31 formula.
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43 **Hemodynamic parameters**

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45 Diastolic and systolic arterial blood pressures were measured with a carotid artery catheter,
46 digitized, then analyzed using the PowerLab data acquisition system (Powerlab/8SP,
47 ADInstruments).
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Systemic inflammation

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2 Cytokine assay: Splenocytes were isolated from fresh harvested spleens and stimulated using
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4 Concanavalin-A (2 µg/ml). Interferon-gamma (IFN-γ) and interleukins IL-4 and IL-10 from
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6 supernatants collected 48-72h after stimulation were measured using an enzyme-linked
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8 immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems).
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Proliferation Assay

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16 Splenocytes were cultured in 96-well plates at a concentration of 5×10^6 cells/ml. Culture
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18 medium consisted of RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM L-
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20 glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% heat-inactivated FBS. Cells
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22 were stimulated in triplicates with graded concentrations of the mitogenic factor
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24 concanavalin-A (Sigma). Forty-eight hours later, cell proliferation was determined using a
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26 non-radioactive MTS cell proliferation assay (Promega).
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Vascular inflammation

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36 Macrophage and T-cell infiltration in the atherosclerotic lesions was assessed on
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38 immunostained cryosectioned aortic roots, using anti-mouse macrophage (Mac-3, clone
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40 M3/84, BD Pharmingen) and CD3 (#A0452, DakoCytomation) antibodies. Collagen was
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42 assessed with sirius red staining. Both stainings were quantified from 5 sections (8 µm
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44 thickness) per animal, separated by 80 µm from each other, using computer image analysis
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46 (MetaMorph6 software, Zeiss microscope)¹⁷, and expressed as a percentage of the lesion size
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48 (ratio macrophage or T-cell area to atherosclerosis area).
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Statistical Analysis

Results were expressed as means \pm SEM and analyzed using parametric (ANOVA and t-tests) or nonparametric tests (Kruskal-Wallis and Mann-Whitney) according to normality and variance homogeneity. Statistical significance was set at $p < 0.05$.

RESULTS

1. Effects of IH in high-cholesterol diet-fed mice

IH aggravated atherosclerosis in both the descending thoraco-abdominal aorta and aortic roots (Figure 1A). Total cholesterol levels were very high, twice the levels measured in SCD animals, but without differences between hypoxic and normoxic animals (Table 1). Therefore, although atherogenesis may have been promoted by extremely high levels of lipids, these last results suggested that the aggravating effect of IH on atherosclerotic lesions was likely not mediated by dyslipidemia only.

2. Effects of IH in standard-chow diet-fed mice

Atherosclerotic plaque size

IH aggravated atherosclerosis as shown by larger lesions in thoraco-abdominal aortas from the 2 hypoxic groups of SCD-fed mice (Figure 1A). In the aortic roots, the proatherogenic effect of IH was observed in 20-week-old animals only (Figure 1B). Lesion size in the aortic roots was similar between older SCD-fed animals and 15-week-old HCD-fed hypoxic mice suggesting that the combination of IH and HCD had mutually potentiated their respective detrimental effects. (Figure 1B).

Dyslipidemia

IH increased both total and LDL-cholesterol levels in SCD-fed mice, without affecting HDL-cholesterol (Figures 2A,B,C). The increases in cholesterols were similar in 15 and 20-week-old animals. In contrast, triglyceride significantly increased only in 20-week-old hypoxic mice, whereas body weight was similar in hypoxic 15 and 20-week-old mice at the end of IH exposure. As usually occurring with the experimental model of IH, the body weight of the two

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SCD-fed groups decreased under IH and remained lower than that of normoxic animals at the end of exposure (Table 2).

Blood pressure

Hematocrit significantly increased in the two groups of SCD-fed mice. Whereas arterial blood pressure measurements were very close between 15-week-old groups, blood pressure values tended to be higher in older hypoxic mice, with +8.3 mmHg for mean BP, +8.9 mmHg for diastolic BP and +7.5 mmHg for systolic BP (Table 2).

Inflammatory alterations

Systemic inflammation

IH caused systemic inflammation in the two groups of SCD-fed mice, as shown by the increase in splenocyte proliferation (Figure 3A) and the trend ($p=0.06$) for lower splenocyte IL-10 secretion (Figure 3B). In contrast, splenocyte secretion of IFN γ and IL-4 was unaffected by IH (data not shown).

Vascular inflammation

The atherosclerotic plaque composition was assessed in 20-week-old mice, as they presented the more advanced atherosclerotic lesions. IH significantly increased T-lymphocytes (CD3 positive cells) within atherosclerotic lesions (Figure 4). In contrast, collagen staining (data not shown) and macrophage recruitment (Figure 4) in aortic roots were unaffected by IH.

DISCUSSION

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2 In view of the known relationship between sleep apnea syndrome and atherosclerosis
3 development^{4, 5, 18}, our data strongly support the role of the hypoxic component in OSA-
4 related atherosclerosis. In the present study, we confirmed that IH accelerates atherosclerosis
5 development in ApoE^{-/-} mice, fed either on a SCD or HCD. We showed that the
6 proatherogenic effect occurred after only 14 days of IH and involved inflammatory,
7 dyslipidemic and hemodynamic alterations.
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The proatherogenic role of IH

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22 There are growing evidence that the hypoxic component of OSA seems to play a major role in
23 the development of atherosclerosis in apneic patients. The severity of nocturnal oxygen
24 desaturation has been found as the best predictor for carotid wall hypertrophy, plaque
25 occurrence and volume in OSA-patients^{4, 5, 19}. Using atherosclerotic-prone mice fed on a high-
26 cholesterol diet, Jun et al. recently showed that long term exposure to IH (4 to 12 weeks)
27 accelerated atherosclerotic plaque growth¹¹. In the present study, we confirmed the
28 proatherogenic role of IH and further evidenced that a shorter exposure to IH, i.e. only 14
29 days, without a high-cholesterol diet, also led to atherosclerosis aggravation.
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Dyslipidemic alterations due to IH

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46 Atherosclerosis is initiated by imbalance of lipid metabolism leading to atherogenic
47 dyslipidemia, including hypercholesterolemia, and contributing to retention, accumulation and
48 oxidation of lipoproteins in the arterial wall²⁰. Several studies reported such anomalies of lipid
49 metabolism in both OSA-patients⁸ and mice exposed to IH⁷, IH-related atherosclerosis
50 appearing to be closely associated with lipid alterations^{10, 11, 21}. However in our study, 14 days
51 of IH in HCD-fed mice accelerated atherosclerosis development independently of total
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1 cholesterol levels. Indeed, even the normoxic mice exhibited extremely high levels of total
2 cholesterol that were not further increased in hypoxic animals. These results suggest that the
3 proatherogenic effect of IH was not mediated by dyslipidemia. However besides quantitative
4 alterations, we cannot rule out qualitative cholesterol alterations due to IH, such as LDL and
5 HDL oxidation. Because cholesterol levels found in HCD-fed mice were not representative of
6 biological alterations usually measured in OSA-patients but rather in familial dyslipidemia,
7 we assessed the proatherogenic effect of IH in SCD-fed animals. And again, whereas IH
8 aggravated atherosclerosis more severely in 20 compared to 15-week-old animals, IH
9 similarly increased total and LDL-cholesterols in these two hypoxic SCD-fed groups. The
10 similarity in aortic root lesion size between hypoxic 20-week-old SCD-fed animals and 15-
11 week-old HCD-fed mice suggests that the combination of IH and HCD may have mutually
12 potentiated each other. However, the successive discrepancies between the proatherogenic
13 effect of IH and dyslipidemia levels suggest that IH could have exerted its aggravating effect
14 on atherosclerosis through other contributing factors than dyslipidemia only.

36 **Blood pressure alterations due to IH**

37 Hemodynamic alterations that have been extensively described in OSA²² are likely to be
38 involved in atherosclerosis progression. About 60% of OSA-patients develop systemic
39 hypertension²², even in the absence of any other risk factors when including both clinical and
40 masked hypertension^{4, 23}. Using animal models^{15, 24} and also applying IH in healthy humans²⁵
41 suggest that IH is a major factor determining blood pressure increase. We have previously
42 shown in C57BL6 mice that blood pressure surges appear at each hypoxic episode, and are
43 associated with increased sympathetic activation¹⁴. These hemodynamic changes are well
44 known to induce functional and structural alterations of the vascular wall that could contribute
45 to vascular remodeling and atherogenesis^{15, 26}. However in the present study, we found a

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slight elevation in blood pressure in 20-week-old mice, whereas hemodynamic parameters were unaffected in 15-week-old mice. This suggests that blood pressure alterations may have modestly contributed to atherosclerosis acceleration in our model.

IH-induced inflammation and atherosclerosis

In addition to metabolic and hemodynamic impairments, inflammation is considered as a key factor in atherosclerosis¹³; a growing body of evidence suggests that OSA is also a chronic low grade inflammatory disease^{9, 27}. Whereas the mechanisms linking OSA and inflammation are not fully elucidated, experimental models suggest a major role of IH in OSA-related inflammation and subsequent vascular alterations¹⁵. In the present study, we found that 14 days of IH induced systemic inflammation, as shown by the increase in splenocyte proliferation rate and the reduced IL-10 secretion. Splenocyte proliferation directly reflects leukocyte activation²⁸ and is consistent with data previously reported in OSA-patients. Specific lymphocyte activation, with enhanced CD8⁺ T-cell cytotoxicity²⁹ and increased cytokine production by both CD4⁺ and CD8⁺ T-cell subtypes³⁰ were found in OSA-patients. The reduced IL10 secretion by splenocytes also strongly suggests a shift of T-helper cell 2 toward T-helper cell 1 (Th2/Th1 balance) and several studies have demonstrated the anti-atherogenic and anti-inflammatory role of IL10 in mice (for review, see³¹). Therefore in our study, leukocyte activation and reduced IL10 secretion in hypoxic ApoE^{-/-} mice could have contributed to accelerate atherogenesis¹³. This significant role of IH-associated inflammation in atherogenesis was also strengthened by the enhanced CD3 T-cell infiltration within atherosclerotic plaques of hypoxic animals. This last result suggests that, besides enlarging atherosclerosis lesions, IH induces a more inflammatory and vulnerable plaque phenotype²⁰.

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In conclusion, we demonstrated that short exposure to IH without HCD is sufficient to accelerate atherosclerosis in ApoE^{-/-} mice. The proatherogenic effect of IH was associated with lipidic, inflammatory and modest hemodynamic alterations. This confirms that sleep apnea, through its hypoxic component, could initiate or aggravate several systemic and organ-specific biological factors that are required for the development of atherosclerosis. These findings further support the need for early diagnosis of vascular remodelling in OSA-patients using sub-clinical cardiovascular markers such as carotid intima media thickness, whilst biomarkers enabling to identify at-risk patients remain to be determined. In light of our results, the assessment of the inflammatory response in OSA-patients could represent a valuable candidate biomarker.

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TABLES AND FIGURE LEGENDS

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4
5 **Table 1.** Weight and biological alterations induced by IH in HCD-fed mice. * $p < 0.05$ vs
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7 N.

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11 **Table 2.** Weight and hemodynamic alterations induced by IH in SCD-fed mice. * $p < 0.05$
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13 vs N, $p < 0.05$ vs day 0.

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19 **Figure 1.** Atherosclerotic lesion measurements in 15-week-old HCD-fed mice and in 15
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21 and 20-week-old SCD-fed mice. A) Thoraco-abdominal aortic lesions
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23 measured as percentage of total aorta surfaces; * $p < 0.05$ vs N. B)
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25 Atherosclerotic lesions in aortic roots, expressed in μm^2 ; * $p < 0.05$ vs N. C)
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27 Representative photographs of aortic root lesions.
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34 **Figure 2.** IH-induced dyslipidemia in 15 and 20-week-old SCD-fed mice. A) Total
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36 cholesterol; B) LDL-cholesterol; C) HDL-cholesterol; D) Triglycerides;
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38 * $p < 0.05$ vs N.
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44 **Figure 3.** IH-induced inflammatory response in 15 and 20-week-old SCD-fed mice. A)
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46 Splenocyte proliferation in response to 0.5 to 5 $\mu\text{g}/\text{ml}$ concanavalin-A (ConA)
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48 after 14 days of IH or air (N). B) Quantification of IL-10 secretion from
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50 splenocytes of mice exposed to IH or air (N).
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56 **Figure 4.** Atherosclerotic plaque composition in 20-week-old SCD-fed mice.
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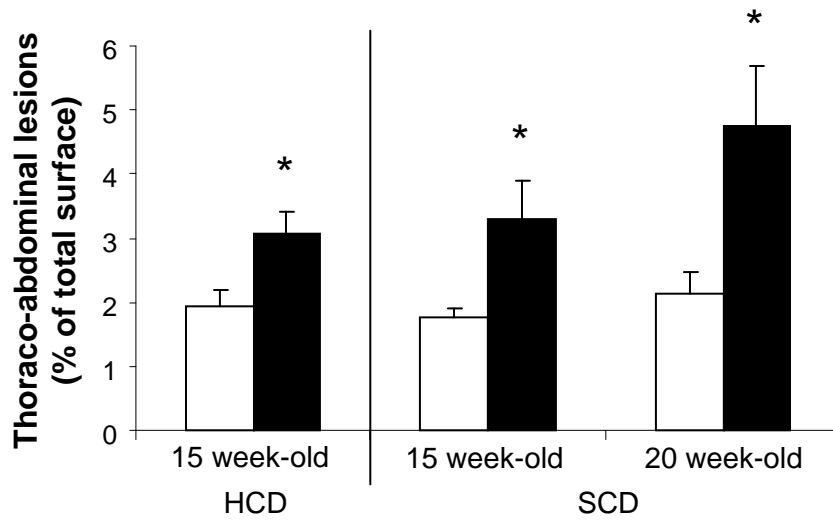
Quantification of T-lymphocyte (CD3 positive cells) and macrophage (Mac-3 positive cells) recruitment in aortic root lesions, expressed in percentage of lesion size; * $p < 0.05$ vs N. Representative photographs of T-cell and macrophage staining in IH and N groups.

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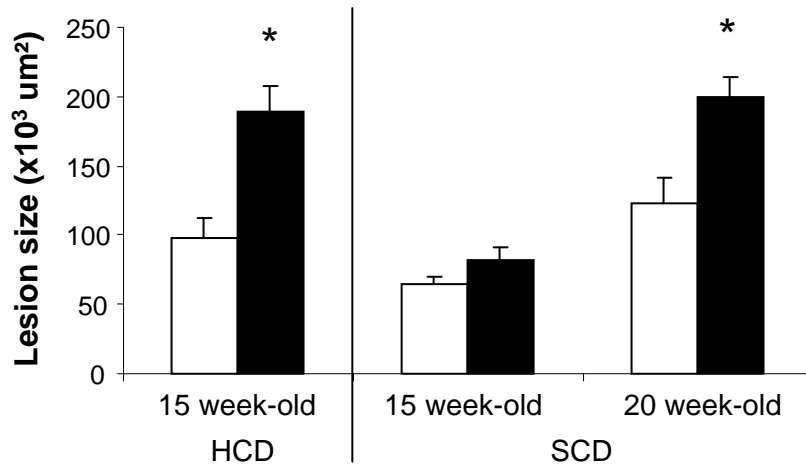
Figure 1

□ N ■ IH

A



B



C

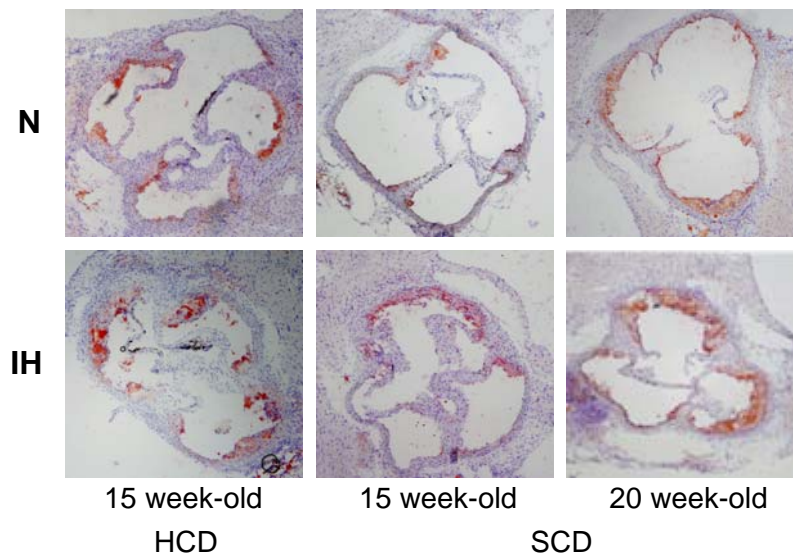
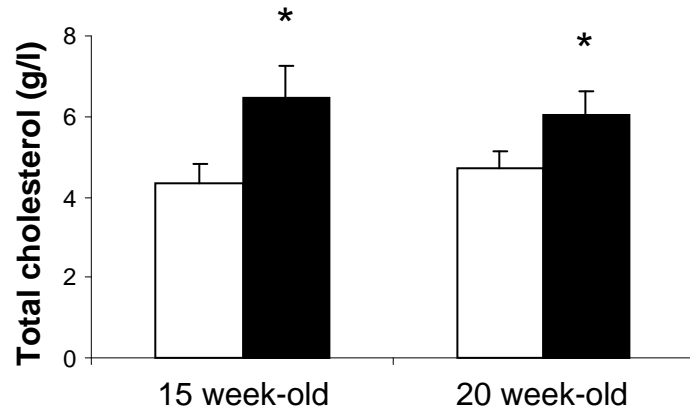
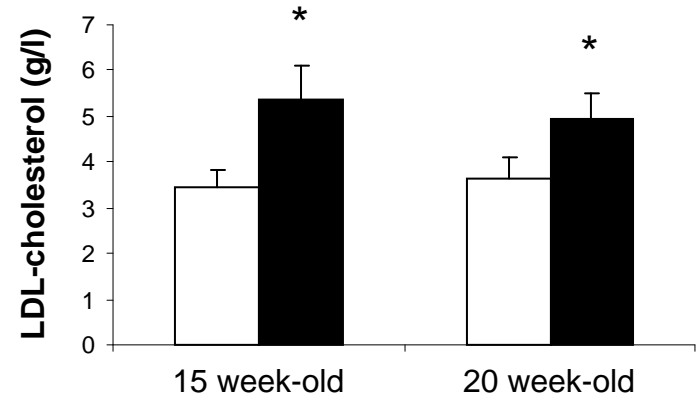


Figure 2

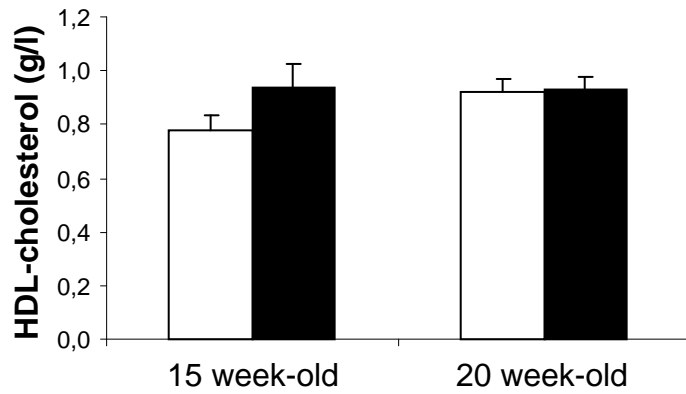
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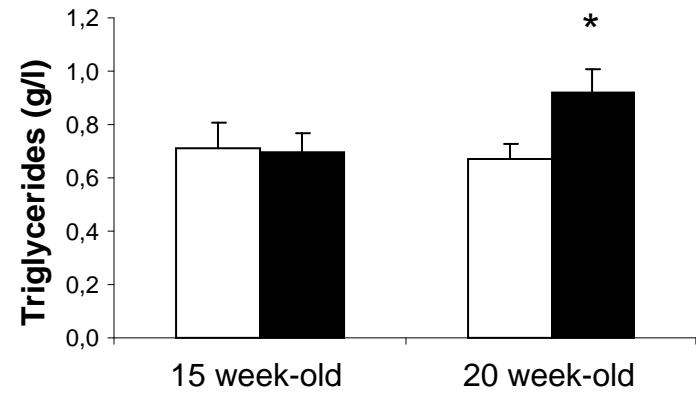
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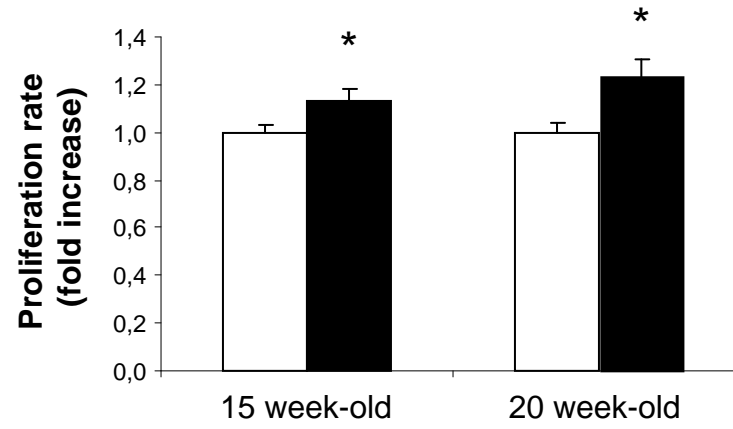
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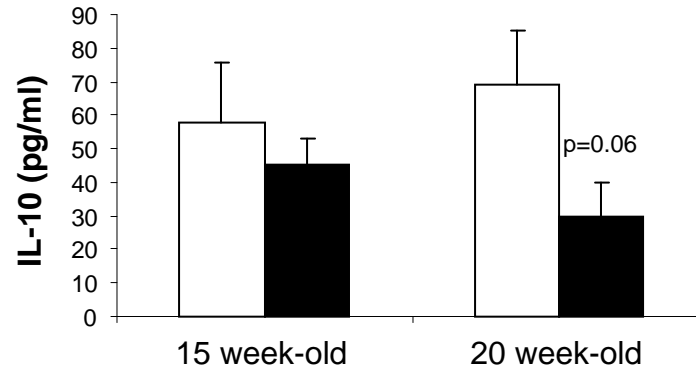
□ N ■ IH

Figure 3

A



B



□ N ■ IH

Figure 4

□ N ■ IH

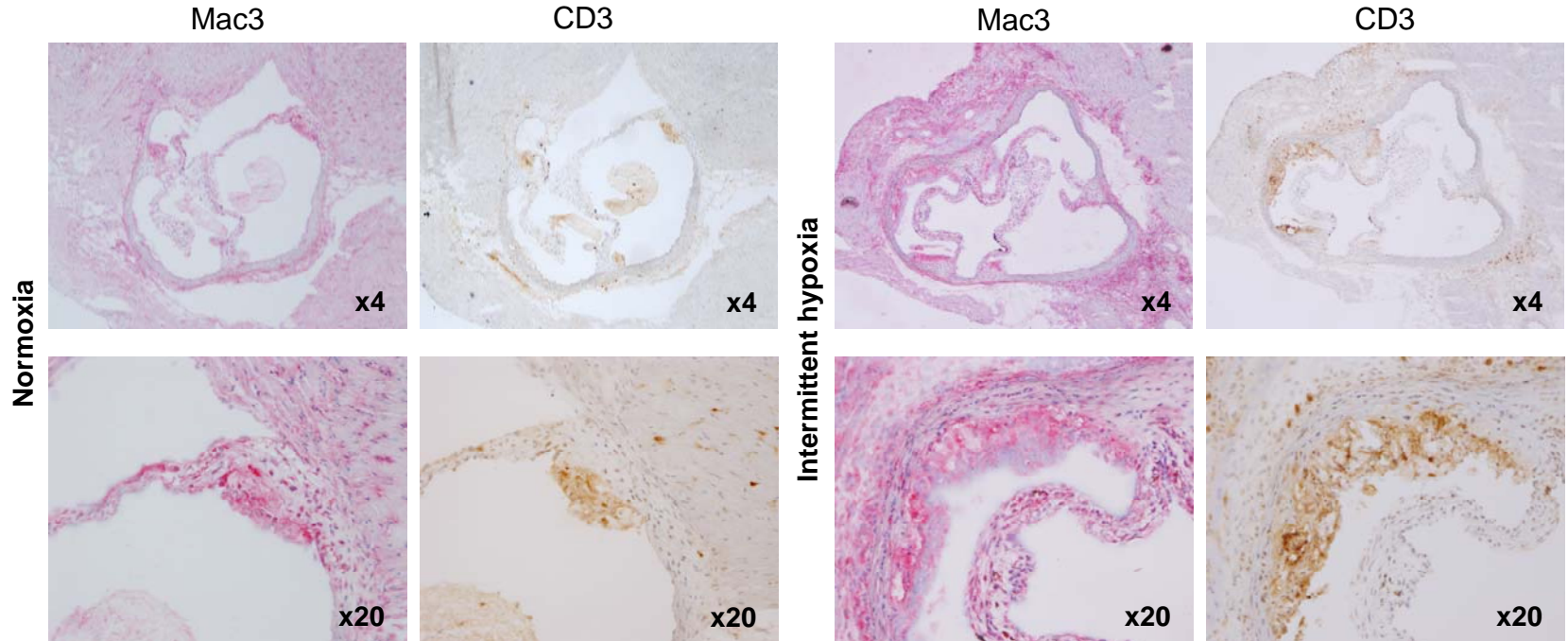
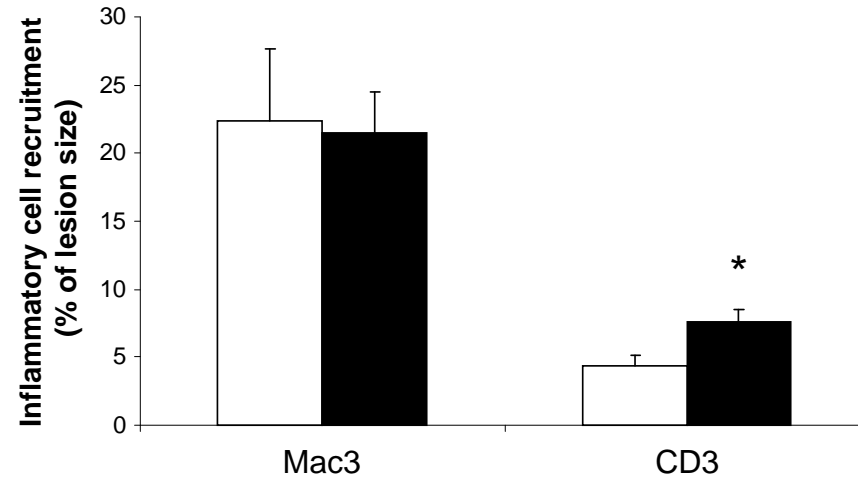


Table 1

	N	IH
Age of mice (weeks)	15	15
n	7	9
Mice weight (g)		
<i>Day 0</i>	26,3 ± 0,8	25,4 ± 0,6
<i>Day 8</i>	25,7 ± 0,6	25,9 ± 0,4
<i>Day 14</i>	25,5 ± 0,8	25,4 ± 0,5
Parameters at 14 days		
Hematocrit (%)	44,9 ± 0,3	48,4 ± 0,9 *
Total cholesterol (g/l)	12,0 ± 0,5	12,4 ± 1,0

Table 2

	N	IH	N	IH
Age of mice (weeks)	15	15	20	20
n	14	13	9	7-9
Mice weight (g)				
<i>Day 0</i>	28,2 ± 0,5	29,4 ± 0,5	30,7 ± 0,3	30,1 ± 0,4
<i>Day 8</i>	27,8 ± 0,5	25,3 ± 0,4 *,\$	30,3 ± 0,3	27,6 ± 0,4 *,\$
<i>Day 14</i>	28,6 ± 0,5	27,2 ± 0,4 *,\$	30,9 ± 0,4	27,7 ± 0,3 *,\$
Parameters at 14 days				
Hematocrit (%)	46,3 ± 0,5	50,1 ± 0,8 *	49,4 ± 1,7	52,4 ± 1,3 *
Blood pressure				
<i>Mean BP (mmHg)</i>	74,5 ± 2,3	74,5 ± 1,9	71,7 ± 3,4	80,0 ± 3,1
<i>Diastolic BP (mmHg)</i>	68,7 ± 2,4	68,5 ± 1,9	67,4 ± 3,4	76,3 ± 3,4

Systolic BP (mmHg)

$86,0 \pm 2,3$

$86,4 \pm 2,4$

| $80,1 \pm 3,5$

$87,5 \pm 2,6$