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Elodie Gautier-Veyret

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## **THÈSE**

Pour obtenir le grade de

### **DOCTEUR DE LA COMMUNAUTÉ UNIVERSITÉ GRENOBLE ALPES**

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Présentée par

**Elodie GAUTIER-VEYRET**

Thèse dirigée par **Françoise STANKE-LABESQUE**

préparée au sein du **Laboratoire HP2 INSERM U1042**  
dans l'École Doctorale Chimie et Sciences du Vivant

## **Rôle des eicosanoïdes dans l'athérogénèse associée au syndrome d'apnées obstructives du sommeil : approches clinique et expérimentale**

Thèse soutenue publiquement le **28 novembre 2016**,  
devant le jury composé de :

**Mr Jean-Louis PEPIN**

PU-PH, Université Grenoble Alpes, président

**Mr Philippe DEVILLIER**

PU-PH, Université de Versailles, rapporteur

**Mr Vincent RICHARD**

PU-PH, Université de Rouen, rapporteur

**Mme Claire ARNAUD**

CR, Université Grenoble Alpes, examinateur

**Mr Wilfried LE GOFF**

CR, Université Pierre et Marie Curie, examinateur

**Mme Françoise STANKE-LABESQUE**

PU-PH, Université Grenoble Alpes, directeur



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## **Résumé/Abstract**

## Résumé

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Le syndrome d'apnées obstructives du sommeil (SAOS) affecte 5 à 20 % de la population générale et est associé à des complications cardiovasculaires, ce qui en fait un véritable problème de santé publique. Les épisodes itératifs d'obstruction pharyngée nocturne qui le caractérisent aboutissent à une hypoxie intermittente, elle-même impliquée dans ces complications cardiovasculaires.

A ce jour, les mécanismes reliant SAOS et athérosclérose restent méconnus. De plus, le traitement de référence du SAOS par pression positive continue, présente dans certaines populations une efficacité limitée sur les conséquences cardiovasculaires du SAOS, d'où la nécessité de développer de nouvelles thérapeutiques ciblant spécifiquement le processus athéromateux. Des perturbations du métabolisme de l'acide arachidonique à l'origine d'une augmentation de la synthèse d'eicosanoïdes pro-inflammatoires ont déjà été décrites au cours du SAOS et étaient liées au processus athéromateux. Le but de ce travail a donc été de préciser par une approche translationnelle le rôle de certains eicosanoïdes jusqu'alors peu étudiés, à savoir le thromboxane  $A_2$  et les cystéinyl-leucotriènes dans l'athérogénèse associée au SAOS et de les évaluer en tant que potentielles cibles thérapeutiques.

Nous avons démontré une activation des voies du thromboxane  $A_2$  et des cystéinyl-leucotriènes au cours du SAOS, et précisé la part relative de l'hypoxie intermittente et des facteurs confondants dans cette activation. Ces voies métaboliques étaient par ailleurs associées au processus athéromateux. Enfin, des traitements pharmacologiques ciblant spécifiquement ces médiateurs étaient capables dans un modèle murin de SAOS de ralentir la progression de l'athérosclérose induit par l'hypoxie intermittente. Ces travaux démontrent donc l'implication du thromboxane  $A_2$  et des cystéinyl-leucotriènes dans l'athérosclérose associée au SAOS, et positionnent ces deux voies métaboliques comme des cibles thérapeutiques potentielles pour traiter les conséquences cardiovasculaires du SAOS.

Mots-clés : syndrome d'apnées obstructives du sommeil, eicosanoïdes, athérosclérose, hypoxie intermittente

## Abstract

---

Obstructive sleep apnea (OSA) is a major public health problem since it affects 5 to 20% of general population and is associated with increased cardiovascular morbidity and mortality. Repetitive nocturnal pharyngeal obstructions lead to intermittent hypoxia, which is responsible of early atherosclerosis and cardiovascular complications.

Nevertheless, the underlying mechanisms linking OSA and atherosclerosis remain poorly understood. In addition, continuous positive airway pressure (CPAP) application, which is the gold standard treatment of OSA, has poor effects on OSA cardiovascular consequences in some populations, highlighting the need of alternative therapeutic strategies. Changes of several eicosanoid biosynthesis have already been described during OSA and associated with vascular remodeling. The aim of this work was to determine through a translational approach the contribution of thromboxane A<sub>2</sub> and cysteinyl-leukotrienes on OSA related-atherogenesis and also to evaluate these pathways as potential therapeutic targets.

We have shown an activation of both thromboxane A<sub>2</sub> and cysteinyl-leukotriene pathways in OSA and determined the respective contribution of chronic intermittent hypoxia and confounders in this activation. Thromboxane A<sub>2</sub> and cysteinyl-leukotriene pathways were associated with vascular remodeling. Finally, pharmacological treatment by cyclooxygenase type 1 inhibitor or CysLT<sub>1</sub> receptor antagonist reduced IH-related atherosclerosis progression in an OSA mouse model.

Overall, these works have demonstrated the implication of both thromboxane A<sub>2</sub> and cysteinyl-leukotriene activation in OSA-related atherosclerosis, but also the therapeutic interest to inhibit these pathways to treat the cardiovascular consequences of OSA.

Key-words: obstructive sleep apnea, eicosanoids, atherosclerosis, intermittent hypoxia.



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## Liste des abréviations

SAOS : syndrome d'apnées obstructives du sommeil

COX : cyclooxygénase

CRP : protéine C réactive

CysLTs : Cystéinyl-leucotriènes

EIM : épaisseur intima-media

FiO<sub>2</sub> : fraction partielle en oxygène

FLAP : *five lipoxygenase activating protein*

FMD: flux-mediated dilatation

HI: hypoxie intermittente

HTA : hypertension artérielle

IAH : index d'apnées-hypopnées

LT : leucotriène

LTB<sub>4</sub> : leucotriène B<sub>4</sub>

LTC<sub>4</sub> : leucotriène C<sub>4</sub>

LTD<sub>4</sub> : leucotriène D<sub>4</sub>

LTE<sub>4</sub> : leucotriène E<sub>4</sub>

11dTXB<sub>2</sub>: 11-dehydrothromboxane A<sub>2</sub>

PPC: pression positive continue

ROS: *reactive oxygen species*

TXA<sub>2</sub> : thromboxane A<sub>2</sub>

TXB<sub>2</sub> : thromboxane B<sub>2</sub>

VOP : vitesse de l'onde de pouls

## **Introduction générale**

Cinq à 20 % de la population générale des pays industrialisés sont aujourd'hui touchés par le syndrome d'apnées obstructives du sommeil (SAOS). Ce syndrome se caractérise par des épisodes répétés de collapsus pharyngé au cours du sommeil aboutissant à une fragmentation du sommeil, une augmentation des efforts respiratoires et une hypoxie intermittente (HI). Ce syndrome est par ailleurs associé à un remodelage vasculaire précoce à l'origine d'une morbi-mortalité cardiovasculaire augmentée.

Le traitement de référence du SAOS consiste aujourd'hui en l'application d'une pression positive continue (PPC) dans les voies aériennes supérieures de manière à éviter leur obstruction. Bien qu'efficace sur les événements obstructifs et l'hypersomnolence diurne, cette thérapeutique présente plusieurs limites. De par son caractère contraignant, l'adhésion du patient est difficile à obtenir ; ainsi seuls 65 à 80 % des patients sont observants. De plus, l'efficacité de la PPC sur les conséquences cardiovasculaires du SAOS est limitée dans certaines populations, notamment chez les patients obèses. Or l'obésité est très fréquente dans la population apnéique et est elle-même associée à un risque cardiovasculaire augmenté. Au final, de nombreux patients diagnostiqués SAOS ne sont pas traités efficacement d'où l'absolue nécessité de développer des alternatives thérapeutiques à la PPC.

Les eicosanoïdes regroupent une vaste famille de médiateurs lipidiques issus du métabolisme de l'acide arachidonique. Bon nombre de ces médiateurs possèdent des propriétés proathérogènes telles que la capacité à induire la prolifération et la contraction des cellules musculaires lisses vasculaires ou encore l'expression de molécules d'adhésion. Des travaux antérieurs menés au sein du laboratoire ont déjà mis en évidence des perturbations du métabolisme de l'acide arachidonique au cours du SAOS avec notamment des concentrations augmentées de leucotriène B<sub>4</sub> (LTB<sub>4</sub>), leucotriène E<sub>4</sub> (LTE<sub>4</sub>) et isoprostanes. Ces perturbations étaient associées non seulement aux paramètres polysomnographiques mais également au remodelage vasculaire, suggérant que ces médiateurs pourraient jouer un rôle dans le processus athéromateux associé au SAOS. En revanche,

certaines eicosanoïdes tels que le thromboxane A<sub>2</sub> (TXA<sub>2</sub>) n'ont été que très peu étudiés au cours du SAOS, tandis que d'autres tels que les cystéinyl-leucotriènes (CysLTs) l'ont été sans que leur rôle dans le processus athéromateux n'ait été investigué.

L'objectif premier de ce travail a été de caractériser les voies métaboliques du TXA<sub>2</sub> et des CysLTs au cours du SAOS et de déterminer leur rôle dans l'athérosclérose propre au SAOS. Le second objectif a été d'évaluer ces deux voies métaboliques en tant que cibles thérapeutiques potentielles de manière à envisager des alternatives thérapeutiques pour le patient apnéique en impasse thérapeutique.

Le manuscrit débute par une partie bibliographique consacrée au SAOS, ses complications cardiovasculaires et les mécanismes physiopathologique sous-jacents, ainsi qu'à sa prise en charge thérapeutique. Cette partie comprend notamment deux revues de la littérature : la première consacrée au rôle des leucotriènes (LTs) dans le processus athéromateux associé au SAOS ; la seconde consacrée à l'efficacité des différentes stratégies thérapeutiques sur les complications vasculaires associées au SAOS, avec une attention particulière pour les traitements pharmacologiques.

La seconde partie de ce manuscrit expose les différents travaux réalisés sous forme de deux articles scientifiques originaux accompagnés d'une revue de la littérature qui positionne ce travail dans l'état de l'art scientifique international.

Enfin, la troisième et dernière partie constitue une analyse critique et une synthèse des résultats de cette thèse ouvrant vers les perspectives et les éventuelles retombées thérapeutiques qui en découlent.

## **Première partie : données de la littérature**



## 1. Le syndrome d'apnées obstructives du sommeil (SAOS) - Généralités

### 1.1. Définitions

Le SAOS est défini par l'association de signes cliniques et d'évènements respiratoires nocturnes à dominante obstructive en nombre supérieur à 5 par heure, ou par la présence d'au moins 15 évènements respiratoires à dominante obstructive par heure de sommeil indépendamment de tout symptôme ou signe clinique (1) (Figure 1).

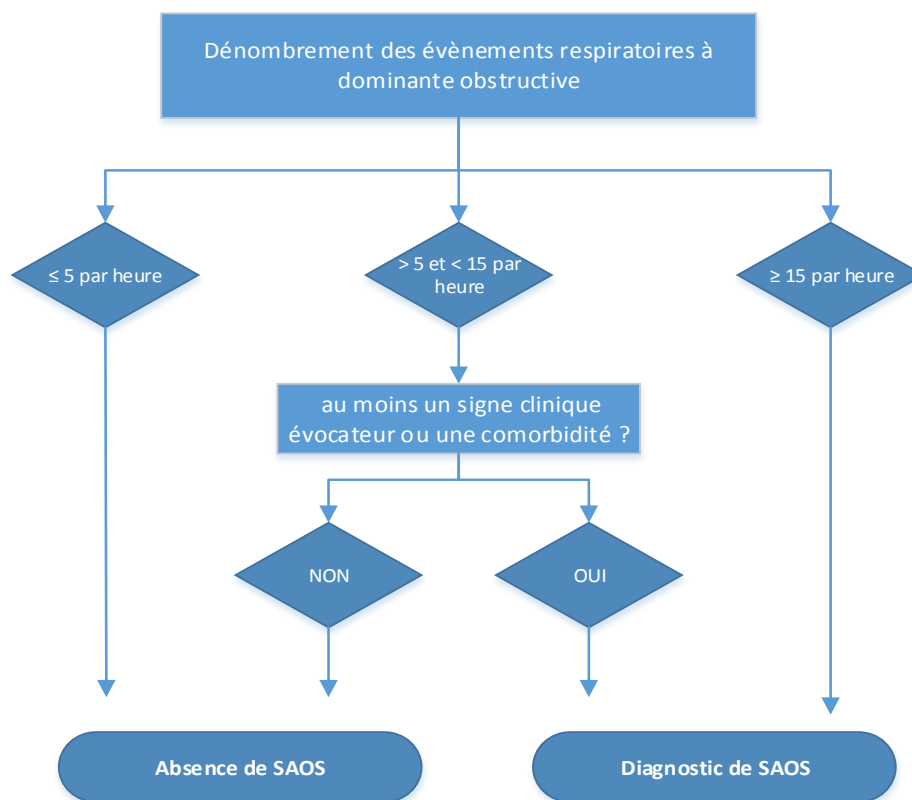


Figure 1. Critères diagnostiques du SAOS d'après Levy et al (1)

Les signes cliniques ou symptômes retenus pour le diagnostic du SAOS regroupent à la fois des symptômes nocturnes tel que le ronflement, des manifestations diurnes telles que l'hypersomnolence, et des comorbidités telles que la présence d'une hypertension artérielle (HTA) ou encore de troubles de l'humeur (Tableau 1)

Signes cliniques/Symptômes	Comorbidités
<i>Diurnes</i>	
Hypersomnolence diurne	Hypertension artérielle
Sommeil non réparateur	Trouble de l'humeur
Fatigue	Dysfonction cognitive
Insomnie	Coronaropathie
<i>Nocturnes</i>	
Eveils en sursaut	Accident vasculaire cérébral
Etouffements nocturnes	Insuffisance cardiaque
Ronflement	Fibrillation atriale
Pauses respiratoires	Diabète de type 2

Tableau 1. Signes cliniques ou comorbidités retenus pour le diagnostic d'un SAOS, d'après Levy et al (1)

Les évènements obstructifs respiratoires regroupent les apnées, les hypopnées et les éveils respiratoires, chacun de ces évènements ayant été définis selon des critères standardisés par l'*American Academy of Sleep Medicine* (2).

Une apnée est définie par la cessation totale du débit aérien durant plus de 10 secondes. Elle est dite obstructive si les efforts respiratoires persistent, par opposition à l'apnée centrale caractérisée par la disparition des mouvements respiratoires. Une apnée peut également être qualifiée de mixte, si elle évolue d'une origine centrale vers une origine obstructive.

Une hypopnée est définie par une diminution d'au moins 50 % du flux aérien durant plus de 10 secondes par rapport à une période de référence stable ou une diminution « significative » de la ventilation associée à une diminution de la saturation artérielle en oxygène de plus de 3 % par rapport à une période de référence stable et/ou un microéveil.

L'ensemble de ces évènements obstructifs respiratoires est mis en évidence et comptabilisé au cours d'une étude du sommeil qui peut prendre la forme d'une polygraphie de ventilation ou d'une polysomnographie, ce dernier constituant l'examen de référence.

Le dénombrement de ces évènements respiratoires permet de calculer l'indice d'apnées-hypopnées (IAH) défini par la somme des apnées et hypopnées obstructives par heure de sommeil. Cet index, largement utilisé, permet notamment d'évaluer la sévérité du SAOS. Les patients sont ainsi classés comme SAOS léger, modéré et sévère pour des IAH respectivement compris entre 5 et 15, entre 15 et 30 et strictement supérieur à 30 évènements par heure.

La sévérité du SAOS peut en outre être évaluée par la sévérité de l'hypersomnolence diurne quantifiée le plus souvent à l'aide de l'échelle de somnolence d'Epworth, ou encore par l'index de désaturation en O<sub>2</sub>.

### ***1.2. Epidémiologie***

La prévalence du SAOS varie selon les études entre 5 et 20 % en population générale, avec une augmentation nette de ce chiffre après 60 ans. Chez l'homme, cette prévalence est estimée aux alentours de 5 à 26 %, contre 6 à 15 % pour les femmes. Ces fourchettes de prévalence assez larges sont le résultat de plusieurs études menées dans des populations variées et utilisant des procédures (polysomnographie/polygraphie) et des critères de diagnostic (seuil d'IAH retenu et prise en compte ou non des signes cliniques évocateurs de SAOS) différents (figure 2).

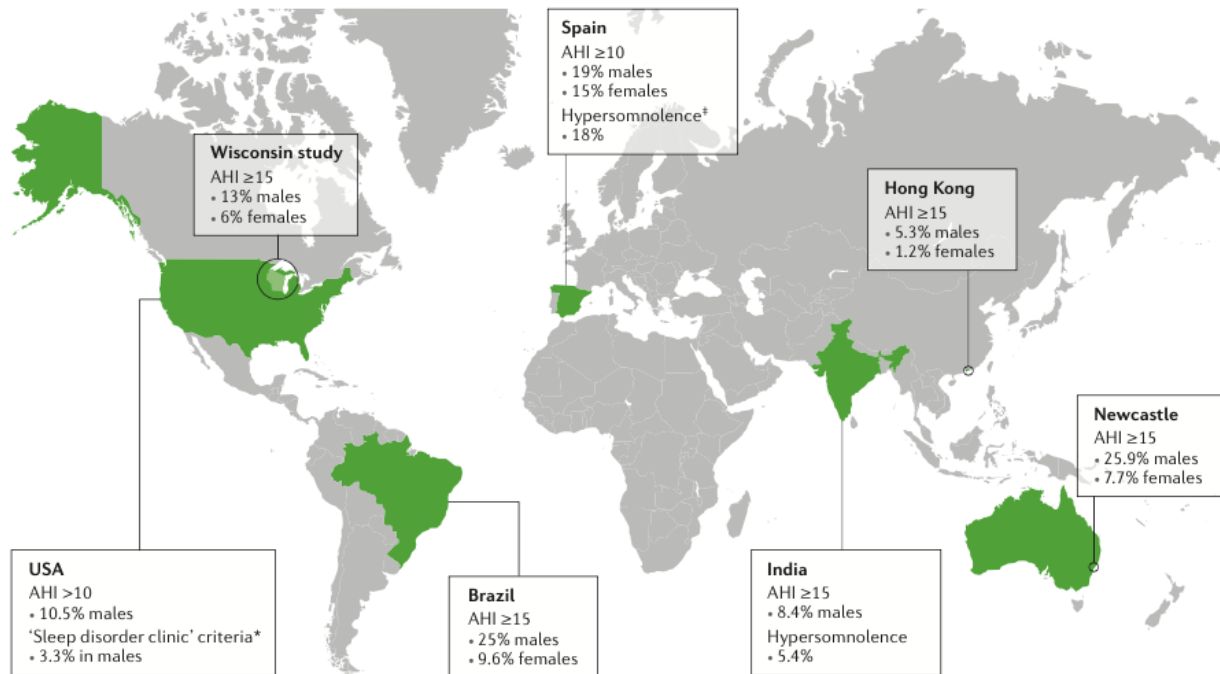


Figure 2. Prévalence du syndrome d'apnées obstructives du sommeil d'après Levy et al (1)

En dépit de ces différences, le SAOS apparaît clairement comme une des pathologies chroniques respiratoires les plus fréquentes (1), ce qui en fait un véritable problème de santé publique.

Les principaux facteurs associés à la présence d'un SAOS sont les suivants : le sexe masculin, l'âge avancé, la surcharge pondérale ou obésité, la configuration anatomique, la consommation d'éthanol, la prise de médicaments myorelaxants (exemple : benzodiazépines) ou encore l'obstruction nasale saisonnière.

Parmi ces différents facteurs, l'obésité semble jouer un rôle de plus en plus important. En effet, l'augmentation de la prévalence du SAOS décrite depuis le début des années 1990 serait à mettre en lien avec l'épidémie d'obésité qui touche le monde entier.

### 1.3. Physiopathologie

Au cours de l'inspiration, les muscles inspiratoires induisent de par leur contraction une pression négative dans les voies aériennes, ce qui est responsable de l'entrée de l'air dans les poumons. Cette pression négative a tendance à réduire le diamètre des voies aériennes supérieures qui ne sont constituées d'aucune structure rigide et demeurent donc particulièrement déformables. A cette pression négative intraluminaire, vient s'ajouter une pression positive extraluminale exercée par les tissus mous entourant les voies aériennes supérieures. A l'état normal et notamment au cours de l'éveil, la perméabilité des voies aériennes supérieures est maintenue par la contraction des muscles dilatateurs du pharynx (cf. Figure 3).

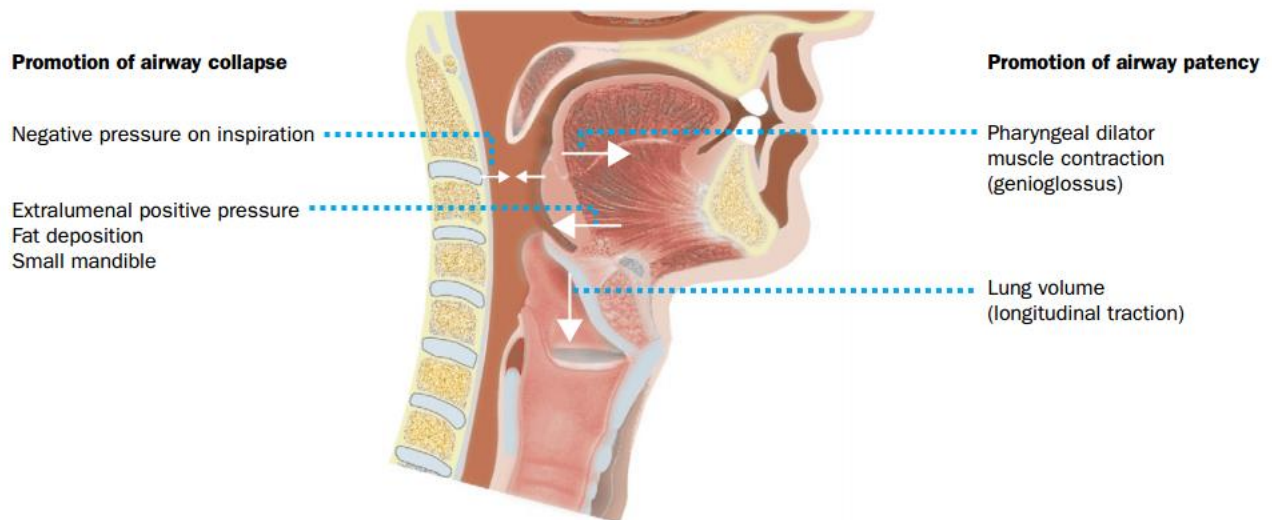


Figure 3. Illustration des différentes forces qui s'exercent sur les voies aériennes supérieures, d'après Malhotra et al (3)

Au cours du sommeil, l'activité musculaire diminue en particulier pour les muscles dilatateurs du pharynx, ce qui peut aboutir à un déséquilibre des forces exercées sur les voies aériennes supérieures entraînant une diminution de leur calibre. Ce déséquilibre des forces survient plus

particulièrement avec certaines conditions anatomiques ou fonctionnelles tels qu'un rétrécissement des voies aériennes supérieures (en lien avec une obésité ou des malformations cranio-faciales), une perte d'efficacité des muscles dilatateurs du pharynx ou encore une altération des réflexes protecteurs des voies aériennes supérieures.

La diminution du diamètre des voies aériennes supérieures entraîne une diminution de la ventilation alvéolaire, aboutissant en cascade à une stimulation des centres nerveux de la respiration, une augmentation des efforts respiratoires et donc une augmentation de la pression négative intraluminaire. Au final, l'ensemble de ces événements aboutit à un collapsus pharyngé et une apnée dite obstructive. Les événements obstructifs se terminent fréquemment par des microéveils salvateurs, qui du fait de la répétition des apnées/hypopnées sont responsables d'une fragmentation du sommeil (figure 4).

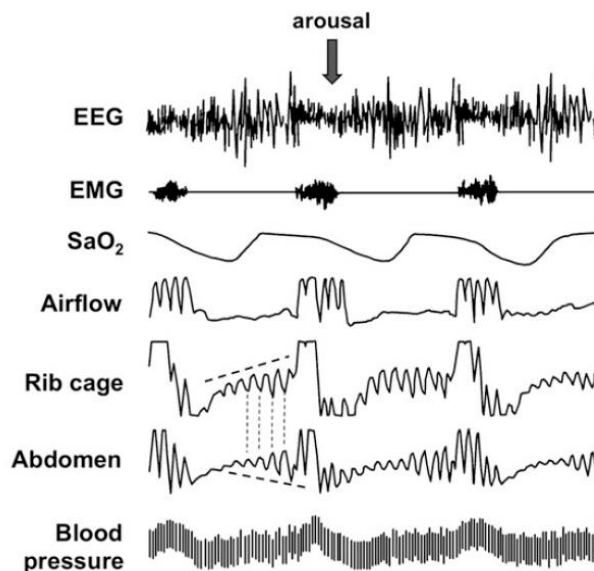


Figure 4. Exemple d'enregistrement polysomnographique d'un patient présentant un SAOS d'après Dematteis et al (4)

Trois événements obstructifs sont représentés sur cet enregistrement avec à chaque fois, un arrêt du flux d'air, une augmentation des efforts respiratoires, une diminution de la saturation en oxygène et la survenue d'un microéveil.

## **2. Conséquences vasculaires du SAOS**

De nombreux travaux ont démontré depuis déjà plusieurs années une augmentation de la morbi-mortalité cardiovasculaire avec le SAOS (5,6); à tel point que ce syndrome est maintenant identifié comme un facteur de risque cardiovasculaire à part entière (7). Bien que les patients apnéiques présentent à la fois des pathologies cardiaques et vasculaires, nous ne traiterons dans la suite de ce chapitre que des altérations vasculaires associées au SAOS.

### **2.1.1. Hypertension artérielle**

L'association entre le SAOS et l'HTA est décrite depuis de nombreuses années. On sait aujourd'hui qu'environ 60 % des patients souffrant d'un SAOS présentent une HTA (8) et inversement, la prévalence du SAOS est évaluée à 30 % au sein de la population hypertendue (9) pour atteindre jusqu'à 70 % chez les patients présentant une HTA réfractaire (10).

Aussi, le risque relatif de développer une HTA est 3 fois plus important chez un individu apnéique non traité (11), tandis qu'un traitement bien conduit par PPC (défini par une utilisation > 4 heures par nuit) induit une réduction modeste mais significative des chiffres tensionnels (12,13).

L'élévation de la pression artérielle observée chez les sujets apnéiques est à prédominance diastolique et survient essentiellement la nuit (14). Son origine est multifactorielle avec de nombreux mécanismes intermédiaires identifiés (détaillés ci-après), le plus important étant probablement l'hyperactivité sympathique (15).

### **2.1.2. Dysfonctions vasculaires**

Le SAOS est associé à une dysfonction vasculaire globale avec d'une part une rigidité artérielle plus spécifiquement localisée sur les vaisseaux de gros calibre (dit de conductance) (16,17) et d'autre part une dysfonction endothéliale (18). La rigidité artérielle consiste en une perte d'élasticité des

vaisseaux de conductance et est classiquement évaluée par la mesure de la vitesse de l'onde de pouls (VOP), tandis que la dysfonction endothéliale consiste en une atteinte de la monocouche de cellules endothéliales qui tapisse la membrane interne du vaisseau (intima). Cette atteinte se traduit par une vasodilatation dépendante de l'endothélium altérée en lien avec une synthèse endothéliale de monoxyde d'azote diminuée. La technique la plus communément utilisée pour étudier la fonction endothéliale demeure la dilatation médiée par le flux (FMD, *flow-mediated dilatation*), cette dernière évaluant uniquement les vaisseaux de gros calibre (par opposition aux techniques évaluant la fonction endothéliale dans la microcirculation).

La dysfonction endothéliale (18) demeure un des marqueurs les plus précoces du processus athérogène (19) et constitue, au même titre que la rigidité artérielle (16,17), un marqueur prédictif de la survenue d'accidents cardiovasculaires (18).

De nombreuses études ont mis en évidence une altération de la fonction endothéliale chez les patients SAOS (cf. revue récente de Hoyos et al (18)) et chez des animaux soumis à une HI (20). Cette altération semblait corrélée à la sévérité du SAOS (21,22), et le lien de causalité a pu être démontré par des études interventionnelles démontrant une amélioration de la dysfonction endothéliale sous PPC (12,22,23).

### **2.1.3. Remodelage vasculaire**

Depuis les premières données publiées en 1998 mettant en évidence davantage de plaques d'athérome dans les carotides de patients apnéiques comparativement à des sujets contrôles, l'association entre le SAOS et l'athérosclérose a été confirmée par de nombreux travaux (24–26). Cependant, ces études étaient limitées par la présence de facteurs confondants eux-mêmes associés à la pathologie athéromateuse (exemple : HTA, diabète, tabagisme). Ce n'est qu'en 2005 que Drager et al démontre, dans une cohorte de patients jeunes indemnes de toute comorbidité, une corrélation entre les marqueurs précoces de remodelage vasculaire (épaisseur intima-media (EIM) et diamètre



carotidien) et les paramètres polysomnographiques (IAH et saturation minimale en O<sub>2</sub>) (27), résultats qui seront confirmés dans la même année par deux autres équipes dont la nôtre (28,29). La preuve du caractère causal du SAOS dans ce remodelage vasculaire arrivera quelques années plus tard avec la démonstration d'une réduction de l'EIM après 4 mois d'un traitement par PPC (30).

L'ensemble de ces altérations vasculaires combinées aux pathologies cardiaques sont autant de phénomènes qui expliquent l'incidence augmentée des événements cardiovasculaires chez les sujets apnéiques.

### ***3. Mécanismes impliqués dans les complications vasculaires du SAOS***

D'un point de vue physiopathologique, le SAOS est caractérisé par des épisodes itératifs de désaturation en O<sub>2</sub> - réoxygénation aboutissant à une HI, mais également par une augmentation des efforts respiratoires et une fragmentation du sommeil. A l'heure actuelle, il est admis que l'HI est le principal stimulus impliqué dans les conséquences cardiovasculaires du SAOS. Ainsi, ce chapitre ne traitera que des mécanismes intermédiaires influencés par l'HI.

#### ***3.1. Hyperactivité sympathique***

L'activation du système nerveux sympathique décrite chez les patients atteints de SAOS (31) fait suite à l'activation des chémorécepteurs périphériques situés au niveau des corps carotidiens et de la crosse aortique qui détectent, à chaque événement obstructif, la baisse de la pression partielle en O<sub>2</sub> dans le sang. Cette hyperactivité sympathique a été objectivée chez le patient apnéique par la mesure de l'activité nerveuse sympathique du nerf péronier qui se trouvait augmentée (32), ainsi que par des concentrations de catécholamines majorées (33). Elle a par ailleurs été reproduite dans différents modèles expérimentaux d'HI, que ce soit le rongeur (34), comme le volontaire sain exposé

pendant 14 jours à une HI nocturne (15) (cf. revue (35)). Cette réponse sympathique qui s'exerce à la fois au niveau vasculaire et cardiaque (36) contribue à une élévation de la pression artérielle et à une diminution de la variabilité de la fréquence cardiaque, l'ensemble contribuant à une augmentation du risque cardiovasculaire (37).

### **3.2. Stress oxydant**

Une production importante d'espèces réactives de l'oxygène (ROS, *reactive oxygen species*) à l'origine d'un stress oxydant est décrite depuis de nombreuses années au cours du SAOS (38,39). Ce stress oxydant résulte d'un déséquilibre entre la production de substances pro-oxydantes qui est considérablement augmentée (40) et les capacités antioxydantes de l'organisme qui sont quant à elles diminuées au cours du SAOS (41). Cette génération accrue de ROS survient préférentiellement dans les phases de réoxygénation qui font suite à chaque évènement obstructif et résulte simultanément d'une dysfonction mitochondriale, de l'activation de deux enzymes : la NADPH oxydase et la xanthine oxydase, ou encore du découplage de la NO synthase (42).

Si cette association a longtemps été sujet à controverse du fait de nombreux facteurs confondants, il existe à ce jour des éléments convaincants soutenant l'hypothèse d'un stress oxydant au cours du SAOS (cf. revue de Lavie (42)). A titre d'exemple, alors que de nombreuses études s'intéressant aux marqueurs de la peroxydation lipidique (notamment aux isoprostanes) avaient abouti à des résultats discordants (43-48), quelques travaux aux méthodologies plus rigoureuses (appariement des témoins et patients SAOS sur l'âge et l'indice de masse corporelle) ont objectivé une augmentation des concentrations d'isoprostanes (45,46,48), de TBARS (thiobarbituric acid - reactive species) et peroxydes (49), et ceci indépendamment de toute autre comorbidité. Les niveaux d'isoprostanes étaient par ailleurs liés à la sévérité de l'hypoxie (45,46,48) et corrigés par la PPC (45), suggérant que le SAOS joue un rôle prépondérant dans ce phénomène de peroxydation lipidique.

Ce stress oxydant contribue aux altérations cardiovasculaires du SAOS de manière directe en induisant notamment une dysfonction endothéliale (50) et de l'athérosclérose (44), mais également indirectement en promouvant l'inflammation et l'hyperactivité sympathique (1).

### ***3.3. Dysfonction métabolique***

La dysfonction métabolique décrite au cours du SAOS regroupe à la fois des perturbations de l'homéostasie glucidique et des anomalies lipidiques.

De nombreuses études ont suggéré un lien entre le SAOS et l'intolérance au glucose ou insulino-résistance, mais l'obésité constituait encore une fois un facteur confondant majeur dans la plupart de ces travaux. Néanmoins, les données obtenues chez des modèles animaux de SAOS ou dans certaines études cliniques supportent l'hypothèse d'une détérioration de l'équilibre glycémique directement en lien avec l'HI. Ainsi, l'insulino-résistance est plus marquée chez des patients apnéiques comparativement à des sujets contrôles et ceci indépendamment de l'obésité (51,52), tandis qu'une réduction de l'utilisation du glucose par les muscles a été décrite chez la souris *wild-type* soumis à l'HI (53).

L'HI semble également induire des anomalies lipidiques telles qu'une élévation des concentrations plasmatiques de cholestérol, une augmentation de la synthèse de triglycérides et phospholipides que ce soit chez le patient comme chez l'animal (54), l'ensemble de ces anomalies étant possiblement impliquées dans l'athérogénèse (54).

### ***3.4. Sécrétion de substances vasoactives***

L'HI aboutit non seulement à une diminution de la production de monoxyde d'azote à l'origine d'une dysfonction endothéliale (cf. chapitre 2.1.2 relatif aux dysfonctions vasculaires du SAOS) mais également à une augmentation de la production d'endothéline-1, un puissant vasoconstricteur ; l'ensemble contribuant en partie à l'élévation de la pression artérielle. Des études ont ainsi montré

des concentrations circulantes d'endothéline-1 ou de son précurseur majorées chez des patients SAOS comparativement à des sujets contrôles et la réduction de ces concentrations avec un traitement par PPC (55–57). Bien que ces résultats vont à l'encontre de précédents travaux ne démontrant pas d'augmentation de l'endothéline-1 au cours du SAOS (58), ils corroborent l'activation de la voie HIF-1/endothéline-1 décrite au niveau cardiovasculaire dans des modèles animaux d'HI (59).

### ***3.5. Coagulopathie***

Le SAOS semble être associé à un état d'hypercoagulabilité résultant de divers phénomènes dont une activation plaquettaire, une augmentation de la viscosité sanguine ou encore des concentrations majorées de substances prothrombotiques telles que le fibrinogène (60,61). Bien que les mécanismes aboutissant à ces différents phénomènes ne soient pas identifiés, il semblerait que l'hyperagrégabilité plaquettaire décrite au cours du SAOS soit liée à la libération accrue de catécholamines qui par la stimulation des récepteurs alpha ont un effet pro-agrégant (60).

### ***3.6. Inflammation***

Au même titre que l'athérosclérose, le SAOS est aujourd'hui décrit comme une pathologie inflammatoire de bas grade. Cette inflammation a été caractérisée au niveau systémique par de nombreuses études démontrant des concentrations circulantes majorées de différents médiateurs de l'inflammation (cytokines pro-inflammatoires et molécules d'adhésion) chez des sujets apnéiques comparativement à des sujets contrôles, et ceci indépendamment de l'obésité (cf. revue de Ryan et al (62).

Alors que les études cliniques relatives à l'interleukine 6 ou à la protéine C réactive (CRP) ont souvent été limitées par la présence de comorbidités (63,64), des données solides existent pour une cytokine pro-inflammatoire clé, à savoir le *tumor necrosis factor  $\alpha$*  (TNF $\alpha$ ). Ainsi, de nombreux

travaux ont objectivé des concentrations majorées de TNF $\alpha$  chez des sujets apnéiques comparativement à des sujets contrôles appariés sur l'âge et l'indice de masse corporelle (64-67). Cette augmentation semblait liée au SAOS puisque les niveaux de TNF $\alpha$  étaient corrélés aux paramètres polysomnographiques (66) et significativement diminués par la PPC (64-67).

En parallèle de cette inflammation systémique, une inflammation vasculaire caractérisée, entre autres, par d'avantage d'interactions entre leucocytes et cellules endothéliales a également été décrite au cours du SAOS. Ainsi, chez des rongeurs exposés à l'HI, le roulement leucocytaire et l'adhésion des monocytes à la paroi vasculaire sont augmentés comparativement aux animaux normoxiques (68,69). Un phénomène similaire a également été décrit pour des monocytes de sujets apnéiques, ce dernier étant accompagné d'une augmentation de molécules d'adhésion (70). Au final, ces différents phénomènes conduisent à une dysfonction endothéliale, un remodelage vasculaire et *in fine* à l'athérosclérose (62,69,71,72).

En outre, des travaux antérieurs (dont la plupart ont été menés au sein de notre laboratoire) ont mis en évidence des perturbations du métabolisme de l'acide arachidonique au cours du SAOS, avec notamment des concentrations majorées de LTB<sub>4</sub>, LTE<sub>4</sub> ou encore d'isoprostanes. L'implication de ces eicosanoïdes dans les conséquences vasculaires du SAOS est développée dans une partie distincte intitulée « métabolisme de l'acide arachidonique et SAOS ».

Bien que la pathogénèse de ce syndrome inflammatoire reste encore non élucidée à ce jour, il semblerait que le facteur de transcription NF-KB, régulateur majeur de l'expression de nombreux gènes de l'inflammation joue un rôle prépondérant. L'activité de ce dernier est en effet augmentée sous IH que ce soit dans des modèles *in vitro* (65) et *in vivo* (69,73), comme dans les monocytes de patients SAOS (74), contribuant ainsi à l'activation de la cascade inflammatoire (65).

## 4. Métabolisme de l'acide arachidonique et SAOS

### 4.1. Présentation générale

L'acide arachidonique est un acide gras poly-insaturé à 20 atomes de carbone présent dans les membranes phospholipidiques. Sous l'action de la phospholipase A<sub>2</sub>, ce dernier est libéré des membranes et peut alors être métabolisé en différents composés regroupés sous le terme générique 'eicosanoïdes' (Figure 5).

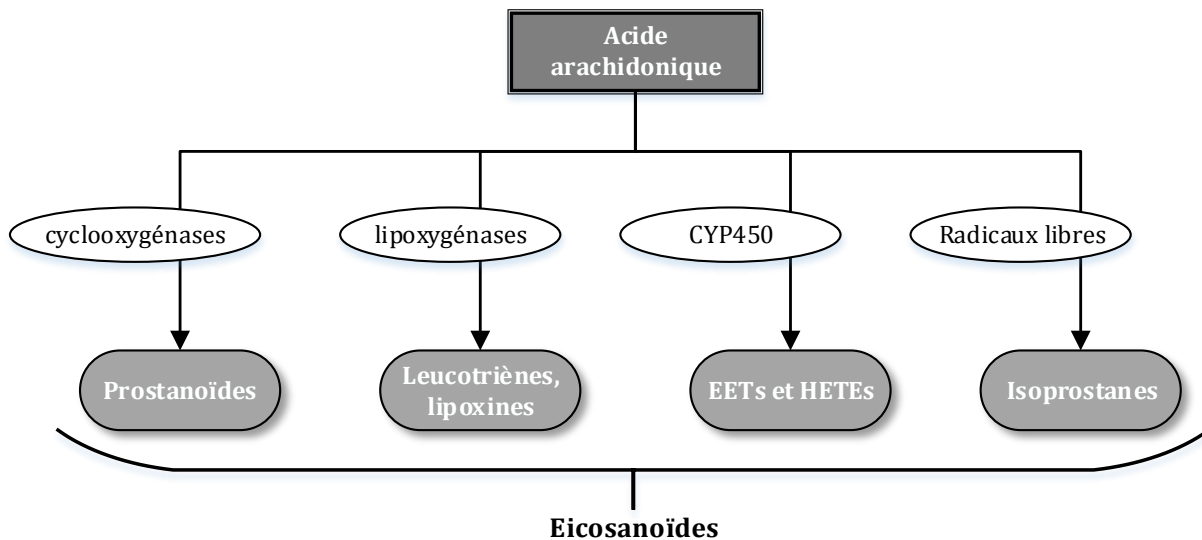


Figure 5. Représentation schématique des voies de métabolisation de l'acide arachidonique.

EETs, acides eicosatétraoïques ; HETEs, acide hydroxyeicosatétraoïques.

Les eicosanoïdes regroupent ainsi une vaste famille de composés parmi lesquels on distingue les **prostanoïdes** (incluant les prostaglandines, le TXA<sub>2</sub> et la prostacycline) issus de l'action des cyclooxygénases (COX), les **leucotriènes** (LTs) et **lipoxines** produits par différentes lipoxygénases, les **acides eicosatétraoïques** (EETs et HETEs) résultant de l'action des époxygénases du cytochrome P450 et enfin les **isoprostanes** issus de l'action des radicaux libres sur l'acide arachidonique.

L'ensemble de ces eicosanoïdes sont impliqués dans le processus inflammatoire, mais dans la suite de ce chapitre, nous développerons uniquement les voies métaboliques en lien avec nos objectifs de recherche, à savoir la voie du TXA<sub>2</sub> et celle des LTs.

#### ***4.2. Voie du thromboxane A<sub>2</sub>***

Le TXA<sub>2</sub> est un métabolite de l'acide arachidonique appartenant à la sous-famille des prostanoïdes. Il est synthétisé de façon prédominante par les plaquettes suite à l'action de la cyclooxygénase de type 1 (COX-1) puis de la thromboxane synthase (Figure 6). Le TXA<sub>2</sub> est doté de propriétés proathérogènes puisqu'en se fixant à son récepteur TP, il est capable d'activer les plaquettes, d'induire la contraction et la prolifération des cellules musculaires lisses ou encore de promouvoir l'expression de molécules d'adhésion (75). Ses effets biologiques sont contrebalancés par ceux de prostacycline, autre prostanoïde issu de l'action successive des COX et de la prostacycline synthase, puisque la fixation de cette dernière sur son récepteur IP induit une vasodilatation, une diminution de l'expression des molécules d'adhésion ainsi qu'une diminution de l'agrégation plaquettaire (75) (Figure 6).

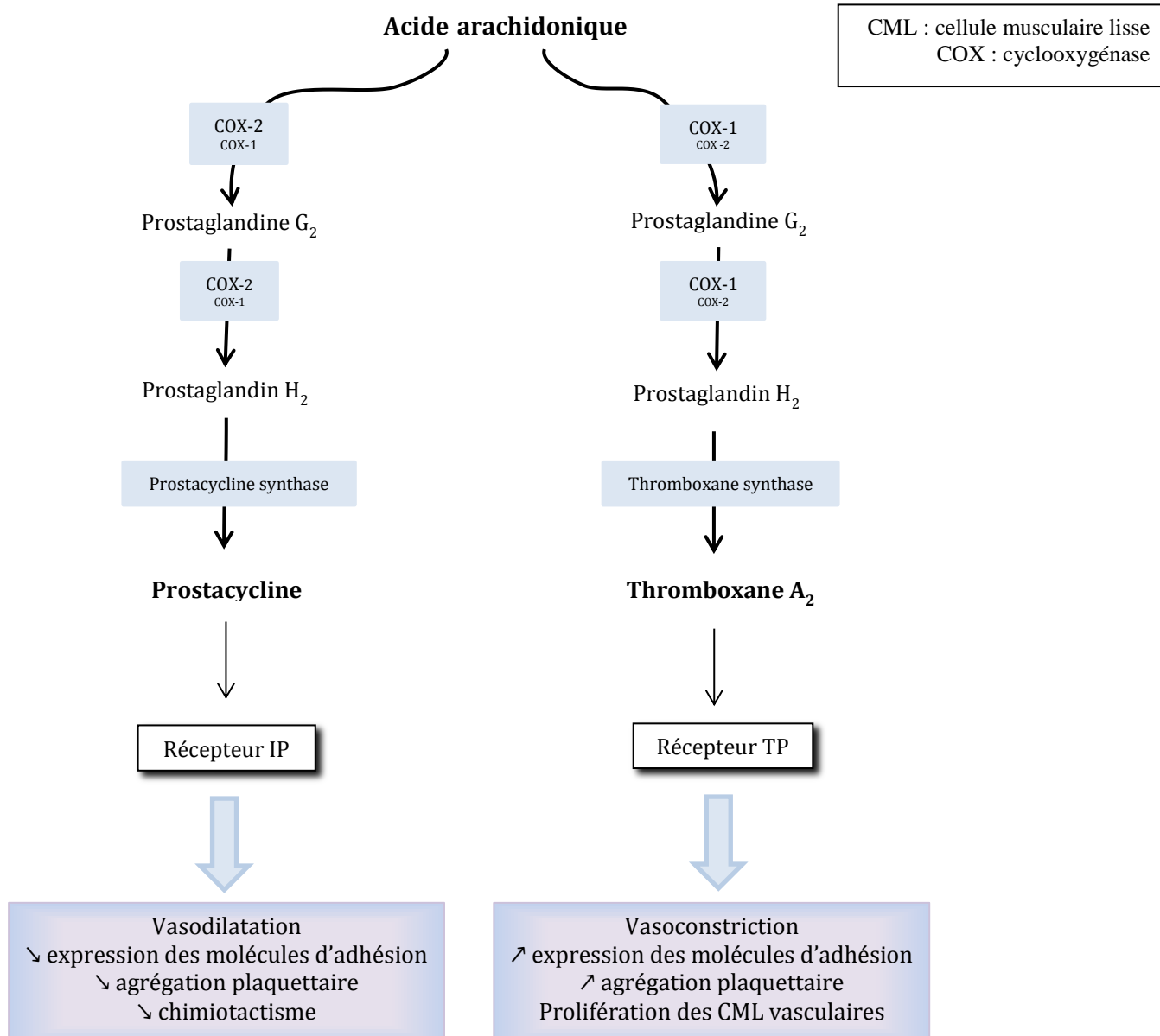


Figure 6. Biosynthèse et effets biologiques du thromboxane A<sub>2</sub> et de la prostacycline

Jusqu'à récemment, les données relatives à la voie du TXA<sub>2</sub> au cours du SAOS étaient assez pauvres et dans certains cas contradictoires (76,77). Aussi, si l'implication du TXA<sub>2</sub> dans l'athérogénèse hors HI n'était plus à démontrer (75), le rôle de ce dernier dans l'athérosclérose induit par l'HI restait à préciser.



### **4.3. Voie des leucotriènes**

Les LTs sont d'autres métabolites de l'acide arachidonique issus de l'action conjointe de la 5-lipoxygénase et de sa coenzyme activatrice la *5-lipoxygenase activating protein* (FLAP). Cette première étape aboutit à la formation d'un composé instable, leucotriène A<sub>4</sub> qui sera rapidement biotransformé en LTB<sub>4</sub> via l'action de la LTA<sub>4</sub> hydrolase, ou en CysLTs (LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>) suite à l'action séquentielle de la LTC<sub>4</sub> synthase et de dipeptidases.

Les LTs sont dotés de propriétés pro-inflammatoires et proathérogènes qu'ils exercent en se fixant sur des récepteurs spécifiques. Le LTB<sub>4</sub> via sa fixation sur ses récepteurs BLT<sub>1</sub> et BLT<sub>2</sub> agit notamment comme un puissant chémoattractant facilitant l'adhésion et le recrutement des leucocytes (75). Les CysLTs sont quant à eux capables d'induire la prolifération et contraction des cellules musculaires lisses, ainsi que l'activation des monocytes/macrophages en se fixant sur leurs récepteurs CysLT<sub>1</sub> et CysLT<sub>2</sub> (75). Le rôle des LTs dans l'athérogénèse a déjà été démontré par de nombreuses études expérimentales utilisant tantôt des approches génétiques, tantôt des interventions pharmacologiques (75).

Des précédents travaux (dont une grande partie a été réalisée au sein du laboratoire HP2) se sont déjà largement intéressés à la voie des LTs au cours du SAOS (78-80), et en particulier au rôle du LTB<sub>4</sub> dans le processus athéromateux lié au SAOS (79,80). Ainsi, une production accrue de LTB<sub>4</sub> (79,80) a été décrites chez des patients apnéiques comparativement à des sujets contrôles appariés sur l'âge, le sexe et l'indice de masse corporelle. Les concentrations de LTB<sub>4</sub> étaient par ailleurs corrélées aux marqueurs de remodelage vasculaire, suggérant que ces eicosanoïdes pourraient être impliqués dans le processus athéromateux associé au SAOS. L'ensemble de ces données a été récapitulé au sein d'une revue (Publication n°1).

Publication n°1

**“Leukotrienes as a molecular link between obstructive sleep  
apnoea and atherosclerosis”**

F Stanke-Labesque, JL Pepin, E Gautier-Veyret, P Levy, M Bäck.

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# Leukotrienes as a molecular link between obstructive sleep apnoea and atherosclerosis

Françoise Stanke-Labesque<sup>1,2,3,4\*</sup>, Jean-Louis Pépin<sup>1,3,4</sup>, Elodie Gautier-Veyret<sup>1,3,4</sup>, Patrick Lévy<sup>1,3,4</sup>, and Magnus Bäck<sup>5,6,7\*</sup>

<sup>1</sup>Université Grenoble Alpes, Grenoble F-38041, France; <sup>2</sup>Laboratory of Pharmacology, Grenoble University Hospital, BP 217, F-38043 Grenoble Cedex 9, France; <sup>3</sup>INSERM U1042, HP2, Grenoble F-38041, France; <sup>4</sup>CHU, Grenoble 38043, France; <sup>5</sup>Department of Medicine, Karolinska Institutet, Stockholm 17176, Sweden; <sup>6</sup>Department of Cardiology, Karolinska University Hospital, Stockholm 17176, Sweden; and <sup>7</sup>Karolinska University Hospital, CIMM L803, 17176 Stockholm, Sweden

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## Abstract

Leukotrienes are biologically active lipid mediators of inflammation involved in atherogenesis. Obstructive sleep apnoea (OSA) patients exhibit early atherosclerosis and activation of the leukotriene pathway. In OSA patients, the production of leukotrienes is increased in relation to OSA severity and *in vitro* exposure of immune cells to intermittent hypoxia increases leukotriene pathway transcription. Moreover, the leukotriene transcriptional pathway is associated with early vascular remodelling. Lastly, obesity is a major confounding factor for leukotriene activation in OSA. The aim of this review was to focus on the intricate network of leukotrienes, chronic intermittent hypoxia, and atherosclerosis, with an emphasis on the role of leukotrienes in the early atherosclerosis observed in OSA patients.

## Keywords

Leukotrienes • Intermittent hypoxia • Obesity • Atherosclerosis

## 1. Introduction

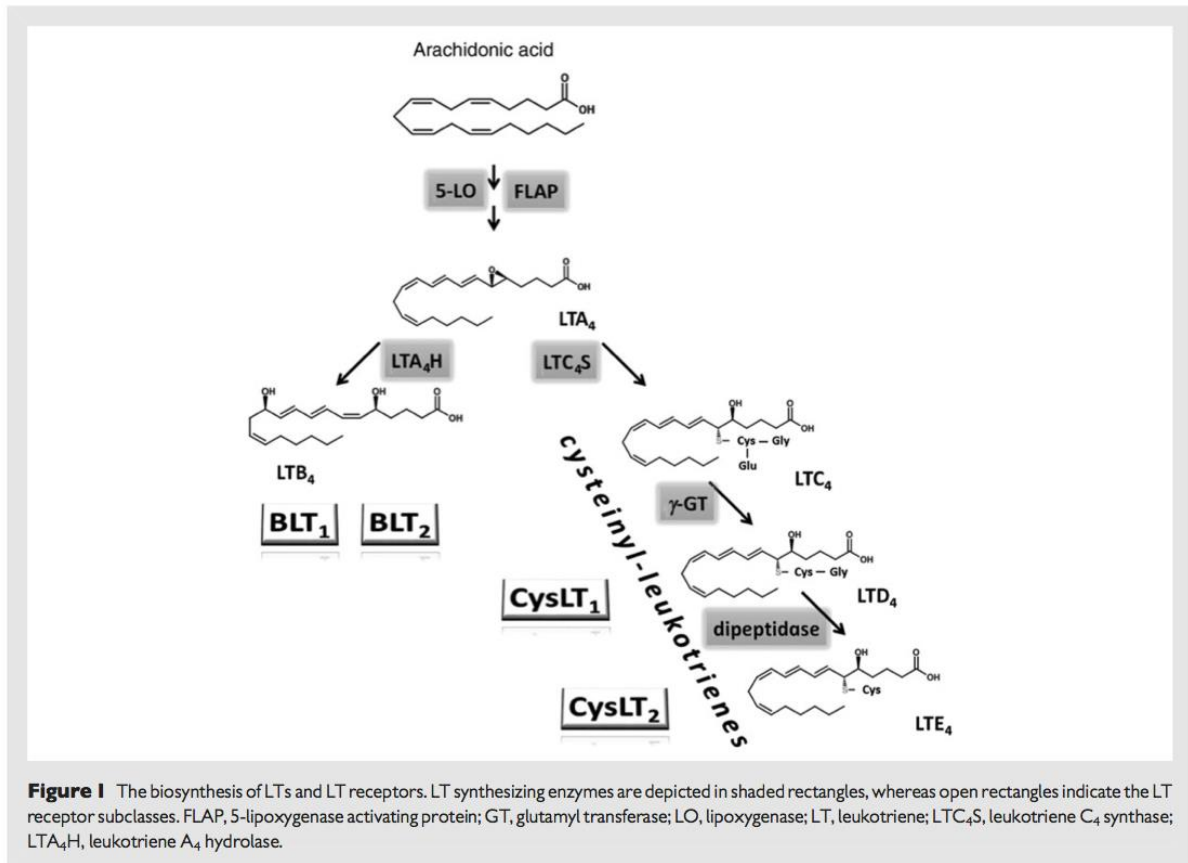
Obstructive sleep apnoea (OSA) is a worldwide public health problem with a prevalence estimated between 5 and 20% of the general population.<sup>1</sup> OSA is characterized by recurrent episodes of partial or complete upper airway obstruction occurring during the sleep, leading to chronic intermittent hypoxia which harms the cardiovascular system. OSA is associated with an increased prevalence of fatal and non-fatal cardiovascular events<sup>2</sup> and is an independent risk factor for death from any cause.<sup>3</sup> Among the intermediary mechanisms that could explain the link between OSA and cardiovascular morbidity, the role of early atherosclerosis has been proposed since even after adjustment for confounding factors, OSA *per se* is associated with atherosclerosis.<sup>4,5</sup> In addition, the intensity of the vascular remodelling is more specifically related to the amount of nocturnal oxygen desaturation.<sup>4–6</sup> Lastly, 4 months of continuous positive airway pressure (CPAP) application seems sufficient to partly reverse early atherosclerosis,<sup>7</sup> confirming the link between OSA-related chronic intermittent hypoxia and atherosclerosis.

It is well established that atherosclerosis is an inflammatory disease,<sup>8,9</sup> thus the mechanistic links between OSA and atherosclerosis could likely be mediated by inflammatory processes induced by chronic intermittent hypoxia. Among several potential inflammatory mediators involved,<sup>8</sup> leukotrienes (LTs) have been described to play a major role in the onset and the progression of atherosclerosis. LTs are lipid mediators derived from arachidonic acid liberated from cell membrane

phospholipids by cytosolic phospholipase A<sub>2</sub>. On activation of the enzyme 5-lipoxygenase (5-LO),<sup>10</sup> which interacts with nuclear membrane-bound 5-LO-activating protein (FLAP),<sup>11</sup> arachidonic acid is converted to leukotriene A<sub>4</sub> (LTA<sub>4</sub>) in inflammatory cells<sup>12</sup> (Figure 1). In polymorphonuclear neutrophil granulocytes (PMNs) and in monocytes/macrophages, LTA<sub>4</sub> is converted by LTA<sub>4</sub> hydrolase (LTA<sub>4</sub>H) into LTB<sub>4</sub> which modulates transcription, or is secreted to mediate autocrine or paracrine effects through the BLT<sub>1</sub> and BLT<sub>2</sub> receptors.<sup>13</sup> LTB<sub>4</sub> is a potent chemoattractant, facilitating leucocyte adhesion and recruitment, and its role in the pathogenesis of atherosclerosis is well established.<sup>14</sup> LTA<sub>4</sub> can also be conjugated with glutathione through the enzyme LTC<sub>4</sub> synthase (LTC<sub>4</sub>S) to produce leukotriene C<sub>4</sub> (LTC<sub>4</sub>). LTC<sub>4</sub> can be converted to leukotriene D<sub>4</sub> (LTD<sub>4</sub>) by  $\gamma$ -glutamyl transpeptidase which then can be metabolized by dipeptidase to produce leukotriene E<sub>4</sub> (LTE<sub>4</sub>) as shown in Figure 1. The production of Cysteinyl LTs (CysLTs), i.e. LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>, can occur in macrophages, but also in mast cells, platelets, and structural cells of the vascular wall as a result of transcellular metabolism of LTA<sub>4</sub> (see review in Sala et al.<sup>15</sup>). CysLTs activate receptors denoted CysLT<sub>1</sub> and CysLT<sub>2</sub> on leucocytes and structural cells in the vascular wall<sup>13</sup> to promote smooth muscle cells contraction and alterations of gene expression.<sup>16</sup>

OSA is characterized by systemic and airway inflammation. Increased urinary excretion of LTE<sub>4</sub><sup>17,18</sup> and increased LT concentration in exhaled breath condensate<sup>19</sup> from OSA provide evidence for the contribution of the LT pathway in OSA-associated inflammation. Based on an

\* Corresponding author. Tel: +33 476765492 (F.S.-L.) / +46 851770000 (M.B.); fax: +33 476768938 (F.S.-L.) / +46 338313147 (M.B.); Email: fstanke@chu-grenoble.fr (F.S.-L.) / magnusbac@ki.se (M.B.)  
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association of the LT pathways with both OSA<sup>18–22</sup> and atherosclerosis,<sup>14,23</sup> the present review will focus on the role of the LT pathway as a potential molecular link between OSA and atherosclerosis.

## 2. LT pathway activation in relation to intermittent hypoxia

The activation of the LT pathway by intermittent hypoxia has been suggested by both experimental and clinical studies. In OSA patients, the mean SaO<sub>2</sub><sup>20</sup> and the percentage of time spent with SaO<sub>2</sub> <90%<sup>21</sup> are important determinants of increased neutrophil LTB<sub>4</sub> production. These data suggested that intermittent hypoxia, leading to oxygen desaturation, may play a major role to increase LTB<sub>4</sub>. The desaturation-re-oxygenation sequence, that is a typical for the majority of respiratory events in OSA patients, leads to an oxidative/nitrosative stress with production of reactive oxygen species<sup>24</sup> and reactive nitrogen species.<sup>25</sup> The increased levels of reactive oxygen species contribute to generate adhesion molecules,<sup>26</sup> to activate leucocytes,<sup>27</sup> and to produce systemic inflammation.<sup>28</sup> Since 5-LO activity is regulated by the cellular redox status and reactive oxygen species,<sup>29</sup> the increased production of reactive species in leucocytes from OSA patients<sup>30</sup> could contribute to the activation of the LT pathway in OSA. Moreover, *in vitro* exposure of either PMNs<sup>21</sup> or monocyte THP-1 cells<sup>31</sup> to intermittent hypoxia

leads to an increase in LTB<sub>4</sub> production. These observations suggest that the intermittent hypoxia associated with OSA may induce a systemic activation of immune cells, which potentially could participate in an accelerated atherosclerosis. These observations are supported by studies in animal models for other diseases, such as second organ ischaemia-reperfusion injury of the lung, which enhances the production of LTB<sub>4</sub> *in vivo*.<sup>32</sup> Enhancement of arachidonic acid release through increased phospholipase activity has also been implicated in other pathological conditions related to hypoxia, such as hypoxic pulmonary vasoconstriction.<sup>33</sup> In addition, an up-regulation of the mRNA levels of the LT pathway components has been reported in leucocytes<sup>21</sup> and endothelial cells<sup>34</sup> exposed to hypoxia. Indeed, *in vitro* exposure of PMNs to intermittent hypoxia (four cycles of 35 min hypoxia followed by 25 min re-oxygenation) induces increased FLAP and LTA<sub>4</sub>H mRNA compared with normoxic conditions.<sup>21</sup> Similar results were obtained in a study on monocyte THP-1 cells, in which longer intermittent hypoxia *in vitro* also increased expression levels of LTB<sub>4</sub> synthesizing enzymes.<sup>31</sup> Lastly, a recent study in apolipoprotein E (ApoE<sup>-/-</sup>) mice demonstrated that acute bouts of hypoxia increased LTC<sub>4</sub>S and CysLT<sub>1</sub> receptor mRNA levels in the myocardium.<sup>35</sup> Although the pathways involved in the up-regulation of LT synthesizing enzyme mRNA levels by intermittent hypoxia remain to be established, severe hypoxic conditions *in vitro* enhance FLAP mRNA as well as a number of other proteins regulated by HIF-1α.<sup>34</sup> This hypoxia-mediated FLAP

expression is regulated by HIF-1 $\alpha$  and NF- $\kappa$ B which activate transcription of FLAP after binding to hypoxia-response elements and NF- $\kappa$ B-binding motif in the FLAP promoter.<sup>34</sup>

Although these observations suggest that intermittent hypoxia directly participates to LT pathway activation in OSA, *in vitro* experiments may not completely mimic the repetitive desaturation-re-oxygenation sequences in OSA. However, the proof of concept for this suggestion has been obtained through studies of cells isolated from subjects with OSA. In summary, PMNs derived from OSA patients exhibit increased production of LTB<sub>4</sub> compared with those derived from controls when challenged by the calcium ionophore A23187 *ex vivo*.<sup>20,21</sup> In the initial study including only cardiovascular disease-free OSA patients, LTB<sub>4</sub> production by stimulated PMNs was significantly correlated to the main parameters evaluating hypoxia severity, including mean nocturnal SaO<sub>2</sub>, minimal nocturnal SaO<sub>2</sub>, percentage of total sleep time spent with SaO<sub>2</sub> < 90% and apnoea-hypopnoea index (AHI).<sup>20</sup> These findings have subsequently been extended to a larger cohort of OSA patients presenting cardiovascular comorbidities as seen in clinical practice.<sup>21</sup> In the latter study, A23187-induced LTB<sub>4</sub> production was enhanced in PMNs derived from severe OSA patients (AHI > 30) and correlated with AHI and percentage of total sleep time with SaO<sub>2</sub> < 90%.<sup>21</sup> Interestingly, the enhanced production of LTB<sub>4</sub> in PMNs derived from OSA patients was associated with a significant increase in FLAP mRNA and protein levels, with a major contributing role of chronic intermittent hypoxia as suggested by the significant association of FLAP expression with an AHI.<sup>21</sup> Taken together, these studies suggest that intermittent hypoxia, leading to oxygen desaturation, may play a major role in the increased LT production evidenced in OSA patients, and that transcriptional activation in leucocytes may be a determinative mechanism in the up-regulation induced by chronic intermittent hypoxia.

### 3. LTB<sub>4</sub> in OSA in relation to measures of atherosclerosis

Importantly, calcium ionophore-induced LTB<sub>4</sub> production in PMNs correlates with systolic and diastolic carotid diameters in severe hypoxic OSA patients without a medical history of cardiovascular disease, suggesting a link between LTB<sub>4</sub> and subclinical atherosclerosis in the form of vascular remodelling.<sup>20</sup> Also in this respect, the transcriptional regulation of the LTB<sub>4</sub> pathway may be directly involved, as suggested by the significant correlations between PMN mRNA and protein levels for FLAP with the mean carotid luminal diameter and mean intima-media thickness of common carotid arteries.<sup>21</sup> Similarly, PMN 5-LO mRNA was associated with carotid luminal diameter and intima-media thickness.<sup>21</sup> Furthermore, 5-LO mRNA levels are greater in PMNs derived from subjects with atherosclerotic plaques vs. those without plaque and higher in patients with carotid wall hypertrophy.<sup>21</sup> Finally, LTA<sub>4</sub>H protein in PMNs is associated with right carotid luminal diameter and left intima-media thickness.<sup>21</sup>

### 4. Mechanisms of LT signalling in OSA-associated atherosclerosis

The increased LTB<sub>4</sub> production in OSA<sup>20,21</sup> in combination with the potent effects of LTB<sub>4</sub> as an activating and chemoattractant factor for leucocytes, and vascular smooth muscle cells implicate LTB<sub>4</sub> as a potential mediator of aggravated atherosclerosis in OSA. The potential mechanisms may include LTB<sub>4</sub>-induced effects on monocytes/

macrophages, PMNs, and structural cells of the vascular wall. Mice lacking ApoE<sup>-/-</sup> exhibit hyperlipidaemia and develop spontaneous atherosclerosis. Exposure of these mice to chronic intermittent hypoxia induces an accelerated atherosclerosis.<sup>36</sup> In addition, either pharmacological or genetic targeting of the BLT<sub>1</sub> receptor for LTB<sub>4</sub> reduces the atherosclerosis burden in ApoE<sup>-/-</sup> knock-out mice.<sup>37</sup> Indeed, deletion of the gene encoding the BLT<sub>1</sub> receptor prevents the acceleration of atherosclerosis induced by chronic intermittent hypoxia exposure in ApoE<sup>-/-</sup> mice,<sup>31</sup> suggesting an important role of the LTB<sub>4</sub>-BLT<sub>1</sub> pathway in intermittent hypoxia-induced atherosclerosis. A recent study suggested that the blockage of the BLT<sub>2</sub> receptor could improve endothelial function and reduce the release of reactive oxygen species in the aorta from ApoE<sup>-/-</sup> mice fed with a high-cholesterol diet.<sup>38</sup> However, this pharmacological treatment failed to reduce the development of atherosclerosis. Since this notion has not been evaluated in chronic intermittent hypoxia, the involvement of the BLT<sub>2</sub> receptor in intermittent hypoxia-induced atherogenesis remains to be established.

LTB<sub>4</sub>-induced activation of human monocytes derived from patients with OSA has been associated with the release of pro-inflammatory cytokines [IL-6 and monocyte chemoattractant protein 1 (MCP-1)]<sup>39</sup> and reactive oxygen species. In line with these findings, lipopolysaccharide-stimulated monocytes derived from patients with severe OSA exhibit elevated production of MCP-1 and matrix metalloproteinases (MMP)-9 after sleep when compared with before sleep.<sup>39</sup> An increased LTB<sub>4</sub> production induced by chronic intermittent hypoxia in OSA could hence promote LTB<sub>4</sub>-induced leucocyte recruitment and activation in the context of atherosclerosis. A major role of the LTB<sub>4</sub> pathway in intermittent hypoxia-induced monocyte recruitment and differentiation has also been supported by the observation that pre-treatment of THP-1 cells with the BLT<sub>1</sub> receptor antagonist U75302 reduces the intermittent hypoxia exposure-mediated monocyte recruitment, whereas pre-treatment with either U75302 or a BLT<sub>2</sub> receptor antagonist reduces intermittent hypoxia exposure-mediated monocyte differentiation to macrophages.<sup>31</sup> PMN-derived production of LTB<sub>4</sub> may also act as a source of LTB<sub>4</sub> acting on lymphocytes, leading to a subsequent release of pro-inflammatory cytokines, i.e. interferon- $\gamma$  or interleukin 17.<sup>13</sup> However, autocrine effects on PMNs may also participate in LTB<sub>4</sub>-induced pro-atherosclerotic signalling. In addition to being a major PMN chemotactic agent, LTB<sub>4</sub> stimulates neutrophil release of proteolytic enzymes, such as lysozyme, myeloperoxidase, and MMPs, which may participate in the atherosclerosis process.<sup>40</sup> Interestingly, also other pathological processes associated with PMNs have been reported in OSA patients. For example, patients with moderate-to-severe OSA exhibit delayed PMN apoptosis and an increased expression of the CD15 subgroup of the adhesion molecule L-selectin.<sup>41</sup> In this context, it is interesting to note that LTB<sub>4</sub> induces delayed neutrophil apoptosis,<sup>41</sup> suggesting that the increased LTB<sub>4</sub> production in OSA could contribute to the increased PMN survival in OSA patients. The latter suggestion opens up for additional potential beneficial effects of targeting the LTB<sub>4</sub> pathway in OSA patients.

Finally, the correlation of expression levels of LTB<sub>4</sub> synthesizing enzymes in PMNs with vascular wall hypertrophy, measured as intima-media thickness, is consistent with the direct chemotactic and proliferative effects of LTB<sub>4</sub> on vascular smooth muscle cells.<sup>42</sup> In animal models, BLT receptor antagonism has been shown to reduce the intimal hyperplasia associated with thickening of the arterial wall after vascular injury.<sup>43</sup> Furthermore, inhibition of LT biosynthesis decreases neutrophil deposition at the sites of arterial injury,<sup>44</sup> hence reinforcing the notion that increased PMN production of LTB<sub>4</sub> may have implications

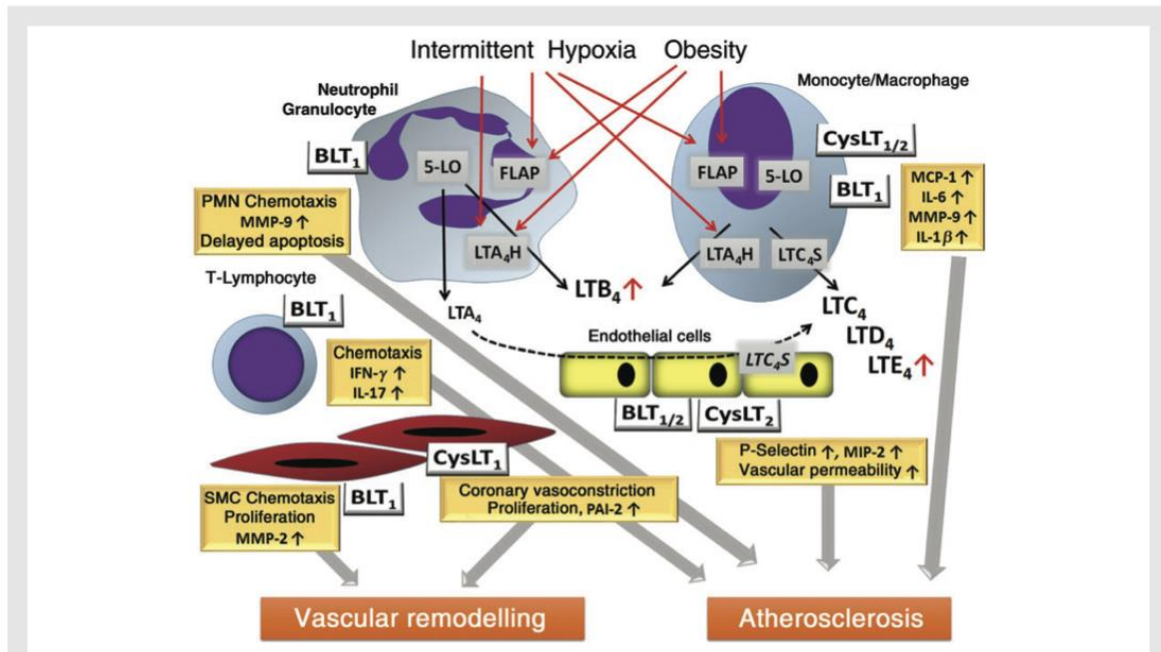
for vascular wall hypertrophy and smooth muscle cell proliferation. Interestingly, OSA has been shown to be an independent predictor for clinical and angiographic outcomes after percutaneous coronary interventions, with restenosis rates being significantly higher in OSA patients compared with coronary patients without OSA.<sup>45</sup>

Whereas most mechanistic studies of the LT pathway in OSA focused on LTB<sub>4</sub>, OSA is also associated with increased CysLTs concentration,<sup>18</sup> and several CysLT-induced effects could potentially also be involved in the link between OSA and atherosclerosis. For example, CysLTs induce a pro-inflammatory gene expression in monocytes<sup>13</sup> and vascular smooth muscle cells,<sup>16</sup> as well as, for example, an increased expression of endothelial P-selectin or macrophage inflammatory protein 2, a chemokine that attracts neutrophils. In addition, CysLT signalling induce smooth muscle cell proliferation and constrict atherosclerotic coronary arteries.<sup>46</sup> Similarly, CysLTs have been shown to activate the release of RANTES, a potent leukoattractant chemokine, by platelets through CysLT<sub>1</sub> receptor activation in healthy subjects.<sup>47</sup> Since both RANTES<sup>48</sup> and CysLTs concentration<sup>18</sup> are increased in OSA, the role of CysLTs in platelet activation and atherothrombosis in OSA could be hypothesized.

Overall, data suggest a role of the LT pathway in the early stages of intermittent hypoxia-related atherosclerosis and vascular remodelling, with monocytes, PMNs, and probably platelets as sources, and several cell types as potential targets both in terms of immunological reactions and direct effects on structural cells within the vascular wall, as depicted in Figure 2.

## 5. Obesity as a significant contributor to LT pathway activation in OSA

A classical issue in clinical research addressing cardiovascular consequences associated with OSA is confounding factors. Including obese OSA with severe desaturation is generally criticized owing to the prominent role of a body mass index (BMI). This confounding factor may also interfere with studies of the LT pathway, as suggested by studies in both children<sup>17,22</sup> and adults with OSA.<sup>18</sup> In the latter studies, urinary LTE<sub>4</sub> levels, an established analytical parameter for monitoring endogenous synthesis of CysLTs, were significantly enhanced in obese subjects with OSA, and the BMI remains an independent predictor of urinary LTE<sub>4</sub> concentration in obese (BMI  $\geq 30$  kg/m<sup>2</sup>) or overweight (25 < BMI < 30 kg/m<sup>2</sup>) OSA patients, even after adjustment for OSA severity. Interestingly, overweight and obesity were gradually associated with a significant increase of FLAP mRNA levels in peripheral mononuclear cells derived from patients with OSA.<sup>18</sup> Such an activation of the LT pathway transcriptional profile in relation to the obesity pathway was also reported in PMNs from OSA patients, where FLAP and LTA<sub>4</sub>H protein expression were significantly associated with BMI.<sup>21</sup> In addition to monocytes and PMNs, also adipocytes have the capability to synthesize LTB<sub>4</sub> and CysLTs.<sup>49,50</sup> Levels of FLAP and 5-LO mRNA are about eight- and two-fold up-regulated, respectively,



**Figure 2** Biochemical mechanisms linking OSA to LT pathway activation, inflammation, and vascular remodelling. The illustration depicts the role of intermittent hypoxia and obesity in the activation of the LT pathway in polymorphonuclear neutrophil granulocytes and monocytes/macrophages. Trans-cellular LT biosynthesis is exemplified by dotted arrow. Red arrows indicate increased expression by intermittent hypoxia and/or obesity, grey arrows indicate link to vascular remodelling and atherosclerosis. Open rectangles represent LT receptor expression, and yellow boxes indicate examples of the cellular responses induced by LT receptor activation. FLAP, 5-lipoxygenase activating protein; LO, lipoxygenase; LT, leukotriene; LTC<sub>4</sub>S, leukotriene C<sub>4</sub> synthase; LTA<sub>4</sub>H, leukotriene A<sub>4</sub> hydrolase; SMC, smooth muscle cell; PAI-2, plasminogen activator inhibitor.

in an obese mice model [mice lacking the obesity gene product leptin (ob/ob)] compared with C57BL/6 mice and immuno-histochemical studies revealed adipose tissue FLAP protein localization to infiltrating macrophages.<sup>49</sup> With respect to a major role of adipose tissue in the underlying inflammation associated with the pathogenesis of metabolic abnormalities and cardiovascular risk in OSA patients,<sup>51</sup> all these data suggested that adipose tissue-derived LTs could also contribute to the increased cardiovascular risk in OSA patients.

## 6. LT activation in childhood sleep apnoea

Paediatric OSA has both distinct and similar characteristics to adult OSA. The activation of the LT pathway has been reported in separated independent studies performed in children with obstructive sleep disorder breathing (SDB) in whom hypertrophy of adenotonsillar tissues is the predominant aetiological factor involved in paediatric OSA. Nevertheless, the pattern of intermittent hypoxia and the effects of hypoxia/re-oxygenation are similar to adult OSA, and in exhaled breath condensate from snoring children, the concentrations of LTB<sub>4</sub> and CysLTs are increased in children with AHI >5 compared with children with AHI <5. However, it should be mentioned that BMI was also higher in the SDB group in this study.<sup>19</sup> Furthermore, upper airway lymphoid tissues of paediatric patients with SDB displayed enhanced expression of CysLT<sub>1</sub> and CysLT<sub>2</sub> receptors and elevated concentration of CysLTs and LTB<sub>4</sub> compared with children with recurrent tonsillitis,<sup>52,53</sup> that may contribute to the proliferation of adenotonsillar cells. Indeed, the addition of LTD<sub>4</sub> to tonsillar cell culture induces a dose-dependent proliferative response that was partly blocked by either the CysLT<sub>1</sub> receptor antagonist montelukast or the dual CysLT<sub>1</sub> and CysLT<sub>2</sub> receptor antagonist BAYu9773.<sup>54</sup> Compared with subjects with recurrent tonsillitis, children with OSA had significantly higher expression of the CysLT<sub>1</sub> receptor in small-size CD19<sup>+</sup>B-lymphocytes and CD3<sup>+</sup>T-lymphocytes.<sup>55</sup> All these data suggested that LT pathway activation may contribute to the local proliferative and inflammatory pathway within the tonsils and adenoids in SDB at least in part through the release of pro-inflammatory cytokines.<sup>54</sup> The active inflammation of the lower airways in OSA children is reflected by enhanced urinary levels of LTE<sub>4</sub>, being gradually increased with respect to OSA severity.<sup>17</sup> Urinary LTE<sub>4</sub> levels were also slightly correlated to adenotonsillar size and multiple linear regression analysis revealed that LogLTE<sub>4</sub> and adenotonsillar size were significant predictors of AHI.<sup>17</sup> The increased concentration of CysLTs in urine, exhaled breath condensate, and adenotonsillar tissues of children with OSA suggested that CysLT receptor antagonists could be useful in treating OSA. An open-label intervention study in children showed that 16-week treatment with the CysLT<sub>1</sub> receptor antagonist montelukast improved the underlying respiratory disturbances during the sleep and therefore could be of benefit in the management of children with mild SDB.<sup>52</sup> These findings were subsequently confirmed by a randomized double-blind study.<sup>56</sup>

## 7. Effects of CPAP on LT pathway activation

Studies showing that CPAP treatment could decrease LT production provide supportive evidence of the role of intermittent hypoxia severity in LT pathway activation. In non-obese OSA patients and in those with a normal BMI (BMI ≤25 kg/m<sup>2</sup>), both the production of LTB<sub>4</sub> by

stimulated PMNs<sup>20</sup> and the urinary excretion of LTE<sub>4</sub><sup>18</sup> were respectively reduced after a 3-month period of CPAP in compliant patients while, during the same time, LTB<sub>4</sub> production remained unchanged in control subjects or non-observant OSA patients. These data suggested that, in the absence of confounding factors, LT pathway activation could be reversed by CPAP treatment. However, CPAP treatment failed to reduce LTE<sub>4</sub> urinary concentration in overweight and obese OSA patients,<sup>18</sup> further underlining the role of obesity as a confounder in the association of LTs with chronic intermittent hypoxia. Since LTB<sub>4</sub> increases chemokine secretion and MMPs, it is interesting to mention that the production of MCP-1 and MMP-9 by monocytes is also significantly decreased after long-term nasal CPAP treatment.<sup>39</sup> Likewise, CD15 expression is attenuated, whereas apoptosis markers are increased in neutrophils derived from nasal CPAP-treated patients.<sup>41</sup>

## 8. Could LT modifiers being interesting in the treatment of OSA-associated atherosclerosis?

Pharmacological inhibition of FLAP has been shown to reduce lesion size in different atherosclerosis-prone mice (see review in Riccioni and Back<sup>57</sup>). In a randomized double-blind trial vs. placebo performed in patients with acute coronary syndrome, the 5-LO inhibitor VIA-2291 reduced urinary LTE<sub>4</sub> concentration and reduced the non-calcified plaque volume.<sup>58</sup> In a registry-based nation-wide Swedish cohort of almost 7 million subjects followed for 3.5 years, the use of the CysLT<sub>1</sub> receptor montelukast reduced the risk of myocardial infarction in male, and reduced the risk of stroke in subjects not on angiotensin-modifying drugs.<sup>59</sup> Taken together, these studies highlight the potential interest of LT pathway inhibition in the prevention of cardiovascular disease. However, the effect of pharmacological inhibition of the LT pathway in the prevention of intermittent hypoxia-induced human atherogenesis still remains to be demonstrated. An *in vitro* study on THP-1 cells showed that BLT<sub>1</sub> receptor blockade markedly attenuated chronic intermittent hypoxia-induced MCP-1 expression,<sup>31</sup> suggesting that disruption of the LTB<sub>4</sub>-BLT<sub>1</sub> pathway could prevent intermittent hypoxia-induced monocyte recruitment. Similarly, BLT<sub>1</sub> and BLT<sub>2</sub> receptor blockage prevented intermittent hypoxia-induced CD14 and CD18 expression,<sup>31</sup> suggesting that the blockage of the LTB<sub>4</sub> pathway could prevent intermittent hypoxia-induced monocyte differentiation.

CPAP treatment is regarded as the cornerstone of treatment for OSA. An observational study showed that CPAP reduced the risk of fatal and non-fatal cardiovascular events in men with severe OSA.<sup>2</sup> These results were in line with the findings that 4 months of effective treatment with CPAP significantly improved validated markers of atherosclerosis in relatively young patients without significant comorbidities and on no medication.<sup>7</sup> However, a recent randomized, parallel, 6-month controlled trial demonstrated that the impact of CPAP in obese mild-to-moderate OSA patients presenting cardiovascular and/or metabolic morbidities was more relative.<sup>60,61</sup> Moreover, ~30–40% of OSA patients refuse<sup>2</sup> or are non-compliant to treatment with CPAP, highlighting the need of pharmacological strategy in association with CPAP in patients at high cardiovascular and metabolic risk.<sup>62</sup>

Of course, the activation of the LT pathway itself could not be the sole mechanism of vascular remodelling in OSA patients. But in contrast to the LT pathway, the cyclooxygenase (COX)-dependent pathway of arachidonic acid metabolism has been less extensively studied in OSA patients. For example, thromboxane A<sub>2</sub> (TXA<sub>2</sub>), which is predominantly

generated by platelets through COX type 1 isoform and thromboxane synthase, induces vasoconstriction, vascular smooth muscle cell proliferation, platelet activation and increases the expression of adhesion molecules binding on its TP receptors.<sup>46</sup> We recently demonstrated the involvement of the TXA<sub>2</sub> pathway in the acceleration of atherosclerosis in ApoE<sup>-/-</sup> mice exposed to intermittent hypoxia.<sup>36</sup> However, the role of hypoxia in the TXA<sub>2</sub>-dependant pathway remains to be established in OSA since the urinary concentrations of 11-dehydro-thromboxane B<sub>2</sub>, a stable metabolite of TXA<sub>2</sub>, were similar in healthy subjects and OSA patients free of cardiovascular risk factor and carefully matched for age and BMI,<sup>36</sup> suggesting that OSA itself might not be associated with thromboxane pathway activation. Other potential mechanisms including decreased nitric oxide availability have been detailed elsewhere to explain OSA-associated atherosclerosis.<sup>63</sup>

## 9. Conclusion

As discussed in this review, there is a growing body of evidences from both experimental *in vitro* or *in vivo* animal model studies and clinical studies, suggesting that the LT pathway could represent a major molecular link between OSA and atherosclerosis. To summarize (Figure 2), both intermittent hypoxia and obesity, that are characteristic features of OSA, induce a transcriptional activation of FLAP in PMNs and monocytes, respectively, leading to an increased production of LTB<sub>4</sub> and CysLTs. This enhanced production of LTs contributes to the underlying systemic and vascular inflammation associated with OSA. With respect to their biological effects, LTs may play a major role in the early vascular remodeling, and atherosclerosis development in OSA patients. Thus, LT modifiers may provide interesting novel therapeutic drugs for OSA-associated atherosclerosis.

**Conflict of interest:** none declared.

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## ***5. Prise en charge du SAOS***

Le traitement de première intention du SAOS consiste aujourd'hui en l'application d'une PPC dans les voies aériennes supérieures de manière à éviter leur obstruction. Bien qu'efficace sur les évènements obstructifs et l'hypersomnolence diurne, cette thérapeutique présente certaines limites. Tout d'abord, de par son caractère contraignant, la tolérance et donc l'observance de la PPC est médiocre. De plus, son efficacité sur la morbi-mortalité cardiovasculaire semble limitée chez certains patients apnéiques, notamment ceux présentant des comorbidités cardiovasculaires. Des alternatives thérapeutiques incluant mesures hygiéno-diététiques ou encore traitements mécaniques, chirurgicaux ou pharmacologiques sont aujourd'hui disponibles sans pour autant que leur effets notamment sur le système cardiovasculaire ne soient élucidés.

L'ensemble des thérapeutiques actuellement proposées dans la prise en charge du SAOS ont fait l'objet de l'écriture d'une revue dont le but a été de résumer les effets de ces différents traitements sur les perturbations vasculaires à la fois fonctionnelles et structurales induites par le SAOS avec un intérêt particulier pour les traitements pharmacologiques.

Publication n°2

**“Precision medicine to treat vascular disease related to  
obstructive sleep apnea: a place for pharmacological approaches  
beyond continuous positive airway pressure”**

E Gautier-Veyret, JL Pépin, F Stanke-Labesque.

*Soumis*

Precision medicine to treat vascular disease related to obstructive sleep apnea: the place of  
pharmacological approaches beyond continuous positive airway pressure

Elodie Gautier-Veyret<sup>1,2,3</sup>, Jean-Louis Pépin<sup>1,2,3</sup>, Françoise Stanke-Labesque<sup>1,2,3</sup>

<sup>1</sup>Univ. Grenoble Alpes, HP2, F-38041 Grenoble, France

<sup>2</sup>INSERM U1042, 38041 Grenoble, France

<sup>3</sup>Centre Hospitalier Universitaire des Alpes, 38043, Grenoble, France.

**Corresponding author:**

Dr Elodie Gautier-Veyret

Address: Laboratory of Pharmacology, Grenoble Alps University Hospital, 217, F-38043 Grenoble  
Cedex 9, France.

Tel: 33.4.76.76.54.92 Fax: 33.4.76.76.89.38

E-mail: [Egautier@chu-grenoble.fr](mailto:Egautier@chu-grenoble.fr)

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**Abstract (word count: 174)**

Obstructive sleep apnea (OSA) is characterized by recurrent episodes of partial or complete upper airway obstruction, occurring during sleep, leading to chronic intermittent hypoxia (IH), which harms the cardiovascular system. OSA is associated with both functional and structural vascular alterations that contribute to an increased prevalence of fatal and non-fatal cardiovascular events. OSA is a heterogeneous disease with respect to the severity of hypoxia, the presence of daytime symptoms, obesity, and cardiovascular comorbidities. Various clusters of OSA phenotypes have been described leading to more highly personalized treatment. The aim of this review is to describe the various therapeutic strategies including continuous positive airway pressure (CPAP), oral appliances, surgery, weight loss, and especially pharmacological interventions that have been evaluated to reduce vascular alterations in both OSA patients and preclinical animal models. Conventional therapies, predominantly CPAP, have a limited impact on vascular alterations in the presence of co-morbidities. A better knowledge of pharmacological therapies targeting IH-induced vascular alterations will facilitate the use of combined therapies and is crucial for designing clinical trials in well-defined OSA phenotypes.

**Key-words:** obstructive sleep apnea, treatment, pharmacology, cardiovascular diseases, intermittent hypoxia.

## **Lists of abbreviations**

AHI, apnea-hypopnea index

ApoE<sup>-/-</sup>, Apolipoprotein E knock-out

BMI, body mass index

BP, blood pressure

CPAP, continuous positive airway pressure

CVD, cardiovascular disease

FMD, flux-mediated dilatation

IH, intermittent hypoxia

IMT, intima-media thickness

MAD, mandibular advancement device

OA, oral appliance

OSA, obstructive sleep apnea

RAAS, renin-angiotensin aldosterone system

RCT, randomized controlled trial

TNF $\alpha$ , tumor necrosis factor alpha

UPPP, uvulopalatopharyngoplasty

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## 1. Introduction

Obstructive sleep apnea (OSA) is a common clinical disorder characterized by recurrent nocturnal pharyngeal collapses which lead to sleep fragmentation, increased respiratory efforts, and intermittent hypoxia (IH). OSA is associated with increased cardiovascular morbidity and mortality (Baguet et al., 2012; Lévy et al., 2015) and is an independent cardiovascular risk factor (Somers et al., 2008). The hypoxic component of OSA is now clearly identified as the main determinant of cardiovascular complications (Dematteis et al., 2009) as IH itself has been shown to accelerate the atherogenic process in animal models (Arnaud, et al., 2011a; Gautier-Veyret et al., 2013) and OSA patients free of overt comorbidities (Baguet et al., 2005). The proposed underlying mechanisms linking OSA and cardiovascular diseases (CVD) are numerous and complex, including sympathetic over activity, oxidative stress, metabolic dysfunction, and inflammation, but are still insufficiently understood (see review (Lévy et al., 2015)).

Since its first description in 1981 (Sullivan et al., 1981), the application of continuous positive airway pressure (CPAP) during sleep remains the first line treatment of OSA. It normalizes ventilation during sleep and reduces daytime sleepiness (Basner, 2007; Bratton et al., 2015a; Sharples et al., 2015) but its effectiveness in reducing OSA-related cardiovascular consequences seems to be limited, especially in minimally symptomatic patients with obesity and comorbidities (Craig et al., 2012; Pépin et al., 2012). Moreover, initial refusal of CPAP occurs in 15% of patients and compliance is difficult to achieve in 20 to 35% (Pépin et al., 1999; Weaver et al., 2007). Other therapeutic options including mandibular advancement devices (MADs), upper airway surgery, weight loss, or pharmacological strategies (White, 2016) may also be proposed in addition or as alternative to CPAP (Lévy et al., 2015). Beyond CPAP and oral appliances (OAs), the effects of therapeutic interventions on OSA-related vascular alterations remain poorly studied, particularly among the clusters of OSA phenotypes currently described to propose personalized medicine (Bailly et al., 2016; Destors et al., 2015; Lacedonia et al., 2016; Vavougiou et al., 2015; Ye et al., 2014). Along



these lines, several pharmacological interventions summarized in figure 1 have been evaluated to treat vascular diseases associated with OSA.

The objective of this review is to provide an overview of the clinical efficacy of conventional OSA treatments, especially CPAP, on OSA-related vascular alterations and to put in perspective the various pharmacological approaches that have been studied, or are in progress, to reduce OSA-related vascular consequences.

## **2. Effects of standard OSA therapies on OSA-related vascular alterations**

### **2.1. CPAP, the reference OSA treatment**

CPAP acts as a pneumatic splint of the upper airway, restoring patency and avoiding pharyngeal collapses during sleep. Several cohort studies have suggested that CPAP treatment is associated with a reduction of cardiovascular events (Buchner et al., 2007; Campos-Rodriguez et al., 2012; Marin et al., 2012; Marin et al., 2005), whereas others showed no effect (Craig et al., 2012; Lamberts et al., 2014). The largest randomized controlled trial evaluating CPAP versus non active intervention showed no effect in intention-to-treat analysis, but post hoc analysis suggested that CPAP treatment may reduce the incidence of hypertension or cardiovascular events in patients with a CPAP adherence of 4 h/night or longer (Barbe et al., 2012). The efficacy of CPAP for reducing the incidence of cardiovascular events was recently assessed in two meta-analysis (Guo et al., 2016; Kim et al., 2016). The first by Kim et al. concluded that CPAP treatment may decrease the risk of cardiovascular events with a more pronounced effect on stroke (Kim et al., 2016). The second by Guo et al. demonstrated a global trend for decreased cardiovascular risk in patients treated with CPAP (Guo et al., 2016). However, the benefit of CPAP must be put in the context of patient compliance, the presence/absence of comorbidities, and OSA severity. We have thus further detailed the vascular effects of CPAP according to OSA severity (mild OSA defined by an apnea-hypopnea

index (AHI) < 15 events/h, moderate: AHI between 15 and 30 events/h and severe: AHI > 30 events/h).

### ***2.1.1. Moderate to severe OSA***

In moderate to severe OSA, the vascular benefits of CPAP on endothelial function (Muñoz-Hernandez et al., 2015; Korcarz et al., 2016; Wons & Kohler, 2015), arterial stiffness (Buchner et al., 2012; Drager et al., 2007; Pépin et al., 2013; Phillips et al., 2013a; Seetho et al., 2015; Korcarz et al., 2016; Vlachantoni et al., 2013; Wons & Kohler, 2015), and blood pressure (BP) (Barbé et al., 2010; Fatureto-Borges et al., 2016; Kohler et al., 2011; Korcarz et al., 2016; Wons & Kohler, 2015) and subsequent vascular remodeling (Drager et al., 2007; Hui et al., 2012; Sharma et al., 2011) have been replicated in numerous studies. These vascular benefits translated into a reduction of cardiovascular events in severe (Campos-Rodriguez et al., 2012; Marin et al., 2005b) and moderate to severe OSA patients (Buchner et al., 2007; Campos-Rodriguez et al., 2012; Martínez-García et al., 2012) in several observational studies. The study of Barbé reproduced the benefit of CPAP on the occurrence of hypertension and cardiovascular events only in the subgroup of patients using CPAP for more than 4 h per night, but not in the intention-to-treat analysis (Barbe et al., 2012). However, this latter study was conducted in moderate to severe patients who were minimally symptomatic (Barbe et al., 2012). How these results can be generalized to sleepy OSA patients remains unknown. Indeed, it has been suggested that the extent of vascular benefits of CPAP are not the same in all moderate to severe OSA patients. A RCT conducted in moderate to severe OSA patients [median and interquartile range of AHI of 31(20-41) events/h], without overt CVD, demonstrated that 12 weeks of CPAP therapy had no effect on endothelial function and a non-significant trend towards a lowering of arterial stiffness (Jones et al., 2013). These discrepancies within the existing literature (Phillips et al., 2013a; Vlachantoni et al., 2013) may be explained by less severe vascular dysfunction in the particular subgroup of OSA patients (Jones et al., 2013) who are free of CVD and exhibit fewer

nocturnal desaturations (Ryan, 2013). The influence of baseline CVD on the response to CPAP is also supported by the results of the SAVE study in which CPAP provided no benefit in terms of the reduction of cardiovascular events in patients with moderate to severe OSA in secondary cardiovascular prevention (McEvoy et al., 2016; Mokhlesi & Ayas, 2016). Thus identification of biomarkers could be of interest to predict individually CPAP efficacy. In this line, analysis of clusters of 3 micro-ribonucleic acids seems able to predict the blood pressure response to CPAP treatment in patients with OSA and resistant hypertension (McEvoy & Michael, 2015; Sánchez-de-la-Torre et al., 2015). Finally, CPAP modalities (Pépin et al., 2016) and timing (Mokhlesi & Ayas, 2016) may also have a substantial impact as a large recent RCT has suggested that the use of fixed pressure more effectively reduces 24-h BP than auto-adjusting CPAP (Pépin et al., 2016).

### ***2.1.2. Mild OSA***

The benefits of CPAP to treat or prevent CVD in mild OSA have not been elucidated to date. To our knowledge, only two randomized controlled studies investigating the effects of CPAP on BP in mild OSA have been conducted and provided contradictory results (Barnes et al., 2002; Weaver et al., 2012). The demonstration of any benefit of CPAP on vascular function is probably difficult in this population (Ryan, 2013) as cardiovascular morbidity and mortality do not seem to increase in mild to moderate OSA patients (Marin et al., 2005a).

### ***2.1.3. Minimally symptomatic***

The link between OSA and vascular dysfunction in minimally symptomatic OSA patients is a subject of debate, as demonstrated by the numerous discussions (Chang, 2009; Lorenzi-Filho & Drager, 2008; Ng & Chan, 2009; Phillips et al., 2009; Yee et al., 2009) in response to the paper of Kohler et al. demonstrating altered vascular function (endothelial function and arterial stiffness) in this

subgroup of patients (Kohler et al., 2008). Data on the benefits of CPAP on cardiovascular outcomes in this population are scarce. A meta-analysis published in 2014 concluded that CPAP is ineffective in reducing BP in this population, except in patients using CPAP for > 4 hours per night (Bratton et al., 2014). Two RCTs conducted in non-sleepy OSA patients did not establish any beneficial effect of CPAP on cardiovascular risk (Barbe et al., 2012; Craig et al., 2012). These negative results may be due to lower CPAP compliance in this population relative to more symptomatic OSA patients (Marshall et al., 2006). Indeed, there was no significant impact of CPAP on cardiovascular risk for the overall population in the MOSAIC trial, where the mean use of CPAP was only 2h39min per night (Craig et al., 2012). However, endothelial function was improved by CPAP in a subset of adherent patients (Kohler et al., 2013). The importance of CPAP compliance was again recently confirmed by the RICCADSA study that showed a reduction of long-term cardiovascular outcomes only after adjustment for CPAP usage (Peker et al., 2016).

## **2.2. Oral appliances**

OAs, such as MADs aim to widen the upper airways to reduce their collapsibility. This therapy is indicated for mild to moderate OSA and for patients who refuse or do not tolerate CPAP. While OAs are less efficient than CPAP in reducing AHI and sleepiness (Bratton et al., 2015a), OA compliance is better, resulting in similar outcomes as CPAP (Kohler et al., 2013; Phillips et al., 2013b; Trzepizur et al., 2009): A meta-analysis showed a similar reduction in BP using the two approaches (Bratton et al., 2015b). Conversely, a RCT conducted in moderate to severe OSA patients did not demonstrate any effect of one-month MAD use on 24-h BP (Phillips et al., 2013b), but in this study, neither CPAP nor MAD use improved BP either, which may be related to the relatively small prevalence of untreated hypertension (Phillips et al., 2013b). A reduction of arterial stiffness from baseline was however reported with OAs in this RCT (Phillips et al., 2013b), and was recently confirmed in an observational study conducted in mild to moderate OSA patients (Galic et al., 2015). Several small (n

from 16 to 30 subjects) prospective observational studies have also shown that MAD use may improve endothelial function in mild to moderate (Galic et al., 2015) or moderate to severe OSA (Itzhaki et al., 2007; Lin et al., 2015; Trzepizur et al., 2009), but this result has not been confirmed in a RCT.

### **2.3. Upper airway Surgery**

Various surgical techniques have been proposed to treat OSA. Maxillofacial surgery results in a high success rate, but few patients are eligible, mostly young, non-obese patients with craniofacial anomalies (Bettega et al., 2000; Lévy et al., 2015). Palatal surgery (including uvulopalatopharyngoplasty (UPPP) and adenotonsillectomy) is another option consisting of excising pharyngeal soft tissues. These surgical approaches exhibit low efficacy and sometimes long-term deterioration, which limits their use (Lévy et al., 2015). The effects of surgical treatment on cardiovascular outcomes have been poorly investigated. Some observational studies have shown an improvement of vascular function (endothelial function and/or BP) after adenotonsillectomy in OSA children (Chan et al., 2015) or after UPPP in adults with moderate to severe OSA (Lee et al., 2009; Peng et al., 2016; Yang et al., 2012). In addition, a reduction of intima-media thickness (IMT) was also reported in moderate to severe OSA patients following UPPP and nasal surgery. These beneficial effects are limited to the approximately 50% of patients who respond to upper airway surgery (Caples et al., 2010).

### **2.4. Weight loss and exercise**

Weight loss is systematically recommended to manage OSA, especially in overweight or obese patients, as OSA and obesity are intrinsically linked (Young et al., 2005). Two meta-analysis showed that weight loss through lifestyle and/or dietary intervention is associated with an improvement of

polysomnographic parameters (Araghi et al., 2013; Thomasouli et al., 2013), an effect confirmed in two RCTs (Chirinos et al., 2014; Johansson et al., 2009). Nevertheless, weight loss itself is insufficient to normalize OSA parameters (Araghi et al., 2013; Ng et al., 2015), underlying the interest of combined therapies. Comparisons of CPAP, weight loss, or the combined interventions demonstrated an additive reduction of BP for the combined therapy, which was also observed for metabolic parameters (triglyceride levels and insulin resistance) (Chirinos et al., 2014).

Bariatric surgery is another way to achieve weight loss in patients with morbid obesity. Despite the ability of this procedure to induce rapid and pronounced weight loss, the improvement of polysomnographic parameters is not greater than those obtained with classical weight loss (Dixon et al., 2012).

Despite the benefit of weight loss on polysomnographic and metabolic parameters (Chirinos et al., 2014; Johansson et al., 2009; Ng et al., 2015), its influence on vascular alterations of OSA patients is poorly described. In a small observational uncontrolled study (n = 33), Iguchi et al. reported a decrease in arterial stiffness after short-term weight loss (through lifestyle modification) in overweight or obese OSA patients (Iguchi et al., 2013). In contrast, weight loss induced by bariatric surgery had no impact on endothelial function of OSA patients newly treated by CPAP (Bakker et al., 2013). This may be due to the fact that the body mass index (BMI) remains relatively high, even after the surgical procedure (median [lower and upper quartile] post-surgery BMI: 32.7[30.1-38.7] kg/m<sup>2</sup>).

Among lifestyle modifications, exercise is another intervention proposed to treat OSA. Exercise was associated with an improvement of OSA parameters and sleepiness (Aiello et al., 2016), but the vascular benefits of exercise remain to be established.

In summary, CPAP appears to have a limited effect on improving OSA-induced vascular alterations, in particular in co-morbid or obese patients. Important data are missing in the field. i) The impact of

OAs, weight loss, and surgery on vascular parameters remains largely unexplored. Further studies are needed to address this issue, which will make it possible to better identify patient phenotypes for which additional therapies are needed. ii) The vascular benefits of CPAP are not the same among OSA phenotypes, and biomarkers predicting the response to therapies should be validated (Sánchez-de-la-Torre et al., 2015). Although the effects of CPAP are discussed in this review by distinguishing OSA severity based on AHI and sleepiness, OSA phenotypes appear to be more complex (Bailly et al., 2016; Lacedonia et al., 2016). The presence/absence of cardiovascular comorbidities, as well as hypoxia severity (assessed by the oxygen desaturation index), should be taken into account when proposing a personalized medicine strategy. iii) CPAP alone is not able to completely restore vascular function (Seetho et al., 2015), even in patients in whom CPAP therapy improves OSA-related vascular alterations, underlying the need for combined therapy. Some trials have already demonstrated greater efficacy for multi-modality intervention such as CPAP treatment and weight loss (Chirinos et al., 2014) or anti-hypertensive treatment and CPAP (Pépin et al., 2010; Thunström et al., 2016). An aggressive risk factor management strategy adapted to OSA phenotypes needs to be validated. Along these lines, pharmacological approaches to treat OSA-related vascular alterations have been studied in OSA patients or OSA animal models.

### **3. Pharmacological approaches targeting OSA-related vascular alterations**

In the last decade, the pathophysiology of OSA-related vascular alterations has been widely studied and several key-mechanisms have been identified. These mechanisms include sympathetic nervous system activation, low grade inflammation, oxidative stress, and metabolic dysfunctions; each of these intermediate mechanisms can be targeted by specific pharmacological treatment. Tables 1 and 2 summarize the studies that have investigated the vascular effects of pharmacological treatment in OSA patients (Table 1) or preclinical OSA models (Table 2).

### **3.1. Antihypertensive drugs**

The effects of classical antihypertensive drugs on OSA-related vascular alterations have been evaluated as hypertension and OSA are closely related. In line with the pathophysiology of OSA-related vascular changes (Lévy et al., 2015), medications targeting the renin-angiotensin-aldosterone system (RAAS),  $\beta$ -blockers, calcium-channel blockers, and diuretics have been evaluated. A recent review reported that drugs acting mainly on the sympathetic nervous system, namely  $\beta$ -blockers and drugs targeting RAAS (angiotensin II receptor blocker, angiotensin converting enzyme inhibitor and mineralocorticoid receptor antagonist) may be useful in treating OSA-associated hypertension (Diogo & Monteiro, 2014), a conclusion that has been reinforced by additional recent studies. In an open, randomized parallel-design trial, a six-week treatment with the angiotensin II type 1 receptor antagonist, losartan, reduced BP in newly diagnosed hypertensive OSA patients, either in the presence or absence of CPAP treatment (Thunström et al., 2016). In addition to its effect on BP elevation, an experimental study has also shown that losartan prevented IH-induced endothelial dysfunction (Marcus et al., 2012). Other authors investigated the effects of aldosterone receptor antagonists on vascular dysfunction in OSA (Kraśńska et al., 2016; L. Yang et al., 2016). The addition of spironolactone (L. Yang et al., 2016) or eplerenone (Kraśńska et al., 2016) to the usual hypertensive therapy reduced the BP in patients with moderate to severe OSA and resistant hypertension. Treatment with eplerenone was also associated with an improvement of arterial stiffness (Kraśńska et al., 2016). These studies, however, have several methodological limitations as eplerenone was evaluated in an observational uncontrolled trial (Kraśńska et al., 2016) whereas the other study, evaluating the effect of spironolactone, was an open RCT where the control group did not receive a placebo (Yang et al., 2016). The efficacy of drugs targeting the RAAS to reduce BP in OSA patients is not surprising as alterations of the RAAS was identified as a major determinant of hypertension in OSA (see reviews (Diogo & Monteiro, 2014; Lévy et al., 2013)).



$\beta$ 1-adrenergic receptor antagonists may also be of interest for the treatment of OSA-associated hypertension (Diogo & Monteiro, 2014) but there have been no new clinical studies since the review of Diogo (Diogo & Monteiro, 2014) to confirm the superiority of this class of antihypertensive drugs to others. In contrast, an animal study did not show any effect of carvedilol, a  $\beta$ -blocker with pleiotropic effects (vasodilatory, antioxidant and antiproliferative effects) on IH-induced hypertension (Diogo et al., 2015).

The vascular benefits of diuretics and calcium channel blockers in OSA patients are more mixed (Diogo & Monteiro, 2014). Calcium channel blockers have been associated with a negative impact on both sleep quality and duration (Nerbass et al., 2011) and diuretics seem to be ineffective in OSA patients without fluid retention (Owen & Reisin, 2013).

Most of the studies on the benefits of antihypertensive drugs in OSA did not take into account the various phenotypes of OSA patients, especially in terms of the presence or absence of comorbidities (Diogo & Monteiro, 2014). For example, the positive effects of diuretics need to be better evaluated in OSA patients with fluid retention, although the benefits in OSA patients seem to be limited (Diogo & Monteiro, 2014). Indeed, diuretics could have beneficial effects both on OSA parameters and BP (Bucca et al., 2007; Kasai et al., 2014) through its effects on fluid retention and thus on the phenomena of fluid shift described in OSA (White et al., 2014). Additional RCTs to further examine the beneficial effects of antihypertensive drugs (ie, angiotensin II receptor blocker,  $\beta$ -blockers, and calcium channel blockers) on vascular parameters, in particular BP, are ongoing, but the results are not yet available (Table 3).

We and others have evaluated the vascular effects of endothelin-receptor antagonism in OSA. Bosentan, a mixed endothelin receptor antagonist currently indicated for the treatment of pulmonary hypertension, prevented IH-induced increases in BP in rodents (Belaidi et al., 2009; de Frutos et al., 2010) and vascular remodeling induced by the exposure to IH in mice (de Frutos et al., 2010; Gras et al., 2016). This treatment was also evaluated in a cross-over randomized controlled

pilot study conducted in 16 mildly hypertensive patients with severe OSA. A reduction of BP was observed after a 4-week course of treatment with bosentan, but was comparable to that obtained with CPAP and was very small (Joyeux-Faure et al., 2016). Given the influence of endothelin-1 on IH-induced vascular damage, further studies are needed to determine the interest of targeting this component for preventing or reversing the vascular alterations associated with OSA (Belaidi et al., 2016).

### **3.2. Anti-inflammatory drugs**

Atherosclerosis (Hansson, 2005) and OSA (Ryan et al., 2005) are associated with low-grade inflammation and there is growing evidence supporting the contribution of OSA/inflammation to atherosclerosis (Arnaud et al., 2011b; Gautier-Veyret et al., 2013; Ma et al., 2016; Song et al., 2015). Several anti-inflammatory drugs have been evaluated for the treatment of IH-induced vascular alterations. We demonstrated, for example, that the inhibition of cyclooxygenase-1 by a four-week course of treatment with SC-560 reduced the size of atherosclerotic lesions in apolipoprotein E deficient (ApoE<sup>-/-</sup>) mice chronically exposed to IH (Gautier-Veyret et al., 2013). The administration of monoclonal antibodies targeting specific inflammatory cytokines shows promise for the treatment of atherosclerosis (Tousoulis et al., 2016). Administration of a monoclonal antibody that targets the cytokine regulated upon activation normal T-cell expressed and secreted / CC chemokine ligand 5 in wild-type mice attenuated IH-induced increases of the IMT (Arnaud et al., 2011a). Neutralization of tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) with infliximab, a monoclonal antibody directed against TNF $\alpha$ , prevented both IH-induced endothelial dysfunction and atherosclerosis when administered during the entire period of exposure of ApoE<sup>-/-</sup> mice to IH (Tuleta et al., 2014). Currently, no trials investigating the vascular benefits of anti-inflammatory therapies have been conducted in OSA patients. Such pharmacological approaches are of particular interest in the context of OSA as the soluble TNF $\alpha$  receptor etanercept induced a notable improvement of

sleepiness and AHI in a pilot study conducted in eight obese men suffering from severe OSA (Vgontzas et al., 2004).

### **3.3. Anti-oxidative drugs**

Numerous studies have proposed oxidative stress as a link between OSA and CVD pathogenesis (see review (Lavie, 2012)). Several molecules with anti-oxidative properties have been evaluated for the reduction of vascular alterations associated with OSA. In animal studies, endothelial dysfunction has been improved by treatment with allopurinol (Dopp et al., 2011), L-glutathione (Tuleta et al., 2014), or melatonin (Hung et al., 2013). This effect was accompanied by the prevention of IH-induced increase of systolic BP for melatonin (Ramond et al., 2013; Yeung et al., 2015) and a reduction of IH-induced atherogenesis for L-glutathione (Tuleta et al., 2014). Prevention of IH-induced increases of BP have also been described for the superoxide dismutase mimetic Tempol (Ramond et al., 2013; Troncoso Brindeiro et al., 2007) and the NADPH oxidase inhibitor apocynin (Schulz et al., 2014). Among these compounds, only allopurinol has been evaluated in humans in a small cross-over RCT. This study extended the preclinical findings as allopurinol also increased flux-mediated dilatation (FMD) relative to placebo in moderate to severe OSA patients (El Solh et al., 2006). Other clinical studies also found positive vascular effects for other antioxidant components. A single injection of vitamin C improved endothelial function both in conductance (Grebe et al., 2006) and resistance vessels (Büchner et al., 2011) in untreated moderate to severe apneic patients, whereas a 6-week course of treatment with carbocysteine had no effect on this parameter, but reduced IMT in moderate to severe OSA (Wu et al., 2016). The effects of some of these antioxidant drugs (ie allopurinol and melatonin) on vascular outcomes are currently being investigated in severe OSA patients (Table 3).

### 3.4. Lipid lowering drugs

Dyslipidemia is a common clinical disorder observed in apneic patients which contributes to the pathogenesis of IH-related vascular alterations (Drager et al., 2010). Lipid-lowering therapy also decreases the incidence of cardiovascular events in high cardiovascular risk patients (Baigent et al., 2005). Few studies have investigated the effect of lipid lowering therapy on the pathogenesis of CVD in OSA. Treatment of rats chronically exposed to IH with the 3-Hydroxyl-3-Methylglutaryl Coenzyme A Reductase inhibitor, atorvastatin, prevented the IH-induced increase in systolic pressure and corrected IH-related endothelial dysfunction and increases of the IMT (Totoson et al., 2013). In a multicenter cross-over RCT conducted in severe OSA patients, treatment with this statin for 12 weeks had a beneficial effect on BP, but no impact on endothelial function, arterial stiffness, or carotid atherosclerosis (Joyeux-Faure et al., 2014). The mechanisms by which statins exert their vascular effects in OSA remain unclear because in addition to their action on lipids, statins also have pleiotropic effects.

Ezetimibe, another lipid lowering drug that reduces cholesterol absorption was tested in ApoE<sup>-/-</sup> mice chronically exposed to IH (Kato et al., 2015). A reduction of the atherosclerotic lesion size was observed in hypoxic mice treated by ezetimibe (Kato et al., 2015), but this effect may not be relevant in the context of IH-induced atherosclerosis. Indeed, mice were fed a high-fat-diet in this study, which is known to more strongly induce atherosclerosis than chronic exposure to IH (Song et al., 2015). Ezetimibe also reduced the size of atherosclerotic lesions in normoxic mice to the same extent as those of mice chronically exposed to IH, making it unlikely that ezetimibe has a specific preventive effect on IH-induced atherosclerosis (Kato et al., 2015). Another pharmacological target could be the lipoprotein lipase inhibitor angiopoietin-like 4 which is up-regulated after IH. Its neutralization using monoclonal antibodies prevented atherosclerosis in ApoE<sup>-/-</sup> mice (Drager et al., 2013). Overall, the vascular benefits of lipid lowering drugs in OSA remain poorly understood and the consequences of their side effects are unknown (González et al., 2016).

In addition to dyslipidemia, OSA-related metabolic dysfunction also includes an alteration of glucose homeostasis (Lévy et al., 2015). Some antidiabetic drugs such as metformin (Fisman & Tenenbaum, 2015) or gliptin (Wu et al., 2015) appear to have beneficial effects on vascular alterations in diabetes, but such pharmacological interventions have yet to be evaluated in the context of OSA.

### **3.5. Medications for obesity**

An evaluation of the effect of obesity medications on OSA symptoms and vascular alterations may be useful as OSA and obesity are strongly associated and the efficiency of CPAP is poor at best in reducing vascular alterations in obese OSA patients. Liraglutide is a glucagon-like peptide-1 analog belonging to the incretinomimetic class. In addition to the treatment of type 2 diabetes, it has recently received marketing approval in Canada for weight reduction at a daily dose of 3 mg, which reduces appetite. A randomized, controlled double blind trial showed that a 32-week course of treatment with liraglutide, combined with diet and exercise, decreased systolic BP by 3.4 mm Hg from baseline, but not diastolic BP, in obese moderate to severe OSA patients (Blackman et al., 2016). However, these effects were observed in a population where only 42% of the patients were hypertensive. Although liraglutide treatment improved polysomnographic parameters, its vascular benefit/risk balance has not been determined in the light of the frequent adverse effects associated with this drug.

Other drugs, such as antiepileptics currently in development for weight reduction, have been evaluated in obese OSA patients (Eskandari et al., 2014; Winslow et al., 2012), but except for a reduction of BP obtained using a combination of phentermine and topiramate (Winslow et al., 2012), the benefits of such therapy are unknown.

### **3.6. Testosterone replacement**

Androgen deficiency is associated with increased cardiovascular morbidity. This biochemical deficiency is frequently observed in men with OSA or obesity. A double-blind RCT performed in obese OSA men demonstrated that an 18-week course of treatment with testosterone combined with a hypocaloric diet decreased arterial stiffness by 3.2% relative to placebo (Hoyos et al., 2012). This strategy needs to be evaluated in a larger cohort of obese OSA men to consider all potential benefits and risks, especially since a worsening of OSA parameters was described after 6-7 weeks of this treatment in the same study (Killick et al., 2013).

### **3.7. Pharmacological treatment to prevent pharyngeal collapse**

Several medications including antidepressants (fluoxetine, paroxetine, mirtazapine) or potassium channel blockers (Grace et al., 2014; Wirth et al., 2013) have been previously evaluated for reducing obstructive events, but none has yet received marketing approval for OSA. These various pharmacological approaches have been recently reviewed (White, 2016) but their impact on vascular alterations associated with OSA remain to be investigated.

## **4. Conclusion**

The efficacy of CPAP is limited by poor adherence and inconstant vascular benefits. As a result, therapeutic alternatives, especially pharmacological therapies, have been evaluated (see figure 1). Although the vascular effects of CPAP have been relatively well-described for different OSA phenotypes, the impact of other therapeutic interventions on vascular parameters remains largely unexplored. Further research is thus warranted to address this issue, keeping in mind that personalized treatment of OSA will probably be established based on combined strategies as already advocated by some trials (Chirinos et al., 2014; Pépin et al., 2010; Thunström et al., 2016).

**Conflict of Interest statement**

The authors declare that there are no conflicts of interest.

## **Figure legend**

Figure 1. Scheme of various therapeutic interventions with a specific focus on pharmacological strategies evaluated to treat obstructive sleep apnea-related vascular alterations. All therapeutic interventions noted in this figure are discussed in this review, especially pharmacological treatments which are detailed in tables 1 and 2.

CPAP, continuous positive airway pressure; RAAS, renin-angiotensin-aldosterone system.



Table 1. Studies investigating vascular effects of drugs in OSA patients\*

drug	Mode of action	Treatment modalities	Study design	Study population	Effects on vascular parameters
<b>losartan</b>	angiotensin II receptor antagonist	50 mg/d for 6 weeks	Open randomized parallel-design trial: losartan alone (n = 23) vs. losartan + CPAP (n = 25)	Newly diagnosed hypertensive and OSA patients	↓ BP without any additive effect with CPAP, except in adherent patients (Thunström et al., 2016)
<b>spironolactone</b>	mineralocorticoid receptor antagonist	20 mg/d (up to 40 mg/d if required at week 4) for 12 weeks	Open blank-controlled randomized trial‡: usual hypertensive therapy vs. usual hypertensive therapy + spironolactone (n = 15 in each arm)	Moderate to severe OSA patients with resistant hypertension	↓ BP (Yang et al., 2016)
<b>epplerenone</b>	mineralocorticoid receptor antagonist	50 mg/d for 3 months	Observational uncontrolled trial (n = 31)	Moderate to severe OSA patients with resistant hypertension	↓BP, PWV, aortic AIHR75, and AI (Kraśnińska et al., 2016)
<b>bosentan</b>	mixed endothelin receptor antagonist (ET <sub>A</sub> and ET <sub>B</sub> )	125 mg/d for 4 weeks	RCT cross-over design: bosentan vs. CPAP (n = 16)	Mildly hypertensive patients with severe OSA	↓ BP with a trend for a greater effect than CPAP (Joyeux-Faure et al., 2016)
<b>carbocysteine</b>	antioxidant	1500 mg/d (500 mg tid) for 6 weeks	Observational uncontrolled trial† (n = 40)	Moderate to severe OSA patients	↓ IMT ↔ FMD (Wu et al., 2016)
<b>vitamin C</b>	antioxidant	single intravenous injection of 0.5 g	Case-control study (n = 10 cases)	Untreated severe OSA patients	↑ FMD (Grebe et al., 2006)
<b>vitamin C</b>	antioxidant	single intra-arterial infusion of 625 mg	Case-control study (n = 11 cases)	Male moderate to severe OSA patients	↑ Ach-induced FBF †† (Büchner et al., 2011)
<b>allopurinol</b>	antioxidant (xanthine oxidase inhibitor)	300 mg/d for 2 weeks	RCT cross-over design: allopurinol vs. placebo (n = 12)	Moderate to severe OSA not treated	↑ FMD (El Solh et al., 2006)
<b>atorvastatin</b>	HMG-CoA reductase inhibitor	40 mg/d for 12 weeks	RCT parallel-design: atorvastatin (n = 25) vs. placebo (n = 26)	Severe OSA patients	↓ BP ↔PAT, PWV and carotid atherosclerosis (IMT and luminal diameter) (Joyeux-Faure et al., 2014)

<b>testosterone</b>	androgenic hormone	1000 mg at 0, 6, and 12 weeks	RCT parallel-design: testosterone (n = 33) vs. placebo (n = 34), both combined with lifestyle modification program	Obese OSA men	↓ aortic AI by 3.2% (Hoyos et al., 2012)
<b>liraglutide</b>	glucagon-like peptide-1 analog	3 mg for 32 weeks combined with diet and exercise	Double blind RCT: liraglutide (n = 180) vs placebo (n = 179)	Obese moderate to severe OSA patients	↓systolic BP ↔diastolic BP (Blackman et al., 2016)
<b>phentermine + topiramate</b>	Phentermine: sympathomimetic amine Topiramate: inhibitor of carbonic anhydrase§	15 mg/d of phentermine + extended release topiramate 92 mg/d for 28 weeks	Double blind RCT: phentermine + topiramate (n = 22) vs placebo (n = 23), both combined with lifestyle-modification counseling	Obese moderate to severe OSA patients	↓ systolic BP (Winslow et al., 2012)

Abbreviations: RCT, randomized controlled study; BP, blood pressure; PAT, peripheral arterial tone; FBF, forearm blood flow; FMD, flux-mediated dilatation; AIHR75, augmentation index at a heart rate of 75 bpm; AI, augmentation index; PWV, pulse wave velocity; IMT, intima-media thickness; CPAP, continuous positive airway pressure; HMG-CoA, hydroxyl-methyl-glutaryl Coenzyme A.

\*Because studies investigating the efficacy of anti-hypertensive drugs on blood pressure in OSA were recently reviewed (Diogo & Monteiro, 2014), this table does not include the works discussed in that review, except those in which study vascular outcomes are different from blood pressure or those which were published after its publication

† This study was a RCT comparing treatment with carbocysteine versus CPAP but vascular parameters were evaluated only in the carbocystein group, so we consider this study as an observational uncontrolled trial for these specific endpoints.

‡ Control subjects did not receive any placebo, so physicians and patients were not blinded to the conditions.

§ Additional mechanisms of action are described for this drug: blockade of voltage-dependent sodium channels, increase of the activity of the neurotransmitter GABA, and antagonism of the AMPA subtype of the glutamate receptor.

†† Ach-induced FBF is a marker of endothelial function of resistance vessels.

Table 2. Studies investigating vascular effects of drugs in animal models of OSA\*

drug	Mode of action	Treatment modalities	Animal model	OSA model	Effects on vascular parameters
<b>losartan</b>	angiotensin II receptor antagonist	25-30 mg/kg/d in drinking water from week n-1 to week 4†	adult male Sprague-Dawley rats on SCD	15 cycles/h (10-21% FiO <sub>2</sub> ) 12 h/d for 28 days	Prevention of IH-induced endothelial dysfunction (Marcus et al., 2012)
<b>bosentan</b>	mixed endothelin receptor antagonist (ET <sub>A</sub> and ET <sub>B</sub> )	100 mg/kg/day in food	7 to 9-week old C57BL/J6 mice on SCD	1 cycle/min (5-21% FiO <sub>2</sub> ) 8 h/d for 2 weeks	Prevention of IH-induced increase of IMT (Gras et al., 2016)
<b>bosentan</b>	mixed endothelin receptor antagonist (ET <sub>A</sub> and ET <sub>B</sub> )	30 mg/kg/day in drinking water	adult male BalB/C wild-type mice ‡	20 cycles/h (5-21% FiO <sub>2</sub> ) 7 h/d for 7 days	Prevention of IH-induced increase of BP and IMT (de Frutos et al., 2010)
<b>carvedilol</b>	β-blocker	10, 30 or 50 mg/kg/d by oral gavage from day 36 to day 60	60-75-day-old male Wistar rats on SCD	5.6 cycles/h (5-21% FiO <sub>2</sub> ) 10.5 h/d for 60 days	No effect of treatment on IH-induced BP increase (Diogo et al., 2015)
<b>SC-560</b>	COX-1 inhibitor	15 mg/d in food from week 4 to week 8	14-week-old male ApoE <sup>-/-</sup> mice on SCD	1 cycle/min (5-21% FiO <sub>2</sub> ) 8 h/d for 8 weeks	Reduction of IH-induced atherosclerosis (Gautier-Veyret et al., 2013)
<b>infliximab</b>	anti-TNFα monoclonal antibody	10 mg/kg, IP injection, 1x/week	10 to 12-week-old male and female ApoE <sup>-/-</sup> mice on HFD	33 cycles (5-21% FiO <sub>2</sub> ) 8 h/d for 6 weeks	Prevention of IH-induced endothelial dysfunction and atherosclerosis (Tuleta et al., 2014)
<b>anti-RANTES</b>	anti-RANTES monoclonal antibody	250 mg, IP injection, 3x/week	8-week-old male C57BL6 mice on SCD	1 cycle/min (5-21% FiO <sub>2</sub> ) 8 h/d for 14 days	Prevention of IH-induced IMT increase (Arnaud et al., 2011a)
<b>apocynin</b>	antioxidant (NADPH oxidase inhibitor)	30 mg/kg/d, IP injection	8 to 9-week old male C57BL/6J mice‡	15 cycle/h (7-21% FiO <sub>2</sub> ) 8 h/d, 5d/week for 6 weeks	Prevention of IH-induced increase of BP (Schulz et al., 2014)
<b>allopurinol</b>	antioxidant (xanthin oxidase inhibitor)	65 mg/kg/d, oral gavage from week n-1 to week 2†	adult male Sprague-Dawley rats on SCD	15 cycles/h (10-21% FiO <sub>2</sub> ) 12 h/d for 14 days	Prevention of IH-induced endothelial dysfunction (Dopp et al., 2011)

<b>L-glutathione</b>	antioxidant	250 mg/kg, IP injection, 3x/week	10 to 12-week-old male and female ApoE <sup>-/-</sup> mice on HFD	33 cycles (5-21% FiO <sub>2</sub> ) 8 h/d for 6 weeks	Prevention of IH-induced endothelial dysfunction and atherosclerosis (Tuleta et al., 2014)
<b>melatonin</b>	antioxidant	10 mg/kg/d, IP injection	28-day-old male Sprague-Dawley rats on SCD	1 cycle/min (5-21% FiO <sub>2</sub> ) 8 h/d for 14 or 21 days	Prevention of IH-induced systolic BP increase and endothelial dysfunction (Hung et al., 2013)
<b>melatonin</b>	antioxidant	10 mg/kg/d, IP injection	28-day-old male Sprague-Dawley rats on SCD	1 cycle/min (5-21% FiO <sub>2</sub> ) 8 h/d for 28 days	Prevention of IH-induced systolic BP (Yeung et al., 2015)
<b>tempol</b>	antioxidant	1 mmol/L in drink water	male Sprague-Dawley rats ‡	20 cycles/h (5-21% FiO <sub>2</sub> ) 7 h/d for 14 days	Prevention of IH-induced BP increase (Troncoso Brindeiro et al., 2007)
<b>atorvastatin</b>	HMGCoA reductase inhibitor	10 mg/kg/d, IP injection from day 0 to day 14 (protocol 1) or from day 14 to day 21 (protocol 2)	male Wistar rats on SCD	1 cycle/min (5-21% FiO <sub>2</sub> ) 8 h/d for 14 days (protocol 1) or 21 days (protocol 2)	Prevention of IH-induced systolic BP increase (protocol 1) Correction of IH-induced endothelial dysfunction and IMT increase (protocol 2) (Totoson et al., 2013)
<b>ezetimibe</b>	NPC1L1 protein inhibitor	5 mg/kg/d in food	8-week-old male ApoE <sup>-/-</sup> mice on HFD	1 cycle/min (5-21% FiO <sub>2</sub> ) 8 h/d for 10 days	Prevention of IH-induced atherosclerosis (Kato et al., 2015)
<b>Anti-angptl4</b>	anti-angptl4 monoclonal antibody	30 mg/kg/week, IP injection	ApoE <sup>-/-</sup> mice ‡	1 cycle/min (6.5-21% FiO <sub>2</sub> ) 12 h/d for 4 weeks	Prevention of IH-induced atherosclerosis (Drager et al., 2013)

Abbreviations: angptl4, angiotensin-like 4; ApoE<sup>-/-</sup>, apolipoprotein E knock-out; COX, cyclooxygenase; SCD, standard-chow diet; HFD, high-fat-diet; FiO<sub>2</sub>, fraction of inspired oxygen; IP, intraperitoneal; RANTES, regulated upon activation normal T-cell expressed and secreted.

\*Because studies investigating the efficacy of anti-hypertensive drugs on blood pressure in OSA were recently reviewed (Diogo & Monteiro, 2014), this table does not include the works discussed in that review, except those which study vascular outcomes different from blood pressure or which were published after its publication

† rats received pharmacological treatment or vehicle starting 1 week before and continuing through period of intermittent hypoxia or normoxia exposure.

‡ diet was not detailed in this study.

Table 3. Ongoing studies assessing the effects of several drugs on intermittent hypoxia-related vascular alterations

Number of clinical trial	intervention	Study design	Vascular outcome*	Study population	Estimated completion date	Sponsor
<b>NCT01637623</b>	losartan vs allopurinol vs placebo	Double-blind parallel-design phase 2 RCT	PWV, BP, cerebral and forearm blood flow, AI, FMD	Severe OSA patients with hypertension	February 2016	University of Wisconsin, Madison National Heart, Lung, and Blood Institute
<b>NCT02484300</b>	melatonin vs placebo	Double-blind parallel-design RCT	BP	Male severe OSA patients	July 2017	University of California, San Diego National Institutes of Health
<b>NCT01771406</b>	nebivolol vs CPAP	Crossover RCT	<b>BP</b> , FMD	OSA patients with hypertension	February 2016	Universitaire Ziekenhuizen Leuven
<b>NCT02408172</b>	amlodipine vs metoprolol	Single blind parallel design phase 2/3 RCT †	<b>BP</b>	OSA patient with hypertension	June 2017	First Affiliated Hospital of Harbin Medical University

Abbreviations: RCT, randomized controlled study; BP, blood pressure; FMD, flux-mediated dilatation; AI, augmentation index; PWV, pulse wave velocity.

\*bold characters indicated that the vascular outcome is the primary outcome of the study.

†phase 2 for amlodipine and phase 3 for metoprolol.

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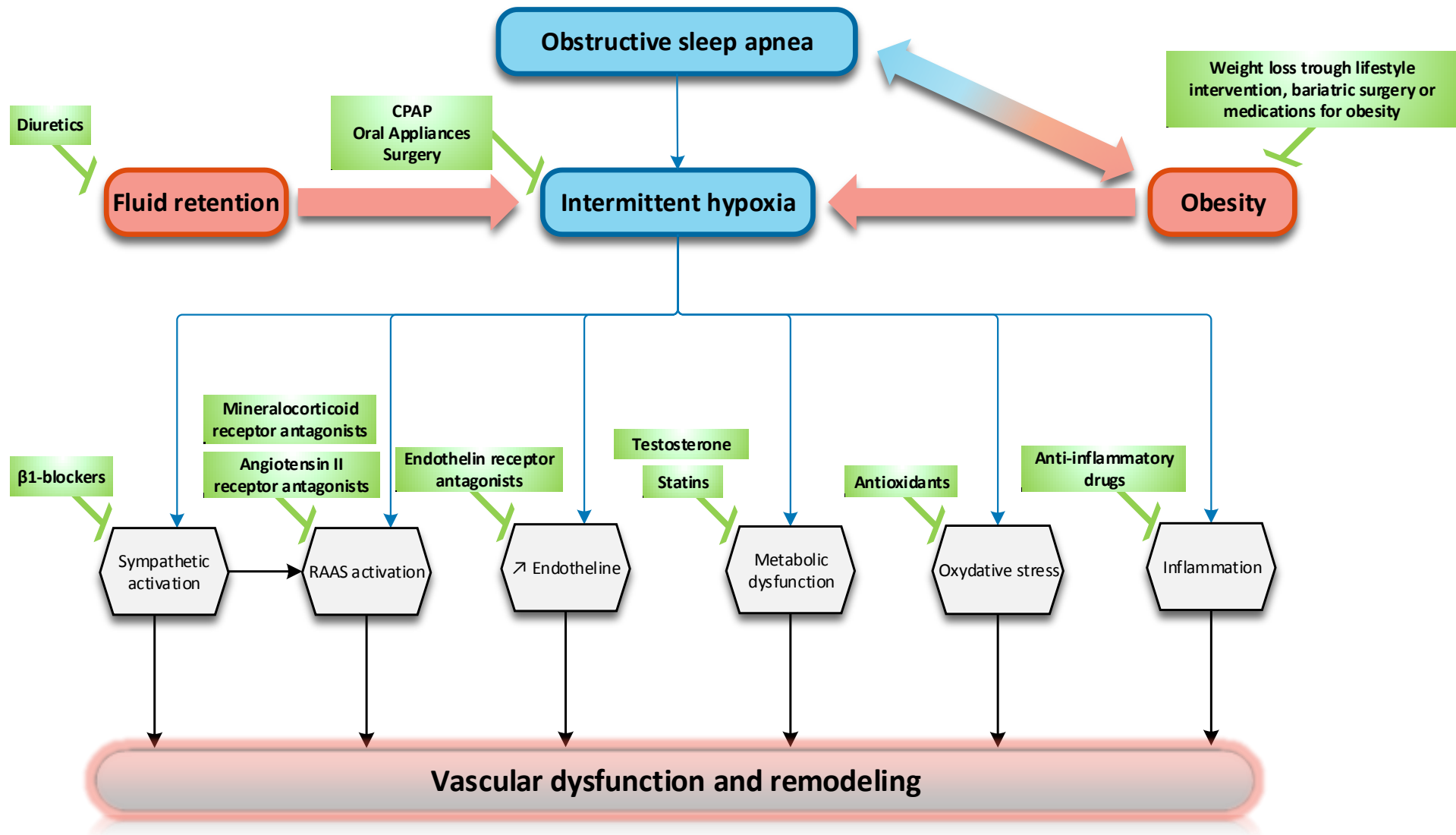



Figure 1

 Therapeutic interventions evaluated to treat OSA-related vascular damages

## **Deuxième partie : travaux de thèse**



## **1. Présentation de la thèse**

Les complications cardiovasculaires du SAOS ne sont que partiellement prévenues par le traitement de référence par PPC. La faible observance des patients pour ce traitement explique en partie son manque d'efficacité, mais d'autres facteurs tels que la présence d'autres comorbidités cardiovasculaires semble également influencer la réponse au traitement.

En parallèle, les connaissances physiopathologiques des altérations vasculaires induites par le SAOS se sont considérablement étoffées ces dernières années. Des travaux précédemment menés au laboratoire ont mis en évidence des perturbations du métabolisme de l'acide arachidonique au cours du SAOS (46,78-80) et ont notamment décrit un lien entre augmentation de la production de LTB<sub>4</sub> ou d'isoprostanes et remodelage vasculaire.. Les eicosanoïdes représentant une vaste famille de composés, bon nombre d'entre eux avaient jusqu'alors été peu étudiés au cours du SAOS, c'est le cas notamment du TXA<sub>2</sub> et des CysLTs.

L'objectif premier de ce travail a donc été de **caractériser les voies métaboliques du thromboxane A<sub>2</sub> et des cystéinyl-leucotriènes au cours du SAOS et d'évaluer leur implication dans l'athérosclérose associée au SAOS.**

Ces eicosanoïdes sont aujourd'hui clairement identifiés comme des déterminants du processus athéromateux en dehors du SAOS et sont actuellement évalués comme cibles thérapeutiques pour lutter contre l'athérosclérose (75,204,205). Certaines stratégies pharmacologiques inhibant spécifiquement un eicosanoïde ont déjà abouti à une amélioration de la dysfonction endothéliale (206,207) ou une réduction du volume des plaques d'athérome (208) notamment chez le patient en prévention secondaire. En revanche, l'intérêt de telles interventions pharmacologiques pour lutter contre les complications cardiovasculaires du SAOS et notamment l'athérosclérose reste à ce jour inconnu.

Ainsi, le second objectif a été d'évaluer **les voies métaboliques du thromboxane A<sub>2</sub> et des cystéinyl-leucotriènes en tant que cibles thérapeutiques pour lutter contre l'athérosclérose associée au SAOS.**

La finalité de ce travail est dans le futur de proposer des alternatives thérapeutiques pharmacologiques au patient apnéique en impasse thérapeutique pour lequel le risque cardiovasculaire est augmenté.

## **2. Matériel et Méthodes**

### *2.1. Etudes expérimentales*

Les études animales ont été menées en accord avec la Convention Européenne pour la protection des animaux vertébrés utilisés à des fins expérimentales ou scientifiques (Conseil de l'Europe, Traité européen ETS 120 du 18 mars 1996, Strasbourg) et le guide pour l'utilisation et le soin des animaux de laboratoire (NIH Publication no. 85-23, revised 1996).

L'athérosclérose étant la complication vasculaire induite par le SAOS que nous souhaitons étudier, les études expérimentales ont été menées sur un modèle animal classique d'athérosclérose, à savoir la souris déficiente pour l'apolipoprotéine E (ApoE<sup>-/-</sup>). Ces souris ont été soumises à l'HI ou la normoxie au sein du dispositif précédemment décrit (4). Un système d'électrovannes distribue de l'air et de l'azote à l'intérieur des cages de manière à faire varier la fraction partielle en O<sub>2</sub> (FiO<sub>2</sub>) au sein des cages. Les animaux exposés à l'HI étaient soumis à un cycle d'hypoxie par minute : exposition à un mélange d'air et d'azote pendant 30 secondes permettant d'atteindre une FiO<sub>2</sub> à 5%, puis à 30 secondes d'air pour rétablir une FiO<sub>2</sub> de 21%. Ces cycles étaient répétés 8 heures par jour à heures fixes durant toute la durée de l'exposition et ceci au cours de la journée (période où les rongeurs dorment).

Les souris normoxiques étaient quant à elles soumises au même flux de gaz, de manière à reproduire exactement les mêmes conditions d'hébergement, sans que la composition de l'air ne varie au cours du temps.

## *2.2. Etudes cliniques*

Les études cliniques ont été menées chez des sujets (patients et contrôles) après recueil de leur consentement libre et éclairé. Ces sujets avaient été inclus dans différents protocoles de recherche clinique qui avaient obtenu au préalable l'avis favorable du Comité de Protection des Personnes.

L'ensemble des sujets ont bénéficié d'un enregistrement polysomnographique au laboratoire du sommeil du Centre Hospitalier Universitaire Grenoble Alpes. Le diagnostic de SAOS était retenu selon les critères précédemment détaillés (1). Le matin suivant l'étude du sommeil, des prélèvements sanguins et urinaires étaient réalisés pour détermination des différents paramètres biologiques.

Selon les protocoles dans lesquels les sujets étaient inclus, ces derniers bénéficiaient d'une échographie carotidienne (selon les modalités décrites précédemment (28)) au cours de laquelle l'EIM des carotides droites et gauches était mesurée et la présence de plaque d'athérome décelée.

Les voies métaboliques du TXA<sub>2</sub> et des CysLTs étaient évaluées respectivement par la quantification au niveau urinaire du 11-dehydrothromboxane B<sub>2</sub> (11dTXB<sub>2</sub>) et du LTE<sub>4</sub>. Ces différents dosages ont été réalisés par chromatographie liquide couplée à la spectrométrie de masse en tandem selon des techniques précédemment décrites (78).

## **3. Résultats et publications**

### *3.1. Voie du thromboxane A<sub>2</sub>*

L'implication du TXA<sub>2</sub> dans l'athérogénèse n'étant plus à démontrer (75), de nombreuses approches pharmacologiques ciblant spécifiquement ce prostanoïde ont déjà été proposées pour lutter contre les maladies cardiovasculaires (75,204). En revanche, dans le contexte du SAOS, les prostanoïdes et plus précisément le TXA<sub>2</sub> ont été relativement peu étudiés (76,77); alors même

que des altérations d'autres métabolites de l'acide arachidonique ont déjà été décrites (46,78,79).

L'objectif de cette première étude originale a été d'étudier la voie du TXA<sub>2</sub> au cours du SAOS et de préciser son rôle dans l'athérogénèse associée au SAOS.

La voie du TXA<sub>2</sub> a été évaluée chez des souris ApoE<sup>-/-</sup> exposées à l'HI pendant 8 semaines et une intervention pharmacologique ciblant spécifiquement le TXA<sub>2</sub> a permis d'apprécier l'implication de cette voie métabolique dans le processus athéromateux lié à l'HI. En parallèle, la voie du TXA<sub>2</sub> et son lien avec le remodelage vasculaire associé au SAOS ont également été évalués chez des patients apnéiques présentant ou non d'autres facteurs de risque cardiovasculaire.

Publication n°3

**“Intermittent hypoxia activated cyclooxygenase pathway: role  
in atherosclerosis”**

E Gautier-Veyret, C Arnaud, M Bäck, JL Pépin, M H. Petri, JP Baguet, R  
Tamisier, P Lévy, F Stanke-Labesque.

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## Intermittent hypoxia-activated cyclooxygenase pathway: role in atherosclerosis

Elodie Gautier-Veyret<sup>1,2</sup>, Claire Arnaud<sup>1</sup>, Magnus Bäck<sup>3</sup>, Jean-Louis Pépin<sup>1,4,5</sup>, Marcelo H. Petri<sup>3</sup>, Jean-Philippe Baguet<sup>4,6</sup>, Renaud Tamisier<sup>1,4,5</sup>, Patrick Lévy<sup>1,4,5</sup> and Françoise Stanke-Labesque<sup>1,2,4</sup>

**Affiliations:** <sup>1</sup>INSERM, U1042, Grenoble, <sup>2</sup>CHU, Hôpital A. Michallon, Laboratoire de Pharmacologie, BP217, Grenoble, <sup>4</sup>Université Grenoble 1, Faculté de Médecine, UFR1, Grenoble, <sup>5</sup>CHU, Hôpital A. Michallon, Pôle rééducation et Physiologie, BP217, Grenoble, <sup>6</sup>CHU, Hôpital A. Michallon, Clinique de Cardiologie, BP217, Grenoble, France, <sup>3</sup>Dept of Medicine, Karolinska Institute and University Hospital, CMM L8, 03, Stockholm, Sweden.

**Correspondence:** F. Stanke-Labesque, Laboratory of Pharmacology, Grenoble University Hospital, BP 217, F-38043 Grenoble Cedex 9, France. E-mail: FStanke@chu-grenoble.fr

**ABSTRACT** Intermittent hypoxia, the main stimulus of obstructive sleep apnoea (OSA), induces inflammation, leading to early atherosclerosis. Whether the cyclooxygenase (COX) pathway contributes to intermittent hypoxia-induced atherosclerosis remains to be determined.

We studied the effects of 8-weeks of intermittent hypoxia exposure on COX-pathway gene expression and atherosclerosis, and the influence of COX-1 inhibition by SC-560 on atherosclerosis progression in aortas of apolipoprotein E<sup>-/-</sup> mice. Urinary 11-dehydrothromboxane B<sub>2</sub> (11-dTXB<sub>2</sub>) was assessed in 50 OSA subjects free of cardiovascular risk factor matched for age and body mass index with 25 controls, and 56 OSA with cardiovascular risk factor.

Intermittent hypoxia significantly increased atherosclerotic lesion sizes, mRNA levels of COX-1 and thromboxane synthase (TXBS). Lesion sizes correlated to COX-1 ( $r=0.654$ ,  $p=0.0003$ ) and TXBS ( $r=0.693$ ,  $p<0.0001$ ) mRNA levels. COX-1 inhibition reduced lesion progression in intermittent hypoxia mice only ( $p=0.04$ ). Urinary 11-dTXB<sub>2</sub> was similar in OSA subjects free of cardiovascular risk factor and controls, but was increased by 13% ( $p=0.007$ ) in OSA subjects with cardiovascular risk factor compared with those without.

Although OSA itself was not associated with increased urinary 11-dTXB<sub>2</sub> concentration, the COX-1 pathway was activated in intermittent hypoxia-exposed mice and in OSA subjects presenting with cardiovascular risk factor, and may contribute to intermittent hypoxia-induced atherogenesis. COX-1 inhibition could be of clinical interest in the prevention of cardiovascular morbidity in OSA.



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COX-1 inhibition could be of clinical interest in the prevention of cardiovascular morbidity in obstructive sleep apnoea <http://ow.ly/1UIb5>

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## Introduction

Obstructive sleep apnoea (OSA) is characterised by recurrent episodes of partial or complete upper airway obstruction occurring during sleep, leading to chronic intermittent hypoxia (CIH). This hallmark is the main factor involved in cardiovascular remodelling in OSA [1]. In OSA patients, early signs of atherosclerosis correlated to hypoxia severity [2], even after adjustment for confounding factors. OSA is associated with increased cardiovascular morbidity and mortality, and is identified as an independent cardiovascular risk factor (CVRF) [3]. Moreover, exposure of apolipoprotein E-deficient (ApoE<sup>-/-</sup>) mice [4, 5] and C57BL/6J mice [6] to CIH accelerated atherogenesis progression.

Atherosclerosis is a chronic inflammatory disease. Among the many inflammatory mediators involved in atherogenesis, we previously demonstrated the contribution of arachidonic acid-derived metabolites in OSA patients. Notably, OSA patients exhibited elevated levels of leukotriene B<sub>4</sub> [7, 8] and cysteinyl-leukotrienes [9] that were associated with vascular remodelling. However, the cyclooxygenase (COX)-dependent pathway of arachidonic acid metabolism was poorly studied in OSA patients, and its implication in CIH-induced atherogenesis is unknown.

Thromboxane A<sub>2</sub> (TXA<sub>2</sub>) and prostacyclin (PGI<sub>2</sub>) are two COX-derived metabolites of arachidonic acid metabolism. TXA<sub>2</sub> is predominantly generated by platelets through COX type 1 isoform (COX-1) and thromboxane synthase (TXBS). TXA<sub>2</sub> is quickly metabolised to thromboxane B<sub>2</sub> (TXB<sub>2</sub>) and 11-dehydrothromboxane B<sub>2</sub> (11-dTXB<sub>2</sub>), two metabolites respectively quantifiable in plasma and urine. TXA<sub>2</sub> binding on its TP receptors induces platelet activation, vasoconstriction, vascular smooth muscle cell proliferation and increases expression of adhesion molecules [10]. The production of PGI<sub>2</sub> mainly depends on endothelial COX-2 and prostacyclin synthase (PGIS). PGI<sub>2</sub> is rapidly metabolised to 6-keto-prostaglandin F<sub>1</sub>α (6-keto-PGF<sub>1</sub>α in plasma and 2,3-dinor-6-ketoprostaglandin F<sub>1</sub>α (PGI-M) excreted in urine. In contrast to TXA<sub>2</sub>, PGI<sub>2</sub> binding on its IP receptors inhibits platelet aggregation and vasoconstriction, and reduces chemotaxis and expression of adhesion molecules [10]. Thus, TXA<sub>2</sub> and PGI<sub>2</sub> have antagonist properties.

A recent growing body of evidence suggests a major role of the COX pathway in the pathogenesis and progression of atherosclerosis. In fact, pharmacological inhibition of COX pathway [11, 12] or genetic deletion [13, 14] delayed atherosclerosis in different mouse models of atherosclerosis. Furthermore, the urinary 11-dTXB<sub>2</sub>/PGI<sub>2</sub>-M ratio is enhanced in elderly subjects with past history of serious cardiovascular event (stroke and myocardial infarction) [15]. As selective COX-2 inhibitors may have nonfavourable effects on this ratio and increase cardiovascular risk [16], the role of COX-1 inhibition has received less attention. In addition, data in OSA are very limited and conflicting, showing either an increased [17] or a decreased [18] urinary ratio 11-dTXB<sub>2</sub>/PGI<sub>2</sub>-M. Thus, the aim of the present study was to characterise the COX pathway in ApoE<sup>-/-</sup> mice exposed to CIH and in OSA patients, and to investigate its link with CIH-induced atherogenesis and OSA-associated early vascular remodelling.

## Materials and methods

### Experimental animal study

Male ApoE<sup>-/-</sup> mice (14 weeks old) were purchased from the Charles River Laboratories (L'Arbresle, France). All animal procedures 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, March 18, 1986) and to the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised 1996).

In a first series of experiments, mice were randomised to 8 weeks of CIH (cyclic 21–25% inspiratory oxygen fraction (F<sub>I</sub>O<sub>2</sub>), 60-s cycle for 8 h·day<sup>-1</sup>) or normoxic air as previously described (n=15 in each group) [1]. In a second series of experiments, mice were exposed to 8 weeks of CIH or normoxic air (n=20 in each group), and randomised to receive either placebo or the selective COX-1 inhibitor SC-560 (Interchim, Montluçon, France) (15 mg·kg<sup>-1</sup> daily [12]) in their food, for the last 4 weeks of exposure. All mice were fed with a normal diet, *ad libitum*, during all experiments.

At the end of exposure, blood was collected under anaesthesia (ketamine/xylazine 100 mg·kg<sup>-1</sup>/10 mg·kg<sup>-1</sup> by intraperitoneal injection) for haematocrit and lipid measurements. Entire aortas were harvested. Abdominal aortas were placed in RNAlater (Life Technologies, Villebon-sur-Yvette, France), frozen in liquid nitrogen and stored at -80°C until analysis. Immediately after their sampling, thoracic aortas were placed in Tyrode solution (137 mM NaCl, 2.7 mM KCl, 0.41 mM NaHPO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 5 μM MgCl<sub>2</sub>, 11.9 mM NaHCO<sub>3</sub> and 5.5 mM glucose) for prostanoid secretion measurements.

### Atherosclerotic lesion size quantification

Atherosclerotic lesions of aortic roots were studied by Oil-Red-O (Sigma Aldrich, Saint Quentin-Fallavier, France) staining, as previously described [5]. For each aorta, lipid deposition was quantified from five

sections (8- $\mu\text{m}$  thickness), separated by 80  $\mu\text{m}$  from each other, using computer image analysis (NisElement; Nikon Instruments Inc., Melville, NY, USA).

#### *Aortic secretion of prostanoids*

Thoracic aortas were incubated for 15 min in Tyrode solution maintained at 37°C, aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, in presence of calcium ionophore A23187 (Sigma Aldrich, Saint Quentin Fallavier, France) at 10<sup>-6</sup> M. Supernatants were immediately frozen at -80°C for prostanoid measurement, while aortas were dried for measurement of dry tissue weight.

#### *COX-pathway gene expression*

Total mRNA was isolated from aorta using the RNeasy kit (Qiagen, Hilden, Germany) as previously described [19] and reverse-transcribed using Superscript II (Invitrogen, Carlsbad, CA, USA) with random hexamers according to the manufacturer's instructions. Quantitative TaqMan PCR was performed on a 7900 HT using primer/probe pairs designed with Assay-On-Demand (both Applied Biosystems; Life Technologies SAS, Saint Aubin, France) (online supplementary table S1). Data were normalised to 18S ribosomal protein mRNA and expressed as 2<sup>- $\Delta\text{CT}$</sup> .

#### *Clinical study*

The local ethics committee approved this study according to the Declaration of Helsinki. All participants gave written informed consent.

113 newly diagnosed OSA patients were consecutively entered into the study between January 2007 and April 2011, as well as 25 controls. Patients were referred to the sleep laboratory of Grenoble University Hospital for symptoms suggesting OSA. Exclusion criteria were past history of stroke or myocardial infarction, known hypertension, and treatment with nonsteroidal anti-inflammatories, aspirin, steroids, antidiabetic, antihypertensive and lipid-lowering drugs.

#### *Design*

The specific contribution of CIH on 11-dTXB<sub>2</sub> urinary concentrations was assessed in 25 controls carefully matched for age and body mass index (BMI) with 50 nonobese OSA patients (*i.e.* one healthy subject for two OSA subjects). All subjects were free of any known CVRFs. A sample size of 50 OSA patients and 25 controls was calculated as adequate to show an increase of 30% in urinary 11-dTXB<sub>2</sub> excretion with at least 90% power and at the 5% significance level. This sample size calculation was based on preliminary data obtained on healthy subjects showing a mean concentration of 11-dTXB<sub>2</sub> of 647 pg·mL<sup>-1</sup> with a standard deviation of 287 pg·mL<sup>-1</sup>. As the dispersion of 11-dTXB<sub>2</sub> was higher in patients with cardiovascular disease than in controls, we chose a mixed model including one healthy for every two OSA subjects.

The influence of CVRFs (which comprise obesity (BMI >30 kg·m<sup>-2</sup>); hypertension (clinical diastolic blood pressure (BP) >90 mmHg and systolic BP >140 mmHg); dyslipidemia (low-density lipoprotein (LDL) cholesterol >4.13 mmol·l<sup>-1</sup> or association of total cholesterol >5.16 mmol·l<sup>-1</sup> and high-density lipoprotein (HDL) cholesterol <1.03 mmol·l<sup>-1</sup>); smoking; and metabolic syndrome (defined by criteria of International Diabetes Federation [20])) on urinary excretion of 11-dTXB<sub>2</sub> was evaluated in the whole cohort of 113 OSA subjects. Patients were stratified on the presence or not of one of these CVRFs.

The influence of continuous positive airway pressure (CPAP) on urinary 11-dTXB<sub>2</sub> concentration was studied in 14 OSA subjects free of any CVRF and 21 OSA patients with CVRF(s) who were adherent to CPAP. CPAP adherence was defined as CPAP daily use for >4 h [9].

All subjects underwent an overnight polysomnography as described [9]. Sleep apnoea was defined as an apnoea/hypopnoea index (AHI)  $\geq 5$  events·h<sup>-1</sup> of sleep and symptoms or respiratory disturbance index (RDI), including flow limitation episodes >15 events·h<sup>-1</sup> [21].

Urine sample for 11-dTXB<sub>2</sub> quantification and venous blood for biochemical measurements were collected at 07:00 h at the end of the nocturnal polysomnographic recordings and were stored at -80°C until later analysis.

Carotid ultrasonography was performed in 97 OSA patients and 24 controls as previously described [2].

#### *Biological measurements*

Plasma cholesterol, triglycerides, glucose and high-sensitivity C reactive protein (hsCRP) concentrations were determined using enzymatic colorimetric methods on a Dimension Vista analyser (Siemens AG, Erlangen, Germany). LDL cholesterol was calculated using the Friedewald formula (total cholesterol-HDL



cholesterol-(triglycerides/5)). Urinary 11-dTXB<sub>2</sub> of patients, TXB<sub>2</sub> and 6-keto-PGF1 $\alpha$  in supernatants of mice were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS) as previously described [9, 22]. The limits of quantitation of TXB<sub>2</sub> and 6-keto-PGF1 $\alpha$  were 14 pg·mL<sup>-1</sup> and 7.25 pg·mL<sup>-1</sup>, respectively, and their inter- and intra-assay coefficients of variation were <12% for both analytes.

#### Statistical analysis

Statistical analyses were performed using NCSS97 (Kaysville, Utah) or SAS (SAS 9.1, Cary, NC, USA) for the mixed model. Data were expressed as median and 10th and 90th percentiles. Normal distribution was tested using Kolmogorov–Smirnov nonparametric test. Comparisons between normoxic air and CIH mice were made using the t-test. Comparisons between OSA patients and controls matched for age and BMI (one control for two OSA subjects) were made using a mixed model. Noncontinuous variables were compared using Fisher's test. Comparisons between more than three groups were made using the Kruskal–Wallis test and subsequent pairwise comparisons were made with the Bonferroni multiple-comparison test. For patients treated by CPAP, differences between baseline and post-CPAP values were analysed using a paired t-test or the Wilcoxon signed-rank test. Relationships between two variables were studied with single regression. A multiple linear regression analysis was performed, taking into account the variables that correlated with dependant variable urinary 11-dTXB<sub>2</sub> in humans. A p-value of <0.05 was considered significant.

## Results

### Experimental animal study

#### Weight, lipid levels and haematocrit

After 8-weeks of exposure, bodyweight and plasma cholesterol levels were similar in CIH and normoxic air mice (table 1). Haematocrit was significantly higher in CIH mice compared with normoxic air mice (table 1).

#### Atherosclerotic lesion sizes

Atherosclerotic lesions on aortic roots were higher (p=0.008) in CIH mice compared with normoxic mice (CIH 66 532 (32 741–163 224) *versus* normoxic air 37 675 (6798–75 596)  $\mu$ m<sup>2</sup>; p=0.03). Lesion sizes correlated with plasma total cholesterol in normoxic air mice (r=0.501, p=0.04) but not in CIH mice.

#### COX-pathway gene expression

COX-1 and TXBS aortic mRNA levels were significantly increased in CIH group compared with normoxic air, whereas COX-2, PGIS, TP and IP receptors aortic mRNA levels were not significantly different between both groups (fig. 1a). mRNA levels of COX-1 and TXBS significantly correlated with lesion sizes (fig. 1b).

#### Aortic prostanoid secretion

A23187-stimulated 6-keto-PGF1 $\alpha$  and TXB<sub>2</sub> secretions and 6-keto-PGF1 $\alpha$ /TXB<sub>2</sub> ratio were similar in aorta from CIH and normoxic air mice (table 2). 6-keto-PGF1 $\alpha$  and TXB<sub>2</sub> concentrations were highly correlated (r=0.842, p<0.0001).

#### Effects of COX-1 inhibition

In both normoxic air and CIH groups, treatment with SC-560 significantly decreased TXB<sub>2</sub> and 6-keto-PGF1 $\alpha$  aortic secretion *versus* placebo (table 2). There was no difference between groups for total cholesterol and bodyweight (table 2). Treatment with SC-560 significantly reduced lesion size by 35% in CIH mice whereas it had no effect in normoxic air mice (fig. 2).

TABLE 1 Bodyweight, plasma cholesterol level and haematocrit of apolipoprotein E-deficient [ApoE<sup>-/-</sup>] mice exposed for 8 weeks to chronic intermittent hypoxia (CIH) or normoxia

	Normoxia	CIH	p-value
Subjects n	15	15	
Bodyweight after exposure g	31.7 [28.5–33.9]	30.3 [27.8–32.6]	0.08
Total cholesterol mmol·L <sup>-1</sup>	12.2 [5.24–18.7]	14.6 [7.10–19.1]	0.46
Haematocrit %	45.0 [41.4–47.0]	47.0 [44.2–55.0]	<b>0.006</b>

Data are presented as median [10th–90th percentiles].

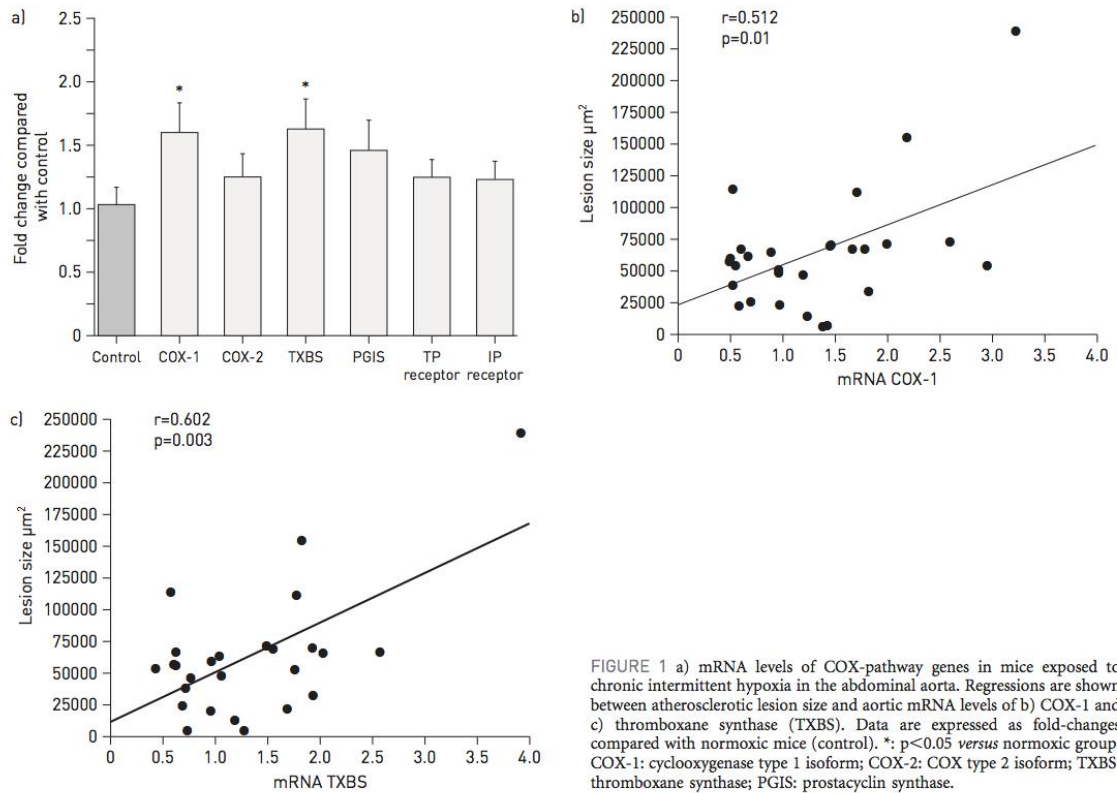


FIGURE 1 a) mRNA levels of COX-pathway genes in mice exposed to chronic intermittent hypoxia in the abdominal aorta. Regressions are shown between atherosclerotic lesion size and aortic mRNA levels of b) COX-1 and c) thromboxane synthase (TXBS). Data are expressed as fold-changes compared with normoxic mice (control). \*:  $p < 0.05$  versus normoxic group. COX-1: cyclooxygenase type 1 isoform; COX-2: COX type 2 isoform; TXBS: thromboxane synthase; PGIS: prostacyclin synthase.

TABLE 2 Bodyweight, plasma cholesterol level, haematocrit and aortic prostanoid secretion of apolipoprotein E-deficient (ApoE<sup>-/-</sup>) mice exposed for 8 weeks to chronic intermittent hypoxia (CIH) or normoxia, with treatment by SC-560 or placebo during the last 4 weeks of exposure

	Normoxia		CIH	
	Placebo	SC-560	Placebo	SC-560
Subjects n	10	9	10	10
Bodyweight after exposure g	28.8 (25.8–31.0)	28.9 (27.1–32.6)	28.9 (25.9–31.5)	31.2 (28.4–32.0)
Total cholesterol mmol·L <sup>-1</sup>	14.2 (9.13–20.2)	11.1 (8.44–17.6)	15.3 (12.8–21.0)	12.3 (8.46–16.1)
Haematocrit %	44.5 (42.5–45.0)	45.0 (41.8–47.0)	46.0 (45.0–47.6) <sup>#</sup>	45.5 (43.5–48.5)
Aortic secretion of TXB <sub>2</sub> ng·L <sup>-1</sup> ·mg <sup>-1</sup>	55.2 (35.3–94.7)	3.13 (1.80–22.6) <sup>†</sup>	56.7 (44.0–80.8)	7.86 (2.65–11.86) <sup>†</sup>
Aortic secretion of 6-keto-PGF1α ng·L <sup>-1</sup> ·mg <sup>-1</sup>	778.3 (559.7–1059.1)	91.8 (45.9–285.7) <sup>†</sup>	802.1 (638.3–1152.8)	107.9 (68.1–198.6) <sup>†</sup>
Aortic 6-keto-PGF1α/TXB <sub>2</sub> ratio	13.4 (9.9–21.6)	24.1 (9.3–40.8)	14.0 (11.6–18.1)	17.0 (10.9–26.7)

Data are presented as median [10th–90th percentiles]. TXB<sub>2</sub>: thromboxane; 6-keto-PGF1α: 6-keto-prostaglandin F1α. <sup>#</sup>:  $p < 0.05$  normoxic mice treated by placebo versus hypoxic mice treated by placebo; <sup>†</sup>:  $p < 0.05$  normoxic mice treated by placebo versus normoxic mice treated by SC-560; <sup>‡</sup>:  $p < 0.05$  hypoxic mice treated by placebo versus hypoxic mice treated by SC-560.

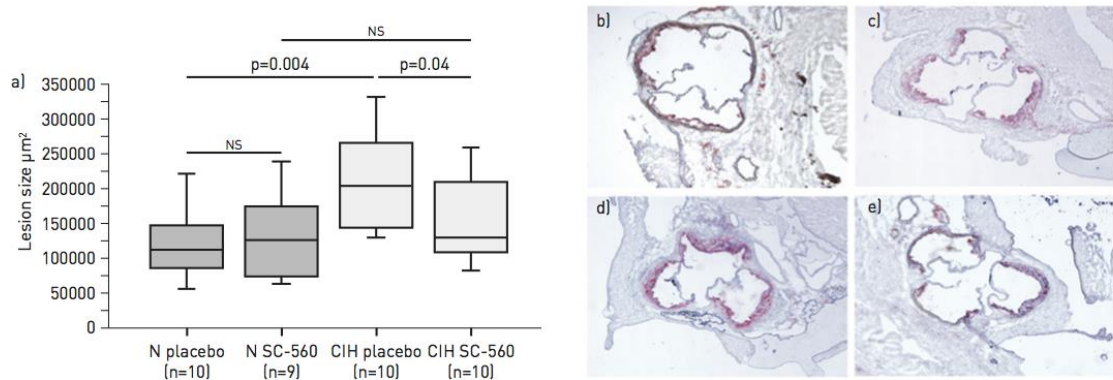


FIGURE 2 Effects of cyclooxygenase type 1 isoform (COX-1) inhibition on atherosclerosis in mice exposed to chronic intermittent hypoxia (CIH) or normoxia (N). a) Lesion sizes. Data are presented as interquartile range (boxes), data range (whiskers) and median (horizontal line). NS: nonsignificant. Representative photographs of Oil-Red-O staining for b) normoxia placebo, c) normoxia SC-560, d) CIH placebo and e) CIH SC-560.

TABLE 3 Baseline characteristics of controls and obstructive sleep apnoea (OSA) patients free of any known cardiovascular risk factor, matched for age and body mass index (BMI)

	Controls	OSA patients	p-value
Subjects n	25	50	
Males %	76.0	88.0	0.20
BMI kg·m <sup>-2</sup>	25.0 (21.6–27.3)	24.9 (21.8–27.9)	0.83
Age years	51.2 (42.7–62.6)	51.1 (43.8–60.9)	0.99
Clinical systolic BP mmHg	127 (112–148)	125 (110–146)	0.72
Clinical diastolic BP mmHg	85 (76–97)	80 (69–93)	0.29
AHI events·h <sup>-1</sup>	5.0 (0.0–9.2)	35.4 (14.9–59.4)	<0.0001
RDI events·h <sup>-1</sup>	6.6 (1.0–15.7)	35.9 (24.1–71.6)	<0.0001
Minimal SaO <sub>2</sub> %	90.0 (85.4–92.5)	84.5 (75.3–90.0)	<0.0001
SaO <sub>2</sub> <90% % TST	0.00 (0.00–1.00)	1.00 (0.00–13.2)	<0.0001
RAI events·h <sup>-1</sup>	4.9 (1.9–11.0)	28.0 (12.0–49.1)	<0.0001
Plasma glucose mmol·L <sup>-1</sup>	4.7 (4.3–5.3)	5.1 (4.5–6.1)	0.03
Plasma insulin µU·mL <sup>-1</sup>	4.2 (2.2–7.11)	5.48 (2.85–8.57)	0.11
HOMA-IR index	0.86 (0.50–1.68)	1.21 (0.63–2.22)	0.13
Total cholesterol mmol·L <sup>-1</sup>	4.77 (3.41–6.17)	5.68 (4.41–6.62)	0.004
LDL cholesterol mmol·L <sup>-1</sup>	2.76 (1.57–3.92)	3.55 (2.13–4.36)	0.003
HDL cholesterol mmol·L <sup>-1</sup>	1.52 (0.98–2.40)	1.52 (1.10–2.13)	0.94
Triglycerides mmol·L <sup>-1</sup>	0.89 (0.52–1.90)	1.11 (0.68–1.84)	0.07
hsCRP mg·L <sup>-1</sup>	1.00 (0.38–1.74)	0.95 (0.30–3.30)	0.61
Right carotid IMT mm	593 (515–653)	604 (508–805)	0.44
Left carotid IMT mm	622 (527–780)	647 (515–872)	0.32
Mean carotid IMT mm	606 (523–710)	620 (539–838)	0.52
Overweight/normal BMI %	48/52	46/56	0.87
Smoking %	0	0	1
Hypertension %	4	6	1
Metabolic syndrome %	0	0	1
Dyslipidemia %	0	20	0.03
Urinary 11-dTXB <sub>2</sub> pg·mg <sup>-1</sup> creatinine	609.0 (265.8–945.3)	629.3 (266.4–933.1)	0.56

Data are presented as median (10th–90th percentiles), unless otherwise stated. BP: blood pressure; AHI: apnoea/hypopnoea index; RDI: respiratory disturbance index; SaO<sub>2</sub>: arterial oxygen saturation; TST: total sleep time; RAI: respiratory arousal index; HOMA-IR: homeostasis model assessment of insulin resistance; LDL: low-density lipoprotein; HDL: high-density lipoprotein; hsCRP: high-sensitivity C reactive protein; IMT: intima-media thickness; 11-dTXB<sub>2</sub>: 11-dehydrothromboxane. Bold indicates statistical significance.

TABLE 4 Baseline characteristics of obstructive sleep apnoea (OSA) patients without (CVRF-) or with (CVRF+) associated cardiovascular risk factors

	OSA		p-value
	CVRF-	CVRF+	
Subjects n	57	56	
Males %	84.5	84.0	1
BMI kg·m <sup>-2</sup>	25.6 [21.5–28.4]	28.2 [23.0–34.6]	<0.0001
Age years	51.3 [44.5–65.3]	53.1 [39.9–66.6]	0.72
Clinical systolic BP mmHg	124 [110–138]	132 [118–160]	0.0002
Clinical diastolic BP mmHg	80 [65–90]	88 [72–108]	<0.0001
AHI events·h <sup>-1</sup>	30.0 [17.0–50.0]	40.2 [19.4–67.0]	0.0004
RDI events·h <sup>-1</sup>	35.8 [24.3–64.3]	51.0 [27.7–80.3]	0.0002
Minimal SaO <sub>2</sub> %	85.0 [76.0–90.0]	82.0 [74.9–88.2]	0.04
Mean SaO <sub>2</sub> %	94.0 [92.0–96.0]	93.0 [90.0–95.0]	0.0009
SaO <sub>2</sub> <90% % TST	1.0 [0.0–11.5]	4.0 [0.0–35.0]	0.002
RAI events·h <sup>-1</sup>	27.9 [12.4–44.2]	35.0 [12.6–59.2]	0.003
Plasma glucose mmol·L <sup>-1</sup>	4.9 [4.5–5.9]	5.2 [4.7–6.4]	0.02
Plasma insulin μU·mL <sup>-1</sup>	4.8 [2.7–9.0]	6.4 [3.7–12.2]	0.0002
HOMA-IR index	1.0 [0.6–2.1]	1.5 [0.9–3.1]	0.0001
Total cholesterol mmol·L <sup>-1</sup>	5.47 [4.02–5.91]	5.81 [4.45–6.73]	0.006
LDL cholesterol mmol·L <sup>-1</sup>	3.35 [2.01–3.95]	3.66 [2.59–4.55]	0.008
HDL cholesterol mmol·L <sup>-1</sup>	1.55 [1.14–2.19]	1.42 [0.98–1.91]	0.13
Triglycerides mmol·L <sup>-1</sup>	1.01 [0.62–1.59]	1.27 [0.79–2.11]	0.002
hsCRP mg·L <sup>-1</sup>	1.30 [0.20–3.42]	1.30 [0.60–3.74]	0.50
Right carotid IMT mm	596 [498–821]	602 [457–815]	0.66
Left carotid IMT mm	613 [512–876]	663 [527–897]	0.20
Mean carotid IMT mm	608 [520–848]	628 [531–848]	0.21
Obesity/overweight/normal BMI %	0/46/54	39/34/27	<0.0001
Smoking %	0	2	0.001
Hypertension %	0	25	<0.0001
Metabolic syndrome %	0	30	<0.0001
Dyslipidemia %	0	50	<0.0001
Urinary 11-dTXB <sub>2</sub> pg·mg <sup>-1</sup> creatinine	612.6 [289.0–866.3]	677.9 [389.5–1247.6]	0.008

Data are presented as median [10th–90th percentiles], unless otherwise stated. BMI: body mass index; BP: blood pressure; AHI: apnoea/hypopnoea index; RDI: respiratory disturbance index; SaO<sub>2</sub>: arterial oxygen saturation; TST: total sleep time; RAI: respiratory arousal index; HOMA-IR: homeostasis model assessment of insulin resistance; LDL: low-density lipoprotein; HDL: high-density lipoprotein; hsCRP: high-sensitivity C reactive protein; IMT: intima-media thickness; 11-dTXB<sub>2</sub>: 11-dehydrothromboxane. Bold indicates statistical significance.

#### Clinical study

##### 11-dTXB<sub>2</sub> and OSA

Baseline characteristics of the 25 controls and the 50 OSA patients matched for age and BMI are described in table 3. There was no significant difference for plasma insulin, hsCRP, HDL cholesterol, homeostatic model assessment insulin resistance index (HOMA-IR), BP, carotid intima-media thickness (IMT) and sex ratio. As expected, polysomnographic parameters were different between OSA and control groups. Plasma glucose, total and LDL cholesterol levels were significantly increased in OSA patients *versus* controls. Urinary 11-dTXB<sub>2</sub> was not significantly different between OSA patients and controls (table 3).

##### 11-dTXB<sub>2</sub>, OSA and cardiovascular risk factor

Clinical, biological and polysomnographic parameters of OSA patients with or without CVRFs are shown in table 4. The OSA group with CVRFs had polysomnographic parameters, BP, BMI, plasma triglycerides, total and LDL cholesterol, glucose, insulin and HOMA-IR higher than the OSA group free of CVRFs (table 4). Conversely, these two groups were similar with regard to age, sex ratio, carotid IMT, HDL cholesterol and hsCRP (table 4). Urinary 11-dTXB<sub>2</sub> was significantly increased in OSA patients with CVRFs compared with OSA patients free of CVRFs (fig. 3a). In OSA patients with or without CVRFs, 11-TXB<sub>2</sub>

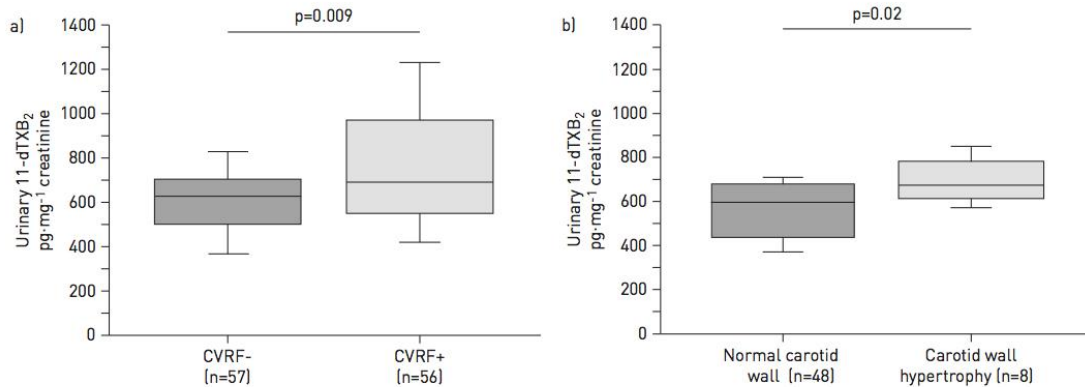


FIGURE 3 a) Urinary 11-dehydrothromboxane B<sub>2</sub> (11-dTXB<sub>2</sub>) concentrations in obstructive sleep apnoea (OSA) patients with (CVRF+) and without (CVRF-) known cardiovascular risk factor. b) Urinary 11-dTXB<sub>2</sub> concentrations in OSA patients with or without carotid wall hypertrophy. Data are presented as interquartile range (boxes), data range (whiskers) and median (horizontal line).

levels were similar in patients with mild-to-moderate (AHI <30 events·h<sup>-1</sup>) and severe OSA (AHI ≥30 events·h<sup>-1</sup>) (data not shown).

To determine the CVRF involved in the increase of urinary 11-dTXB<sub>2</sub>, simple regressions were performed. Hypertension ( $r=0.190$ ,  $p=0.05$ ) and obesity ( $r=0.242$ ,  $p=0.01$ ) were weakly associated with increased urinary excretion of 11-dTXB<sub>2</sub>. In multiple regression model, obesity remained the sole independent predictive factor of urinary 11-dTXB<sub>2</sub> ( $r=0.257$ ,  $p=0.04$ ).

#### Effect of CPAP treatment on urinary 11-dTXB<sub>2</sub> concentration

CPAP treatment for at least 8 weeks significantly decreased AHI, RDI and respiratory arousal index, increased minimal nocturnal arterial oxygen saturation (S<sub>a</sub>O<sub>2</sub>) and mean nocturnal S<sub>a</sub>O<sub>2</sub>, and decreased the percentage of time spent with a mean S<sub>a</sub>O<sub>2</sub> <90%. CPAP treatment induced no change on 11-dTXB<sub>2</sub> urinary concentration in OSA without or with CVRF (online supplementary tables S2 and S3).

#### 11-dTXB<sub>2</sub>, OSA and vascular remodelling

In OSA patients free of CVRF, vascular hypertrophy (defined by carotid IMT >0.8 mm) was associated with increased urinary 11-dTXB<sub>2</sub> concentrations compared with subjects without vascular hypertrophy ( $p=0.02$ ) (fig. 3b).

## Discussion

Our study demonstrated for the first time an activation of the COX pathway in ApoE<sup>-/-</sup> mice exposed to CIH and also in OSA patients with other CVRFs; this activation is associated with increased atherosclerotic lesions in mice and with early markers of atherosclerosis in OSA patients.

We showed that CIH mice had higher atherosclerotic lesions than normoxic air mice, suggesting that, in our model, CIH might have accelerated atherosclerosis development. This result is in accordance with previous works in ApoE<sup>-/-</sup> mice exposed to CIH from 2 to 4 weeks [4, 5] or in C57BL/6J mice exposed to CIH for 12 weeks [6].

In the present study, plasma cholesterol levels were not significantly different between normoxic air and CIH groups and atherosclerotic lesion sizes correlated with plasma cholesterol in normoxic air mice, but not in CIH mice. These data suggested that, in our model, CIH-induced atherosclerosis might be independent of lipid disorders, and contrast with previous works showing that concomitant exposure to a high-fat high-cholesterol diet and CIH aggravate both atherosclerosis and dyslipidemia in ApoE<sup>-/-</sup> mice [4] and in C57BL/6J mice [6, 23]. However, we recently showed that CIH also exerts pro-atherogenic effects through other contributing factors, notably the inflammatory process [5]. In agreement with this later hypothesis, our data showed an activation of the thromboxane pathway in CIH mice, as mRNA levels of COX-1 and TXBS were increased in aortic tissue of CIH mice. Furthermore, the correlation between these mRNA levels and atherosclerotic lesion size was consistent with direct effects of TXA<sub>2</sub> on macrophages [24]. Unexpectedly, the aortic secretion of TXB<sub>2</sub> and 6-keto-PGF1 $\alpha$ , as well as the TXB<sub>2</sub>/6-keto-PGF1 $\alpha$  ratio were similar in CIH and normoxic air mice. These results could be explained by the fact that we measured prostanoid secretion

after aorta stimulation with A23187, and not the basal aortic production. A previous study performed in LDL r-knockout (r-KO) mice demonstrated that the acceleration of atherogenesis in response to high fat diet was associated with increased basal levels of TXB<sub>2</sub> and 6-keto-PGF1 $\alpha$  in the aortic arch [25], but measurements of prostanoids were performed by ELISA, which is not a specific method.

We chose to measure TXB<sub>2</sub> and 6-keto-PGF1 $\alpha$  upon aorta stimulation with A23187 to obtain detectable prostanoid levels by our analytical technique (LC-MS/MS), which is highly specific but probably less sensitive. However, in our model, A23187 appears to stimulate TXB<sub>2</sub> and 6-keto-PGF1 $\alpha$  release to the same extent, as both levels were highly correlated, which could explain the similar TXB<sub>2</sub>/6-keto-PGF1 $\alpha$  ratio in aorta from CIH and normoxic air mice. We acknowledge that comparisons of basal TXB<sub>2</sub> and 6-keto-PGF1 $\alpha$  production and Western blot analysis of COX-1 and TXBS would have been of interest with regard to the increased mRNA levels of COX-1 and TXBS induced by CIH, and that their absence may represent a limitation of our study.

Moreover, treatment with the selective COX-1 inhibitor SC-560 during the last 4 weeks of CIH exposure reduced atherosclerosis progression in CIH mice, providing further evidence for a CIH-dependent activation of the COX pathway. We already demonstrated that 4 weeks of CIH exposure were sufficient to induce atherosclerosis in ApoE<sup>-/-</sup> mice [5], thus it was of interest to explore the effects of COX-1 inhibition on established atherosclerotic lesions. As previously described [12], treatment with SC-560 had no effect on established atherosclerotic lesions in normoxic air mice, although it was effective in inhibiting COX-1 pathway as assessed by the measurement of aortic TXB<sub>2</sub> and 6-keto-PGF1 $\alpha$  secretion. Collectively, these data demonstrated that in ApoE<sup>-/-</sup> mice, the atherogenic process was accelerated by CIH exposure, at least in part through COX-1 pathway activation. CIH activates leukocytes [26] and OSA patients display leukocyte activation [7, 8, 27]. By regulating the interaction between leukocytes, smooth muscle cells and endothelial cells, TXA<sub>2</sub> promotes and PGI<sub>2</sub> prevents the initiation and progression of atherogenesis [14].

To extend the thromboxane-pathway activation observed in CIH mice to OSA patients, we measured the urinary excretion of 11-dTXB<sub>2</sub>, a validated biomarker of systemic TXA<sub>2</sub> production [10]. Urinary 11-dTXB<sub>2</sub> concentrations of OSA patients free of CVRF were not different to those of controls carefully matched for age and BMI, two major confounding factors often present in studies focused on the underlying inflammation associated to OSA. These data suggested that OSA itself was not associated with an increased urinary 11-dTXB<sub>2</sub> excretion, an hypothesis confirmed by the observation that CPAP treatment had no influence on urinary 11-dTXB<sub>2</sub> level. However, increased urinary 11-dTXB<sub>2</sub> concentrations have previously been described in patients with cardiovascular diseases including obesity [28] and hypertension [29]. Consistent with these data, we showed that OSA patients with an associated CVRF had higher urinary concentrations of 11-dTXB<sub>2</sub> than OSA patients free of a known CVRF. Furthermore, among the studied CVRFs, obesity was the sole independent predictor of urinary 11-dTXB<sub>2</sub> excretion, which is in agreement with a previous study demonstrating an increased urinary 11-dTXB<sub>2</sub> excretion in obese females [28]. This result provided further evidence for the major role of obesity in arachidonic acid metabolism activation in OSA patients, as we previously described for the 5-lipoxygenase pathway [9]. Finally, urinary 11-dTXB<sub>2</sub> excretion was associated with carotid wall hypertrophy in OSA patients. Again, these findings were consistent with the proliferative effects of TXA<sub>2</sub> on vascular smooth muscle cells [10], although it must be kept in mind that vascular remodelling is a complex process implying many mediators and that COX pathway activation might not be the sole mechanism of vascular remodelling in OSA patients with CVRFs.

In conclusion, our study showed an activation of the COX-1 pathway in ApoE<sup>-/-</sup> mice exposed to CIH, which contributed to the acceleration of the atherogenic process induced by this stimulus. Such an activation of the COX-1 pathway was also found in OSA patients with associated CVRFs. This study extends the activation of the arachidonic acid metabolism previously described for the leukotriene pathway [7, 9] to the COX-1 pathway in OSA in relation to vascular remodelling. These findings open the field to the interest of new pharmacological approaches (dual COX and 5-lipoxygenase inhibitor [30]) in the prevention of cardiovascular morbidity in OSA patients.

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Ces travaux ont mis en évidence une activation de la voie de la COX-1 chez des souris ApoE<sup>-/-</sup> soumises à une IH et chez des patients SAOS présentant au moins un autre facteur de risque cardiovasculaire parmi les suivants : HTA, tabagisme, dyslipidémie, syndrome métabolique, obésité. Cette activation semblait impliquée dans le processus athéromateux puisque les niveaux d'ARNm des enzymes intervenant dans la synthèse du TXA<sub>2</sub> (à savoir la COX-1 et la thromboxane synthase) étaient corrélés à la taille des lésions d'athérosclérose des souris soumises à l'HI; et que chez le patient apnéique, les concentrations urinaires de 11dTXB<sub>2</sub> étaient majorées en cas d'hypertrophie carotidienne (définie par une EIM >0,8 μm). Enfin, chez la souris ApoE<sup>-/-</sup>, le traitement pharmacologique par un inhibiteur sélectif de la COX-1 réduisait la progression de l'athérosclérose induit par l'HI.

Bien que cette première approche pharmacologique ait présenté des résultats encourageants, de nombreuses autres stratégies thérapeutiques ciblant le TXA<sub>2</sub> ont déjà été proposées pour lutter contre l'athérosclérose (hors HI). Ces stratégies visaient tantôt à réduire la production de TXA<sub>2</sub> (par une approche pharmacologique (209–211) ou nutritionnelle (212)), tantôt à bloquer les récepteurs TP (213–215) ou encore à combiner ces deux stratégies (206,207,216–218). Néanmoins, les effets de telles interventions thérapeutiques sur le processus athéromateux induit par l'HI restent à préciser.

Nous avons donc écrit une revue de la littérature dont l'objectif a été de synthétiser dans une première partie les connaissances relatives à l'activation de la voie du TXA<sub>2</sub> au cours du SAOS et son implication dans le processus athéromateux; avant de discuter, dans un second temps, l'intérêt potentiel des différentes approches thérapeutiques ciblant la voie du TXA<sub>2</sub> pour lutter contre l'athérosclérose induit par l'HI.



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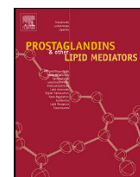
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## Prostaglandins and Other Lipid Mediators



## Could the thromboxane A<sub>2</sub> pathway be a therapeutic target for the treatment of obstructive sleep apnea-induced atherosclerosis?



Elodie Gautier-Veyret<sup>a,b,c</sup>, Laetitia Van Noolen<sup>a,b,c</sup>, Patrick Lévy<sup>a,b,c</sup>, Jean Louis Pepin<sup>a,b,c</sup>,  
Françoise Stanke-Labesque<sup>a,b,c,\*</sup>

<sup>a</sup> Univ Grenoble Alpes, F-38041 Grenoble, France

<sup>b</sup> INSERM U1042, HP2, F-38041 Grenoble, France

<sup>c</sup> Centre Hospitalier Universitaire de Grenoble, Hôpital Albert Michalon, F-38043 Grenoble, France

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### ABSTRACT

Obstructive sleep apnea (OSA) is characterized by recurrent nocturnal episodes of intermittent hypoxia. This disease is associated with premature atherosclerosis and consequently with increased cardiovascular morbidity and mortality. Atherosclerosis is a chronic inflammatory disease characterized by the activation of some components of the cyclooxygenase pathway. In particular, OSA is associated with activation of the thromboxane A<sub>2</sub> (TXA<sub>2</sub>)-pathway, in which obesity seems to be a major confounding factor. Moreover, TXA<sub>2</sub>-pathway activation is related to the vascular remodeling associated with OSA. In view of the modest effect of the conventional treatment of OSA by continuous positive airway pressure on the cardiovascular risk in obese OSA patients, the identification of new therapeutic targets to treat OSA-induced atherosclerosis seems essential. As disruption of the TXA<sub>2</sub>-pathway has been suggested to be of potential interest to prevent atherosclerosis progression, we have reviewed the recent findings on the intricate interaction between the TXA<sub>2</sub>-pathway, chronic intermittent hypoxia and atherosclerosis and suggest promising therapeutic strategies to treat OSA-related atherogenesis, including pharmacological and/or nutritional approaches.

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### Introduction

Obstructive sleep apnea (OSA) is a worldwide public health problem affecting 5–20% of the general population [1]. OSA is characterized by recurrent episodes of nocturnal upper airway collapses, leading to intermittent hypoxia (IH) that is detrimental for the cardiovascular system. Cardiovascular morbidity and mortality are in fact increased in patients suffering from OSA [2] and OSA is now recognized as an independent cardiovascular risk factor [3].

Several studies have suggested that IH *per se* may promote early vascular remodeling. Indeed, OSA patients free of cardiovascular risk factors present early signs of atherosclerosis [4,5] that correlate to hypoxia severity [4,5] and are partially reversed by continuous positive airway pressure (CPAP) treatment [5]. Experimental animal studies have provided further evidence for the role of IH in the development of atherosclerosis. Chronic exposure to IH induced preatherosclerotic remodeling in lean mice [6] or an acceleration of atherogenesis in apolipoprotein E-deficient (ApoE<sup>-/-</sup>) mice [7–9], all these experiments being performed in the absence of high-fat-diet.

**Abbreviations:** AA, arachidonic acid; AHI, apnea hypopnea index; ApoE<sup>-/-</sup>, apolipoprotein E-deficient; BMI, body mass index; COX, cyclooxygenase; CPAP, continuous positive airway pressure; DHA, docosahexanoic acid; EPA, eicosapentanoic acid; IH, intermittent hypoxia; IMT, intima-media thickness; OSA, obstructive sleep apnea; PGI<sub>2</sub>, prostacyclin (prostaglandin I<sub>2</sub>); PUFA, polyunsaturated fatty acid; 6-ketoPGF<sub>1α</sub>, 6-ketoprostaglandin F<sub>1α</sub>; TXA<sub>2</sub>, thromboxane A<sub>2</sub>; TXB<sub>2</sub>, thromboxane B<sub>2</sub>; TXBS, thromboxane synthase; VSMC, vascular smooth muscle cells.

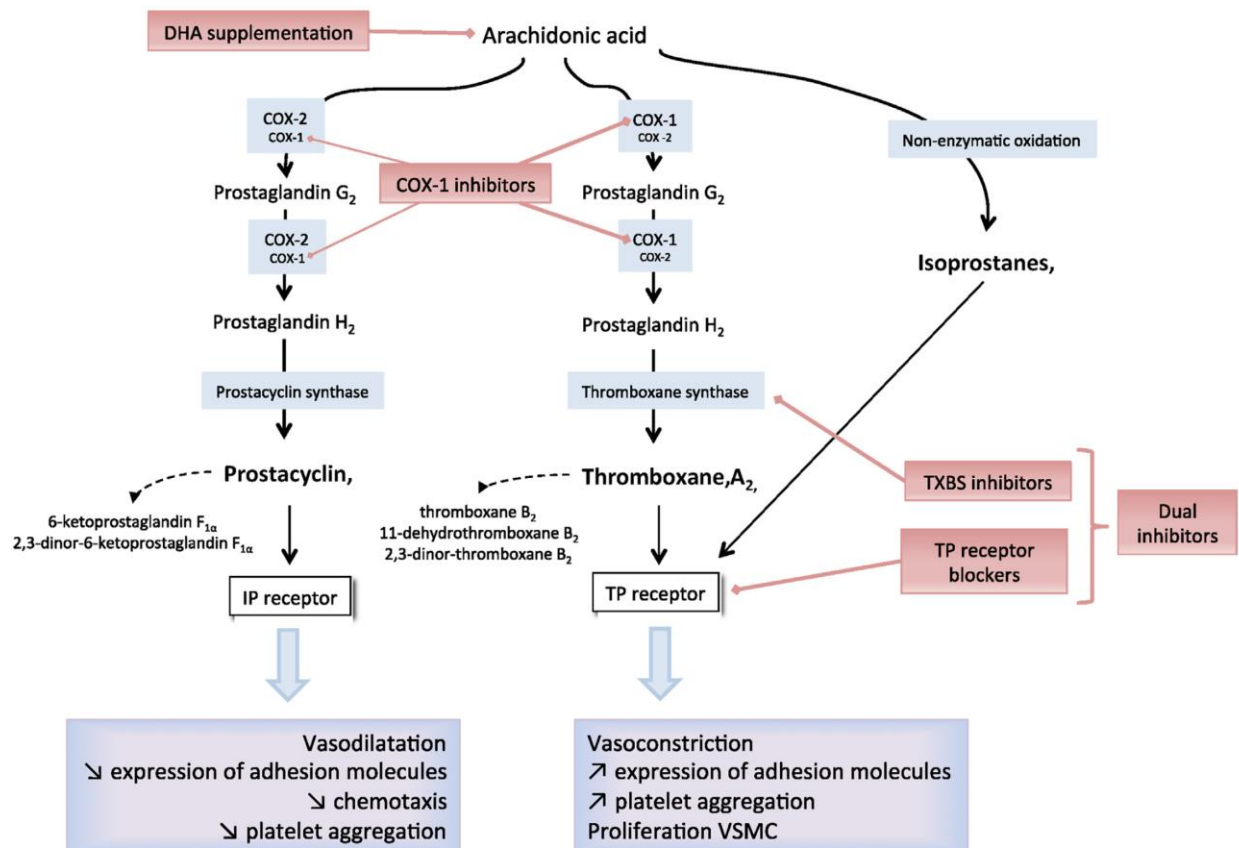
\* Corresponding author at: CHU, Hôpital Albert Michallon, Laboratoire de Pharmacologie-Toxicologie, CS10217, F-38043 Grenoble Cedex 9, INSERM U1042, France. Tel.: +33 4 76 76 54 92; fax: +33 4 76 76 89 38.

E-mail address: fStanke@chu-grenoble.fr (F. Stanke-Labesque).

Atherosclerosis [10] and OSA [11,12] are diseases associated with chronic low grade inflammation. Thus, several works have supported the hypothesis that the underlying inflammation induced by IH may be a determining factor that could contribute to IH-induced vascular remodeling [6–9,13–19].

Among the numerous inflammatory mediators, the cyclooxygenase (COX)-pathway has been shown to play a major role in the onset and progression of atherosclerosis [20–22]. Thromboxane A<sub>2</sub> (TXA<sub>2</sub>) and prostacyclin (PGI<sub>2</sub>) are two COX-derived metabolites of arachidonic acid (AA). TXA<sub>2</sub> is predominantly generated by

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**Fig. 1.** Biosynthesis and biological effects of thromboxane A<sub>2</sub>, prostacyclin and isoprostanes, and different therapeutic approaches to disrupt the thromboxane A<sub>2</sub> pathway. The enzymes synthesizing prostanoids are depicted in blue rectangles, whereas the white shaded rectangles indicate prostanoid receptors. The dotted arrows indicate the rapid degradation of thromboxane A<sub>2</sub> and prostacyclin into various metabolites. Biological effects induced by receptor activation are indicated in large blue rectangles and the different therapeutic approaches are indicated in red rectangles. COX, cyclooxygenase; TXBS, thromboxane synthase; DHA, docosahexaenoic acid; VSMC, vascular smooth muscle cell.

platelets through the COX type 1 isoform (COX-1) and thromboxane synthase (TXBS), while PGI<sub>2</sub> predominately results from the activity of the endothelial COX type 2 isoform (COX-2) and prostacyclin synthase (Fig. 1). As TXA<sub>2</sub> and PGI<sub>2</sub> are unstable, they are rapidly metabolized into various metabolites that are quantifiable in plasma and/or urine: 6-ketoprostaglandin F<sub>1α</sub> (6-ketoPGF<sub>1α</sub>) and 2,3-dinor-6-ketoPGF<sub>1α</sub> for PGI<sub>2</sub>, thromboxane B<sub>2</sub> (TXB<sub>2</sub>) and its derivatives 2,3-dinor-TXB<sub>2</sub> and 11-dehydroTXB<sub>2</sub> for TXA<sub>2</sub> (Fig. 1). TXA<sub>2</sub> and PGI<sub>2</sub> exert their biologic effects through binding to distinct receptors, the TP and IP receptors, respectively, both expressed on platelets, vascular smooth muscle cells (VSMC), monocytes and endothelial cells. From a biological point of view, TXA<sub>2</sub> and PGI<sub>2</sub> have antagonist properties. TXA<sub>2</sub> induces platelet activation, VSMC proliferation and vasoconstriction, and the expression of adhesion molecules, while PGI<sub>2</sub> reduces platelet aggregation, chemotaxis and vasoconstriction (Fig. 1) (see review of Capra et al. [21]).

In addition to TXA<sub>2</sub>, others lipid mediators, i.e. the isoprostanooids, are able to bind to TP receptors [21,23], mimicking the biological effects of TXA<sub>2</sub> (Fig. 1). The isoprostanooids derived from AA via non-enzymatic oxidation include several isoprostanes: F-isoprostane, E-isoprostane and D-isoprostane.

Few studies have investigated the role of the TXA<sub>2</sub>-pathway in OSA-induced atherosclerosis; although this pathway has already been proposed as a therapeutic target for the treatment of cardiovascular diseases (see reviews [20,21,23]). The present review

will focus on the role of the TXA<sub>2</sub>-pathway as a potential molecular link between OSA and atherosclerosis, and on the therapeutic interest of targeting TXA<sub>2</sub> to reduce or prevent IH-induced atherosclerosis.

### Thromboxane A<sub>2</sub> pathway and OSA/IH-induced atherosclerosis

#### Thromboxane A<sub>2</sub> pathway activation in relation to intermittent hypoxia

The activation of the COX-pathway by IH has been demonstrated in clinical and animal studies, both at the systemic and tissue levels. We demonstrated that urinary 11-dehydroTXB<sub>2</sub> concentrations in OSA patients free of cardiovascular comorbidities were not different from those of controls carefully matched for age and body mass index (BMI) [8]. Similarly, 11-dehydroTXB<sub>2</sub> levels were similar in controls and mild-to-moderate OSA patients treated with antihypertensive drugs [24]. All these results suggest that OSA *per se* might not activate the TXA<sub>2</sub>-pathway. This hypothesis is also supported by the fact that CPAP treatment for 3 nights [25] or for at least 8 weeks [8] does not affect urinary TXA<sub>2</sub> metabolite concentrations. In line with these results, a recent study conducted in healthy volunteers showed that acute exposure to IH for 6 h induced no change in urinary excretion of TXA<sub>2</sub> metabolites [26]. Taken

together, these data suggest that acute exposure to IH or OSA itself might not be sufficient to trigger the activation of the TXA<sub>2</sub>-pathway in the absence of comorbidity.

In contrast, an increase in urinary 11-dehydroTXB<sub>2</sub> concentrations has been described in OSA patients exhibiting cardiovascular comorbidities [8,26]. Indeed we demonstrated that urinary 11-dehydroTXB<sub>2</sub> concentrations were increased in a cohort of OSA patients presenting various cardiovascular risk factors, including obesity, arterial hypertension, dyslipidemia, smoking or metabolic syndrome [8]. Among all these cardiovascular risk factors, obesity and arterial hypertension were associated with enhanced urinary excretion of 11-dehydroTXB<sub>2</sub> (simple regression analysis) and obesity remained the sole independent predictive factor of increased urinary 11-dehydroTXB<sub>2</sub> (multiple regression analysis) [8]. In another study, OSA patients exhibited enhanced urinary TXA<sub>2</sub> metabolite concentrations compared to controls [26]; but these OSA patients were much older and displayed higher blood pressure and BMI compared to controls. In line with previous results describing higher concentrations of TXA<sub>2</sub> metabolites in obese [27–30] or hypertensive patients [31], the increased TXA<sub>2</sub> metabolite levels observed in OSA patients [26] is likely to be related to higher BMI and blood pressure rather than to OSA itself.

Since PGI<sub>2</sub> and TXA<sub>2</sub> have antagonist properties, the TXA<sub>2</sub>/PGI<sub>2</sub> ratio has been proposed as a surrogate biological marker of atherosclerosis [32], although its relevance should be considered with caution since PGI<sub>2</sub> counteracts not only most of the biological properties of TXA<sub>2</sub> but also those of others prostanoids such as prostaglandin E<sub>2</sub>. Furthermore, data concerning this ratio (or its inverse) in OSA patients are limited and conflicting. Indeed, both an increase [33] and a decrease [25] of the 6-ketoPGF<sub>1α</sub>/TXB<sub>2</sub> ratio have been reported in OSA patients and these results must be regarded with caution due to the very small number of patients included in these studies ( $n=11$  and  $n=7$ , respectively). Then again, these discrepancies could probably be due to the presence of confounding factors such as obesity (the BMI of OSA patients in the studies of Krieger et al. [33] and Kimura et al. [25] were  $28.7 \pm 4.3$  and  $31 \pm 2.0$  kg/m<sup>2</sup>, respectively). More recently, Beaudin et al. reported no difference in urinary PGI<sub>2</sub> metabolite concentrations and PGI<sub>2</sub>/TXA<sub>2</sub> ratios between OSA patients and controls, and between pre- and post-6 hour-IH exposure of healthy subjects [26]. On the contrary, higher plasma concentrations of 6-ketoPGF<sub>1α</sub> were found in mild-to-moderate OSA patients compared to controls, but no comparison of the 6-ketoPGF<sub>1α</sub>/TXB<sub>2</sub> ratio was done [24]. Nevertheless, in the latter study, the efficient treatment by mandibular advancement splints during 6 months had no effect on the increased production of PGI<sub>2</sub>, suggesting that this increase was independent of OSA [24]. A possible explanation could be the higher frequency of antihypertensive drug use by the OSA patients of this study, probably including angiotensin converting enzyme inhibitors that are known to increase PGI<sub>2</sub> production [34].

In contrast to the clinical data, experimental studies in OSA animal models have strongly suggested that the TXA<sub>2</sub>-pathway is activated in OSA. Experimental models of sleep-disordered breathing are numerous, including both surgical and non-surgical models. Surgical models consist of inserting a collapsible endotracheal tube in tracheostomized animals [35], mimicking the obstructive apneas of OSA. While this approach is limited by the invasive nature of the procedure, variations of oxygen content in the flushed air permit the separate investigation of the effects of increased respiratory effort and IH [35]. The more widely used nonsurgical models consist of placing animals in devices in which the oxygen content of the air can be varied [8,36]. These nonsurgical models result in exposure to IH which can be modulated in terms of duration of daytime exposure, the frequency of desaturating events or the severity of induced hypoxia (for more details on experimental models of OSA, see the review by Dematteis et al. [37]).

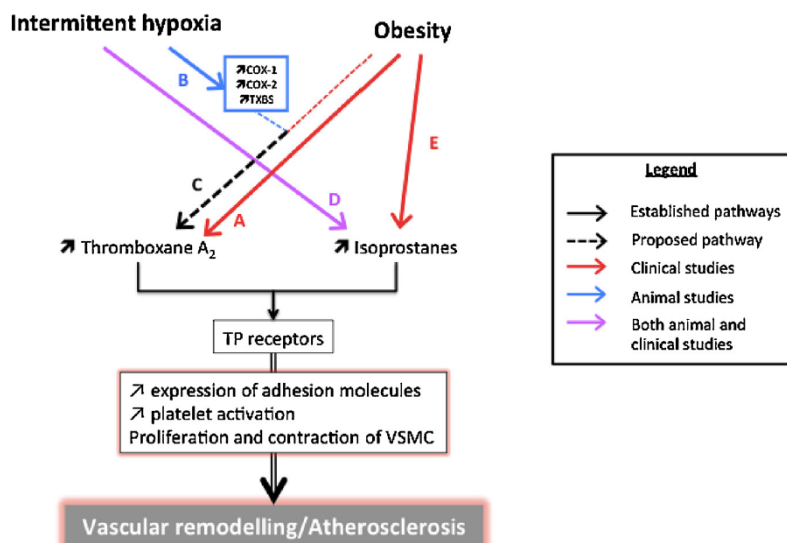
In rats subjected to obstructive apnea for 3 h [35] or exposed to IH for 6 h per day during 4 weeks [36], the plasma TXB<sub>2</sub> concentrations and the TXB<sub>2</sub>/6-ketoPGF<sub>1α</sub> ratios were increased. Nacher et al. used an animal model to determine the respective contributions of IH and respiratory effort in the activation of the TXA<sub>2</sub>-pathway. They demonstrated that the increased plasma TXB<sub>2</sub>/6-ketoPGF<sub>1α</sub> ratio was related to IH and not to respiratory effort [35], suggesting that the hypoxic component of OSA may play a major role in triggering TXA<sub>2</sub> production.

In parallel to these systemic studies, the influence of IH exposure on TXA<sub>2</sub>-pathway activation was investigated at the tissue level. In the cortical region of rat brain, the levels of COX-1 mRNA were unchanged in rats exposed to IH, while the expression of COX-2 was enhanced at both transcriptional and protein levels; this increase being associated with neuronal apoptosis and neurobehavioral deficit [38]. Little is known about possible IH-related COX-pathway activation in the cardiovascular system, whereas the influence of COX-derived mediators in the development of cardiovascular diseases has been largely demonstrated [20–22]. An increase in COX-2 transcriptional expression in the myocardium and cardiomyocytes of rats exposed to IH was demonstrated [39], but prostanoids were not measured in parallel. In aorta of ApoE<sup>-/-</sup> mice exposed to IH for 8 weeks, COX-1 and TXBS mRNA were up-regulated [8] but not associated with an *in situ* increase in prostanoid release, since concentrations of TXB<sub>2</sub> and 6-ketoPGF<sub>1α</sub> in the supernatants of aortic rings stimulated with A23187 were similar in mice exposed to IH or normoxia [8].

These discrepancies between clinical and experimental studies could be explained by the greater severity of hypoxia in animal models compared to OSA patients. The intensity of hypoxia evaluated by minimal oxygen saturation was notably greater in animal models (60–80%) [37] compared to OSA patients (approximately 80%) [8,24]. Moreover, in the OSA animal model of Nacher [35] and Farré [36], rats exhibited 60 hypoxic events per hour, whereas in humans the respiratory disturbance index was around 30 to 35 events per hour [8,26]. Another possible explanation could be that TXA<sub>2</sub> production was evaluated by the quantification of either urinary or plasma metabolites of TXA<sub>2</sub> in clinical and animal studies, respectively. While the measurement of urinary metabolites reflects the systemic production of TXA<sub>2</sub>, the measurement of plasma metabolites seems more closely related to TXA<sub>2</sub> production by platelets [40] and can be altered by residual production during blood collection and/or handling [41,42].

To summarize, animal data have suggested that chronic exposure to IH could be sufficient to activate the TXA<sub>2</sub>-pathway; while in OSA patients, the activation of the TXA<sub>2</sub>-pathway could more likely be related to the presence of confounding factors such as obesity or hypertension rather than to the OSA itself. Nevertheless, one study suggested that the hypoxic component of OSA could partly contribute to the activation of the TXA<sub>2</sub>-pathway observed in OSA patients. Indeed, changes in plasma 11-dehydroTXB<sub>2</sub> levels observed after 6 months of OSA treatment by mandibular advancement splints were positively correlated to the improvement of polysomnographic markers of OSA (apnea hypopnea index (AHI) and mean oxygen desaturation), while BMI did not change throughout the 6-months treatment period [24]. Thus we make the hypothesis that IH and obesity act in synergy toward the activation of the thromboxane A<sub>2</sub> pathway in OSA patients (Fig. 2). Further studies are therefore needed to provide definitive evidence for the contribution of IH in TXA<sub>2</sub>-pathway activation and to determine the underlying mechanisms.

Studies concerning isoprostanoid levels in OSA are also numerous and the results conflicting [43–49], and are hampered by the presence of confounding factors such as overweight/obesity [44,47,49], which are associated with increased levels of isoprostanes independently of OSA itself [50–53]. Nevertheless, some



**Fig. 2.** Respective contribution of intermittent hypoxia and obesity to thromboxane A<sub>2</sub>/TP receptor pathway activation during obstructive sleep apnea. Obesity is associated with increased concentrations of thromboxane A<sub>2</sub> metabolites (A) [27–30]; In contrast, the influence of intermittent hypoxia on thromboxane A<sub>2</sub> pathway activation still remains unclear since results are conflicting. Experimental animal data demonstrated that intermittent hypoxia activates the thromboxane A<sub>2</sub> pathway both at a systemic level (plasma thromboxane A<sub>2</sub> metabolite levels [35,36]), or at a tissue level (COX-1 and TXBS mRNA levels in aorta [8], COX-2 mRNA levels in heart [39]) (B). Conversely, in OSA patients, thromboxane A<sub>2</sub> pathway activation is more likely to be strongly dependent on obesity rather than OSA itself [8,24,26]. Thus, it could be hypothesized that obesity and intermittent hypoxia act in synergy towards the activation of the thromboxane A<sub>2</sub> pathway in OSA (C). Both intermittent hypoxia [45,46,48,92] (D) and obesity [50–53] (E) lead to the higher concentrations of 15-F<sub>2t</sub>-isoprostanes observed in OSA patients. Prostacyclin was not mentioned in the figure because very little data is available about the influence of intermittent hypoxia and obesity on this prostanoid. OSA, obstructive sleep apnea; COX, cyclooxygenase; TXBS, thromboxane synthase; VSMC, vascular smooth muscle cell.

studies with rigorous methodology have provided convincing results suggesting that OSA could be associated with increased isoprostanoic concentrations [45,46,48]. Minoguchi et al. demonstrated that OSA patients had increased overnight urinary excretion of 15-F<sub>2t</sub>-isoprostanes compared to controls carefully matched for age and BMI [48]. Interestingly, AHI remained the sole independent predictor of 15-F<sub>2t</sub>-isoprostane levels [46,48], even after adjustment on BMI [48]. Similarly, while the effects of CPAP on isoprostane levels observed in uncontrolled studies [44,47–49] are questionable, a prospective, randomized, placebo controlled, double-blind, crossover study demonstrated its efficacy in normalizing plasma 15-F<sub>2t</sub>-isoprostane levels [45]. Taken together, these data suggest that, in addition to obesity, the hypoxic component of OSA may play a major role in the isoprostane pathway activation observed in OSA patients (Fig. 2).

#### Link between IH-related thromboxane A<sub>2</sub> activation and vascular remodeling/atherosclerosis

TXA<sub>2</sub> is a well-known proatherogenic mediator since it is able to induce VSMC proliferation, contraction and to promote chemotaxis *via* an increased expression of adhesion molecules (Fig. 1) (see reviews [21,23]). Since some experimental studies have demonstrated that IH can activate the TXA<sub>2</sub>-pathway [8,35,36], the later could be implicated in IH-induced atherogenesis. Importantly, the increased aortic COX-1 and TXBS mRNA levels observed in ApoE<sup>-/-</sup> mice exposed to IH for 8 weeks correlated with atherosclerosis lesion size on the aortic roots [8]. Moreover, pharmacological COX-1 inhibition during 4 weeks abrogated the IH related progression of atherosclerosis in these mice [8]. Together these data strongly suggest that TXA<sub>2</sub> could act as an important mediator in IH-induced atherogenesis in ApoE<sup>-/-</sup> mice. In OSA patients, the TXA<sub>2</sub>-pathway seems also to be associated with vascular remodeling

since urinary 11-dehydroTXB<sub>2</sub> levels were increased in OSA patients with carotid hypertrophy (defined by an intima-media thickness (IMT) > 0.8 mm) [8]. These data are consistent with the direct chemotactic and proliferative effects of TXA<sub>2</sub> on VSMC (see reviews [21,23]). Similarly, urinary 15-F<sub>2t</sub>-isoprostane concentrations were correlated with mean carotid IMT [46], consistent with their ability to activate TP receptors [21,23].

#### Therapeutic approaches to treat the OSA/IH-induced atherosclerosis

CPAP, the reference treatment of OSA, abolishes nocturnal apnea but fails to reduce cardiovascular risk in minimally symptomatic obese OSA patients [54], advocating for combined therapies [55] associating CPAP and pharmacological [56] or nutritional strategies targeting intermediary mechanisms leading to OSA related cardiovascular consequences.

#### Pharmacological approach

In a randomized double-blind study, we demonstrated that a 12-week treatment with atorvastatin (40 mg/d) neither improved endothelial function, nor reduced early signs of atherosclerosis, although it lowered blood pressure and improved the lipid profile, in patients with no history of cardiovascular disease [57]. Consistent with these data, the urinary levels of 11-dehydroTXB<sub>2</sub> remained unchanged during this treatment period [57].

However, considering the activation of the TXA<sub>2</sub>-pathway in OSA patients presenting cardiovascular comorbidities [8,26], and the failure of CPAP to reduce urinary 11-dehydroTXB<sub>2</sub> concentrations in OSA patients [8,25], targeting the TXA<sub>2</sub>-pathway could represent an alternative strategy to address early on or to delay the deleterious cardiovascular consequences linked to OSA.

Numerous studies have suggested the interest of disrupting the TXA<sub>2</sub> signaling pathway to reduce atherosclerosis, either by inhibiting TXA<sub>2</sub> production [58–60], blocking TP receptors [61–63] or combining a TXA<sub>2</sub> synthesis inhibitor and a TP receptor antagonist [64–66]. Pharmacological inhibition of TXA<sub>2</sub> biosynthesis with non-steroidal anti-inflammatory preferential COX-1 inhibitor [58], low-dose aspirin [60] or a more selective COX-1 inhibitor (SC560) [59,64] reduced atherosclerosis lesion size. In ApoE<sup>-/-</sup> mice exposed to IH for 8 weeks, COX-1 inhibition by SC-560 during the last 4 weeks of IH exposure reduced the IH related progression of established atherosclerosis [8]. In addition to the up-regulation of COX-1 and TXBS mRNA in aorta from ApoE<sup>-/-</sup> mice exposed to chronic IH and the correlation between these mRNA levels and the atherosclerosis lesion size [8], these results provided further evidence for the role of the COX-1 pathway in IH-induced atherogenesis. Conversely, SC-560 treatment had no influence on established atherosclerotic lesions from ApoE<sup>-/-</sup> mice fed on a high-fat-diet [59] or a normal diet [8]. All these experimental data strongly suggest the potential interest of disrupting the TXA<sub>2</sub>-pathway to reduce IH related atherosclerosis progression. However, further studies are required to investigate the potential efficacy of this pharmacological strategy in OSA.

In contrast, the effects of COX-2 inhibition on atherosclerosis are controversial since increased [67], reduced [68] or unchanged [58,59,69] atherosclerosis lesion sizes have been described after chronic treatment with COX-2 inhibitors. However, the increased risk of cardiovascular events associated with the use of the COX-2 selective inhibitor coxibs [32,70] opposes the putative interest of using these drugs to prevent OSA-induced atherosclerosis.

Blockade of TP receptors constitutes another way of disrupting the TXA<sub>2</sub>-signaling pathway, in order to treat atherosclerosis. This approach has several theoretical advantages over COX-1 inhibition. First, while COX-1 inhibition (notably by low-doses of aspirin) inhibits platelet mediated-TXA<sub>2</sub> production, targeting the TP receptor counteracts the effects of TXA<sub>2</sub> generated not only by platelet COX-1, but also by COX-2 in immature platelets [71] and by monocyte/macrophage COX-2 [72]. Moreover, this strategy inhibits the binding to TP receptor of all its agonists, including TXA<sub>2</sub> and isoprostanes. Overall, a greater inhibition of atherogenesis through TP blockade, compared to that obtained by the inhibition of TXA<sub>2</sub> production, might be expected [20]. Several experimental studies have indeed demonstrated that TP receptor antagonism is more efficient in reducing lesion formation than COX inhibition [61,62,73]. Nevertheless, in a randomized double-blind study conducted in well-treated patients with a history of cerebrovascular events, TP receptor blockade by terutroban for 3 years did not beneficially affect progression of carotid atherosclerosis compared to aspirin [74]. This apparent discrepancy could partly be due to the fact that TP antagonism while efficient in preventing atherosclerotic lesion formation [61–63], does not seem effective against established atherosclerotic lesions as conflicting results have been published [63,73]. Indeed, while in rabbits fed with a high cholesterol diet, the regression of an established lesion was demonstrated by 6-month treatment with a selective TP receptor inhibitor (S18886) [63], the same treatment for 16 weeks in old Apobec-1/LDLr double knock-out mice had no effect on established atherosclerosis [73]. Finally, a better strategy could be the combination of both COX-1 inhibition and TP receptor blockade, as supported by studies conducted in animal models [64] and in patients [75,76].

Currently, no study investigating the effect of TP blockade has been conducted in an experimental model of OSA or in patients suffering from OSA. Regarding TXA<sub>2</sub>-pathway activation [8,26] and the increased isoprostane levels observed in OSA patients [45,46,48], both related to vascular remodeling [8,46], it could be

suggested that TP antagonism represents an interesting pharmacological strategy to reduce IH-induced atherosclerosis.

#### Nutritional approach

The health benefits of *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs) on cardiovascular diseases have been reported by numerous studies (see reviews [77,78]), even if results still remain controversial [77]. *n*-3 PUFAs are a family of biologically active fatty acids. Among them, docosahexaenoic acid (DHA) (C22:6*n*-3), one of the most biologically active compounds derives from the essential fatty acid linolenic acid (C18:3*n*-3) or eicosapentaenoic acid (C20:5*n*-3) (EPA) by an alternating series of desaturation and elongation reactions [79]. *n*-3 PUFAs display a wide range of physiological roles and are directly linked to clinical benefits, mainly by modulating the inflammatory process. These fatty acids can partly inhibit low grade inflammation by reducing leukocyte chemotaxis, the expression of adhesion molecules, and the production of inflammatory cytokines [80]. They are also precursors of anti-inflammatory eicosanoids, including resolvins and protectins [81], leukotriene B<sub>5</sub> [82] and series-3 prostaglandins and thromboxanes [80].

Among the underlying mechanisms involved, the influence of *n*-3 PUFAs on inflammation is mediated by, or associated with, changes in the fatty acid composition of cell membranes [83]. In healthy subjects, oral supplementation with DHA induced a dose-dependent increase of DHA incorporation in polymorphonuclear cell membranes [82]. Similarly, experimental studies on Wistar rats [84], wild type mice [85] or ApoE<sup>-/-</sup> mice [9] demonstrated that oral DHA supplementation was associated with an increase in DHA and EPA incorporation, and a decrease of AA incorporation in different tissues. Since DHA, EPA and AA are competitive substrates of the lipoxygenases and COXs, changes in the eicosanoid profile is also expected. Experimental studies on rabbits fed with a high-fat-diet and supplemented with fish oil reported a decrease in TXB<sub>2</sub> levels in the ascending and abdominal aortas associated with a significant suppression of atherosclerosis [86]. In addition, we demonstrated that oral supplementation with fish oil (containing 80% DHA/4% EPA) of ApoE<sup>-/-</sup> mice exposed to IH for 8 weeks, prevented the IH-induced atherosclerosis progression [9]. Unfortunately, thromboxane B<sub>2</sub>/B<sub>3</sub> productions were not measured. However, in this model, fish oil supplementation induced no change in the mRNA levels of the resolvin pathway components (ALOX15, FRP2 and ChemR23) [9].

The impact of fish oil supplementation on TXB<sub>2</sub> levels has also been studied in several clinical studies reporting a decrease of TXB<sub>2</sub> serum levels in men at risk of cardiovascular disease [87], in hypercholesterolemic patients [88] or in patients with known cardiovascular disease [89]. However, whether this decrease in TXB<sub>2</sub> serum concentrations was associated with improvement of early markers of atherosclerosis was not mentioned in these studies.

To date, no study in humans suffering from OSA and given *n*-3 PUFA supplements has been performed, although some authors have recommended dietary supplementation with omega-3 fatty acids in order to minimize the inflammatory process associated with OSA [90]. One study reported that red blood cell membrane DHA levels were inversely correlated to OSA severity [91], but unfortunately confounding factors such as obesity were not considered. Further studies are obviously needed to confirm these results.

Taken together, experimental animal and clinical data confirm the role of *n*-3 PUFAs in decreasing the availability of AA as a substrate for eicosanoid synthesis. These data suggest the potential interest of *n*-3 PUFAs supplementation in the prevention of cardiovascular morbidity in OSA patients.

## Conclusion

As discussed in this review, there is a growing body of evidence from both experimental animal-model studies and clinical studies, suggesting that the TXA<sub>2</sub>-pathway could represent a molecular link between OSA and atherosclerosis. To summarize, IH and obesity the two major characteristic features of OSA seem to have a synergistic effect on the activation of the TXA<sub>2</sub>-pathway. TXA<sub>2</sub> could therefore contribute to the development of atherosclerosis, particularly in obese OSA patients. Since CPAP treatment fails to reduce cardiovascular risk in minimally symptomatic obese OSA patients [54], multiple modalities of treatment should be proposed to patients [55]. Disrupting the TXA<sub>2</sub>-pathway by pharmacological or nutritional approaches may provide interesting therapeutic strategies to treat atherosclerosis in obese OSA patients.

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En résumé, ces différents travaux ont permis de démontrer que la voie du TXA<sub>2</sub> était activée au cours du SAOS, les facteurs confondants tels que l'obésité étant à l'origine de cette activation. Cette activation contribue à l'athérosclérose associée au SAOS et, à ce titre, la voie du TXA<sub>2</sub> constitue une cible thérapeutique potentielle pour lutter contre l'athérogénèse induite par l'HI. L'inhibition de la COX-1 a d'ores et déjà démontré des effets intéressants pour réduire l'athérosclérose associée au SAOS, mais d'autres stratégies thérapeutiques ciblant le TXA<sub>2</sub> (détaillées dans la revue) sont possibles.

### *3.2. Voie des leucotriènes*

L'écriture de la revue de la littérature consacrée aux LTs (publication n°1) a permis de souligner le rôle du LTB<sub>4</sub> dans l'athérosclérose associée au SAOS, mais aussi de mettre en exergue le manque de données relatives quant au rôle des CysLTs.

Si l'activation de la voie des CysLTs au cours du SAOS n'est plus à démontrer (219–221), les conséquences de cette activation sur le processus athéromateux induit par l'HI restent inconnues ; et ceci alors même que les CysLTs sont doués de propriétés proathérogènes (75) et que leur implication dans l'athérogénèse a déjà été établie (75).

L'objectif premier de cette seconde étude originale a donc été de préciser le rôle de l'HI dans l'activation de la voie des CysLTs et de déterminer le rôle des CysLTs dans l'athérogénèse associée au SAOS. Le second objectif a été d'évaluer l'effet d'un blocage pharmacologique de la voie des CysLTs sur le développement de l'athérosclérose induit par l'HI.

Pour ce travail, l'excrétion urinaire de LTE<sub>4</sub> a été évaluée chez des sujets contrôles et des patients apnéiques nouvellement diagnostiqués. Les déterminants de cette excrétion ont été identifiés parmi les paramètres démographiques, biologiques, polysomnographiques et les antécédents d'évènements cardiovasculaires (infarctus du myocarde, accident vasculaire

cérébral). Afin de compléter cette étude observationnelle chez le patient apnéique, les mécanismes reliant l'HI, la voie des CysLTs et le processus athéromateux ont été étudiés chez la souris ApoE<sup>-/-</sup> soumise à l'HI. Enfin, l'effet d'un traitement pharmacologique par un antagoniste des récepteurs CysLT<sub>1</sub>, le montelukast, a été étudié chez ces souris.

Publication n°5

**“Manipulate leukotriene pathway a new treatment paradigm  
for reducing obstructive sleep apnea cardiovascular  
consequences?”**

E Gautier-Veyret, M Bäck, C Arnaud, E Belaïdi, R Tamisier, P Levy, N Arnol,  
M Perrin, JL Pépin, F Stanke-Labesque.

*Soumis*

Targeting the cysteinyl-leukotriene pathway: a new treatment paradigm for obstructive sleep apnea precision medicine?

Elodie Gautier-Veyret<sup>1,2,3</sup>, Magnus Bäck<sup>4</sup>, Claire Arnaud<sup>1,2</sup>, Elise Belaïdi<sup>1,2</sup>, Renaud Tamisier<sup>1,2,3</sup>, Patrick Lévy<sup>1,2,3</sup>, Nathalie Arnol<sup>3</sup>, Marion Perrin<sup>3</sup>, Jean-Louis Pépin<sup>1,2,3,§</sup>, Françoise Stanke-Labesque<sup>1,2,3,§</sup>

<sup>1</sup>Univ. Grenoble Alpes, HP2, F-38041 Grenoble, France; <sup>2</sup>INSERM U1042, 38041 Grenoble, France; <sup>3</sup>Centre Hospitalier Universitaire des Alpes, 38043, Grenoble, France; <sup>4</sup>Department of Medicine, Karolinska Institute and University Hospital, Stockholm, Sweden; § Co-senior authors.

**Corresponding author:** Pr Françoise Stanke-Labesque. Address: Laboratory of Pharmacology, Grenoble Alpes University Hospital, 217, F-38043 Grenoble Cedex 9, France. Tel: 33.4.76.76.54.92 Fax: 33.4.76.76.89.38 E-mail: [FStanke@chu-grenoble.fr](mailto:FStanke@chu-grenoble.fr)

This article has an online data supplement.

**Abstract (word count: 221)**

**Background** - Obstructive sleep apnea (OSA) characterized by nocturnal intermittent hypoxia (IH) is associated with atherosclerosis and cysteinyl-leukotrienes (CysLT) pathway activation.

**Objectives** - We aimed to identify the determinants of CysLT pathway activation and to precise the role of CysLT in OSA-related atherosclerosis.

**Methods** - Determinants of the urinary excretion of LTE<sub>4</sub> (U-LTE<sub>4</sub>) including history of cardiovascular events, polysomnographic and biological parameters were studied in 170 OSA patients and 29 controls. Mechanisms linking IH, CysLT pathway and IH-related atherogenesis were investigated in Apolipoprotein E deficient (ApoE<sup>-/-</sup>) mice exposed to 8-week IH.

**Results** - In humans, U-LTE<sub>4</sub> was independently influenced by age, minimal oxygen saturation and history of cardiovascular events. U-LTE<sub>4</sub> significantly correlated to left and mean carotid intima-media thickness. In IH mice, atherosclerosis lesion size and mRNA levels of 5-lipoxygenase, 5-lipoxygenase activating protein (FLAP), leukotriene A<sub>4</sub> hydrolase and CysLT<sub>1</sub> receptor were significantly increased. This transcriptional activation was associated with the binding of HIF-1 to the FLAP promoter and was strongly related to the atherosclerosis lesion size. Pharmacological blockade of CysLT<sub>1</sub> receptor by montelukast significantly reduced atherosclerosis progression in IH mice.

**Conclusion** - IH-related CysLT pathway activation contributed to OSA-induced atherogenesis. In the era of personalized medicine, U-LTE<sub>4</sub> might be an interesting biomarker to identify OSA patients for whom CysLT<sub>1</sub> blockade might represent a new therapeutic avenue for reducing their cardiovascular risk.

**Keywords:** obstructive sleep apnea, leukotrienes, atherosclerosis, intermittent hypoxia, pharmacology.

## **List of abbreviations**

ApoE<sup>-/-</sup> : apolipoprotein E deficient

AHI: apnea+hypopnea index

BMI: body mass index

BP: blood pressure

ChIP: chromatin immunoprecipitation

CPAP: continuous positive airway pressure

CysLT: cysteinyl leukotrienes

5-LO: 5-lipoxygenase

FLAP: 5-lipoxygenase activating protein

HIF: hypoxia inducible factor

HRE: hypoxia response element

IH: intermittent hypoxia

IMT: intima-media thickness

LT: leukotriene

LTA<sub>4</sub>: leukotriene A<sub>4</sub>

LTA<sub>4</sub>H: leukotriene A<sub>4</sub> hydrolase

LTC<sub>4</sub>S: leukotriene C<sub>4</sub> synthase

LTE<sub>4</sub>: leukotriene E<sub>4</sub>

OSA: obstructive sleep apnea

## **Introduction**

Obstructive sleep apnea (OSA) characterized by nocturnal intermittent hypoxia (IH) is deleterious for the cardiovascular system and leads to the early development of atherosclerosis (1). Previous data suggest that the leukotriene (LT) pathway is implicated in OSA-related atherosclerosis (2).

LTs are produced in inflammatory cells from arachidonic acid. 5-lipoxygenase (5-LO) interacts with nuclear membrane-bound 5-LO activating protein (FLAP) to convert arachidonic acid into leukotrienes (LT); leukotriene B<sub>4</sub> (LTB<sub>4</sub>) or cysteinyl-LTs (CysLT: leukotrienes C<sub>4</sub>, D<sub>4</sub> and E<sub>4</sub> (LTE<sub>4</sub>)). CysLTs activate the receptors CysLT<sub>1</sub> and CysLT<sub>2</sub> on leukocytes and structural cells of the vascular wall to promote smooth muscle cell contraction, proliferation and alterations in adhesion molecule expression (3). While the role of LTB<sub>4</sub> in OSA-related atherogenesis has been previously demonstrated (4–6), the contribution of the CysLT pathway in OSA-related atherosclerosis remained unexplored. Since the urinary excretion of LTE<sub>4</sub> (U-LTE<sub>4</sub>), a marker of systemic CysLT pathway activation is elevated in OSA patients free of cardiovascular disease (7), CysLT could be implicated in OSA-related atherosclerosis.

We aimed to determine through a translational approach the role of IH in the CysLT pathway activation observed in OSA and its role in IH-related atherogenesis. Firstly, we identified the determinants of U-LTE<sub>4</sub> in a cohort of OSA patients and controls and studied its link with their vascular remodeling. Then, we investigated mechanisms linking IH, LT pathway activation and atherogenesis in apolipoproteinE knock-out (ApoE<sup>-/-</sup>) mice.

## **Material and Methods**

### *Clinical study*

The local ethics committee, per the Declaration of Helsinki, approved this study (“Comité de Protection des Personnes” n° 07-CHUG-8). All participants gave written informed consent and the study was registered (NCT 01089257).



Between January 2007 and December 2012, 170 consecutive newly diagnosed OSA patients and 29 controls were entered in the study (see figure E1 for flow diagram). Patients with symptoms suggesting OSA had been referred to the sleep laboratory of Grenoble University Hospital. There was no overlap with subjects included in our previous study (7). Non-inclusion criteria were: central sleep apnea syndrome, hypercapnic chronic respiratory failure, pregnancy, asthma, rhinitis, arthritis and treatment by CysLT<sub>1</sub> antagonist.

All subjects underwent an overnight polysomnography following the recommendations and scoring rules of the AASM (8). Sleep apnea syndrome was defined as an apnea-hypopnea index (AHI)  $\geq 5$  events per hour of sleep and symptoms or an AHI  $> 15$  events per hour (9).

Biochemical parameters especially U-LTE<sub>4</sub> were measured (additional details are provided in an online data supplement) and history of cardiovascular events (stroke, myocardial infarction) was noted. Carotid ultrasonography was performed in 124 patients and 24 controls (see figure E1 for flow diagram) as previously described (10).

In the subgroup of OSA patients free of past history of cardiovascular event, the 10-year risk of fatal cardiovascular disease score was determined according to the European SCORE project in populations at low risk of cardiovascular disease using the model based on total cholesterol (11). The algorithm proposed by Conroy *et al.* estimates the probability of a fatal cardiovascular event within 10 years from 6 factors: age, sex, cigarette smoking, diabetes mellitus, systolic blood pressure (BP) and total cholesterol (11).

#### *Experimental animal studies*

We exposed 14-week-old male ApoE<sup>-/-</sup> mice to IH or normoxia for 8 weeks in a previously described device (12) (see online data supplement for details). All animal procedures 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 with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

In a first series of experiments, IH induced larger atherosclerotic lesions in ApoE<sup>-/-</sup> mice exposed to 8-week compared to normoxia (n=15 in each group) (13). In these mice, the aortic mRNA levels of 5-LO pathway genes were determined as previously described (14) using primer/probe pairs designed with Assay-On-Demand™ (both Applied Biosystems) (Table E1). Data were normalized to 18S ribosomal protein mRNA and expressed as  $2^{-\Delta\Delta Ct}$ .

A second series of experiments was performed to determine the influence of CysLT<sub>1</sub> receptor antagonism by montelukast on atherosclerosis. Mice similarly exposed to IH or normoxia for 8 weeks were randomized to receive vehicle or montelukast (1mg/kg/d) (15) in their food, for the 4 last weeks of exposure (n=9 or 10 in each group). Atherosclerosis lesion size was investigated in abdominal aortas and hearts were harvested for the *in vivo* chromatin immunoprecipitation (ChIP) assay (see online data supplement for details).

#### *Statistical analysis*

Statistical analyses were performed using SAS 9.4 software (SAS Institute Inc., Cary, NC). If variables have normal distribution (Shapiro-Wilk test) data are expressed as mean ± standard deviation and parametric tests were used; if not, data are expressed as median (25<sup>th</sup>-75<sup>th</sup> percentiles) and non-parametric tests were used. To identify determinants of U-LTE<sub>4</sub>, a logarithmic transformation was applied because distribution of U-LTE<sub>4</sub> was not normal, and simple linear regressions were performed. In case of non-significance, regressions were performed on categorized variables (on the basis of quartiles (example: triglycerides) or previously defined classification (example: presence or absence of obesity defined by a body mass index (BMI) > or ≤ 30 kg/m<sup>2</sup> respectively)) to highlight a potential non-linear relation. After imputation for missing data (using median value of the relevant variable) and study of collinearity (Person's or Spearman tests), variables with a p-value ≤ 0.1 were integrated in a multiple linear model to identify independent determinants of U-LTE<sub>4</sub>. A p-value ≤ 0.05 was considered as statistically significant.

## Results

*Clinical study: determinants of urinary leukotriene E<sub>4</sub> excretion and link with vascular remodeling*

### **Baseline characteristics and urinary leukotriene E<sub>4</sub> excretion in OSA compared to controls**

Baseline characteristics of 170 OSA patients and 29 controls are shown in Table 1. As expected, polysomnographic parameters were significantly different between controls and OSA patients. In addition, OSA patients were older, with a higher proportion of men and had higher BMI, blood pressure (BP), HOMA-IR index, glucose and LDL-cholesterol plasma levels compared to controls. U-LTE<sub>4</sub> was not significantly increased in OSA patients compared to controls in the whole population (Table 1).

### **Determinants of urinary leukotriene E<sub>4</sub> excretion in the whole population**

Since in our previous study, U-LTE<sub>4</sub> was influenced by OSA (especially hypoxia severity) but also obesity (7), determinants of U-LTE<sub>4</sub> were studied in this independent cohort in order to assess the respective influence of OSA and confounders on CysLT pathway. In univariate analysis, U-LTE<sub>4</sub> levels were significantly and positively associated with age, systolic BP, AHI, insulin, HOMA-IR index, secondary cardiovascular prevention but also high sensitive C reactive protein (hsCRP) and triglycerides both stratified on quartiles (Table 2). In addition, significant negative associations were founded between U-LTE<sub>4</sub> and both min SaO<sub>2</sub> and HDL-cholesterol (Table 2).

To determine independent determinants of U-LTE<sub>4</sub>, multivariate analyses were performed (Table 3). The initial model integrated all variables related to U-LTE<sub>4</sub> in univariate analysis with a p-value ≤0.1, except hsCRP, insulin and HOMA-IR index which were highly related to the type of cardiovascular prevention and obesity respectively. In this model, age, min SaO<sub>2</sub> and type of cardiovascular prevention were independent determinants of U-LTE<sub>4</sub>. After a backward selection, age, min SaO<sub>2</sub> and type of cardiovascular prevention remained independent factors of U-LTE<sub>4</sub> (Table 3). To confirm these results, comparisons of U-LTE<sub>4</sub> levels were performed between controls and OSA patients according to the type of cardiovascular prevention (Fig. 1A).

U-LTE<sub>4</sub> was significantly enhanced in OSA patients in primary prevention compared to controls in primary prevention and also in controls in secondary prevention compared to controls in primary prevention (Fig.1A).

### **Determinants of urinary leukotriene E<sub>4</sub> excretion in OSA patients in primary cardiovascular prevention**

To investigate the specific determinants of increased U-LTE<sub>4</sub> observed in OSA patients free of cardiovascular event (see baseline characteristics in Table E2), we used the same methodology as in the whole population. In univariate analysis, age, diastolic and systolic BP, min SaO<sub>2</sub> and HOMA-IR were significantly related to U-LTE<sub>4</sub>, but only age (p=0.02) and min SaO<sub>2</sub> (p=0.04) were identified as independent factors influencing U-LTE<sub>4</sub>.

Since U-LTE<sub>4</sub> was influenced by age (this study) but also by confounders such as diabetes (16) and tobacco (17) which are present in our cohort, we investigated the link between U-LTE<sub>4</sub> and a composite score integrating all these determinants, namely the 10-year cardiovascular score according to the SCORE project (11). This 10-year cardiovascular risk score exhibited a trend towards association with increased U-LTE<sub>4</sub> ( $\beta$  [IC 95%]= 4.18 [-0.67;1.22], p=0.07). But this cardiovascular risk score integrates neither nocturnal hypoxia severity nor BMI while they both influenced the LT pathway activation (present study and others (4, 5, 7, 18)) and the cardiovascular risk (19, 20). Hence we explored several multivariate models to determine the respective influence of hypoxia severity, BMI and the cardiovascular risk score on U-LTE<sub>4</sub> (Table 4). In the model 1 adjusted for BMI U-LTE<sub>4</sub> was not affected by BMI and the 10-year cardiovascular risk score was associated positively with U-LTE<sub>4</sub>, albeit not statistically significant (p=0.09). In the model 2 adjusted for min SaO<sub>2</sub>, the 10-year cardiovascular risk score and min SaO<sub>2</sub> were both independent predictors of U-LTE<sub>4</sub> (Table 4). In the model 3 adjusted for BMI and min SaO<sub>2</sub>, the 10-year cardiovascular risk score and the min SaO<sub>2</sub> were again identified as independent predictors of U-LTE<sub>4</sub> (Table 4). Furthermore, U-LTE<sub>4</sub> levels were different according to subgroup of the cardiovascular risk score (p=0.009) and

tended to be different between the two groups of min SaO<sub>2</sub> (p=0.06) (Fig. 1B).

### **Urinary LTE<sub>4</sub> concentrations in relation to vascular remodeling**

U-LTE<sub>4</sub> was weakly correlated with left and mean intima-media thickness (IMT) (left and mean IMT: r= 0.202, p= 0.014 and r= 0.165, p=0.045 respectively). In addition, U-LTE<sub>4</sub> was not significantly different between subjects with or without atheromatous plaques (presence of plaque (n=61): 64.9 [48.4;99.1] vs absence (n=87): 58.1 [38.4;88.5] pg/mg creatinine; p= 0.14).

*Experimental animal study: 5-LO pathway activation in response to IH and link with atherosclerosis*

### **IH activates 5-LO pathway transcription in aorta**

In IH mice, aortic mRNA levels of 5-LO (p=0.04), FLAP (p=0.01), LTA<sub>4</sub>H (p=0.04) and CysLT<sub>1</sub> receptor (p=0.02) were significantly increased compared to normoxic mice (Fig. 2A).

### **HIF-1 binds to the promoter of the FLAP gene in myocardium**

The CHIP assay enabled to cross-link and immunoprecipitate myocardial hypoxia inducible factor 1 (HIF-1) bound to a myocardial FLAP promoter. Fig. 3A and 3B show the PCR amplification of the DNA sequence previously shown to bind HIF-1 and consequently containing a hypoxia response element (HRE) in the mouse FLAP gene. PCR amplification was obtained only in the immunoprecipitated DNA from the IH group, indicating binding of HIF-1 to the FLAP gene in hypoxic conditions only (Fig. 3B). The absence of amplification in mock conditions confirmed the specificity of the immunoprecipitation technique (Fig. 3B). PCR signals from DNA before immunoprecipitation (input) showed that the results were not due to differences in sample DNA content that was calculated before immunoprecipitation. PCR performed on serial

input DNA dilutions confirmed that the amplification was proportional to DNA quantity (Fig. 3C).

### **mRNA levels of the 5-LO pathway genes are associated with atherosclerosis lesion size in mice exposed to IH**

In IH mice, aortic mRNA levels of 5-LO, FLAP, LTA<sub>4</sub>H, LTC<sub>4</sub>S, CysLT<sub>1</sub>, CysLT<sub>2</sub>, BLT<sub>1</sub> and BLT<sub>2</sub> were significantly associated with atherosclerotic lesion size (Fig. 2B and Table E3); while lesion size in normoxia mice was related to plasma cholesterol levels only (Table E3).

### **Montelukast reduces IH-related atherosclerosis**

Montelukast treatment for 4 weeks did not affect body weight and plasma total cholesterol in mice exposed to IH or normoxia (Table E4). Abdominal aortic lesions were significantly increased in IH mice compared to normoxia mice. Treatment with montelukast significantly reduced abdominal aortic lesions in IH but not in normoxia mice (Fig. 4).

## **Discussion**

Our translational approach allowed us to identify IH as a major determinant of CysLT pathway activation both in OSA patients and mice exposed to IH. In humans, past history of cardiovascular event, age and the 10-year cardiovascular risk also influenced independently U-LTE<sub>4</sub>. CysLT pathway markers were related to vascular remodelling. Finally, pharmacological blockade of CysLT<sub>1</sub> receptor prevented IH-induced atherogenesis in mice.

### **CysLT pathway activation in humans: determinants and link with atherosclerosis**

Our clinical study identified min SaO<sub>2</sub> as an independent determinant of U-LTE<sub>4</sub> both in the whole population and in OSA patients in primary cardiovascular prevention, which is in agreement with the increased U-LTE<sub>4</sub> previously described in OSA patients free of comorbidities compared to age- and BMI- matched controls (7). In addition, age and secondary cardiovascular

prevention were also independently associated with U-LTE<sub>4</sub> in the whole population, which is consistent with inflammation associated with both atherosclerosis (21–23) and aging (24). However, OSA and history of cardiovascular events have no additive influence on U-LTE<sub>4</sub> since U-LTE<sub>4</sub> levels were not significantly different between OSA patients in primary and secondary cardiovascular prevention. Surprisingly BMI influenced weakly U-LTE<sub>4</sub> which is in contrast to our previous study (7). This discrepancy could be explained by the different inclusion criteria since patients with history of cardiovascular events or confounders such as smoking and diabetes were excluded in our previous study but included in the present work, which may have blunted the influence of BMI on U-LTE<sub>4</sub>.

The 10-year risk of fatal cardiovascular disease score (11) has precisely the advantage of integrating several confounders present in our cohort especially presence of diabetes (16) and smoking (17) which are associated with higher U-LTE<sub>4</sub>. Since this composite score has been validated in primary cardiovascular prevention (11) and U-LTE<sub>4</sub> was specifically enhanced in OSA patients free of cardiovascular event, we investigated the link between U-LTE<sub>4</sub> and the cardiovascular risk score in this subgroup. While the 10-year cardiovascular risk was positively associated with U-LTE<sub>4</sub> in univariate analysis without reach significance, this latter was identified as an independent determinant of U-LTE<sub>4</sub> after adjustment on BMI or/and min SaO<sub>2</sub>. Hence, CysLT pathway activation observed in OSA patients free of cardiovascular events was attributable to both hypoxia severity and cardiovascular risk factors included in this 10-year risk of fatal cardiovascular.

LTs are known for their proatherogenic properties (3). LTB<sub>4</sub> stimulates leukocyte chemotaxis and adhesion, while CysLTs promote smooth muscle cell contraction, proliferation and expression of adhesion molecules via CysLT<sub>1</sub> stimulation (3). LTB<sub>4</sub> production by challenged leucocytes is associated with subclinical atherosclerosis in OSA (4) and the present study suggests a link between U-LTE<sub>4</sub> and markers of vascular remodeling. Although these findings together provide strong support for OSA-induced CysLT pathway activation as a driver of atherogenesis in OSA, the observational design of the U-LTE<sub>4</sub> exploration in the human cohort

represents a limitation, which should be acknowledged and which precludes any conclusions in terms of causality. Therefore, we subsequently characterized and explored the CysLT pathway in a mouse model of OSA and atherosclerosis.

### **Mechanisms of CysLT pathway activation in a mouse model of OSA and consequences on atherosclerosis**

IH is clearly known as an inducer of atherosclerosis notably in ApoE<sup>-/-</sup> mice (13, 25). Interestingly, IH induced overall transcriptional upregulation of the LT pathway in aortae with notably increased mRNA levels of FLAP and CysLT<sub>1</sub> receptor. These results are in agreement with the enhanced mRNA levels of LT synthesizing enzymes previously founded after IH (5, 6, 26). However, the mechanisms by which IH activates the transcription of the LT pathway has remained poorly understood. Here, we demonstrated that HIF-1, the activation of which is well described after IH exposure (27), contributes to the activation of the LT pathway by its binding to the FLAP gene promoter in myocardial tissues from IH mice. This result is in accordance with *in vitro* data showing that hypoxia-mediated FLAP expression in endothelial cells is regulated in part by HRE (28). Nevertheless, HIF-1 mediated FLAP activation is probably not sufficient to explain itself the CysLT pathway activation in OSA and others mediators such as NF-κB (6, 28) may act synergistically with HIF-1.

In our experimental study, atherosclerosis lesion size were strongly correlated with mRNA levels of several LT pathway genes, including those specifically involved in CysLT signaling (LTC<sub>4</sub>S and CysLT<sub>1</sub> receptor). These findings are in agreement with the correlation between upregulated mRNA levels of LTB<sub>4</sub> synthesizing enzymes (5) or U-LTE<sub>4</sub> (this study) and early markers of atherosclerosis in OSA patients. Collectively, these data suggest that the LT pathway could be implicated in OSA-related atherogenesis.

### **CysLT<sub>1</sub> receptor antagonism to treat IH-induced atherosclerosis**



As a final proof of concept for the role of CysLT pathway activation in IH-related atherogenesis, we demonstrated a reduction of atherosclerosis by a 4-week treatment with the CysLT<sub>1</sub> receptor antagonist montelukast in mice exposed to IH. In contrast, montelukast had no effect on atherosclerotic lesions in normoxia mice, which provides strong evidence for a specific contribution of the CysLT<sub>1</sub> pathway in IH-induced atherosclerosis. Previous studies of anti-LT treatment in experimental atherosclerosis have generated contradictory findings (14, 15, 29–31), which have led think that the most pronounced effects are observed in models where the LT pathway is selectively up-regulated, such as high fat diet (15, 29, 30, 32), as well as combined genetic alterations of ApoE and either the TGFβ (14) or COX2 pathways (31). The interest of 5-LO pathway disruption in treating atherosclerosis has been suggested by a few clinical studies. In a randomized double blind trial in patients with an acute coronary syndrome, a leukotriene synthesis inhibitor reduced the noncalcified plaque volume (33) and in a nation-wide pharmacoepidemiological study, use of the CysLT<sub>1</sub> receptor antagonist montelukast reduced the risk of myocardial infarction in males, and reduced the risk of stroke in subjects not on angiotensin-modifying drugs (34). Thus, with respect to the activation of the CysLT pathway in OSA (7), the use of montelukast could be clinically highly relevant in this context. This point is of particular interest since continuous positive airway pressure (CPAP), the first line therapy for OSA, has limited efficacy on the cardiovascular consequences of OSA in minimally symptomatic obese OSA patients (35, 36) or in moderate to severe OSA patients with cardiovascular disease (37, 38), highlighting the need for a pharmacological approach in combination with or in substitution of CPAP (39, 40).

There is currently a strong interest in defining different clusters of OSA phenotypes so as to propose more personalized medicine to OSA patients (41–44) and to identify those who will respond favorably to CPAP or pharmacological treatments (precision medicine) (45, 46). In this context, our data suggest the potential interest of U-LTE<sub>4</sub> as a biomarker to identify OSA patients in whom pharmacological blockage of CysLT<sub>1</sub> receptor could be of interest to treat or prevent OSA-related atherogenesis. Obviously CysLTs are not the sole mediators implicated in the

atheromatous process and further researches are needed to validate U-LTE<sub>4</sub> such as a biomarker.

### **Conclusion**

To summarize, as illustrated by the figure 5, we demonstrated 1/ that IH is implicated in the CysLT pathway activation observed in OSA and 2/ that this activation is involved in IH-related vascular remodelling. In the era of precision medicine, pharmacological blockage of the CysLT<sub>1</sub> receptor could be an interesting therapeutic strategy to investigate in OSA patients to treat or to prevent OSA-related atherogenesis.

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## Figure legends

**Fig. 1 Leukotriene E<sub>4</sub> urinary (U-LTE<sub>4</sub>) excretion in controls and OSA patients stratified on the type of cardiovascular prevention (A) and in OSA patients free of cardiovascular event according to the 10-year cardiovascular risk score and the minimal nocturnal oxygen saturation (SaO<sub>2</sub>) (B).**

U-LTE<sub>4</sub> was not logarithmic transformed for this figure. For figure 1A, subgroups of patients were defined by the tertiles for the 10-year cardiovascular risk score and by the median for minimal oxygen saturation (< or > median SaO<sub>2</sub>). Numbers in each histogram bar indicate the numbers of patients (Total number of patients is not equal to 136 due to missing data).

**Fig.2 The leukotriene transcriptional pathway is up regulated in mice exposed to intermittent hypoxia for 8 weeks and is associated with atherosclerosis lesion size.**

A/ Aortic mRNA levels of leukotriene pathway genes in intermittent hypoxia mice compared to normoxia mice. Data are expressed as fold-change compared with normoxic mice (control). B/ Correlations between atherosclerosis aortic root lesion size and aortic mRNA levels of leukotriene pathway genes in intermittent hypoxia mice.

5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; LTA<sub>4</sub>H, leukotriene A<sub>4</sub> hydrolase; LTC<sub>4</sub>S, leukotriene C<sub>4</sub> synthase; N, normoxia; IH, intermittent hypoxia.

**Fig.3 Myocardial *in vivo* chromatin immunoprecipitation (IP) of hypoxia inducible factor-1 (HIF-1) linked to the 5-lipoxygenase activating (FLAP) gene.**

A/ Schematic representation of the FLAP gene with hypoxia response element (HRE).  
B/ PCR amplification of samples on fragmented DNA before IP (Input), after IP without (Mock) and with (IP) anti-HIF-1 antibody in hearts from normoxic (N) or hypoxic (IH) mice. C/ Histogram representing PCR performed on serial DNA dilutions. HRE, hypoxia response element; FLAP, 5-lipoxygenase activating protein; IP, Immunoprecipitation; OD, optic density.

**Fig.4 Atherosclerosis lesion size on abdominal aortas of mice exposed to 8-week normoxia or intermittent hypoxia and treated by montelukast or vehicle.** ORO, Oil-Red-O; IH, intermittent hypoxia; N, normoxia.

**Fig.5 Mechanisms linking intermittent hypoxia, cysteinyl-leukotriene pathway and vascular remodelling.** Intermittent hypoxia (IH) increases mRNA levels of enzymes implicated in leukotriene synthesis, namely 5-lipoxygenase (5-LO) and 5-lipoxygenase activating protein (FLAP). The hypoxia inducible factor 1 (HIF-1), the activation of which is well known after IH (240), binds to the hypoxia response element (HRE) located in the promoter of the FLAP gene, contributing at least in part to FLAP transcriptional activation. Increased expression of 5-LO and FLAP results in increased production of cysteinyl-leukotrienes (CysLTs denoted LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>). After their release, CysLTs bind to CysLT<sub>1</sub> receptor, the expression of which is also increased after IH. Stimulation of CysLT<sub>1</sub> receptors on vascular smooth muscle cells and monocytes or macrophages contributes to vascular remodelling and atherogenesis induced by IH. Blue arrows indicated effects of intermittent hypoxia with continuous arrows indicating



results derived of this study, while dotted arrows concern previous results. AA, acid arachidonic; 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; LTC<sub>4</sub>S, leukotriene C<sub>4</sub> synthase; HIF, hypoxia inducible factor; HRE, hypoxia element response.

**Table1. Baseline characteristics of controls and OSA patients.**

	Controls (n=29)	OSA patients (n=170)	p value
<b>sex</b>			
male	15 (51.7)	138 (81.2)	<b>0.001</b>
female	14 (48.3)	32 (18.8)	
<b>age (yrs)</b>	52.1 [42.8;59.6]	57.3 [50.8;62.5]	<b>0.012</b>
<b>tobacco consumption</b>			
smoking	7 (24.1)	41 (24.3)	0.89
former smoking	11 (37.9)	71 (42.0)	
no smoking	11 (37.9)	57 (33.7)	
<b>body mass index (kg/m<sup>2</sup>)</b>	24.1 [22.8;27.0]	27.4 [24.8;30.1]	<b>&lt;0.0001</b>
<b>systolic BP (mmHg)</b>	122 [110;137]	135 [125;150]	<b>0.002</b>
<b>diastolic BP (mmHg)</b>	79.3 ± 12.9	86.7 ± 13.7	<b>0.007</b>
<b>AHI (events/h)</b>	1.6 [0.7;4.0]	30 [20;41]	<b>&lt;0.0001</b>
<b>RDI (events/h)</b>	7.4 [3.2;11]	41 [29; 55]	<b>&lt;0.0001</b>
<b>minimal nocturnal SaO<sub>2</sub> (%)</b>	90 [89;92]	84 [79-87]	<b>&lt;0.0001</b>
<b>mean nocturnal SaO<sub>2</sub> (%)</b>	95 [93;96]	93 [92;95]	<b>&lt;0.0001</b>
<b>time spent with SaO<sub>2</sub>&lt;90% (%TST)</b>	0 [0;0]	2.2 [0.3;8.0]	<b>&lt;0.0001</b>
<b>RAI (events/h)</b>	5.3 [2.8;9.7]	29 [20;42]	<b>&lt;0.0001</b>
<b>plasma glucose (mmol/l)</b>	4.8 [4.6;5.1]	5.2 [4.8;5.8]	<b>0.002</b>
<b>plasma insulin (μUI/ml)</b>	5.0 [3.5;6.9]	5.8 [4.5;8.4]	<i>0.06</i>
<b>HOMA-IR index</b>	1.0 [0.7;1.6]	1.3 [1.0;2.1]	<b>0.016</b>
<b>total cholesterol (g/l)</b>	1.9 ± 0.4	2.1 ± 0.4	<i>0.07</i>
<b>HDL cholesterol (g/l)</b>	0.6 [0.5;0.7]	0.6 [0.5;0.6]	0.14
<b>LDL cholesterol (g/l)</b>	1.1 ± 0.4	1.3 ± 0.4	<b>0.019</b>
<b>triglycerides (g/l)</b>	0.8 [0.7;1.3]	1.1 [0.8;1.5]	<i>0.06</i>
<b>hsCRP (mg/l)</b>	0.9 [0.6;5.9]	1.6 [0.9;3.3]	<i>0.09</i>
<b>type of cardiovascular prevention</b>			
primary	22 (75.9)	136 (80.0)	0.56
secondary	7 (24.1)	34 (20.0)	
<b>U-LTE<sub>4</sub> (pg/mg creatinine)</b>	54.4 [36.6;80.1]	63.7 [43.7;93.2]	0.23

Abbreviations: BP, blood pressure; AHI, apnea/hypopnea index; RDI, respiratory disturbance index; SaO<sub>2</sub>, arterial oxygen saturation; TST, total sleep time; RAI, respiratory arousal index; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high-sensitivity C reactive protein; U-LTE<sub>4</sub>, urinary excretion of leukotriene E<sub>4</sub>.

Quantitative variables are expressed as mean ± standard deviation or median [25<sup>th</sup>-75<sup>th</sup> percentiles] as if the variables were normally distributed or not. Qualitative variables are expressed as n (%). Bold and italic p values indicate respectively p values ≤ 0.05 and ≤ 0.1.

\*Obesity was defined by a body mass index >30kg/m<sup>2</sup>.

**Table 2. Linear regression between U-LTE<sub>4</sub> and demographic, polysomnographic and metabolic parameters in the whole population**

<b>Variables</b>	<b>Estimate [IC 95%]</b>	<b>p value</b>
<b>sex:</b> F vs M	-0.0036 [-0.1970 ; 0.1898]	0.97
<b>age</b>	0.0117 [0.0037 ; 0.0197]	<b>0.005</b>
<b>tobacco consumption:</b>		0.70
former smoking vs never smoking	0.0516 [-0.1660 ; 0.2691]	0.64
smoking vs never smoking	0.0801 [-0.1092 ; 0.2694]	0.41
<b>obesity*:</b> absence vs presence	-0.1600 [-0.3540 ; 0.0336]	<i>0.10</i>
<b>systolic BP</b>	0.0046 [0.0004 ; 0.0088]	<b>0.03</b>
<b>diastolic BP</b>	0.0034 [-0.0025 ; 0.0094]	0.25
<b>AHI</b>	0.0040 [0.0002 ; 0.0078]	<b>0.04</b>
<b>RDI</b>	0.0034 [-0.0007 ; 0.0075]	<i>0.10</i>
<b>minimal nocturnal SaO<sub>2</sub></b>	-0.0160 [-0.0270 ; -0.0050]	<b>0.004</b>
<b>mean nocturnal SaO<sub>2</sub></b>	0.0107 [-0.0276 ; 0.0490]	0.58
<b>time spent with SaO<sub>2</sub>&lt;90% (%TST)</b>	0.0044 [-0.0024 ; 0.0111]	0.21
<b>RAI</b>	0.0047 [-0.0003 ; 0.0096]	<i>0.06</i>
<b>plasma glucose</b>	0.0134 [-0.0627 ; 0.0894]	0.73
<b>plasma insulin</b>	0.0240 [0.0006 ; 0.04740]	<b>0.05</b>
<b>HOMA-IR index</b>	0.0840 [0.0024 ; 0.1656]	<b>0.05</b>
<b>total cholesterol</b>	-0.0599 [-0.2539 ; 0.1340]	0.54
<b>HDL cholesterol</b>	-0.5406 [-1.0330 ; -0.0483]	<b>0.03</b>
<b>LDL cholesterol</b>	0.0587 [-0.1708 ; 0.2882]	0.61
<b>triglycerides quartiles:</b>		<b>0.03</b>
<i>Q1 vs Q4</i>	-0.2875 [-0.5170 ; -0.0581]	<b>0.014</b>
<i>Q2 vs Q4</i>	-0.1490 [-0.3703 ; 0.0722]	0.19
<i>Q3 vs Q4</i>	0.0295 [-0.1951 ; 0.2540]	0.80
<b>hsCRP quartiles :</b>		<b>0.001</b>
<i>Q1 vs Q4</i>	-0.4660 [-0.7025 ; -0.2294]	<b>&lt;0.0001</b>
<i>Q2 vs Q4</i>	-0.3424 [-0.5649 ; -0.1199]	<b>0.003</b>
<i>Q3 vs Q4</i>	-0.2456 [-0.4726 ; -0.0186]	<b>0.034</b>
<b>type of cardiovascular prevention</b>		
primary vs secondary	-0.3207 [-0.5191 ; -0.1223]	<b>0.002</b>

Abbreviations: F, female; M, male; BP, blood pressure; AHI, apnea-hypopnea index; RDI, respiratory disturbance index; SaO<sub>2</sub>, arterial oxygen saturation; TST, total sleep time; RAI, respiratory arousal index; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high sensitive C reactive protein; Q, quartile.

Bold and italic p values indicate respectively p values ≤ 0.05 and ≤ 0.1.

\*Obesity was defined by a body mass index >30kg/m<sup>2</sup>.

**Table 3. Determinants of U-LTE<sub>4</sub> in different multivariate models in the whole population**

<b>Models and variables</b>	<b>Estimate [IC 95%]</b>	<b>p value</b>
<b>Initial model</b>		
age	0.0089 [0.0006;0.0172]	<b>0.04</b>
systolic BP	0.0008 [-0.0036;0.0052]	0.72
obesity (absence vs presence)	-0.0158 [-0.2202;0.1885]	0.88
min SaO <sub>2</sub>	-0.0112 [-0.0223;-0.0001]	<b>0.05</b>
HDL cholesterol	-0.3050 [-0.8037;0.1938]	0.23
triglycerides quartiles		0.41
Q1 vs Q4	-0.1316 [-0.3718;0.1086]	0.28
Q2 vs Q4	-0.1233 [-0.3436;0.0970]	0.27
Q3 vs Q4	0.0267 [-0.1934;0.2469]	0.81
type of cardiovascular prevention		
primary vs secondary	-0.358 [-0.5545;-0.1614]	<b>0.0004</b>
<b>Final model</b>		
age	0.0096 [0.0016;0.0175]	<b>0.019</b>
min SaO <sub>2</sub>	-0.0133 [-0.0241;-0.0025]	<b>0.016</b>
type of cardiovascular prevention		
primary vs secondary	-0.3187 [-0.5105;-0.1269]	<b>0.001</b>

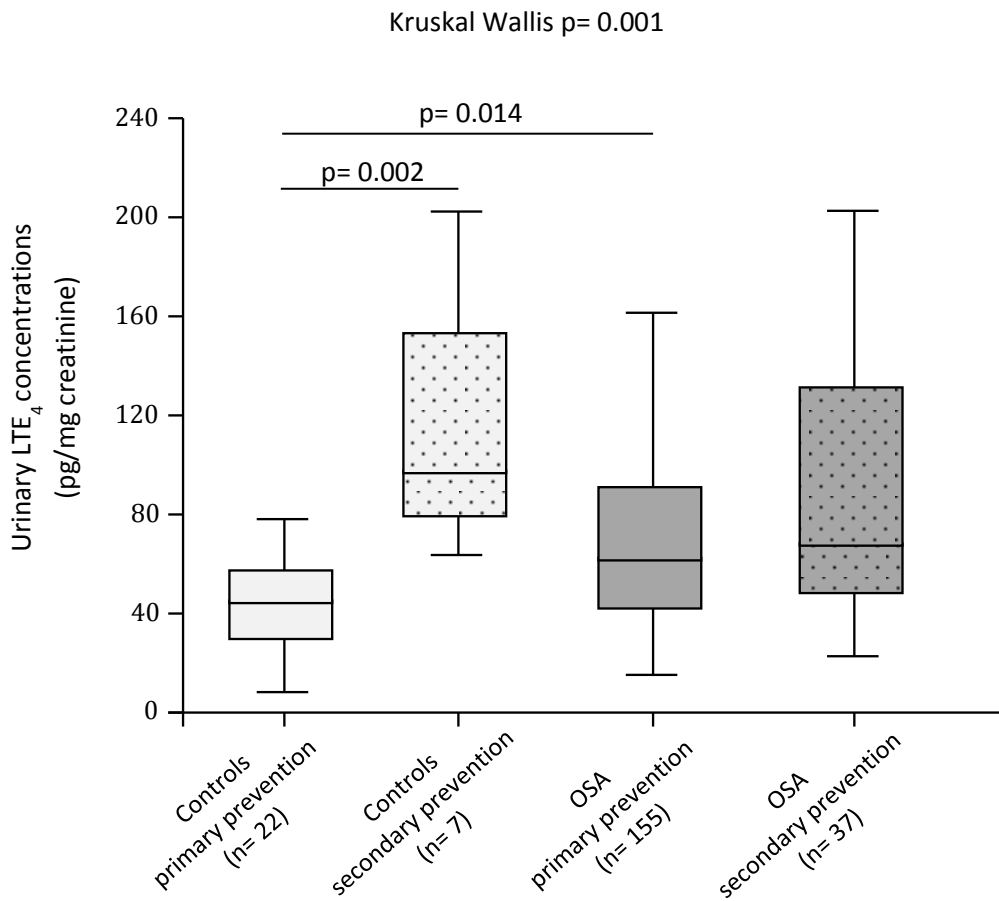
Abbreviations: BP, blood pressure; min SaO<sub>2</sub>, minimal nocturnal arterial oxygen saturation; Q, quartile.

**Table 4. Association between the 10-year cardiovascular risk score, minimal arterial oxygen saturation, body mass index and U-LTE<sub>4</sub> in OSA patient free of history of cardiovascular events in different multivariate models.** Model 1 integrates BMI and cardiovascular risk score, model 2 min SaO<sub>2</sub> and cardiovascular risk score and model 3 min SaO<sub>2</sub>, BMI and cardiovascular risk score. In these models, U-LTE<sub>4</sub> was logarithmic transformed.

	variables	estimate	95% confidence interval	p value
Model 1	BMI	0.05	[-0.04;0.14]	0.25
	cardiovascular risk score	3.81	[-0.67;8.29]	0.09
Model 2	min SaO <sub>2</sub>	-0.06	[-0.11;-0.01]	<b>0.01</b>
	cardiovascular risk score	4.83	[0.63;9.03]	<b>0.02</b>
Model 3	min SaO <sub>2</sub>	-0.06	[-0.10;-0.01]	<b>0.03</b>
	BMI	0.04	[-0.04;0.13]	0.34
	cardiovascular risk score	4.53	[0.28;8.77]	<b>0.04</b>

BMI, body mass index; min SaO<sub>2</sub>, minimal arterial oxygen saturation.

A



B

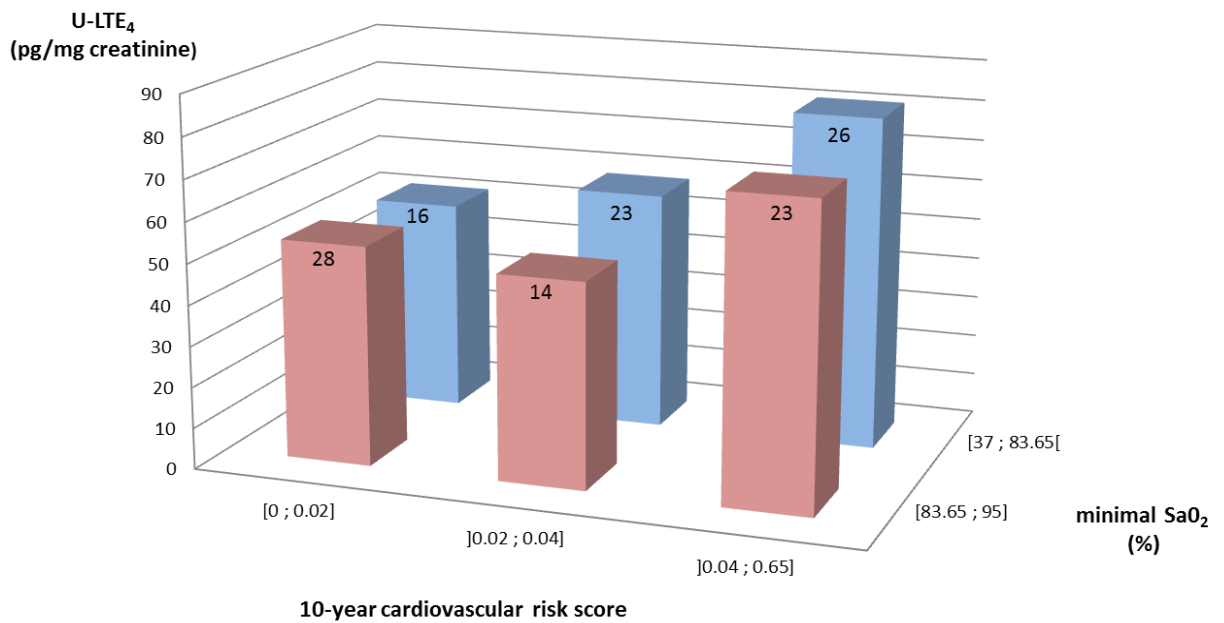
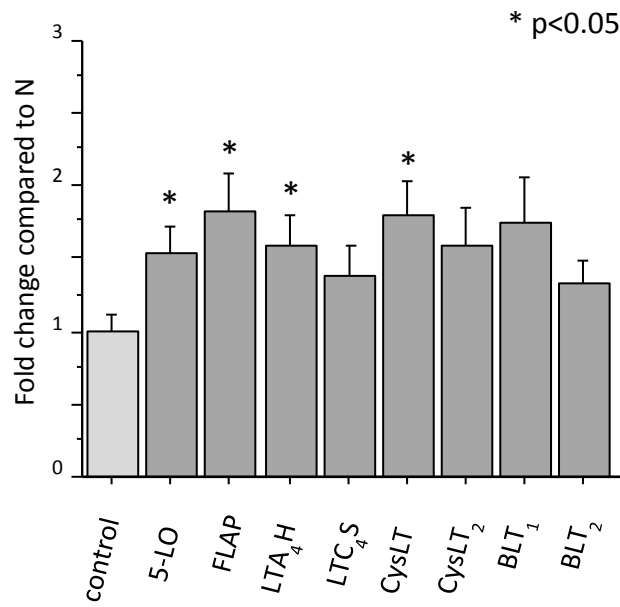
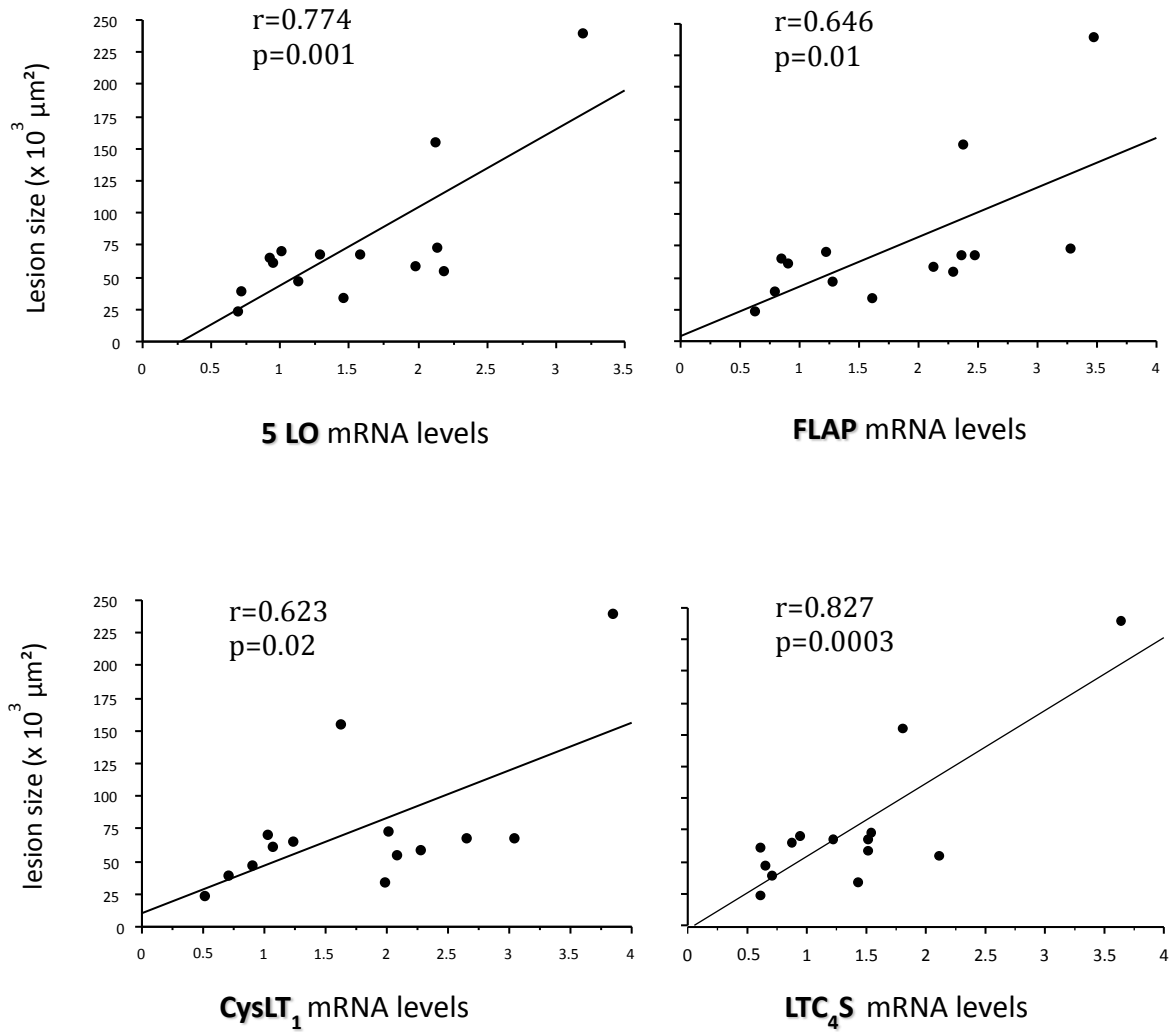
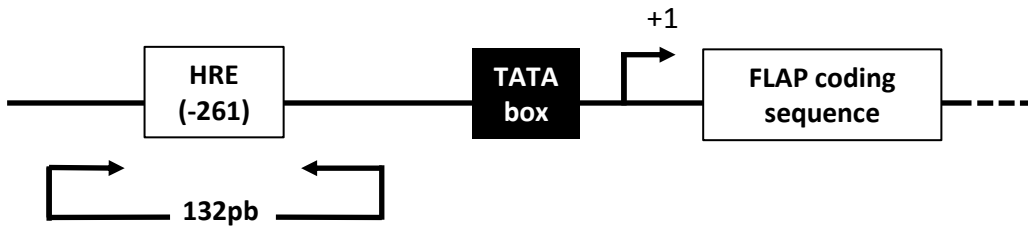


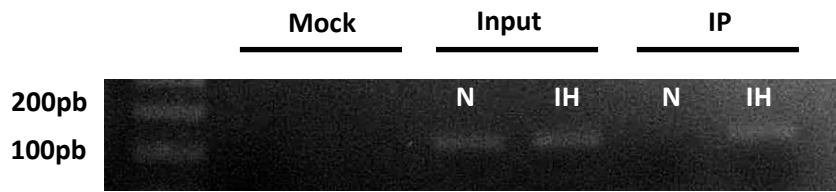
Figure 1

**A****B****Figure 2**

A



B



C

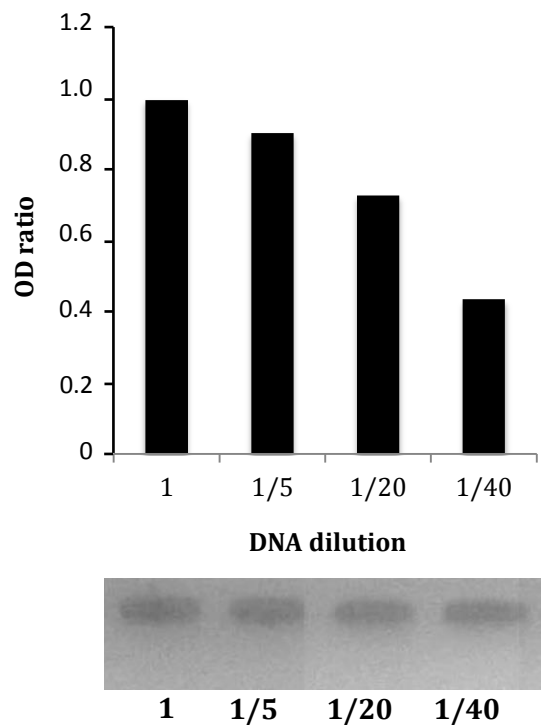


Figure 3



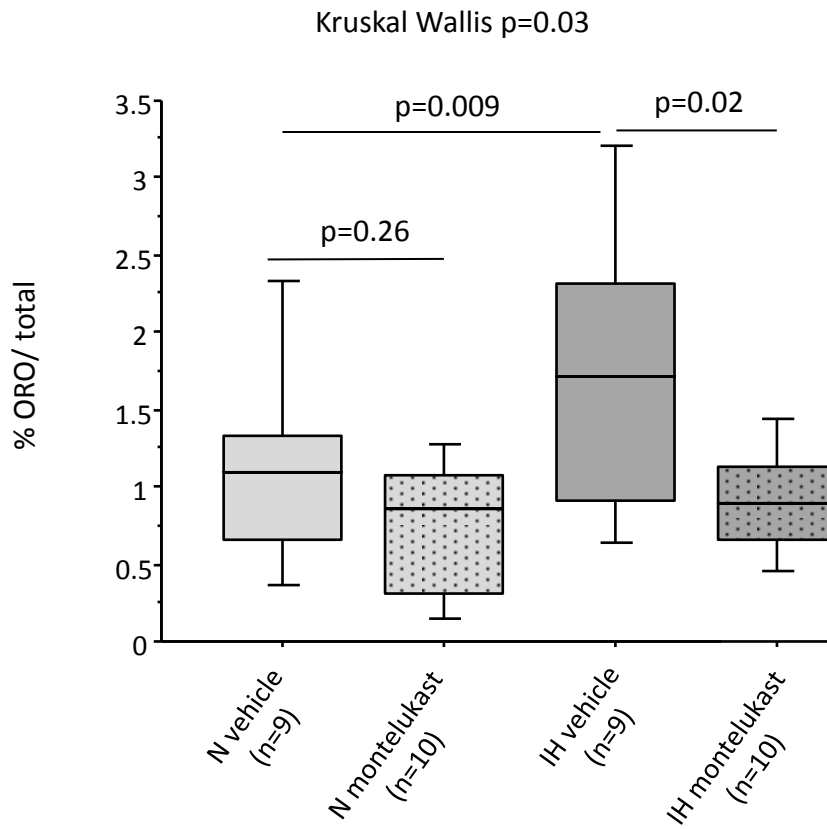


Figure 4

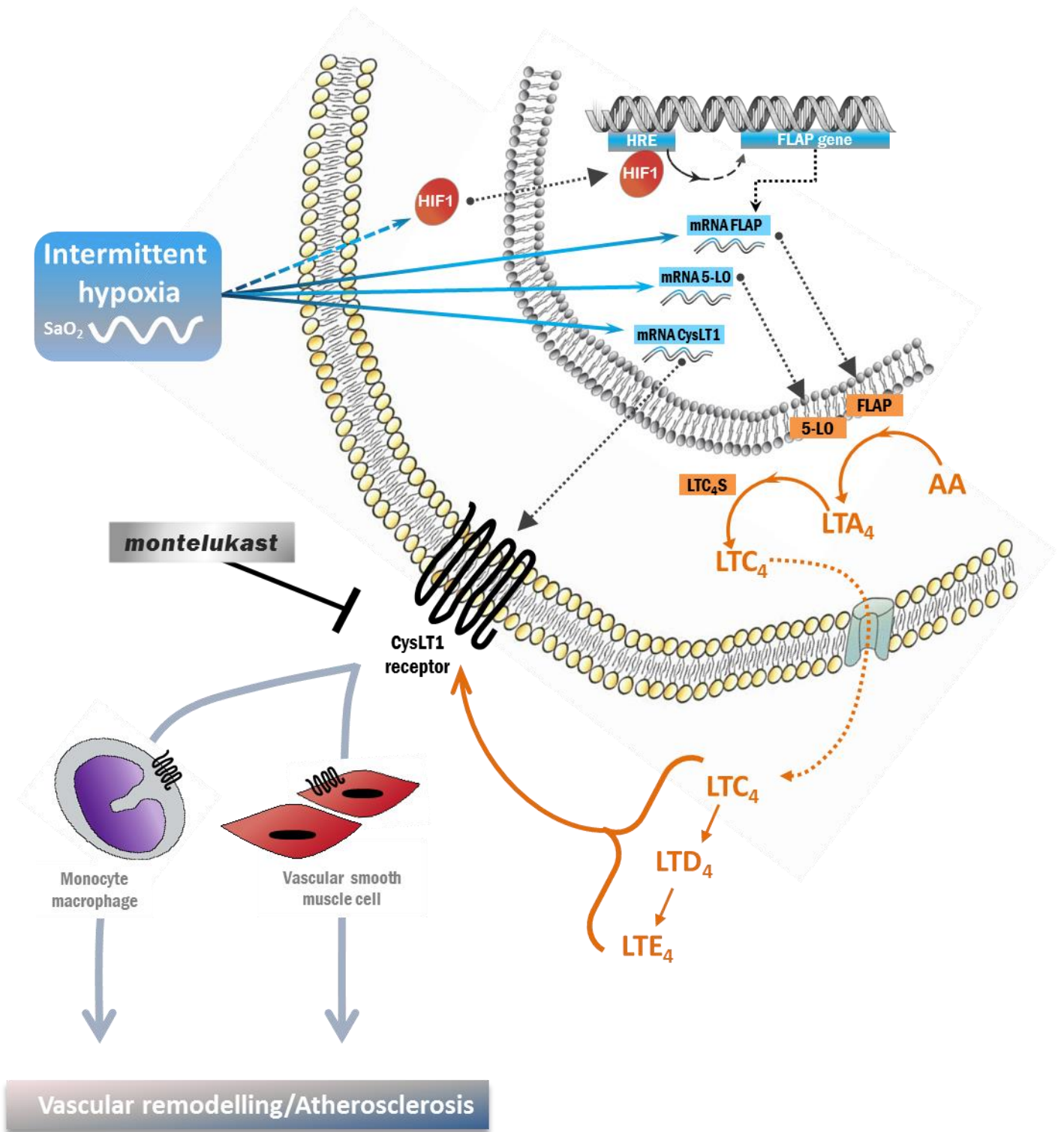


Figure 5

## **Online Data Supplement**

Targeting the cysteinyl-leukotriene pathway: a new treatment paradigm for obstructive sleep apnea precision medicine?

Elodie Gautier, Magnus Bäck, Claire Arnaud, Elise Belaïdi, Renaud Tamisier, Patrick Lévy, Nathalie Arnol, Marion Perrin, Jean-Louis Pépin, Françoise Stanke-Labesque

## Supplemental Materials

### Biochemical measurements in clinical study

Urine samples and venous blood were collected at 7 am at the end of the nocturnal polysomnographic recordings. Biochemical measurements were performed immediately after sampling (plasma glucose, cholesterol, triglycerides, insulin, C reactive protein and urine creatinine) using enzymatic colorimetric methods on a Dimension Vista analyzer (Siemens AG, Erlangen, Germany). Urine samples for LTE<sub>4</sub> measurement were stored at -80 °C until later analysis by liquid chromatography-tandem mass spectrometry as previously described (E1).

### Animal procedures

We exposed 14-week-old male ApoE<sup>-/-</sup> mice to IH or normoxia for 8 weeks in a previously described device (cyclic 21–5% FiO<sub>2</sub>, 60s cycle, normoxic and hypoxic phases both for 30s) (E2). Mice were exposed to IH for 8 hours per day during daytime and were fed with a standard diet *ad libitum* throughout the experimental period. At the end of the experimental period, mice were anesthetized (ketamine/xylazine 100 mg/kg-10 mg/kg, intraperitoneal injection). Blood was collected by cardiac puncture and plasma cholesterol was determined using the infinity kit (Thermo Fischer Scientific®). Entire aortas and hearts were harvested for investigations.

### Atherosclerosis quantification

Atherosclerosis was evaluated by Oil-Red-O staining of abdominal aorta. Abdominal aorta were fixed in 2% paraformaldehyde, washed, opened longitudinally and stained with Oil-Red-O. Images were analysed by manual contouring (abdominal aorta) using computer image analysis (NisElement®) by an investigator who was blinded to the experimental group.

### Myocardial *in vivo* chromatin immunoprecipitation assay of HIF-1

Myocardial samples (~200 mg) from IH and normoxia mice were harvested and treated for 10 min with formaldehyde (1%) at room temperature. Cross-linking was stopped by the addition of

glycine to a final concentration of 125 mM. Tissues were homogenized as previously described (E3, E4). Cross-linked extracts were fragmented enzymatically using Micrococcal Nuclease (Enzymatic Chromatin IP kit, Cell signaling, Ozyme) (input sample). Then, 10 µg of chromatin extract was incubated overnight at 4°C with a ChIP-grade anti-HIF-1α antibody (ab2185; Abcam) or without antibody (mock condition sample). Immunoprecipitated DNA-protein complexes were bound to magnetic beads for 2 h at 4°C and washed in low-salt buffer and high-salt buffer (Enzymatic Chromatin IP kit, Ozyme). After protein elimination with proteinase K and DNA extraction, PCR was performed. PCR primers were selected using previous data characterizing 4 potential HRE (E5), of which one (-261bp) amplifies a 132-bp DNA sequence in mice genomic DNA. The forward primer sequence used was 5'-CTCTGTCTGACCTGAAAGTTTTGGG-3', and the reverse primer sequence was 5'-TGAATCCTGGACCCAAGACCATG-3'. PCR amplification products were analyzed on ethidium bromide-stained 2% agarose gels during the exponential phase of the PCR (39 cycles).

## References

- E1. Stanke-Labesque F, Bäck M, Lefebvre B, Tamisier R, Baguet J-P, Arnol N, Lévy P, Pépin J-L. Increased urinary leukotriene E4 excretion in obstructive sleep apnea: effects of obesity and hypoxia. *J Allergy Clin Immunol* 2009;124:364–70, 370.
- E2. Dematteis M, Godin-Ribuot D, Arnaud C, Ribuot C, Stanke-Labesque F, Pépin J-L, Lévy P. Cardiovascular consequences of sleep-disordered breathing: contribution of animal models to understanding the human disease. *ILARJ* 2009;50:262–81.
- E3. Belaidi E, Beguin PC, Levy P, Ribuot C, Godin-Ribuot D. Prevention of HIF-1 activation and iNOS gene targeting by low-dose cadmium results in loss of myocardial hypoxic preconditioning in the rat. *Am J Physiol Heart Circ Physiol* 2008;294:H901–8.
- E4. Belaidi E, Joyeux-Faure M, Ribuot C, Launois SH, Levy P, Godin-Ribuot D. Major role for hypoxia inducible factor-1 and the endothelin system in promoting myocardial infarction and hypertension in an animal model of obstructive sleep apnea. *J Am Coll Cardiol* 2009;53:1309–17.
- E5. Gonsalves CS, Kalra VK. Hypoxia-mediated expression of 5-lipoxygenase-activating protein involves HIF-1alpha and NF-kappaB and microRNAs 135a and 199a-5p. *J Immunol* 2010;184:3878–88.

## Supplemental Tables

**Table E1. Assay-on-demand™ from Applied Biosystems, used for real-time TaqMan PCR.**

<b>Gene of interest</b>	<b>Abbreviation</b>	<b>Assay number</b>
5-lipoxygenase	5-LO	Mm01182748_m1
5-lipoxygenase activating protein	FLAP	Mm00802100_m1
leukotriene A <sub>4</sub> hydrolase	LTA <sub>4</sub> H	Mm00521826_m1
leukotriene C <sub>4</sub> synthase	LTC <sub>4</sub> S	Mm00521864_m1
leukotriene B <sub>4</sub> receptor 1	BLT <sub>1</sub>	Mm00521839_m1
leukotriene B <sub>4</sub> receptor 2	BLT <sub>2</sub>	Mm00498491_s1
cysteinyl leukotriene receptor 1	CysLT <sub>1</sub>	Mm00445433_m1
cysteinyl leukotriene receptor 2	CysLT <sub>2</sub>	Mm02620584_s1
18S ribosomal protein	18S	Hs99999901_s1

**Table E2. Baseline characteristics of OSA patients free of cardiovascular event**

variables	OSA patients (n=136)
<b>male (%)</b>	82.4
<b>age (yrs)</b>	57.4 [50.8-62.3]
<b>smoking/former smoking/no smoking (%)</b>	45/20/35
<b>body mass index (kg/m<sup>2</sup>)</b>	27.3 [24.8-29.7]
<b>systolic BP (mmHg)</b>	134 [125-150]
<b>diastolic BP (mmHg)</b>	85 [77-95]
<b>AHI (events/h)</b>	30 [20-43]
<b>RDI (events/h)</b>	41 [29-57]
<b>minimal nocturnal SaO<sub>2</sub> (%)</b>	84 [79-87]
<b>mean nocturnal SaO<sub>2</sub> (%)</b>	93 [92-94]
<b>time spent with SaO<sub>2</sub>&lt;90% (%TST)</b>	2.0 [0.3-8.0]
<b>RAI (events/h)</b>	28 [19-41]
<b>plasma glucose (mmol/l)</b>	5.1 [4.8-5.7]
<b>plasma insulin (μUI/ml)</b>	5.8 [4.1-7.8]
<b>HOMA-IR index</b>	1.3 [1.0-1.9]
<b>total cholesterol (g/l)</b>	2.1 [1.9-2.4]
<b>LDL cholesterol (g/l)</b>	1.3 [1.1-1.6]
<b>HDL cholesterol (g/l)</b>	0.6 [0.5-0.7]
<b>triglycerides (g/l)</b>	1.0 [0.8-1.4]
<b>hsCRP (mg/l)</b>	1.4 [0.8-2.5]
<b>10-year cardiovascular risk score (%)</b>	0.027 [0.015- 0.066]
<b>U-LTE<sub>4</sub> (pg/mg creatinine)</b>	62.4 [43.0-90.9]

Data are expressed as median [25<sup>th</sup>-75<sup>th</sup> percentiles] or percentages. BP, blood pressure; AHI, apnea/hypopnea index; RDI, respiratory disturbance index; SaO<sub>2</sub>, arterial oxygen saturation; TST, total sleep time; RAI, respiratory arousal index; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high-sensitivity C reactive protein.



**Table E3. Regression between atherosclerosis lesion size and mRNA levels of leukotriene-pathway genes and plasma total cholesterol after intermittent hypoxia or normoxia.**

	mRNA levels								Total cholesterol
	5-LO	FLAP	LTA <sub>4</sub> H	LTC <sub>4</sub> S	CysLT <sub>1</sub>	CysLT <sub>2</sub>	BLT <sub>1</sub>	BLT <sub>2</sub>	
<b>IH (n=14)</b>	<b>r=0.774</b> <b>p=0.001</b>	<b>r=0.646</b> <b>p=0.01</b>	<b>r=0.676</b> <b>p=0.008</b>	<b>r=0.827</b> <b>p=0.000</b> <b>3</b>	<b>r=0.623</b> <b>p=0.02</b>	<b>r=0.638</b> <b>p=0.01</b>	<b>r=0.633</b> <b>p=0.02</b>	<b>r=0.593</b> <b>p=0.03</b>	r=0.239 p=0.2
<b>N (n=14)</b>	r=-0.210 p=0.5	r=0.112 p=0.7	r=-0.049 p=0.9	r=0.210 p=0.5	r=-0.084 p=0.8	r=0.077 p=0.8	r=0.063 p=0.8	r=0.098 p=0.8	<b>r=0.697</b> <b>p=0.007</b>

Abbreviations: IH, intermittent hypoxia; N, normoxia; 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase activating protein; LTA<sub>4</sub>H, leukotriene A<sub>4</sub> hydrolase; LTC<sub>4</sub>S, leukotriene C<sub>4</sub> synthase.

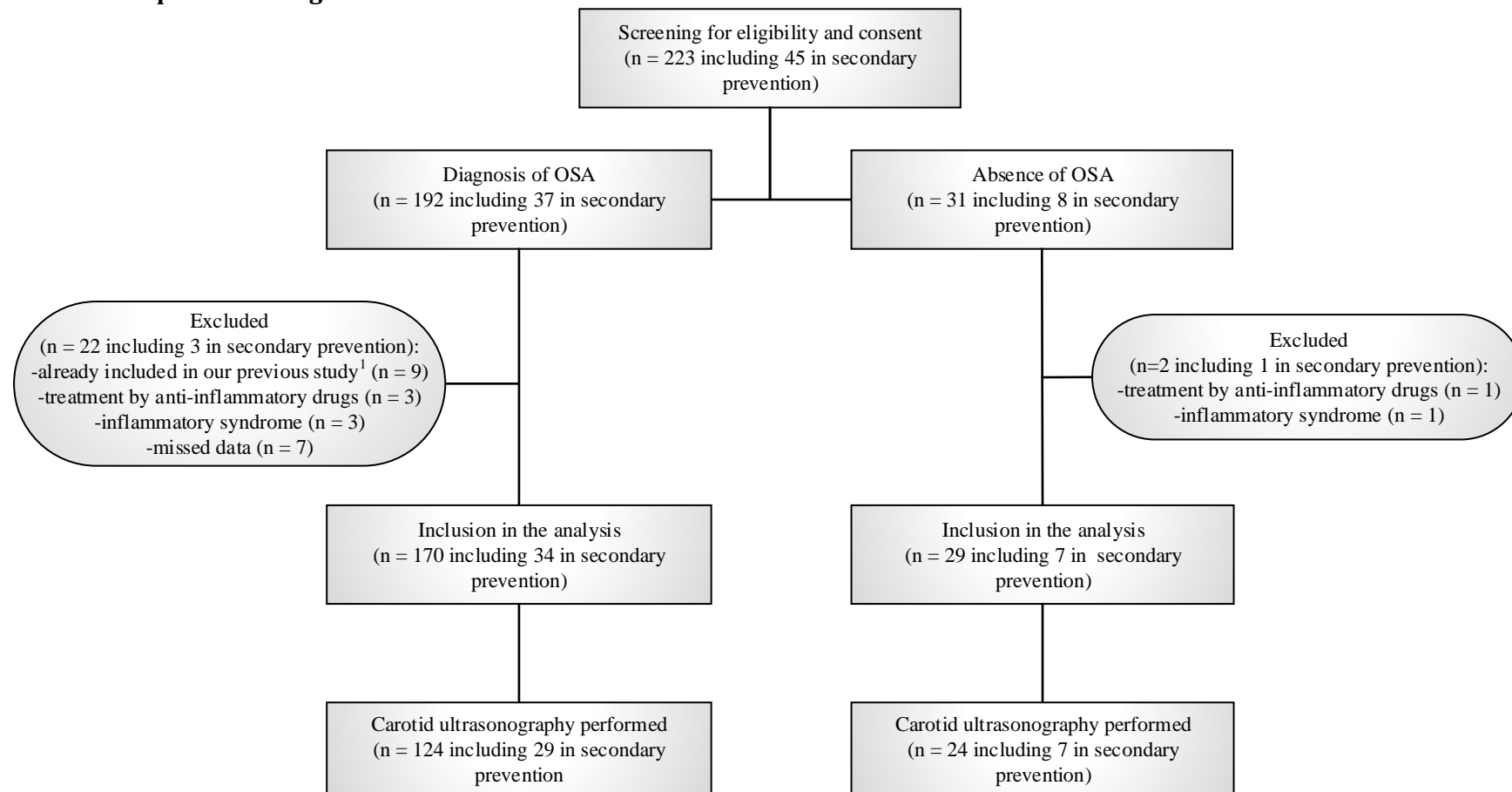
Bold characters indicate significant regressions.

**Table E4. Weight and plasma total cholesterol of mice exposed to intermittent hypoxia (IH) or normoxia (N) and treated with montelukast or vehicle.**

	<b>N vehicle (n=9)</b>	<b>N montelukast (n=10)</b>	<b>IH vehicle (n=9)</b>	<b>IH montelukast (n=10)</b>	p value
weight at day 0 (g)	29.1 [25.7-30.8]	29.1 [24.7-32.3]	29.4 [25.9-30.7]	29.5 [25.7-30.8]	0.7
weight at day 56 (g)	30.3 [27.6-32.2]	30.8 [25.9-33.2]	29.5 [25.0-30.3]	29.1 [26.1-30.7]	0.07
total cholesterol (g/l)	6.1 [3.6-7.2]	5.1 [4.0-7.2]	5.5 [4.0-7.4]	5.8 [3.9-8.1]	0.7

Data are expressed as median [25<sup>th</sup>-75<sup>th</sup> percentiles]. P values were obtained with the Kruskal Wallis test.

**Figure E1. Participant flow diagram**



<sup>1</sup>Stanke-Labesque F, Bäck M, Lefebvre B, Tamisier R, Baguet J-P, Arnol N, Lévy P, Pépin J-L. Increased urinary leukotriene E<sub>4</sub> excretion in obstructive sleep apnea: effects of obesity and hypoxia. *J Allergy Clin Immunol* 2009;124:364–70, 370.

L'implication de l'HI dans l'activation de la voie des LTs et le rôle joué par ces médiateurs dans l'athérosclérose associée au SAOS avaient déjà été démontrés pour le LTB<sub>4</sub> (79,80). Avec cette nouvelle étude, ces connaissances ont été élargies à la voie des CysLTs.

Notre étude a clairement identifié l'hypoxie comme un déterminant majeur de l'activation de la voie des CysLTs au cours du SAOS que ce soit chez l'homme comme dans notre modèle murin d'HI, en démontrant notamment que le facteur de transcription HIF1 se fixait sur le promoteur d'une enzyme clé de la synthèse des LTS, à savoir la FLAP. Chez l'homme, d'autres facteurs tels que l'âge avancé ou un antécédent d'évènement cardiovasculaire semblaient également associés à une excrétion majorée de LTE<sub>4</sub>. Les marqueurs de la voie des CysLTs étaient corrélées aux paramètres de remodelage vasculaire à la fois chez l'homme et la souris, et un traitement par montelukast, antagoniste du récepteur CysLT<sub>1</sub> réduisait l'athérosclérose en lien avec l'HI chez les souris. Ce résultat est en accord avec la réduction du risque d'évènements cardiovasculaires décrite, au cours d'une étude pharmacoépidémiologique suédoise, chez les sujets en prévention secondaire traités par montelukast (246). Ces données sont d'autant plus intéressantes que le montelukast est un médicament peu onéreux commercialisé de longues dates comme traitement adjuvant de l'asthme. A ce titre, il pourrait constituer une alternative thérapeutique à bas coût pour lutter contre l'athérosclérose associée au SAOS, notamment pour les patients peu observants au traitement par PPC ou ceux chez qui le bénéfice de la PPC en terme de réduction du risque cardiovasculaire n'est pas démontré (87,101,116). Dans ce contexte, un projet d'essai multicentrique randomisé contrôlé de traitement par montelukast versus placebo chez le patient apnéique peu symptomatique en prévention secondaire a été déposé au Protocole Hospitalier de Recherche Clinique interrégional 2016.

## **Discussion générale**

Au cours de ces différents travaux, nous avons mis en évidence une activation des voies métaboliques du TXA<sub>2</sub> et des CysLTs au cours du SAOS et l'implication de ces deux voies dans le processus athéromateux induit par l'HI. De plus, des traitements pharmacologiques bloquant spécifiquement la voie du TXA<sub>2</sub> ou des CysLTs étaient capables de ralentir la progression de l'athérosclérose en lien avec l'HI dans un modèle murin de SAOS. L'ensemble de ces résultats suggère donc que des interventions pharmacologiques visant le TXA<sub>2</sub> ou les CysLTs pourraient constituer des stratégies thérapeutiques intéressantes pour ralentir le processus athéromateux des patients apnéiques et donc diminuer leur risque cardiovasculaire.

### **Activation des voies du thromboxane A<sub>2</sub> et des cystéinyl-leucotriènes au cours du SAOS : effet de l'hypoxie intermittente**

Nous avons démontré une activation des voies du TXA<sub>2</sub> et des CysLTs au cours du SAOS que ce soit dans notre modèle murin, comme chez les patients souffrant de SAOS. Chez la souris soumise à l'HI, cette activation a été objectivée par une expression génique aortique augmentée des enzymes impliquées dans la synthèse du TXA<sub>2</sub> et des CysLTs accompagnée d'une augmentation de l'expression du récepteur CysLT<sub>1</sub>. Chez l'homme, nous avons quantifié l'excrétion urinaire de 11dTXB<sub>2</sub> (publication n°3 (82)) et de LTE<sub>4</sub> (publication n°5), marqueurs respectifs de la production systémique de TXA<sub>2</sub> et de CysLTs. Les concentrations urinaires de 11dTXB<sub>2</sub> n'étaient pas significativement différentes entre des volontaires sains et des sujets SAOS indemnes de pathologies cardiovasculaires et appariés sur l'âge et l'indice de masse corporelle (publication n°3 (82)). Ces données, ainsi que le fait que le traitement par PPC n'ait pas d'impact sur les concentrations urinaires de 11dTXB<sub>2</sub> (77,248), suggèrent que le SAOS n'est pas suffisant pour induire à lui seul une activation de la voie du TXA<sub>2</sub>. Bien que cette hypothèse s'oppose aux résultats de précédentes études expérimentales (82,249-251), elle a récemment été confirmée à deux reprises par une équipe polonaise qui rapporte des concentrations similaires des métabolites du TXA<sub>2</sub> chez des volontaires sains et des patients SAOS (252,253).

En revanche, pour ce qui est des LTs, le SAOS -et plus précisément sa composante hypoxique- semble bien être impliqué dans l'activation de cette voie métabolique (221). Des études cliniques antérieures ont en effet rapporté une production accrue de LTB<sub>4</sub> (79,80) et de CysLTs (78) chez les individus apnéiques en lien avec la sévérité de l'hypoxie. Ces concentrations majorées de LTs s'expliquaient en partie par une augmentation de l'expression leucocytaire de la FLAP (80) et étaient corrigées par un traitement bien conduit par PPC (78,79). Notre étude originale sur la voie des CysLTs (publication n°5) confirme donc l'influence de l'HI sur l'activation de la voie des LTs au cours du SAOS. Bien que l'excrétion urinaire de LTE<sub>4</sub> n'était pas significativement différente entre les sujets contrôles et les patients SAOS dans l'ensemble de la population, cette dernière était majorée chez les patients apnéiques en prévention cardiovasculaire primaire en comparaison de celle de volontaires sains, également exempts d'antécédent cardiovasculaire. Parmi les paramètres polysomnographiques, l'IAH et la saturation minimale en O<sub>2</sub> étaient significativement liés aux concentrations de LTE<sub>4</sub> en analyses univariées et la saturation minimale en O<sub>2</sub> constituait un facteur indépendant de l'excrétion urinaire de LTE<sub>4</sub>. Enfin, nos travaux ont également apporté des éléments mécanistiques en démontrant que le facteur de transcription HIF1, dont l'activation est largement démontrée au cours du SAOS (240), était capable de se fixer au promoteur de la FLAP. Ainsi, HIF1 contribuerait au moins en partie à l'expression augmentée de la FLAP observée dans les leucocytes de sujets apnéiques ou de sujets sains exposés *in vitro* à une IH (80).

Outre l'activation des voies du TXA<sub>2</sub> [publication n°3 + 64, 70] et des LTs [33-35, 56, publication n°5], d'autres voies de métabolisation de l'acide arachidonique semblent également activées au cours du SAOS. Ainsi, des concentrations augmentées d'isoprostanes (45,46,48) ou encore de *lipocalin-type prostaglandin D synthase* (L-PGDS) (255), enzyme impliquée dans la synthèse des prostaglandines, ont été décrites au cours du SAOS. La composante hypoxique du SAOS semblait là encore être impliquée dans l'activation de ces voies métaboliques, puisque les concentrations urinaires d'isoprostanes et de L-PGDS étaient corrélées aux paramètres polysomnographiques, en particulier à l'IAH (46,48,255), et étaient diminuées par la PPC (45,255).

L'ensemble de ces données démontre donc que le SAOS induit une altération globale du métabolisme de l'acide arachidonique à l'origine d'une production accrue d'eicosanoïdes. La composante hypoxique contribue à la plupart des perturbations décrites, à l'exception du TXA<sub>2</sub> dont les concentrations du métabolite sont d'une part similaires entre des sujets témoins et des patients SAOS rigoureusement appariés sur l'âge et l'indice de masse corporelle, et d'autre part non diminuées par la PPC.

### **Activation des voies du thromboxane A<sub>2</sub> et des cystéinyl-leucotriènes au cours du SAOS : effet des facteurs confondants**

Si l'activation de la voie du TXA<sub>2</sub> observée chez les patients apnéiques (82,254) ne semble pas être directement imputable au SAOS, celle-ci est probablement à mettre en lien avec les comorbidités fréquemment retrouvées chez les sujets apnéiques. Au cours de notre première étude originale, nous avons en effet mis en évidence une augmentation des concentrations urinaires de 11dTXB<sub>2</sub> chez des patients SAOS présentant d'autres facteurs de risque cardiovasculaire (HTA, syndrome métabolique, obésité, dyslipidémie ou encore tabagisme) comparativement à des patients SAOS indemnes de comorbidités (Publication n°3 (82)). Parmi ces facteurs de risque, l'HTA et l'obésité étaient associées positivement aux concentrations urinaires de 11dTXB<sub>2</sub>, mais l'obésité constituait le seul facteur indépendant. Ces résultats relatifs à la voie du TXA<sub>2</sub> sont en accord d'une part avec l'activation de la voie TXA<sub>2</sub> décrite chez des patients obèses (256–259) ou hypertendus (260) en dehors du SAOS; et d'autre part avec l'impact de l'obésité sur une autre voie métabolique de l'acide arachidonique au cours du SAOS, à savoir celle des LTs (78,80,221).

Notre seconde étude originale focalisée sur la voie des CysLTs (Publication n°5) n'a en revanche pas retrouvé de manière aussi claire l'influence de l'obésité sur cette voie métabolique. Dans cette cohorte, la présence d'une obésité était associée à des concentrations urinaires de LTE<sub>4</sub> majorées sans pour autant atteindre la significativité. Ces résultats sont en désaccord avec notre précédente étude (78) qui avait clairement identifié l'obésité comme un déterminant majeur de



l'excrétion urinaire de  $\text{LTE}_4$  en démontrant d'une part, que l'indice de masse corporelle était associé de manière indépendante à l'excrétion urinaire de  $\text{LTE}_4$  et que d'autre part, la PPC qui réduit les concentrations de  $\text{LTE}_4$  chez les patients SAOS avec indice de masse corporelle normal était sans effet chez les sujets en surpoids ou obèses. Cette discordance entre nos deux études consacrée aux CysLT peut s'expliquer par des critères d'inclusions différents puisque les sujets présentant un diabète, un tabagisme ou encore un antécédent d'évènement cardiovasculaire ont été inclus dans notre présente étude (Publication n°5) alors qu'ils avaient été exclus de la précédente (78), ce qui a pu masqué l'effet de l'obésité. Ces critères d'inclusion moins restrictifs ont néanmoins permis l'identification de nouveaux déterminants de l'activation de la voie des CysLTs puisqu'un âge avancé ou encore le fait d'avoir déjà présenté un évènement cardiovasculaire étaient associés positivement et indépendamment à une excrétion urinaire majorée de  $\text{LTE}_4$ . Ces résultats semblent logiques puisque l'âge (236) comme l'athérosclérose (172) sont associés à une inflammation de bas grade, en témoignent les concentrations élevées de LTs décrites après un infarctus du myocarde (261,262) ou encore de métabolites du  $\text{TXA}_2$  chez des sujets âgés en prévention cardiovasculaire secondaire (263).

Au-delà du  $\text{TXA}_2$  et des CysLTs, d'autres eicosanoïdes semblent également influencés par les comorbidités cardiovasculaires. Ainsi, en dehors du SAOS, les concentrations d'isoprostanes sont influencées conjointement par l'âge (264), le tabagisme (265), la présence d'une obésité (266) ou encore d'une HTA (260), tandis que des concentrations augmentées de L-PGDS ont été décrites chez des patients coronariens (267), hypertendus (268) ou encore diabétiques de type 2 (269).

En résumé, il semblerait que l'altération globale du métabolisme de l'acide arachidonique décrite au cours du SAOS soit en grande partie due aux comorbidités qui lui sont fréquemment associées. L'obésité, l'âge mais également le fait d'avoir déjà présenté un évènement cardiovasculaire jouent notamment un rôle majeur dans l'activation des voies du  $\text{TXA}_2$  et des LTs décrite au cours du SAOS. D'autres comorbidités tels que le diabète ou le tabagisme pourraient également contribuer à ces perturbations, puisque en dehors du contexte d'HI, elles

ont elles-mêmes été associées à des perturbations de ces voies métaboliques (voie des LTs (232,233) et du TXA<sub>2</sub>(270)). Néanmoins, à ce jour, aucune étude n'a évalué l'impact combiné du SAOS et de ces autres comorbidités sur la production de LTs ou du TXA<sub>2</sub>. En revanche, nous avons montré qu'un score composite évaluant le risque d'événement cardiovasculaire à 10 ans (229) et intégrant notamment le diabète et le tabagisme (en plus de l'âge, du sexe, du niveau de pression artérielle et des concentrations en cholestérol) était associé de manière indépendante à l'excrétion urinaire de LTE<sub>4</sub> (Publication n°5), suggérait bien que l'ensemble des comorbidités pourraient impacter le métabolisme de l'acide arachidonique. Bien que les mécanismes ne soient pas complètement élucidés, il semblerait que l'impact de ces différents facteurs confondants soit supérieur à l'effet propre de l'HI spécifique du SAOS notamment pour la voie du TXA<sub>2</sub>.

### **Implication des voies du thromboxane A<sub>2</sub> et des cystéinyl-leucotriènes dans le processus athéromateux associé au SAOS**

Nos différents travaux ont apporté des éléments en faveur de l'implication du TXA<sub>2</sub> et des CysLTs dans le processus athéromateux propre au SAOS. Au cours de nos études animales, l'expression génique aortique des enzymes impliquées dans la synthèse de ces eicosanoïdes (thromboxane synthase et COX-1 pour le TXA<sub>2</sub> (Publication n°3 (82)); 5-lipoxygénase et FLAP pour les CysLTs (Publication n°5)) était corrélée à la taille des lésions d'athérosclérose chez les souris soumises à l'HI. Cette corrélation était également observée entre la taille des lésions d'athérosclérose et les niveaux d'ARNm aortiques des différents récepteurs aux LTs (BLT<sub>1</sub>, BLT<sub>2</sub>, CysLT<sub>1</sub>, CysLT<sub>2</sub>). Chez l'homme, l'excrétion urinaire de 11dTXB<sub>2</sub> et de LTE<sub>4</sub> était associée aux paramètres de remodelage vasculaire, puisque les concentrations de 11-dTXB<sub>2</sub> étaient augmentées en cas d'hypertrophie carotidienne et que l'excrétion urinaire de LTE<sub>4</sub> était faiblement corrélée à l'EIM carotidienne. De plus, l'inhibition pharmacologique de ces deux voies métaboliques que ce soit par l'inhibition de la COX-1, comme le blocage du récepteur CysLT<sub>1</sub> permettait de réduire la progression de l'athérosclérose induit par l'HI dans notre modèle murin. L'ensemble de ces données démontre donc que l'activation des voies du TXA<sub>2</sub> et des

CysLTs observées au cours du SAOS contribue à l'athérogénèse induit par l'HI. Ces résultats sont en accord d'une part avec le rôle déjà connu du LTB<sub>4</sub> dans le processus athéromateux associé au SAOS (79,80,221,223); et d'autre part avec les propriétés pro-athérogènes du TXA<sub>2</sub> et des CysLTs, notamment leurs capacités à induire la contraction et prolifération des cellules musculaires lisses mais également l'expression de molécules d'adhésion (75).

Bien que le rôle joué par le TXA<sub>2</sub> et les CysLTs dans l'athérogénèse ne soit plus à démontrer, notamment dans le contexte de l'HI, il ne faut pas perdre de vue que le développement de l'athérosclérose demeure un processus complexe impliquant de nombreux mécanismes intermédiaires. TXA<sub>2</sub> et CysLTs viennent donc compléter une liste déjà longue de médiateurs impliqués dans le processus athéromateux associée au SAOS.

### **Thromboxane A<sub>2</sub> et cystéinyl-leucotriènes en tant que cibles thérapeutiques pharmacologiques pour lutter contre l'athérosclérose associée au SAOS: quelle stratégie adopter ?**

Puisque les voies du TXA<sub>2</sub> et des CysLTs sont activées au cours du SAOS et qu'elles contribuent à l'athérogénèse, nous avons émis l'hypothèse que ces eicosanoïdes pourraient constituer des cibles thérapeutiques intéressantes pour lutter contre l'athérosclérose associée au SAOS. Dans notre modèle murin, nous avons démontré que des traitements pharmacologiques ciblant spécifiquement le TXA<sub>2</sub> ou les CysLTs, à savoir l'inhibition de la COX-1 par le SC-560 ou l'antagonisme du récepteur CysLT<sub>1</sub> par le montelukast, étaient capables de ralentir de manière significative la progression de l'athérosclérose en lien avec l'HI (Publications n°3 et n°5). Notre hypothèse initiale a donc été vérifiée. Bien que ces premières stratégies aient présenté des résultats encourageants, d'autres interventions pharmacologiques ciblant ces mêmes eicosanoïdes sont possibles (Publication n°4 + 221,271). Pour ce qui est du TXA<sub>2</sub>, l'antagonisme des récepteurs TP pourrait, par exemple, constituer une alternative intéressante puisqu'elle permettrait de contrer à la fois les effets du TXA<sub>2</sub> et des isoprostanes (272); ce qui semble d'autant plus pertinent que les isoprostanes possèdent tout comme le TXA<sub>2</sub> des effets

proathérogènes (75) et que leur production accrue au cours du SAOS (45–47) est corrélée aux paramètres de remodelage vasculaire (46).

Concernant les LTs, des interventions pharmacologiques autres que l'antagonisme du récepteur CysLT<sub>1</sub> ont également déjà été proposées pour lutter contre l'athérosclérose (75,204). Il s'agissait tantôt de bloquer la synthèse des LTs dans leur ensemble (par une inhibition de la 5-lipoxygénase ou de sa protéine co-activatrice, la FLAP) ou de manière plus spécifique (inhibition de la LTA<sub>4</sub>H ou LTC<sub>4</sub>S), tantôt de bloquer les récepteurs aux LTB<sub>4</sub>. Bien que ces différentes approches aient généré des résultats contradictoires que ce soit chez l'animal (230,231,242–244,273), comme chez l'homme (208,246,274), leur intérêt dans le traitement de l'athérosclérose des patients apnéiques demeure une piste de recherche à investiguer. Les discordances observées pourraient, en effet, être dues à des niveaux d'activation de la voie des LT sensiblement différents entre les modèles ou populations d'études ; les effets bénéfiques n'apparaissant potentiellement que dans les situations où la voie des LTs était spécifiquement activée. Dans la mesure où c'est le cas dans le SAOS (221), les thérapeutiques anti-LTs pourraient avoir des effets bénéfiques sur l'athérosclérose.

A la lumière de nos résultats, l'agent pharmacologique idéal pour ralentir le processus athérogène induit par l'HI serait finalement un composé capable de bloquer simultanément le récepteur CysLT<sub>1</sub> et d'inhiber la COX-1 ; mais, un tel composé n'existe pas à l'heure actuelle. En revanche, des approches pharmacologiques ciblant une seule et même voie métabolique mais combinant plusieurs mécanismes d'action ont déjà été proposées et ont abouti à des résultats intéressants. Ainsi, l'antagonisme du récepteur TP associé à l'inhibition de la COX-1 a démontré des effets supérieures à l'inhibition de la COX-1 seul que ce soit en termes de réduction d'athérosclérose chez la souris (216), comme d'amélioration de la fonction endothéliale chez le patient présentant déjà des lésions athéromateuses (206,207). Néanmoins, ce type de stratégie présente un inconvénient majeur, à savoir la nécessité d'avoir recours à deux agents pharmacologiques différents (206,207,216), ce qui multiplie le risque iatrogène. Finalement, des composés combinant plusieurs mécanismes d'actions pourraient lever cette limite en agissant à

différents niveaux d'une voie métabolique (218,275,276). A titre d'exemple, le BM-573 qui est à la fois antagoniste du récepteur TP et inhibiteur de la thromboxane synthase s'est avéré efficace sur le développement précoce de l'athérosclérose (217,218,277).

**Inhibition pharmacologique des voies du thromboxane A<sub>2</sub> ou des cystéinyl-leucotriènes: quel patients concernés ?**

La question suivante à laquelle il convient de répondre est sans doute à quel type de patient apnéique s'adresseraient de telles interventions pharmacologiques. Pour répondre à cette interrogation, il faut prendre en compte à la fois le risque cardiovasculaire que confère la présence du SAOS mais également l'efficacité des thérapeutiques déjà disponibles sur ce risque cardiovasculaire. Ces différents éléments ayant été largement discutés dans la revue sur les traitements pharmacologiques à visée vasculaire du SAOS (publication n°2), les meilleurs candidats à un traitement pharmacologique demeurent probablement d'un côté, les patients apnéiques modérés à sévères en prévention cardiovasculaire secondaire, que ces derniers soit traités ou non par PPC (population cible 1); et d'un autre, les patients présentant un SAOS modéré à sévère, non tolérants à la PPC et n'ayant jamais présenté d'évènements cardiovasculaires mais à haut risque cardiovasculaire (population cible 2).

L'évaluation d'un éventuel traitement pharmacologique dans l'une ou l'autre de ces populations devrait en toute rigueur se baser sur les résultats des études expérimentales. Malheureusement, dans le contexte du SAOS, la plupart des études animales évaluant les effets vasculaires d'agents pharmacologiques ont été conduites de manière à détecter un effet préventif du traitement (cf. table 2 de la publication n°2), et s'inscrivent donc à ce titre, plutôt dans une approche préventive. Or des altérations vasculaires sont bien souvent déjà présentes au moment du diagnostic du SAOS (27,28), d'autant plus si le diagnostic arrive tardivement après un premier accident cardiovasculaire. Pour coller au plus près à cette réalité clinique, nous avons donc fait le choix de traiter des souris qui avaient au préalable déjà été exposées à l'HI pendant 4 semaines, ce qui est suffisant pour induire des lésions d'athérosclérose (71). Puisque l'inhibition de la

COX-1 ou l'antagonisme du récepteur CysLT<sub>1</sub> ont réduit la progression des lésions d'athérosclérose chez la souris soumise à l'HI, nous pensons que des traitements inhibant la voies du TXA<sub>2</sub> ou des CysLTs constitueraient des stratégies intéressantes à évaluer chez des patients SAOS présentant déjà des lésions notables d'athérosclérose. Les essais cliniques menés jusqu'à maintenant sont peu nombreux (206–208,274) et aucun n'a ciblé les patients apnéiques. A titre d'exemple, un traitement de 12 semaines par atreleuton, inhibiteur de la 5-lipoxygénase, a induit une réduction du volume des plaques d'athérome chez des patients présentant un syndrome coronarien aigu récent (208), sans pour autant avoir d'effet sur l'inflammation vasculaire et systémique dans une seconde étude menée dans une population similaire (274). Une étude pharmacoépidémiologique a, par ailleurs, mis en évidence une réduction du risque d'évènements cardiovasculaires chez les sujets en prévention secondaire traités par montelukast (246). De telles interventions seraient d'autant plus intéressantes à évaluer dans la population cible 1 (patients apnéiques en prévention secondaire, traités ou non par PPC) que l'étude SAVE a récemment démontré l'absence d'effet de la PPC sur le nombre évènements cardiovasculaires chez des patients apnéiques présentant déjà une pathologie cardiovasculaire (116). Dans ce sens et dans la continuité de notre étude originale consacrée à la voie des CysLTs (Publication n°5), un projet d'essai multicentrique randomisé de traitement par montelukast versus placebo chez des sujets présentant un SAOS léger à modéré en prévention secondaire a été déposé cette année au PHRC interrégional. Cibler les patients en prévention secondaire semble d'autant plus pertinent que nous avons démontré que ce type de prévention était associé de manière indépendante à une excrétion urinaire majorée de LTE<sub>4</sub> (Publication n°5).

Pour ce qui est de la population cible 2 (patients apnéiques modérés à sévères en prévention cardiovasculaire primaire non tolérants à la PPC), les bénéfices d'interventions pharmacologiques inhibant le TXA<sub>2</sub> et/ou les CysLTs restent à déterminer. En effet, cette population regroupe des patients aux profils hétérogènes (94) avec des degrés d'atteinte vasculaire assez variables. Or la gravité des lésions vasculaires basales semble conditionner l'efficacité des traitements qu'ils soient mécaniques comme la PPC (105, 106 + Publication n°2)

ou pharmacologiques (Publication n° 3). A titre d'exemple, l'antagonisme du récepteur TP bien qu'efficace pour prévenir l'apparition des plaques d'athérome présente des effets variables sur des lésions athéromateuses déjà établies (215,278). Dans la mesure où nous n'avons démontré les effets bénéfiques de tels traitements que chez des souris qui présentaient déjà des lésions vasculaires (plaques d'athérome), l'effet préventif de ces thérapeutiques sur des vaisseaux non ou peu lésés à l'état basal reste à déterminer. C'est pourquoi nous menons actuellement une étude sur le rôle de la voie du TXA<sub>2</sub> dans le remodelage vasculaire précoce induit par l'HI. Pour cela nous évaluons le remodelage vasculaire de souris *wild-type* soumises à l'HI ou la normoxie, et traitées ou non par aspirine. Ce travail apportera quelques éléments de réponse quant à la pertinence d'inhiber la voie du TXA<sub>2</sub> pour prévenir les altérations vasculaires précoces du SAOS. Il permettra également d'investiguer le rôle d'une autre famille d'eicosanoïdes aux propriétés anti-inflammatoires, à savoir les lipoxines. En effet, le traitement par aspirine est connu pour induire la synthèse d'un diastéréoisomère de la lipoxine A<sub>4</sub>, à savoir la 15-épi-lipoxine A<sub>4</sub> encore appelée aspirin-trigger lipoxin (279), dont le rôle dans l'athérosclérose reste assez mal connu.

Enfin, dans l'ère de la médecine personnalisée, la meilleure stratégie serait peut-être de sélectionner les patients candidats à ce type d'intervention pharmacologique sur la base de tests compagnons, dont le but serait d'évaluer l'activation basale des voies métaboliques spécifiquement ciblées par le traitement. La quantification des métabolites du TXA<sub>2</sub> ou des CysLTs au niveau urinaire pourrait, par exemple, être utilisée à ces fins. Une telle démarche semble d'autant plus intéressante que les concentrations urinaires de 11dTXB<sub>2</sub> et de LTE<sub>4</sub> ont été récemment proposées comme biomarqueurs pour prédire respectivement la survenue d'un événement cardiovasculaire après un premier infarctus du myocarde (280) ou la resténose chez des patients atteints d'artériopathie oblitérante des membres inférieurs (234). Le génotypage de certaines enzymes impliquées dans la synthèse des eicosanoïdes, notamment la FLAP et la LTA<sub>4</sub>H pourraient également être positionnés comme test compagnon, puisque certains variants de ces gènes sont associés à un risque majoré d'infarctus du myocarde (281,282). Ainsi, de la même manière que des clusters de miRNA ont été décrits comme étant prédictifs de la réponse

antihypertensive à la PPC (118), on peut imaginer qu'un génotype spécifique de la 5-LO ou de la FLAP, comme des concentrations élevées de 11dTXB<sub>2</sub> ou du LTE<sub>4</sub> pourraient prédire l'efficacité d'un traitement pharmacologique inhibant spécifiquement ces voies métaboliques.



## **Conclusion et perspectives**

Le SAOS est reconnu depuis maintenant de nombreuses années comme un facteur de risque cardiovasculaire à part entière. Le traitement de première ligne par PPC bien qu'efficace sur l'hypersomnolence diurne ne présente qu'une efficacité limitée sur les conséquences cardiovasculaires du SAOS. De nouvelles thérapeutiques visant à traiter ces complications cardiovasculaires sont donc nécessaires pour compléter l'arsenal thérapeutique du SAOS.

Nos travaux ont permis d'identifier de nouvelles cibles thérapeutiques à savoir le TXA<sub>2</sub> et les CysLTs pour traiter les conséquences vasculaires du SAOS (cf. figure 7). Ces médiateurs sont en effet produits en plus grande quantité au cours du SAOS et contribuent au processus athéromateux. L'inhibition de l'une ou l'autre de ces voies métaboliques a permis de réduire la progression de l'athérosclérose induit par l'HI dans un modèle murin de SAOS.

Ces nouvelles cibles thérapeutiques ayant été identifiées, des recherches supplémentaires sont désormais nécessaires pour identifier la meilleure stratégie pharmacologique à adopter, mais également pour préciser quelle population cible de patients apnéiques seraient susceptibles de tirer un bénéfice de telles interventions thérapeutiques.

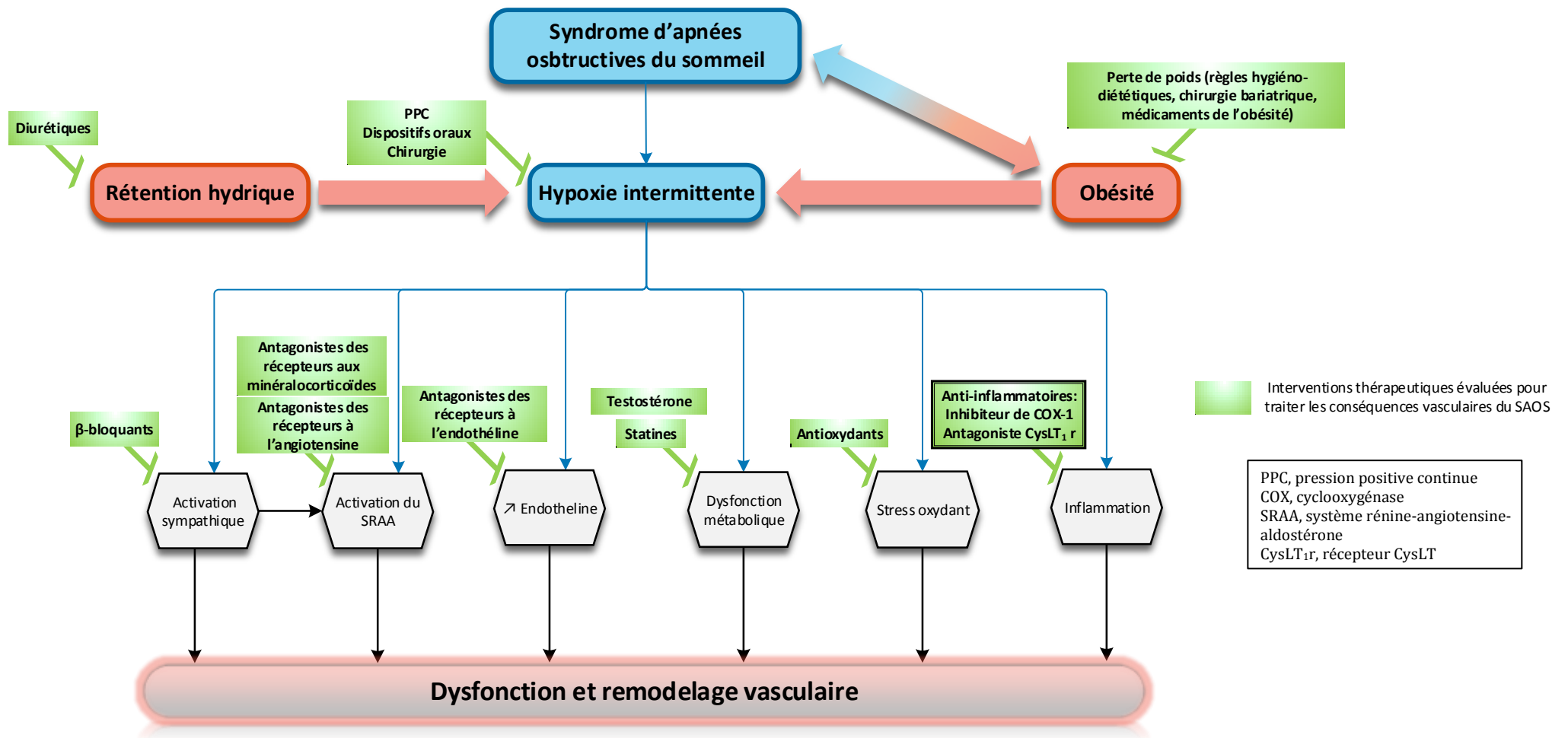


Figure 7. Représentation schématique des interventions thérapeutiques du SAOS détaillant spécifiquement les traitements pharmacologiques visant à réduire les conséquences vasculaires délétères du SAOS.

Parmi les nombreuses interventions pharmacologiques en cours d'évaluation pour traiter spécifiquement les conséquences vasculaires du SAOS, nous avons démontré que des traitements à visée anti-inflammatoire, à savoir l'inhibition de la COX-1 ou l'antagonisme du récepteur CysLT<sub>1</sub> pouvaient réduire la progression de l'athérosclérose et constituaient à ce titre des stratégies intéressantes pour réduire le risque cardiovasculaire des patients souffrant de SAOS.

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