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# Leukotriene $B_4$: early mediator of atherosclerosis in obstructive sleep apnoea?

**B. Lefebvre[*,#,¶], J-L. Pépin[*,#,+], J-P. Baguet[#,§,ƒ], R. Tamisier[*,#,+], M. Roustit[¶], K. Riedweg[¶], G. Bessard[*,#,¶], P. Lévy[*,#,+] and F. Stanke-Labesque[*,#,¶]**

**ABSTRACT: Severity of oxygen desaturation is predictive of early atherosclerosis in obstructive sleep apnoea (OSA). Leukotriene (LT)$B_4$ is a lipid mediator involved in atherogenesis.**

**In 40 non-obese OSA patients, free of a cardiovascular history, and 20 healthy volunteers, the following were evaluated: 1) $LTB_4$ production by polymorphonuclear leukocytes (PMNs) stimulated with A23187; and 2) the relationships between $LTB_4$ production and both OSA severity and infraclinical atherosclerosis markers. The effect of continuous positive airway pressure (CPAP) on $LTB_4$ production was also studied. An overnight sleep study was followed by first-morning blood sampling. Isolated PMNs were stimulated with A23187 in order to induce $LTB_4$ production, which was measured by liquid chromatography–tandem mass spectrometry. Carotid intima-media thickness (IMT) and luminal diameter were measured in subset groups of 28 OSA patients and 11 controls.**

**$LTB_4$ production was increased in OSA patients compared with controls. $LTB_4$ levels correlated with the mean and minimal arterial oxygen saturation ($S_{a,O_2}$). $LTB_4$ production correlated with luminal diameter data in patients with a mean $S_{a,O_2}$ of $\leqslant$94% but not with IMT. Lastly, CPAP significantly reduced $LTB_4$ production by 50%.**

**Leukotriene $B_4$ production is increased in obstructive sleep apnoea in relation to oxygen desaturation. Leukotriene $B_4$ could promote early vascular remodelling in moderate-to-severe hypoxic obstructive sleep apnoea patients.**

**KEYWORDS: Atherosclerosis, leukotriene $B_4$, polymorphonuclear cells, sleep apnoea**

Obstructive sleep apnoea (OSA) is characterised by recurrent episodes of partial or complete upper airway obstruction occurring during sleep. These episodes of upper airway obstruction are usually associated with a desaturation–reoxygenation sequence, which is an acknowledged detrimental stimulus for the cardiovascular system. Recent data indicate that OSA is associated with an increased prevalence of fatal and nonfatal cardiovascular events [1], and is an independent risk factor for death from any cause [2]. Among the intermediary mechanisms that could explain the link between OSA and cardiovascular morbidity, the role of early atherosclerosis has been proposed. It has now been demonstrated that, even after adjustment for confounding factors, OSA *per se* may lead to atherosclerosis, and that the intensity of the vascular damage is more specifically related to the amount of nocturnal oxygen desaturation [3–5]. Moreover, 4 months of continuous positive airway pressure (CPAP) application seems sufficient to partly reverse early atherosclerosis [6].

Leukotriene (LT)$B_4$ is an inflammatory mediator that is derived from the 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism. $LTB_4$ synthesis is initiated by the activation of 5-LO [7] and its subsequent interaction with the nuclear-membrane-bound 5-LO-activating protein (FLAP) [8] of inflammatory cells. In polymorphonuclear leukocytes (PMNs), the activation of 5-LO depends upon intracellular calcium concentration, which is increased by the addition of calcium ionophores [9]. When released from cell membranes by the action of phospholipase $A_2$, arachidonic acid is converted into 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid by 5-LO, which also catalyses its further transformation to $LTA_4$. In PMNs, $LTA_4$ is then converted to $LTB_4$ by $LTA_4$ hydrolase. $LTB_4$ then binds to specific $LTB_4$ receptors (BLTs), namely $BLT_1$ and $BLT_2$, to elicit its biological effects [10], including

AFFILIATIONS
*INSERM, ERI7
#Grenoble University 1, Faculté de Médecine, IFR1,
¶BP217, Pharmacology Laboratory,
+BP217, EFCR Laboratory, and
§BP217, Cardiology Unit, A. Michallon Hospital, Centre Hospitalier Universitaire, and
ƒINSERM 8777, Grenoble, France.

CORRESPONDENCE
F. Stanke-Labesque
Laboratoire de Pharmacologie,
Centre Hospitalier Universitaire,
Hôpital A. Michallon, BP 217,
38043 Grenoble Cedex 9, France.
Fax: 33 476768938
E-mail: FStanke@chu-grenoble.fr

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stimulation of leukocyte chemotaxis, adhesion to vascular endothelium, and degranulation.

A recent growing body of evidence suggests a major role of the 5-LO pathway in the pathogenesis and progression of atherosclerosis. First, stimulated PMNs from individuals with a past history of myocardial infarction produce more $LTB_4$ than PMNs from controls [11]. In addition, expression of the 5-LO pathway (5-LO, FLAP, $LTC_4$ synthase and cysteinyl LT receptors) is increased in atherosclerotic lesions at various stages of development in human aorta and coronary and carotid arteries [12, 13]. Furthermore, recent human genetic studies have shown that a promoter variant of 5-LO is associated with an increase in carotid intima–media thickness (IMT) in healthy subjects [14], and certain FLAP haplotypes have been linked to an almost two-fold increase in risk of myocardial infarction or stroke [11, 15].

Few studies have assessed the role of local $LTB_4$ production in OSA. These studies have been performed in children and have demonstrated an increased concentration of $LTB_4$ in the upper airway lymphoid tissues of paediatric OSA patients compared with those with recurrent tonsillitis, as well as enhanced levels of $LTB_4$ in the exhaled breath condensate of these children [16]. The main objective of the present study was to compare $LTB_4$ production by stimulated PMNs in a group of 40 OSA patients, free of any cardiovascular history and medications, to that of a control group of 20 healthy volunteers. The secondary objectives were: 1) to study the relationship between OSA severity and $LTB_4$ production; 2) to evaluate the relationship between $LTB_4$ production and validated markers of early atherosclerosis (carotid luminal diameter and IMT); and 3) to determine the effect of CPAP on $LTB_4$ production.

## METHODS

### Population

Patients with newly diagnosed OSA (n=47) were prospectively included in the present study, as well as 20 control subjects. Patients were referred to the sleep laboratory of Grenoble University Hospital (A. Michallon Hospital, Grenoble, France) for symptoms suggesting OSA. The controls were healthy volunteers who received no compensation for their participation in the present study. All patients and controls underwent full polysomnography. They were nonsmokers and were free of symptoms or a history of medical or surgical treatment for cardiovascular diseases. Exclusion criteria were as follows: known hypertension, disease potentially affecting blood pressure regulation (Parkinson's disease, renal or cardiac transplantation, and severe cardiac heart failure), atrial fibrillation or frequent premature beats ($\geqslant$10 beats·min$^{-1}$), smoking, shift work, diabetes mellitus, asthma, chronic obstructive pulmonary disease, atopy, rhinitis, arthritis, oral appliances or maxillofacial surgery, or pharmacological treatment that could affect LT level. In order to minimise confounding risk factors for atherosclerosis, subjects aged >60 yrs and those with a body mass index (BMI) of >30 kg·m$^{-2}$ were excluded.

The control group was free of any acute or chronic cardiovascular, inflammatory or sleep disorders, and of any medication. Of the 47 OSA patients, 40 were matched for age, BMI and sex with the 20 control subjects for comparison of $LTB_4$ production, and 15 were treated with CPAP for $\geqslant$3 months.

The present study was approved by the local ethics committee in accordance with the Declaration of Helsinki. All of the participants gave their written informed consent.

### Polysomnography

The diagnosis of OSA was established by full polysomnography, which included recording of oronasal flow and thoracoabdominal movements, ECG, submental and pretibial electromyography, electro-oculography, electroencephalography (EEG) and transcutaneous measurement of arterial oxygen saturation ($S_{a,O_2}$). An apnoea was defined as a complete cessation of airflow for $\geqslant$10 s and a hypopnoea as a reduction in the nasal pressure signal of $\geqslant$50% or a reduction of 30–50% associated with either oxygen desaturation of $\geqslant$3% or an EEG arousal (defined according to the Chicago report [17]), both lasting for $\geqslant$10 s. The apnoea index (AI) was defined as the number of apnoeas (obstructive or mixed) per hour of sleep. The apnoea/hypopnoea index (AHI) was calculated as the total number of apnoeas and hypopnoeas (obstructive or mixed apnoeas plus obstructive hypopnoeas) per hour of sleep. The respiratory disturbance index (RDI) was calculated and defined as the total number of respiratory events (obstructive or mixed apnoeas, obstructive hypopnoeas and inspiratory flow limitation episodes) per hour of sleep (full polysomnography) or per hour of recording (polysomnography without EEG recording). Sleep apnoea was defined as an AHI $\geqslant$5 events·h$^{-1}$ and symptoms or an RDI >15 events·h$^{-1}$ [17, 18].

Venous blood for stimulated PMN experiments was collected at 07:00 h, immediately following nocturnal polysomnographic recordings.

### Carotid ultrasonography

Carotid ultrasonography was performed on 28 OSA patients and 11 controls as previously described [3]. The right common carotid artery was studied in the long axis with a probe incidence permitting good-quality images. The images were recorded at end-diastole and end-systole and then stored on an optical disc for subsequent analysis by a specific validated program (TIMC laboratory of Grenoble University Hospital). The common carotid IMT and luminal diameter were automatically measured. Carotid ultrasonography was performed by the same sonographer, who was blinded to the other study data. Analysis of the carotid parameters, using the specific software, was performed by the same operator for the duration of the present study.

### Isolation of human PMNs

Venous blood was drawn from all OSA patients and control subjects, and collected on citrate as anticoagulant. PMNs were isolated by dextran sedimentation, followed by Ficoll-Paque™ PLUS centrifugation (GE Healthcare, Stockholm, Sweden) as previously described [19]. Contaminating erythrocytes were eliminated by hypotonic lysis, and PMNs were washed in PBS (pH 7.4) containing 0.133 g·L$^{-1}$ $CaCl_2$ and 0.1 g·L$^{-1}$ $Mg^{2+}$ (Sigma, L'Isle d'Abeau, France). PMNs were finally resuspended in the same buffer at a concentration

**TABLE 1** Baseline characteristics of controls, obstructive sleep apnoea (OSA) patients and OSA patients stratified by hypoxia[#]

| | Controls | OSA | | | p-value[¶] |
|---|---|---|---|---|---|
| | | Whole population | Mild hypoxia | Moderate-to-severe hypoxia | |
| **Subjects n** | 20 | 40 | 19 | 21 | |
| **Males n (%)** | 15 (75) | 34 (85) | 16 (84) | 18 (86) | 0.58 |
| **Age yrs** | 46.1±7.2 | 48.6±7.1 | 47.9±8.2 | 49.1±6.1 | 0.21 |
| **BMI kg·m$^{-2}$** | 24.5±2.4 | 25.6±2.8 | 25.1±2.8 | 26.0±2.9 | 0.38 |
| **Clinical SBP mmHg** | 121±11 | 127±13 | 127±10 | 126±16 | 0.26 |
| **Clinical DBP mmHg** | 80±9 | 82±10 | 85±10 | 80±11 | 0.32 |
| **Sleep respiratory parameters** | | | | | |
| AI events·h$^{-1}$ | 0.2±0.7$^{+,\S}$ | 8.4±10.9* | 6.9±9.2 | 9.7±12.4 | <0.0001 |
| AHI events·h$^{-1}$ | 3.6±5.2$^{+,\S}$ | 29.0±16.6* | 24.4±18.2 | 32.0±14.4 | <0.0001 |
| RDI events·h$^{-1}$ | 11.2±5.2$^{+,\S}$ | 40.9±14.2* | 38.8±14.8 | 43.1±12.9 | <0.0001 |
| Mean $Sa,O_2$ % | 95.2±1.7 | 94.2±1.8* | 95.6±1.5 | 92.9±1.0$^{*,+}$ | <0.0001 |
| Minimal $Sa,O_2$ % | 89.2±4.3 | 84.8±6.5* | 88.7±4.2 | 81.4±6.6$^{*,+}$ | <0.0001 |
| $Sa,O_2$<90% %TST | 0.3±0.8 | 3.4±6.7* | 0.6±1.2 | 6.0±8.2$^{*,+}$ | <0.0001 |
| RAI events·h$^{-1}$ | 21.9±10.6$^{+,\S}$ | 37.5±13.1* | 39.3±13.6 | 35.9±14.7 | 0.0002 |
| **Biological parameters** | | | | | |
| Fasting plasma glucose mM | 4.8±0.4 | 5.1±0.6 | 5.1±0.8 | 5.0±0.5 | 0.16 |
| Fasting plasma insulin μUI·mL$^{-1}$ | 5.4±2.5 | 6.7±2.8 | 7.0±3.5 | 6.4±2.2 | 0.23 |
| HOMA-R index | 1.2±0.5 | 1.5±0.7* | 1.6±0.9 | 1.4±0.6 | 0.11 |
| Total cholesterol g·L$^{-1}$ | 1.9±0.3 | 2.1±0.3 | 2.2±0.3 | 2.0±0.2 | 0.03 |
| HDL cholesterol g·L$^{-1}$ | 0.6±0.2 | 0.6±0.2 | 0.6±0.2 | 0.6±0.1 | 0.93 |
| LDL cholesterol g·L$^{-1}$ | 1.1±0.3 | 1.2±0.3 | 1.3±0.2 | 1.2±0.3 | 0.18 |
| Triglycerides g·L$^{-1}$ | 0.8±0.4$^{\S}$ | 1.2±0.1* | 1.2±0.6 | 1.2±0.7 | 0.03 |
| **$LTB_4$ ng·mL$^{-1}$** | 12.0±4.5 | 14.3±4.7 | 12.3±4.2 | 16.1±4.6$^{*,+}$ | 0.003 |
| **Early markers of atherosclerosis n** | 11 | 28 | 12 | 16 | |
| **Systolic carotid diameter μm** | 6167±639 | 6356±657 | 6126±633 | 6507±678 | 0.23 |
| **Diastolic carotid diameter μm** | 5716±597 | 5979±618 | 5756±623 | 6127±620 | 0.14 |
| **Intima–media thickness μm** | 551±85 | 615±148 | 635±520 | 601±100 | 0.35 |

Data are presented as mean±SD, unless otherwise stated. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; AI: apnoea index; AHI: apnoea/hypopnoea index; RDI: respiratory disturbance index; $Sa,O_2$: arterial oxygen saturation; TST: total sleep time; RAI: respiratory arousal index; HOMA-R: homeostasis model assessment of insulin resistance; HDL: high-density lipoprotein; LDL: low-density lipoprotein; LT: leukotriene. [#]: $Sa,O_2$ of >94% in mild hypoxia and $Sa,O_2$ of ⩽94% in moderate-to-severe hypoxia; [¶]: ANOVA or Kruskal–Wallis test for comparison between controls and OSA patient subgroups, with subsequent pairwise comparisons made using the Bonferroni or Kruskal–Wallis multiple comparison test. *: p<0.05 *versus* controls; [+]: p<0.05 *versus* mild hypoxic OSA; [§]: p<0.05 *versus* moderate-to-severe hypoxic OSA.

of $2\times10^6$ cells·mL$^{-1}$. Cellular viability was >98% as judged by the trypan blue exclusion method.

### *Cell stimulation*

PMNs ($2\times10^6$ cells·mL$^{-1}$) were incubated for 15 min at 37°C in the presence of 10 μM A23187 (Sigma) or vehicle as previously described [11]. Incubations were stopped by centrifugation for 5 min at 5,000×*g* at 4°C, and the supernatants were stored at -80°C until subsequent analysis.

### *Quantification of $LTB_4$ by liquid chromatography–tandem mass spectrometry*

Quantification of $LTB_4$ was performed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using a method adapted from a method previously described for $LTE_4$ [20]. The measurement of $LTB_4$ was performed on 400 μL centrifuged supernatant. Deuterated $LTB_4$ ($LTB_4$-d4; 2 ng) was added to each sample as an internal standard. Solid-phase extraction was performed as previously described [20]. Methanolic extracts were dried under nitrogen flow at room temperature and reconstituted in 40 μL mobile phase (methanol and 10 mM ammonium formate; 80:20 volume:volume). After centrifugation, 10 μL of reconstituted extract were injected into the LC-MS/MS system previously described [20]. The chromatographic separation was obtained on a 5-μm Kromasil C8 column (125×2 mm; Macherey-Nagel, Hoerdt, France) maintained at 30°C. The mobile phase consisted of 10 mM ammonium formate (phase A) and methanol (phase B) delivered at a flow rate of 200 μL·min$^{-1}$ as follows: initial 50% B maintained for 6 min, then increased in a linear gradient to 80% B in 6 min and maintained at 80% B for 11 min.

MS/MS acquisitions were made in the negative-ion mode using multiple reaction monitoring, and monitoring the $m/z$ transitions from 335.0 to 195.1 for $LTB_4$ and from 339.1 to 196.9 for $LTB_4$-d4. Calibration curves were constructed using

**TABLE 2** Correlation coefficients (r) between leukotriene $B_4$ production and age, body mass index (BMI) and polysomnographic and metabolic variables in obstructive sleep apnoea patients

| | r | p-value |
|---|---|---|
| **Age** | 0.14 | 0.24 |
| **BMI** | 0.20 | 0.22 |
| **AI** | 0.29 | 0.07 |
| **AHI** | 0.31 | 0.05 |
| **RDI** | 0.22 | 0.18 |
| **Mean $S_{a,O_2}$** | -0.53 | 0.001 |
| **Minimal $S_{a,O_2}$** | -0.51 | 0.001 |
| **$S_{a,O_2}$<90% %TST** | 0.47 | 0.003 |
| **Total arousal index** | 0.02 | 0.89 |
| **Fasting plasma glucose** | -0.14 | 0.41 |
| **Fasting plasma insulin** | 0.05 | 0.75 |
| **HOMA-R index** | -0.028 | 0.86 |
| **Total cholesterol** | -0.28 | 0.09 |
| **HDL cholesterol** | 0.09 | 0.59 |
| **LDL cholesterol** | -0.29 | 0.09 |
| **Triglycerides** | 0.03 | 0.87 |

AI: apnoea index; AHI: apnoea/hypopnoea index; RDI: respiratory disturbance index; $S_{a,O_2}$: arterial oxygen saturation; TST: total sleep time; HOMA-R: homeostasis model assessment of insulin resistance; HDL: high-density lipoprotein; LDL: low-density lipoprotein;

weighted ($1/x$) linear least-square regression. The lower limit of quantification was 60 pg·mL$^{-1}$ for $LTB_4$.

### *Statistical analysis*

Continuous data are presented as mean±SD, and noncontinuous data as n (%). Normality was evaluated using skewness and kurtosis tests. Comparisons between continuous variables were made using an unpaired t-test or Mann–Whitney U-test. Noncontinuous variables were compared using a Chi-squared test. Comparisons between the three groups (two OSA groups and control subjects) were made using ANOVA or the Kruskal–Wallis test; subsequent pairwise comparisons were performed using the Bonferroni or Kruskal–Wallis multiple comparison test. Correlations were analysed using the Pearson or Spearman rank test. A multiple regression analysis was performed taking into account the variables that correlated with the dependant variable $LTB_4$. The differences between baseline and post-CPAP values were analysed by means of a paired t-test or the Wilcoxon signed-rank test. A p-value of $<0.05$ was considered significant.

## RESULTS

### *$LTB_4$ production stimulated by A23187*

The baseline characteristics of the study population are described in table 1. There were no significant differences between controls and OSA patients in terms of age, BMI, sex and blood pressure. Conversely, triglyceride levels were significantly higher in OSA patients than in control subjects. As expected, sleep respiratory disturbance parameters differed significantly between OSA patients and controls. Early markers of atherosclerosis (carotid diameter and IMT) did not differ significantly between groups.

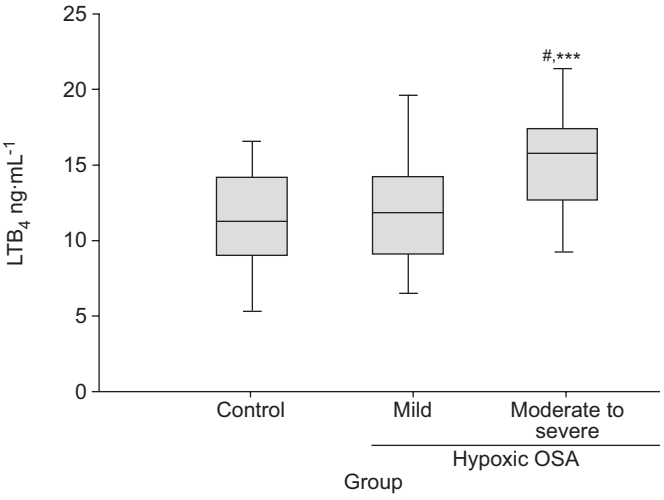


**FIGURE 1.** Boxplot showing leukotriene (LT)$B_4$ release by polymorphonuclear leukocytes from obstructive sleep apnoea (OSA) patients and controls in response to calcium ionophore. Boxes represent median and interquartile range; vertical bars represent range. $LTB_4$ release was significantly higher in the moderate-to-severe hypoxic OSA group (n=21) than in the control group (n=20) or mild hypoxic OSA group (n=19). #: p<0.003 *versus* control. ***: p<0.001 *versus* mild hypoxic OSA group.

PMNs stimulated with 10 μM A23187 produced $LTB_4$, whereas unstimulated PMNs did not (data not shown). The production of $LTB_4$ by PMNs stimulated with A23187 was increased in OSA patients compared with control subjects (14.3±4.7 *versus* 12.0±4.5 ng·mL$^{-1}$; p<0.05).

As shown in table 2, a significant correlation was found between $LTB_4$ production and mean nocturnal $S_{a,O_2}$, minimal nocturnal $S_{a,O_2}$, percentage of time spent with an $S_{a,O_2}$ of <90% and AHI in OSA patients. No significant correlation was observed between $LTB_4$ production and age or metabolic variables (BMI, cholesterol, triglycerides, insulin, fasting glucose or homeostasis model assessment of insulin resistance index; see table 2).

In an attempt to further define the relationship between either $LTB_4$ production and hypoxia or $LTB_4$ production and early signs of atherosclerosis, *post hoc* analyses were performed.

First, multiple regression analysis was conducted taking into account the variables correlated with the dependant variable $LTB_4$ production (AHI and mean $S_{a,O_2}$). Since there was a trend towards a correlation between low-density lipoprotein (LDL) cholesterol and $LTB_4$ production (p=0.09), this variable was also included in the model. This analysis yielded a model in which mean $S_{a,O_2}$ was the strongest independent predictor of $LTB_4$ production (p=0.0006; p=0.026 for LDL cholesterol and p=0.75 for AHI). Therefore, OSA patients were stratified on the basis of mean $S_{a,O_2}$. The median mean $S_{a,O_2}$ in OSA patients (*i.e.* 94%) was used to separate the OSA patients into two groups: mild hypoxic OSA (mean $S_{a,O_2}$ of >94%), and moderate-to-severe hypoxic OSA (mean $S_{a,O_2}$ of ⩽94%). The baseline characteristics of the two groups of OSA patients are detailed

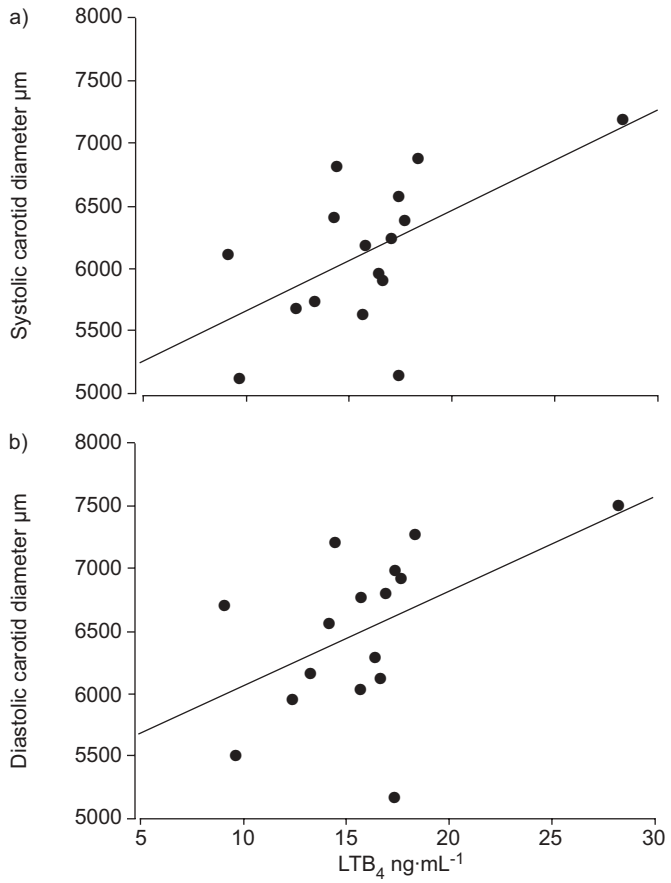


**FIGURE 2.** Leukotriene (LT)$B_4$ release by polymorphonuclear leukocytes in response to calcium ionophore correlated with: a) systolic carotid diameter (r=0.55, p=0.034); and b) diastolic carotid diameter (r=0.54, p=0.036) in moderate-to-severe hypoxic obstructive sleep apnoea.

in table 1. As shown in figure 1 and table 1, production of $LTB_4$ by PMNs stimulated with A23187 was significantly higher in the moderate-to-severe hypoxic OSA group than in the mild hypoxic OSA group and control group.

Secondly, in severe hypoxic OSA patients, the influence of the increased production of $LTB_4$ on early markers of atherosclerosis was investigated. As shown in figure 2, $LTB_4$ production correlated with the systolic (r=0.55; p=0.034) and diastolic (r=0.54; p=0.036) carotid diameters in severe hypoxic OSA. A significant correlation was also found between systolic luminal diameter and $LTB_4$ production (r=0.39; p<0.002) and diastolic diameter (r=0.41; p=0.01) in the whole population. Conversely, IMT did not correlated with $LTB_4$ production in moderate-to-severe OSA patients (p=0.79) or in the whole population (p=0.96).

### *Effect of CPAP on $LTB_4$ production in moderate-to-severe hypoxic OSA patients*

$LTB_4$ production was evaluated in 15 OSA patients (mean age 55±7 yrs) who were successfully treated with CPAP for ⩾3 months (mean duration 178±96 days) and were regularly using their CPAP (5.2±1.3 h·night$^{-1}$). As shown in table 3, CPAP significantly decreased AI, AHI and RDI, increased both minimal and mean nocturnal $S_{a,O_2}$, and decreased the percentage of time spent with an $S_{a,O_2}$ of <90%. No significant change occurred in BMI after treatment with CPAP. Conversely, CPAP significantly decreased A23187-mediated $LTB_4$ production (table 3; fig. 3). During the same period, $LTB_4$ production remained unchanged in control subjects (11.4±1.2 and 12.4±3.2 ng·mL$^{-1}$ at the beginning and end of the 3-month study period, respectively; n=8). The production of $LTB_4$ did not differ significantly between CPAP-treated OSA patients and controls (p=0.26).

**TABLE 3** Characteristics of obstructive sleep apnoea patients# before and after treatment with continuous positive airway pressure (CPAP)

| | Before CPAP | After CPAP | p-value |
|---|---|---|---|
| **BMI kg·m$^{-2}$** | 26.8±2.8 | 27.2±1.8 | 0.28 |
| **AI events·h$^{-1}$** | 7.1±9.3 | 0.4±0.8 | 0.02 |
| **AHI events·h$^{-1}$** | 34.6±15.6 | 2.3±3.2 | <0.001 |
| **RDI events·h$^{-1}$** | 43.8±9.8 | 6.2±5.8 | <0.001 |
| **Mean nocturnal $S_{a,O_2}$ %** | 93.8±1.2 | 95.0±0.8 | <0.001 |
| **Minimal nocturnal $S_{a,O_2}$ %** | 82.9±7.6 | 90.3±3.3 | <0.001 |
| **$S_{a,O_2}$<90% %TST** | 4.8±6.1 | 0.1±0.3 | 0.01 |
| **RAI events·h$^{-1}$** | 42.8±13.1 | 26.0±10.6 | <0.001 |
| **$LTB_4$ ng·mL$^{-1}$** | 15.2±4.6 | 10.2±3.6 | <0.001 |

Data are presented as mean±SD, unless otherwise stated. BMI: body mass index; AI: apnoea index; AHI: apnoea/hypopnoea index; RDI: respiratory disturbance index; $S_{a,O_2}$: arterial oxygen saturation; TST: total sleep time; RAI: respiratory arousal index; LT: leukotriene. #: n=15.

## DISCUSSION

The present study represents the first demonstration of increased production of $LTB_4$ in OSA in relation to nocturnal oxygen desaturation severity. Moreover, in moderate-to-severe hypoxic OSA patients, the enhanced production of $LTB_4$ was associated with an increased carotid luminal diameter. Finally, 3 months of CPAP treatment significantly reduced $LTB_4$ production. These results suggest that $LTB_4$ could be one of the mediators relating oxygen desaturation severity and early vascular changes in OSA patients.

Previous studies demonstrated activation of the $LTB_4$ pathway in patients with cardiovascular diseases. In particular, enhanced production of $LTB_4$ by stimulated PMNs has been reported in patients with a past history of myocardial infarction or stroke [11]. As in this previous study, production of $LTB_4$ was evaluated by challenge with calcium ionophore. A23187 induces a rise in the intracellular calcium level of PMNs and the translocation of 5-LO from the cytosol to the nuclear membrane [9], thereby permitting the direct evaluation of 5-LO pathway activity independently of any receptor-dependent signalling pathway.

A classic issue in clinical research addressing cardiovascular consequences associated with OSA is confounding factors. The inclusion of obese OSA patients with severe desaturation is generally criticised owing to the prominent role of BMI. For

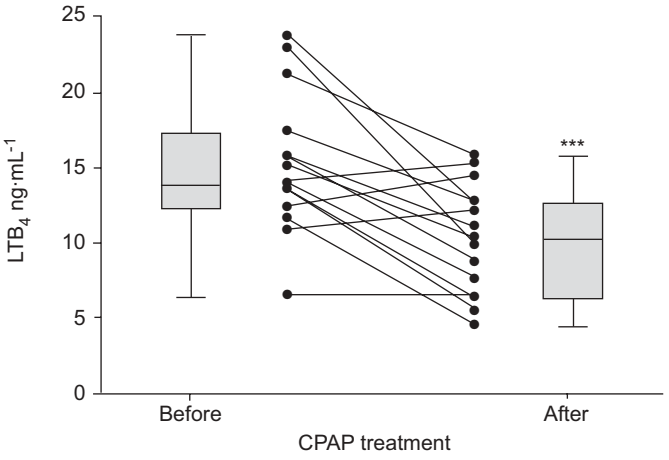


**FIGURE 3.** Boxplot showing effect of treatment with continuous positive airway pressure (CPAP) on leukotriene (LT)$B_4$ production in 15 moderate-to-severe hypoxic obstructive sleep apnoea patients. Boxes represent median and interquartile range; vertical bars represent range. Data for individual patients are also shown. Treatment with CPAP significantly reduced $LTB_4$ production in these patients. ***: $p<0.001$ *versus* before CPAP.

example, several studies addressing oxidative stress or inflammation in obese OSA patients have demonstrated that obesity is the main contributor to these biological changes [21, 22]. The classic means of avoiding this limitation is thus to match controls and OSA patients, which results in the inclusion of OSA patients exhibiting moderate oxygen desaturation. As already mentioned, it was decided to include only carefully selected middle-aged non-obese OSA patients and also to exclude patients exhibiting any cardiovascular events, including known hypertension, myocardial infarction and stroke. This strict selection permitted the rigorous comparison of $LTB_4$ production in controls and OSA patients, being free of any confounding factor, and thus highlighting the specific role of even moderate intermittent hypoxia in $LTB_4$ production in OSA. Although triglyceride levels were significantly higher in OSA patients, and total cholesterol tends to be increased in OSA patients, these factors did not correlate with $LTB_4$ production, suggesting that they may not contribute to the increased production of $LTB_4$ in OSA.

In the present study, it was demonstrated, on multivariate analysis, that the main determinant of increased $LTB_4$ production was mean $Sa,O_2$, suggesting that intermittent hypoxia, leading to oxygen desaturation, may play a major role in the increased $LTB_4$ production evidenced in OSA patients. The desaturation–reoxygenation sequence is a typical pattern coupled with the majority of respiratory events in OSA patients. This sequence leads to oxidative/nitrosative stress, with production of reactive oxygen species [23] and reactive nitrogen species [24], which are the most important free radicals. The increased levels of reactive oxygen species contribute to the generation of adhesion molecules [25], activation of leukocytes [26] and production of systemic inflammation [27]. Since 5-LO activity is regulated by the cellular redox status and reactive oxygen species [28], the increased production of reactive species in leukocytes from OSA patients [29] could contribute to the activation of the $LTB_4$ pathway in OSA. Exposure of isolated PMNs to hypoxia/normoxia sequences is required to provide definite evidence regarding the role of intermittent hypoxia in $LTB_4$ release.

Since increased production of $LTB_4$ in moderate-to-severe hypoxic OSA patients was clearly demonstrated, and since $LTB_4$ is a mediator of atherogenesis [30, 31], the potential relationship between $LTB_4$ production and various markers of early vascular remodelling that have been demonstrated to be associated with infraclinical atherosclerosis was investigated. Carotid imaging was performed in a more limited group of controls and OSA patients but this subgroup did not differ significantly in terms of anthropometric variables and severity of sleep apnoea. Previous studies have reported increased carotid IMT in OSA patients [3, 4, 32]. Having excluded other cardiovascular risk factors in the present carefully selected population is the probable explanation for the nonsignificant difference found between OSA patients and controls regarding IMT. Indeed, previous studies showing early signs of atherosclerosis in OSA have generally been performed in overweight patients (a BMI of $28.1\pm0.6$ and $29.3\pm0.6$ kg·m$^{-2}$ in the studies of MINOGUCHI *et al.* [32] and DRAGER *et al.* [4], respectively). Similarly, an increased IMT was found in OSA by SILVESTRINI *et al.* [33]; however, their studied population included smokers (22%), hypertensive subjects (65%) and patients with diabetes (17%). Finally, in the studies of both DRAGER *et al.* [4] and BAGUET *et al.* [3], only OSA patients exhibiting the most severe oxygen desaturation showed carotid hypertrophy. IMT is an established predictor of atherosclerosis [34], but luminal diameter has also been recommended for measurement since there is evidence for an association between increased diameter and the early stages of vascular remodelling [35–37]. Moreover, DRAGER *et al.* [3] have used the same parameter in assessing atherosclerosis in OSA. Interestingly, whereas carotid IMT was increased only in the most severe patients, carotid diameter was significantly higher in both moderate and severe patients. This suggests that carotid luminal diameter is a more sensitive marker of early atherosclerosis in OSA, and might explain why it was the only parameter that correlated with $LTB_4$ production in the present study. A demonstration of a reduction in carotid diameter under CPAP would have strengthened these data, but such measurements were not available in the present study.

The crucial role of $LTB_4$ in the early stages of atherogenesis is now well established [10]. $LTB_4$ is a potent chemoattractant that facilitates recruitment and endothelial cell adhesion of neutrophils to the inflammatory site and promotes recruitment of inflammatory cells into tissues. Recruitment of leukocytes and leukocyte invasion of the arterial wall are critical steps in the development of atherogenesis. Consistent with these findings, pharmacological blockade of the 5-LO pathway [38] prevents atherosclerosis development in mice, and genetic experiments have identified 5-LO as a major gene contributing to atherosclerosis susceptibility in mice [39].

If the hypoxic stress of OSA is a causal factor in promoting $LTB_4$ pathway activation, then treatment with CPAP should reduce $LTB_4$ formation. In the present study, it was shown that the production of $LTB_4$ by stimulated PMNs is reduced after a 3-month minimum period of CPAP in compliant patients. During the same time, $LTB_4$ production remained unchanged

in control subjects, demonstrating reliability and reproducibility of these measurements. These data are consistent with a recent study showing that 4 months of treatment with CPAP reduces early signs of atherosclerosis [6]. Thus further evidence is provided that, under conditions in which confounding factors and comorbid conditions are minimised, CPAP reduces $LTB_4$ production and could thereby limit atherosclerosis development. With regard to the 40% of OSA patients noncompliant with CPAP treatment, targeting the $LTB_4$ pathway could represent a new therapeutic strategy in the prevention of the cardiovascular consequences of OSA. However, this should be further validated in interventional studies.

In conclusion, leukotriene $B_4$ production is increased in obstructive sleep apnoea patients, and correlates with the severity of oxygen desaturation. The present results are the first to suggest that leukotriene $B_4$ could be a new candidate mediator for explaining the relationship between oxygen desaturation severity and early atherosclerosis in obstructive sleep apnoea patients.

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