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RESEARCH ARTICLE

Intermittent hypoxia increases lipid insulin resistance in healthy humans: A randomized crossover trial

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Summary

Sympathetic overactivity caused by chronic intermittent hypoxia is a hallmark of obstructive sleep apnea. A high sympathetic tone elicits increases in plasma free fatty acid and insulin. Our objective was to assess the impact of 14 nights of chronic intermittent hypoxia exposure on sympathetic activity, glucose control, lipid profile and subcutaneous fat tissue remodelling in non-obese healthy humans. In this prospective, double-blinded crossover study, 12 healthy subjects were randomized, among them only nine underwent the two phases of exposures of 14 nights chronic intermittent hypoxia versus air. Sympathetic activity was measured by peroneal microneurography (muscle sympathetic nerve activity) before and after each exposure. Fasting glucose, insulin, C-peptide and free fatty acid were assessed at rest and during a multisampling oral glucose tolerance test. We assessed histological remodelling, adrenergic receptors, lipolysis and lipogenesis genes expression and functional changes of the adipose tissue. Two weeks of exposure of chronic intermittent hypoxia versus ambient air significantly increased sympathetic activity ($p = 0.04$). Muscle sympathetic nerve activity increased from 24.5 [18.9; 26.8] before to 21.7 [13.8; 25.7] after ambient air exposure, and from 20.6 [17.4; 23.9] before to 28.0 [24.4; 31.5] bursts per min after exposure to chronic intermittent hypoxia. After chronic intermittent hypoxia, post-oral glucose tolerance test circulating free fatty acid area under the curve increased ($p = 0.05$) and free fatty acid sensitivity to insulin decreased ($p = 0.028$). In adipocyte tissue, intermittent hypoxia increased expression of lipolysis genes (adipocyte triglyceride lipase and hormone-sensitive lipase) and lipogenesis genes (fatty acid synthase; $p < 0.05$). In this unique experimental setting in healthy humans, chronic intermittent hypoxia induced high sympathetic tone, lipolysis and decreased free fatty acid sensitivity to insulin. This might participate in the trajectory to systemic insulin resistance and diabetes for patients with obstructive sleep apnea.

Clinical trial registration: URL: http://clinicaltrials.gov. Unique identifier: NCT02058823.

KEYWORDS
fatty acid, intermittent hypoxia, lipolysis, obstructive sleep apnea, sympathetic nervous system

1 | INTRODUCTION

Obstructive sleep apnea (OSA) is a common respiratory disease, with a prevalence estimated to up to 1 billion people worldwide (Benjafield et al., 2019). OSA is characterized by repeated upper airway closure during sleep, leading to sleep fragmentation, increased respiratory efforts, and repetitive hypoxia/reoxygenation sequences called intermittent hypoxia (IH). IH is recognized as the main OSA component responsible for deleterious cardiovascular and metabolic consequences (Randerath et al., 2018; Ryan et al., 2019). Sympathetic tone is increased in patients with OSA (Lévy et al., 2015), as well as in rodent models (Ryan, 2018). In healthy human subjects exposed to IH (Tamisier et al., 2011), 2 weeks of exposure lead to an increase in sympathetic activity, arguing for a major role of IH as a stimulus of sympathetic overactivity.

Obstructive sleep apnea is strongly associated with metabolic disorders, including dyslipidaemias, insulin resistance and type 2 diabetes (Briançon-Marjollet et al., 2015; Gunduz et al., 2018; Lévy et al., 2015; Murphy et al., 2017; Ryan, 2017; Ryan et al., 2020). Healthy volunteers (Louis & Punjabi, 2009) as well as rodents (Briançon-Marjollet et al., 2015; Drager et al., 2010) exposed to IH, as a model of OSA, consistently exhibit insulin resistance and dyslipidaemias (Barros & García-Río, 2019; Thomas et al., 2017). Dyslipidaemia can be caused by excessive lipolysis in the adipose tissue of patients with OSA (Chopra et al., 2017; Gu et al., 2017; Meszaros & Bikov, 2022; Trinh et al., 2021). In rodents, several studies have demonstrated that IH can cause dyslipidaemias through free fatty acid (FFA) release (Jun et al., 2010; Poulain et al., 2014) In addition, IH also induced adipose tissue inflammation and lipolysis (Briançon-Marjollet et al., 2016a; Poulain et al., 2014; Poulain et al., 2015). Lipolysis is a complex and multi-step process. Basically, triglycerides are successively hydrolysed by adipocyte triglyceride lipase (ATGL), hormone-sensitive lipase (HSL; the limiting step of lipolysis) and monoacylglycerol lipase (MGL) to release non-esterified fatty acids. Among the neuroendocrine signals regulating fatty acid metabolism, the main ones are adrenergic stimulation counterbalanced by insulin-induced suppression of lipolysis (Boyda et al., 2013). Lipolysis of the adipose tissue is highly regulated both by sympathetic afferences and direct adrenergic stimulation through circulating catecholamines. In humans, epinephrine and norepinephrine exert lipolytic action through beta-adrenergic receptors beta 1, beta 2 and beta 3 (Lafontan & Langin, 2009; Yang & Tao, 2019). Beta-adrenergic receptors signalling pathway involves stimulatory G-proteins (Gs) and protein kinase A (PKA) AA signalling leading to HSL activity and lipolysis (Frühbeck et al., 2014). Increased sympathetic tone and beta receptors activation in OSA/IH could thus be one of the main mediators of dyslipidaemia and insulin resistance (Briançon-Marjollet et al., 2015; Dewan et al., 2015). FFAs are able to induce insulin resistance through their effect on muscle, liver and adipose tissue itself (Delarue & Magnan, 2007), supporting the hypothesis of a causal link between sympathetic tone, lipolysis and insulin resistance.

In addition, insulin resistance itself could in turn modulate IH-induced lipolysis. Indeed, insulin stimulates lipogenesis in adipose tissue, and powerfully inhibits catecholamine-induced lipolysis. In IH, insulin resistance could thus accelerate the sympathetic nervous system (SNS)-induced lipolysis.

Finally, cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), and adipokines such as leptin and adiponectin, can also participate in lipolytic control (Frühbeck et al., 2014). IH induces adipose tissue inflammation leading to dysregulation of adipokine secretion (Ryan, 2017). Adiponectin, an insulin-sensitizing hormone decreasing lipolysis, is decreased by IH (Magalang et al., 2009), while TNF-α, IL-6 and leptin are increased by IH (Briançon-Marjollet et al., 2016b; Fu et al., 2015; Poulain et al., 2014) and able to induce lipolysis (Frühbeck et al., 2014; van Hall et al., 2003; Zhang et al., 2002).

We hypothesized that sympathetic overactivity might participate in IH-induced lipolysis and decreased FFA sensitivity to insulin, which in turn may promote systemic insulin resistance. To address this question, we have assessed the impact of 14 nights of chronic intermittent hypoxia (CIH), in healthy volunteers, on sympathetic tone, lipid profiles, adipose tissue remodelling and function, and FFA sensitivity to insulin.

2 | METHODS

2.1 | Subjects

This prospective, interventional, randomized, double-blinded and cross-over study was conducted in Grenoble University Hospital Centre. Volunteers were included if they were aged 18 years or older, with a body mass index (BMI) between 20 and 25 kg m^{-2} without OSA (apnea–hypopnea index [AHI] < 15 per hr). They were free of comorbidities without history of alcohol consumption or smoking. All women were tested negative for pregnancy. Baseline characteristics are presented in Table 1.

Each subject underwent two phases of exposure to 14 nights randomized between CIH versus ambient air (AA). The washout period was 6 weeks. Registration was done in clinicaltrial.gov (clinical trial NCT02058823), also including an interventional arm using cardiometabolic drugs. However, due to ethical refusal of this arm, this intervention was not conducted.

Twelve subjects were randomized to each arm (Figure 1). Two subjects withdrew their consent shortly after starting AA exposure, and one subject did not perform post-IH exposure measurement due to non-respect to the protocol rules during exposure. A total of nine subjects and 10 subjects completed IH and AA exposures, respectively. Therefore, analysis was performed on the nine that performed both exposures. One subject was finally not included into sympathetic

TABLE 1 Main anthropometric, sympathetic and respiratory sleep characteristics at inclusion depending on allocation groups, i.e. exposition first to IH or AA.

	All subjects ($n = 9$)	IH first ($n = 6$)	AA first ($n = 3$)	p-Value
Age (years)	22 [21; 24]	23 [21.5; 24]	21 [21; 29]	0.89
Men, *n* (%)	7 (77.80%)	4 (66.70%)	3 (100.00%)	0.50
BMI (kg m^{-2})	22.0 [20.5; 24.9]	21.0 [19.8; 24.4]	22.3 [22.0; 26.2]	0.25
Bursts per min	22.6 [16.4; 29.7]	23.7 [17.1; 31.3]	21.5 [7.4; 25.4]	0.55
Bursts per 100 hb	35.0 [31.2; 44.6]	37.6 [31.7; 49.6]	32.2 [15.7; 42.4]	0.37
AHI, *n* per hr	1.4 [0.5; 3.6]	1.0 [0.2; 6.1]	1.7 [1.4; 5.5]	0.36

Note: Data are presented as median [Q1; Q3] and number (percentage).
Abbreviations: AA, ambient air; AHI, apnea–hypopnea index in number of events per hour of sleep; BMI, body mass index; IH, intermittent hypoxia.

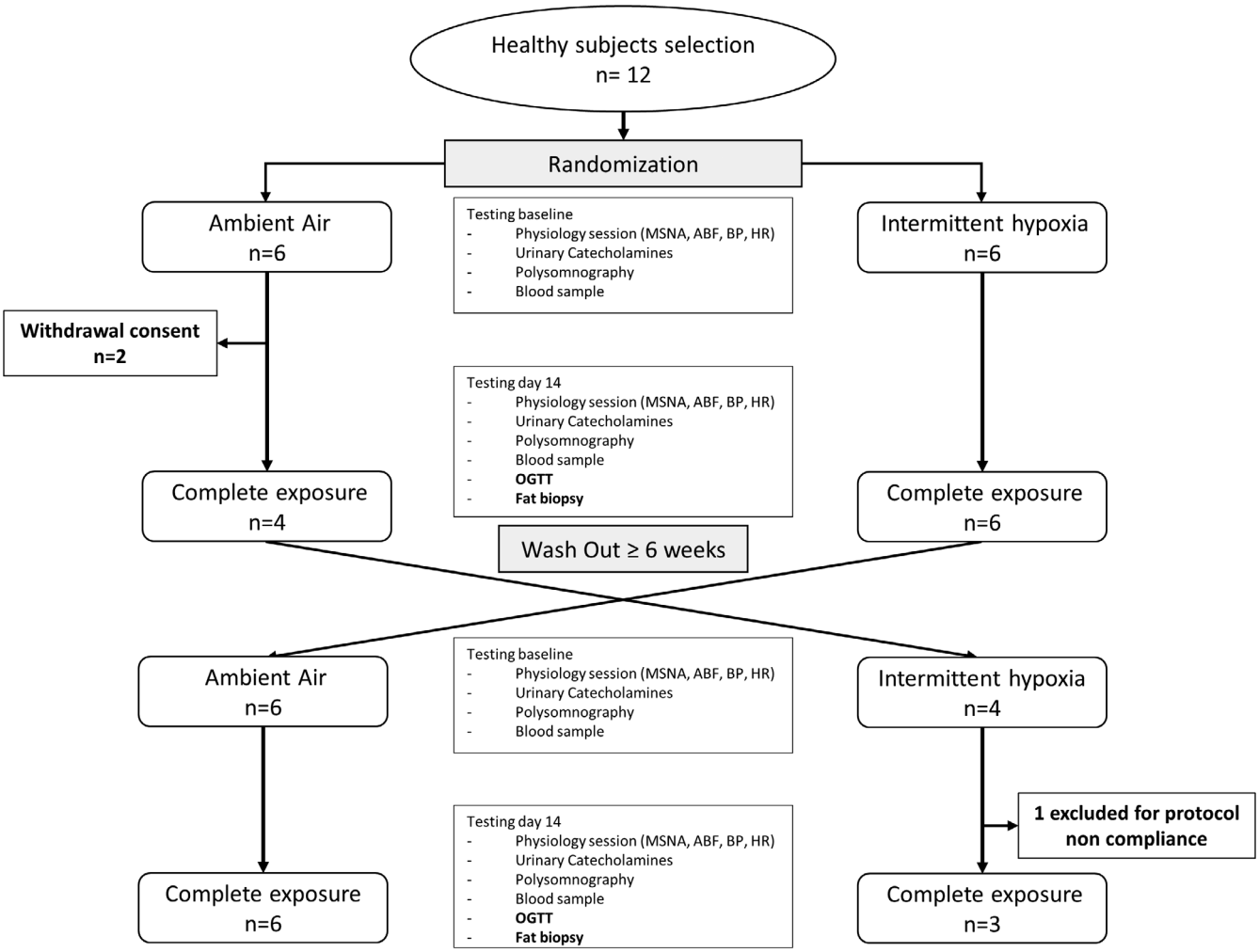

FIGURE 1 Flow chart of the study. A total of 12 subject were randomized, two subjects were withdrawn from the study after starting AA exposure, and one subject was excluded for non-compliance to protocol during the IH exposure. AA, ambient air; ABF, arterial blood flow; BP, blood pressure; HR, heart rate; IH, intermittent hypoxia; MSNA, muscle sympathetic nerve activity; OGTT, oral glucose tolerance test.

analysis due to outlier muscle sympathetic nerve activity (MSNA) values at rest (no sympathetic tone), therefore analysis was performed on eight subjects who completed both exposures. Adipose tissue biopsies were available in eight subjects who completed both exposures.

All participants provided written informed consent approved by the ethical committee Nord Ouest III (10 October 2011, ref CPP 2011-32; EudraCT:2010-020801-34) and CNIL approval.

2.2 | IH exposure

Our model of CIH exposure has been previously described (Tamisier et al., 2009). Briefly, following a 2-night adaptation to the environment (AA), subjects were exposed to 8 hr of IH between 22:00 hours and 06:00 hours for 14 consecutive nights. The IH stimulus was intermittent poikilocapnic hypoxia, i.e. inspiratory oxygen

fraction (FiO_2) was controlled, and carbon dioxide was allowed to fluctuate normally. For all nights, subjects slept with a nasal cannula in a commercially available hypoxia tent (Hypoxico, New York, NY, USA). The tent exposed subjects to an FiO_2 of 0.13. The tent was continuously flushed to limit rebreathing, and the oxygen fraction in the tent was continuously monitored (Maxtec OM-25 MEI; Maxtec, Salt Lake City, UT, USA). The nasal cannula restored oxygen saturation (range 95%–98%) via a 15-s bolus of oxygen every 120 s. Oxygen saturation was monitored continuously (BlueNight; SleepInnov Technology, Moirans, France) and oxygen boluses were adjusted between 1.5 and 2 L min^{-1} to achieve an 85%–95% range of oxygen desaturation re-saturation. The combination of tent and nasal cannula allowed for 30 oxygen desaturation re-saturation sequences per hour.

2.3 | "Sham IH" exposure (AA exposure)

Subjects and investigators were maintained blinded from exposure. Subject slept in the same setting compared with IH exposure. For all nights, subjects slept with a nasal cannula in a commercially available hypoxia tent (Hypoxico, New York, NY, USA). The main door of the tent was closed, while several air vents were maintained open. Oxygen saturation was monitored continuously (BlueNight; SleepInnov Technology, Moirans, France).

2.4 | Measurements

Additional methods are detailed in the online supplement.

2.4.1 | Muscle sympathetic nerve activity

Muscle sympathetic nerve activity was performed at D0 and D14 of each exposure (IH and AA). We obtained peroneal nerve recordings via standard sympathetic microneurographic procedures with tungsten microelectrodes, as described previously (see supplement file for further descriptions; Treptow et al., 2019). MSNA bursts were identified using an algorithm developed by Hamner and Taylor (2001) using Matlab software (The Mathworks, Natick, MA, USA). MSNA was averaged over 5-min periods and expressed as burst frequency (bursts per min and bursts per 100 hb) and burst amplitude (AIU per min and AIU per 100 hb).

2.4.2 | Polysomnography

To assess the impact of IH on sleep, a polysomnography was performed at D2 and D14 of the exposure. A complete description of sleep recording and analysis are provided in the online supplement, and two examples of recordings are shown in Figure 7.

2.4.3 | Biological samples

Fasting plasma triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol concentrations were determined at D0 and D13. At the same time, a 24-hr urine catecholamine assay was performed.

The oral glucose tolerance test (OGTT) for assessing FFA sensitivity to insulin

The OGTT was performed at D13. After a 12-hr overnight fast, participants were given a 75-g OGTT with plasma samples taken at 0, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270 and 300 min for assessment of insulin, glucose and FFA concentrations. The area under the curve (AUC) of glucose, insulin and FFA during the OGTT was determined by the trapezoid method between 0 and 300 min. To evaluate FFA homeostasis, we used two indices that were described initially by our team (Borel et al., 2013). They extrapolate to lipid regulation two widely used plasma glucose/insulin homeostasis indices: FFA-resistance index (RI) that evaluates the fasting resistance of lipids to insulin – it was extrapolated from homeostasis model assessment-insulin resistance (HOMA-IR) using the formula FFA-RI = log (fasting plasma FFA × fasting plasma insulin), with insulin as micro-international units per millilitre and FFA as millimoles per litre; and insulin sensitivity index (ISI) of FFA (FFA-ISI) that is a dynamic index indicating insulin capacity to decrease circulating FFA levels during an OGTT, it was extrapolated from the ISI of Matsuda (ISI Matsuda) using the formula FFA-ISI = $100/\sqrt{}$ [(fasting plasma insulin × fasting FFA) × (mean plasma insulin during OGTT × mean FFA during OGTT)], with insulin as international units per litre and FFA as millimoles per litre.

Adipose tissue sampling

On the day following the last exposure period, a subcutaneous adipose tissue biopsy was realized in the peri-umbilical region. A local cutaneous anaesthesia using xylocaine allowed to introduce a liposuction canula (13 cm/3 mm tulip type) into subcutaneous adipose tissue. Aspiration was manually applied using a 60-ml syringe. The tissue was washed several times in physiological serum, then either snap-frozen in liquid nitrogen then stored at −80°C or fixed in 95% ethanol for mRNA study or immunohistochemistry, respectively.

Ethanol-fixed, paraffin-embedded adipose tissue was sectioned (4 μm) for slide preparation, deparaffinized, and then stained with haematoxylin and eosin to assess tissue morphology.

Adipocyte size was measured from photographs (10 × 40 magnification) using the NIS Elements microscope imaging software (Nikon Instruments Europe BV, Amsterdam, the Netherlands).

Immunohistochemistry (IHC)

To determine the expression of adrenergic receptors by IHC, the paraffin sections were incubated with primary antibodies against β1 and β3 adrenergic receptors (Abcam®) and revealed with avidin-biotin staining system (ABC kit, Vectastain®) combined to Histogreen peroxidase substrate (Linaris). The substrate used leads to green staining of proteins. Quantifications were made using ImageJ (NIH, Bethesda, MD, USA) and NIS (Nikon) software.

TABLE 2 Polysomnographic parameters before and after AA and IH exposures.

	AA		IH		
	Before	After	Before	After	*p*-Value
Total sleep period (min)	444 [416; 484]	446 [400; 468]	435 [416; 456]	457 [423; 461]	0.52
Total sleep time (min)	420 [399; 429]	419 [371; 446]	394 [387; 431]	408 [389; 436]	0.89
WASO (min)	26 [15; 42]	17 [9; 32]	26 [21; 37]	32 [18; 47]	0.25
Stage 1 sleep (min)	27 [17; 49]	27 [15; 32]	31 [24; 45]	42 [23; 61]	0.36
Stage 2 sleep (min)	186 [160; 226]	181 [167; 202]	170 [156; 204]	168 [156; 217]	1.00
Slow-wave sleep (min)	74 [63; 83]	69 [55; 83]	68 [64; 79]	57 [31; 73]	0.52
REM sleep (min)	117 [99; 133]	125 [102; 162]	123 [99; 140]	138 [125; 147]	0.89
Total arousal index (nb/ per hr of sleep)	9.2 [7.5; 11.9]	7.9 [5.4; 9.3]	8.4 [7.5; 9.0]	12.2 [8.8; 19.3]	
Respiratory arousal index (nb per hr of sleep)	1.0 [0.6; 3.3]	1.2 [0.2; 1.8]	0.9 [0.0; 3.1]	5.2 [1.0; 12.1]	
AHI (nb per hr of sleep)	1.7 [1.1; 2.6]	2.8 [0.6; 4.0]	1.7 [0.5; 5.5]	10.8 [4.0; 20.5]	
Respiratory disorder index (nb per hr of sleep)	1.8 [1.3; 2.9]	3.1 [1.4; 4.6]	3.7 [0.9; 9.5]	16.0 [4.0; 22.9]	
Mean SpO_2 (%)	96.8 [95.8; 97.2]	96.5 [95.3; 97.0]	96.9 [96.2; 97.5]	91.4 [90.2; 94.9]	
Nadir SpO_2	93.0 [91.5; 94.0]	93.0 [89.5; 93.5]	94.0 [91.5; 94.5]	81.0 [76.5; 87.5]	
TC90 (min)	0.0 [0.0; 0.0]	0.0 [0.0; 0.0]	0.0 [0.0; 0.3]	137.0 [40.2; 168.7]	
ODI 3% (nb per hr of sleep)	5.0 [1.5; 10.5]	8.0 [3.0; 14.5]	5.0 [2.5; 7.0]	225.0 [75.5; 272.0]	

Note: Data are presented as median [25; 75] and $n = 9$ for all. *p*-Values are the comparison of (delta pre/post for IH) versus (delta pre/post for AA). By design, AHI, TC90, SpO_2, and arousal and ODI were statistically different after AA and IH exposure.
Abbreviations: AA, ambient air; AHI, apnea–hypopnea index; IH, intermittent hypoxia; ODI, oxygen desaturation index; REM, rapid eye movement; SpO_2, digital oxygen pulse saturation; TC90, time of sleep with oxygen saturation below 90%; WASO, wake after sleep onset.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

Total RNA extraction was performed using Trizol™ followed by RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) in accordance with the manufacturer's instructions. cDNA was reverse transcribed from 0.5 µg of total RNA with the iScript™ Reverse Transcription Supermix 100 reaction kit (Bio-Rad). Real-time PCR was conducted using 2 µl of DNAc with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). Gene expression was quantified using the comparative threshold cycle (Ct) method, and RPLP0 and RPL27 mRNA levels were simultaneously analysed as housekeeping genes (de Jonge et al., 2007; Pérez-Gómez et al., 2023). Primers sequences are indicated in Table S1.

2.5 | Statistical analysis and sample size calculation

Sample size was calculated based on our primary objective, which was the measure of SNS activity after IH exposure. We considered an estimated 10 bursts per min increasing of MSNA in healthy subjects as found in our previous study (Tamisier et al., 2009). With a risk α at 0.05 and a power of 90%, we arrived at the required number of 12 subjects.

A crossover analysis was used to compare the evolution IH versus AA. We analysed period effect, treatment effect and carryover effect. Although analysis of crossover design is based on repeated measure, herein we directly compared the differences between periods using a Mann–Whitney test. Data obtained during OGTT and adipose tissue biopsies were compared using a Mann–Whitney test.

Crossover analyses to assess the evolution of IH versus AA were compared as follows, where y1 = Day 0 and y2 = Day 14 for the first period; z1 = Day 0 and z2 = Day 14 for the second period: Group A (IH first): dA = (y2 – y1) – (z2 – z1) and Group B (AA first): dB = (z2 – z1) – (y2 – y1), treatment effect: dA + dB, period effect: dA – dB, and carryover: [y1 + y2 (for group A)] – [y1 + y2 (for group B)]. If a carryover effect was present ($p < 0.05$), the second period of exposure was excluded for statistical analysis. This was the case for four variables, diastolic blood pressure measured during sympathetic recording, nadir oxygen saturation during polysomnography, nighttime diastolic blood pressure measured using 24-hr blood pressure monitoring, and 24-hr epinephrine level on urine sample.

Statistical significance was set at $p < 0.05$. Data are presented as median [Q25; Q75], number (%), unless otherwise notified.

3 | RESULTS

We included nine healthy subjects (Table 1) in the final analysis (Figure 1), among them seven were men (77.8%). They had a median age of 22 years [21; 24] and were non-obese with a BMI of 22.0 [20.5; 24.9] kg m^{-2}. Subjects were randomized in AA first or IH first groups; among our finally included subjects, six subjects started exposure with IH and three with AA (sham exposure; Table 1). There was no change in BMI that were 22.5 [21.2; 23.7]

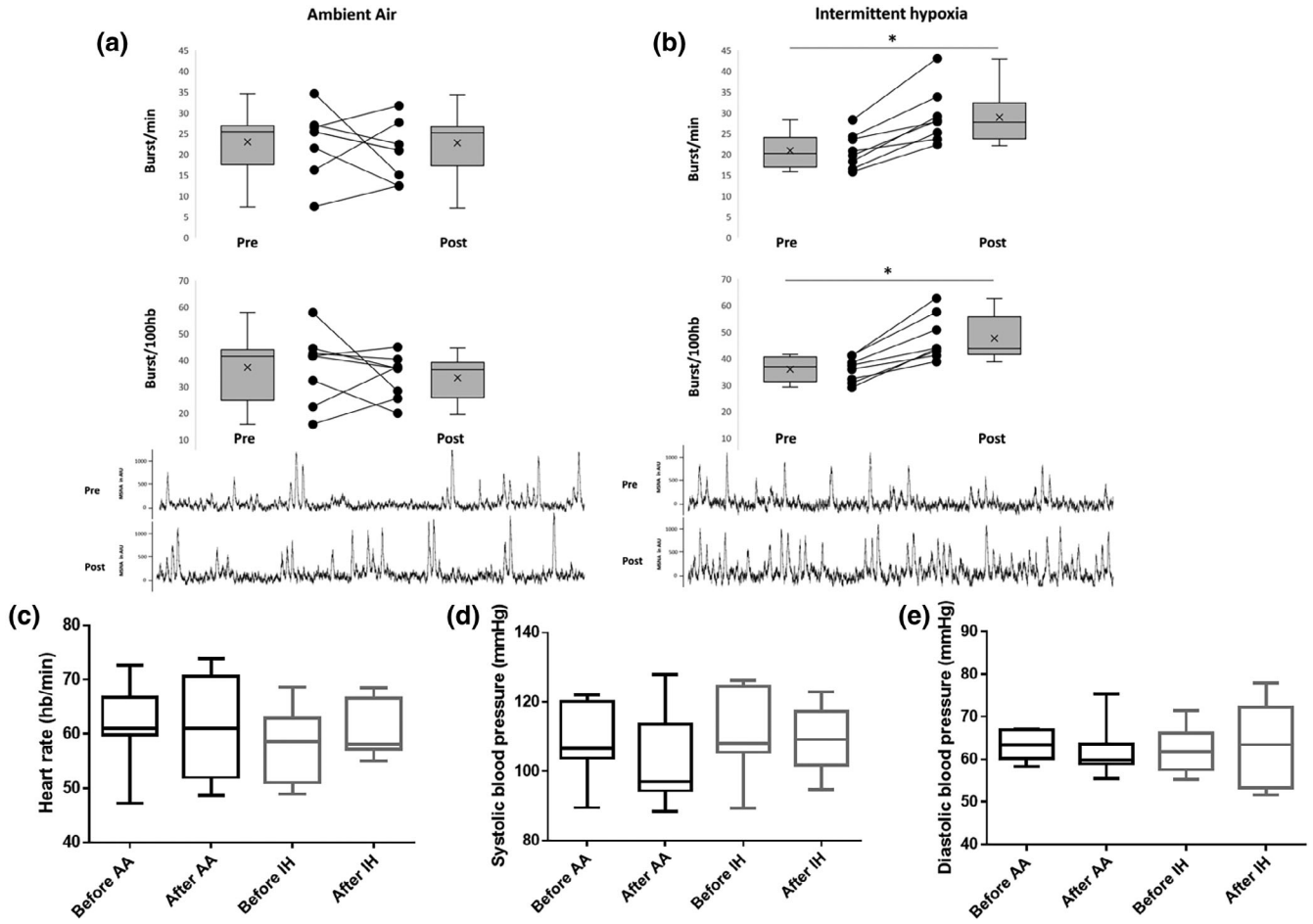

FIGURE 2 SNS activity increases after 14 nights of IH. (a) Sympathetic activity evaluated by MSNA on the peroneal nerve. Before versus after AA and IH (b), expressed as number of bursts per min (upper panel) and number of bursts per 100 hb (medium panel), $^{*}p = 0.037$ for both. Illustrating nerve traces before and after IH and AA. (c) Heart rate (beats per min), (d) systolic blood pressure, and (e) diastolic pressure measured during MSNA recording using finger cuff (CPAP). Data are presented as median ± IQ range and min/max values, $n = 8$ subjects. AA, ambient air; CPAP, continuous positive airway pressure; IH, intermittent hypoxia; MSNA, muscle sympathetic nerve activity; SNS, sympathetic nervous system.

kg m^{-2} and 22.5 [20.1; 24.4] kg m^{-2} after 14 nights of AA and IH, respectively.

Polysomnographic measurements were performed before and after each exposure of either IH or AA (Table 2). By design, there were significant increases of hypoxic stimuli measured by oxygen desaturation index (ODI 3%), time of sleep with SpO_2 below 90%, and mean SpO_2 during sleep. As described before, IH exposure induced ventilatory instability with an increase of AHI per hour of sleep, although sleep fragmentation increase did not reach significance.

3.1 | Primary outcome: IH increases SNS activity

Sympathetic activity increased significantly (Figure 2; Table 3), with a delta value between post-IH and AA of +10.4 [4.0; 13.6] and +16.4 [5.7; 22.7] bursts per min and bursts per 100 hb, respectively ($p = 0.037$ for both). Other parameters assessing sympathetic tone did not increase, i.e. total sympathetic activity, urinary catecholamines levels (Table 3). Ambulatory blood pressure was not modified (Table S2).

3.2 | IH alters FFA metabolism during OGTT

Static morning fasting levels of triglycerides, cholesterol, insulin and glucose were not modified after 14 nights of IH (Table S3; Figure 3). FFAs AUC during the OGTT was significantly higher after 14 nights of IH compared with AA exposure: AUC 77,510 [56,082: 103,527] and 63,685 [40,957: 102,082], respectively ($p = 0.05$; Figure 3d; Table S4). There was a drop in FFA sensitivity to insulin (FFA-ISI) from 114.4 [86.1; 195.3] to 34.7 [32.5; 59.4, $p = 0.028$; Figure 3f] and a tendency of adipocyte size to decrease after IH (Figure 4a,b; $p = 0.08$). However, there was no significant change in FFA-RI (Table S5). Glucose, insulin and C-peptide AUC measurements (Figure 3a–c), and the calculated HOMA-IR (Figure 3e; Table S5) were not different after AA and IH exposure.

TABLE 3 Cardiovascular and MSNA parameters ($n = 8$) and urinary catecholamines ($n = 9$) before and after exposure to AA or IH.

	AA		IH		
	Before	After	Before	After	*p*-Value
HR, hb per min	61.9 [59.9; 65.3]	61.7 [52.8; 66.8]	58.9 [53.9; 64.8]	58.1 [57.2; 65.4]	0.77
SBP, mmHg	105.5 [96.7; 116.1]	96.8 [91.9; 106.5]	107.1 [104.7; 121.1]	109.2 [103.2; 117.1]	0.37
DBP, mmHg	62.0 [59.2; 65.4]	59.7 [57.2; 62.9]	61.7 [56.7; 64.1]	63.5 [53.5; 72.0]	0.14[a]
Peak flow, cm s^{-1}	3.2 [2.6; 4.7]	3.0 [2.5; 3.3]	3.1 [2.6; 4.2]	2.7 [2.0; 4.2]	0.55
MSNA					
Burst frequency (B per min)	24.5 [18.9; 26.8]	21.7 [13.8; 25.7]	20.6 [17.4; 23.9]	28.0 [24.4; 31.5]	0.04
Burst frequency (B per 100 hb)	39.5 [27.3; 43.4]	37.1 [26.9; 38.9]	35.0 [31.6; 39.9]	44.0 [42.2; 54.4]	0.04
Activity (AIU per min)	581.7 [369.4; 716.1]	645.8 [532.6; 845.7]	407.1 [371.3; 512.9]	586.3 [537.9; 778.2]	0.77
Activity (AIU per 100 hb)	939.9 [534.8; 1196.4]	1184.8 [931.1; 1374.8]	710.6 [659.3; 843.5]	1014.8 [868.7; 1255.3]	0.77
Urinary catecholamine (nmol $mmol^{-1}$ of creatinine)					
Norepinephrine	11.52 [7.94; 16.56]	12.42 [11.21; 14.76]	11.58 [9.33; 17.88]	16.42 [7.13; 19.66]	0.89
Epinephrine	2.44 [1.49; 4.74]	3.16 [2.07; 6.03]	3.59 [1.07; 3.87]	2.06 [1.32; 3.81]	0.52[a]
Dopamine	101.3 [80.9; 138.0]	118.1 [93.7; 131.5]	114.5 [101.2; 138.4]	121.0 [80.6; 153.6]	0.69

Note: Data are presented as median [Q1; Q3] and number (percentage).
p-Values are the comparison of (delta pre/post for IH) versus (delta pre/post for AA).
Abbreviations: 100 hb, 100 hear beat; AA, ambient air; DBP, office diastolic blood pressure; HR, heart rate; IH, intermittent hypoxia; MSNA, muscle sympathetic nerve activity; peak flow, doppler popliteal artery blood flow; SBP, office systolic blood pressure.
[a]Carryover effect was detected on the first period, therefore the second period was excluded from analysis.

3.3 | IH induces adipose tissue remodelling with upregulation of lipolysis and lipogenesis genes

3.3.1 | Sympathetic regulation

Adipocyte size tended to decrease after IH in the subcutaneous adipose tissue (Figure 4c; $p = 0.08$). Expression of adrenergic receptors was not modified by IH (Figure 4), although expression of β1-AR tended to increase (Figure 4d; $p = 0.08$). RT-qPCR for α2, β1, β2 and β3 adrenergic receptor genes confirmed that there was no significant change of gene expression (Figure 4f).

3.3.2 | Lipolysis lipogenesis balance and beta-oxidation

Gene expression analysis revealed that IH upregulated the gene expression level of two key enzymes of lipolysis, namely ATGL and HSL (Figure 5a) by 4.6-fold ($p = 0.037$) and 2.5-fold ($p = 0.04$), respectively. MGL (Figure 5a) or perilipin-1 (not reported) gene expression levels were not modified following IH compared with AA. Lipogenesis genes expression was altered by IH with an increase of 3.2-fold ($p = 0.037$; Figure 5b) in fatty acid synthase (FAS) mRNA, but not in other enzymes like acetylCoA carboxylase 1 (ACC1), carbohydrate responsive element binding protein (ChREBP) or acylCoA synthetase 1 and 2 (ACS1 and ACS2; Figure 5b; Table S5).

Related to enzymes involved in fatty acid beta-oxidation, IH increased gene expression levels of acetylCoA carboxylase 2 (ACC2) by 3.1-fold ($p = 0.037$; Figure 5a), but not fatty acid translocase (FAT) or carnitine palmitoyl transferase 1 (CPT1; Figure 5a; Table S5).

3.4 | IH does not modify adipose tissue inflammation markers

Finally, we evaluated several markers of adipose tissue inflammation by RT-qPCR. IH did not modify expression levels of the genes of leptin, adiponectin, TNFα, IL-6 or the macrophage CD68 marker (Figure 6). The gene expression level of hypoxia-inducible factor-1α (HIF-1α) transcription factor subunit was also not modified by IH (Figure 6).

4 | DISCUSSION

Using a unique model of IH exposure in healthy humans without obesity and comorbidity, we demonstrated that IH-related sympathetic overactivity was linked with an increase in FFA circulating levels and a reduction of FFA sensitivity to insulin, but not glucose insulin sensitivity. This is accompanied with modifications of the lipolysis pathway (ATGL and HSL) as well as lipogenesis (FAS). We believe that these mechanisms triggered by IH are the first steps involved in the development of insulin resistance observed in patients with OSA (Figure 7).

Our experimental model is unique with IH exposure in healthy humans free of confounders (i.e. obesity and comorbidities). This is crucial when evaluating metabolic pathways. Individuals were switched from normal air to 13% FiO_2, allowing a range of SaO_2 from

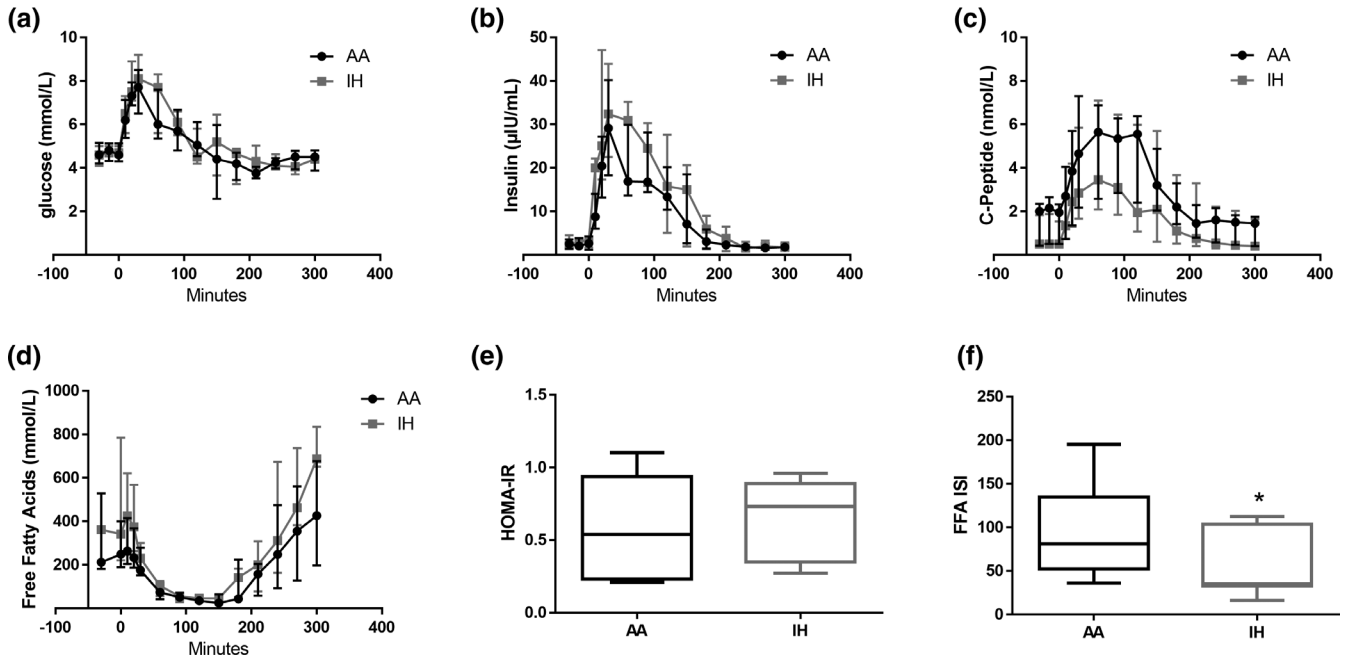

FIGURE 3 IH alters FFA response to OGTT. Evaluation of (a) glycaemia (mmol L^{-1}), (b) insulinaemia (µIU ml^{-1}), (c) C-peptide (mmol L^{-1}) and (d) FFAs (mmol L^{-1}) before and during an OGTT, performed at the end of the AA or IH period. For FFA, the AUC is significantly higher after IH than after AA ($p = 0.05$). (e) Calculated HOMA-IR index and (f) calculated FFA-ISI. *$p = 0.028$ IH versus AA. Data are presented as median ± IQ range and min/max values, $n = 8$ subjects. AA, ambient air; AUC, area under the curve; FFA, free fatty acid; HOMA-IR, homeostasis model assessment-insulin resistance; IH, intermittent hypoxia; ISI, insulin sensitivity index; OGTT, oral glucose tolerance test.

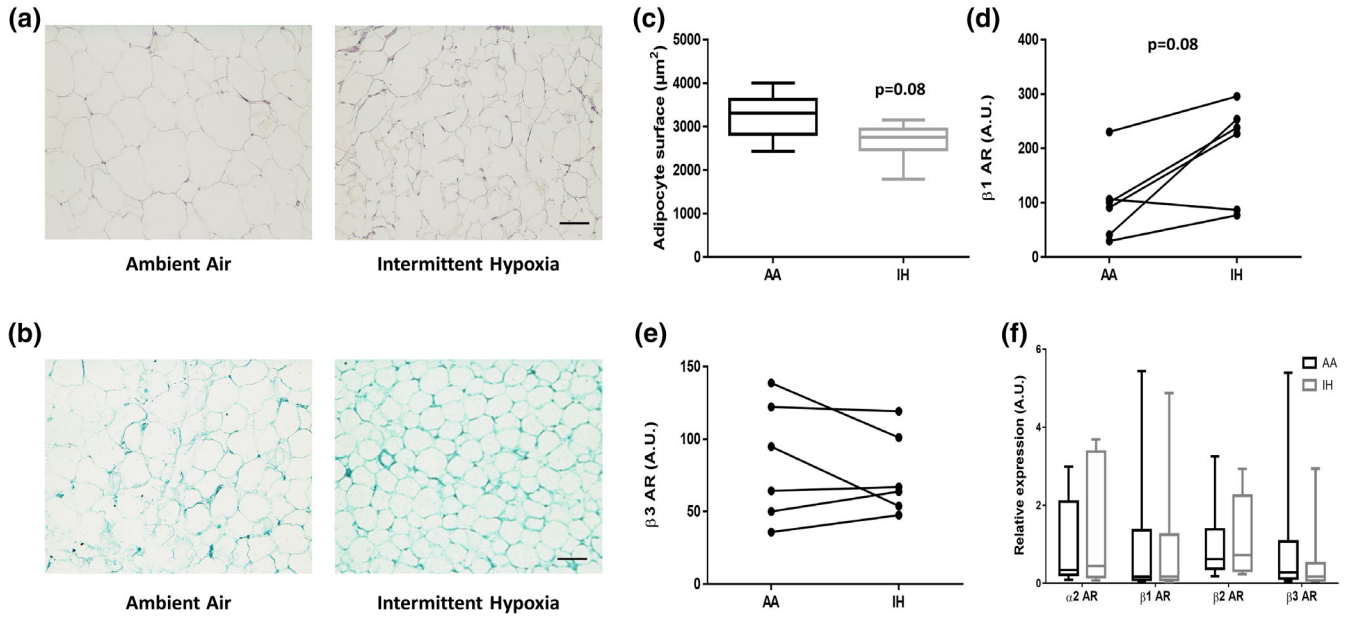

FIGURE 4 Adipose tissue histology and adrenergic receptor expression. (a) Haematoxylin–eosin staining of subcutaneous adipose tissue after AA or IH. (b) Staining of β1-adrenergic receptor on adipose tissue biopsies after AA or after IH. (c) Quantification of adipocyte surface on adipose tissues biopsies, $p = 0.08$ AA versus IH. (d,e) Quantification of adrenergic receptor expression after immunohistochemistry, (d) β1-AR, $p = 0.08$ AA versus IH, and (e) β3-AR. (f) qPCR analysis of mRNA expression for adrenergic receptor genes. Data are presented as median ± IQ range and min/max values, $n = 6$ subjects due to missing biopsies for two participants. Scale bars: 50 µm (a,b). (a,b) Pictures are representative pictures taken from different subjects. AA, ambient air; IH, intermittent hypoxia; qPCR, quantitative polymerase chain reaction.

95% to 85%, 30 cycles per min during 8 hr of sleep. This was first described in our laboratory (Tamisier et al., 2009), and allows the subject to sleep freely while exposed either to sham (AA) or IH. We previously demonstrated an increase in sympathetic tone by using the reference method of muscle sympathetic activity (Gilmartin et al., 2010; Tamisier et al., 2011). The rise in muscle sympathetic tone

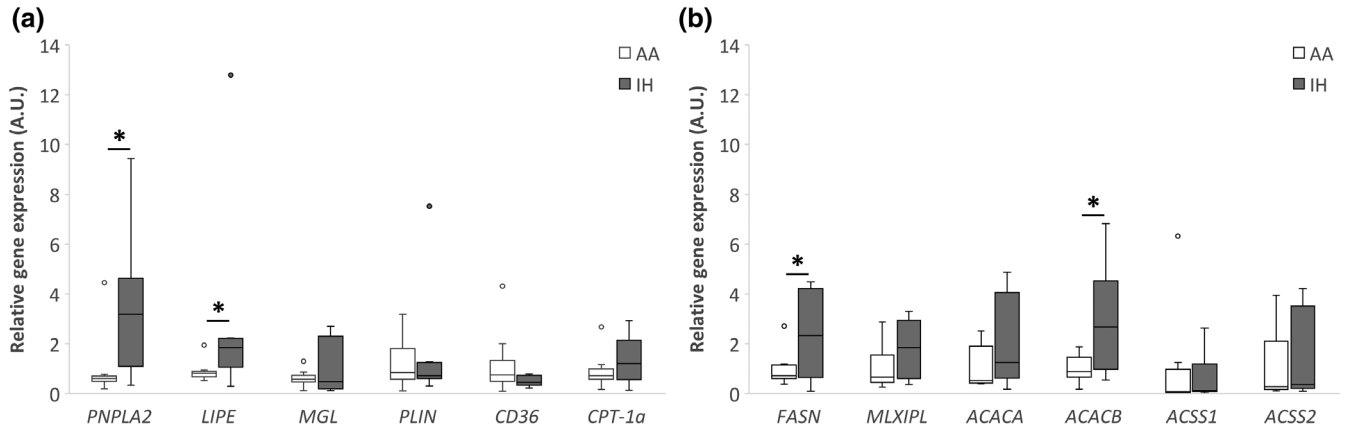

FIGURE 5 Expression levels of key genes of lipid metabolism in adipose tissue after IH. (a) qPCR evaluation of mRNA levels for genes involved in lipolysis and beta-oxidation of fatty acids: *PNPLA2* gene for ATGL, *LIPE* for HSL, MGL, *PLIN* for perilipin-1, *CD36*, *CPT-1a* for CPT1. (b) qPCR evaluation of genes involved in lipogenesis: *FASN* for FAS, *MLXIPL* for ChREBP, *ACACA* for ACC1, *ACACB* for ACC2, *ACSS1* and *ACSS2* for ACS1 and ACS2. *$p < 0.05$ Ambiant Air (AA) versus Intermittent Hypoxia (IH), $n = 8$ subjects. Data are presented as median ± IQ range and min/max values. ACC1, acetylCoA carboxylase 1; ACC2, acetylCoA carboxylase 2; ACS1, acylCoA synthetase 1; ACS2, acylCoA synthetase 2; ATGL, adipocyte triglyceride lipase; ChREBP, carbohydrate responsive element binding protein; CPT1, carnitine palmitoyl transferase 1; FAS, fatty acid synthase; HSL, hormone-sensitive lipase; IH, intermittent hypoxia; MGL, monoacylglycerol lipase; qPCR, quantitative polymerase chain reaction.

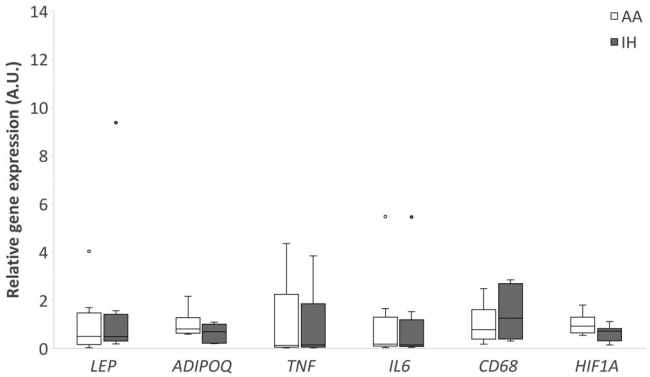

FIGURE 6 Expression levels of key genes of inflammation in adipose tissue after IH. qPCR evaluation of mRNA levels for genes involved in inflammation: *LEP* for leptin, *ADIPOQ* for adiponectin, TNFα, IL-6, CD68 and HIF-1α showed no change after IH. Data are presented as median ± IQ range and min/max values, $n = 8$ subjects. CD68, cluster of differentiation 68; HIF-1α, hypoxia-inducible factor-1α; IH, intermittent hypoxia; IL-6, interleukin-6; qPCR, quantitative polymerase chain reaction; TNF-α, tumour necrosis factor-α.

after IH is of the same magnitude as those reported in patients with OSA versus control or under OSA primary therapy with continuous positive airway pressure (CPAP; Tamisier et al., 2015; Tan et al., 2013). This model is therefore the best to investigate early stages of pathophysiological consequences of a high sympathetic tone state induced by IH.

Metabolism of FFAs is crucial as a primary source of energy. However, excessive FFAs circulating level is implicated in the mechanisms of insulin resistance. Elevations of fatty acids also stimulate insulin secretion, but in a range that is not sufficient to fully compensate for the FFA-induced insulin resistance (Boden, 2005). Finally, this leads to impaired insulin secretion from the pancreatic beta cells (Stefanovski et al., 2020).

Consistently with this knowledge and our hypothesis, our study demonstrates that FFA AUC after OGTT increased, and that FFA-ISI (corresponding to FFA sensitivity to insulin) significantly decreased after IH. This is a dynamic and robust indicator of a decrease in the ability of insulin to reduce circulating FFA levels. These data are consistent with similar alterations of FFA metabolism during OGTT recently reported in patients with sleep-disordered breathing (Stefanovski et al., 2020).

Although MSNA measurements showed an increase in sympathetic activity, characterization of adipose tissue biopsies showed that the expression of adrenergic receptors did not change. Beta1 adrenergic receptors expression tended to increase, suggesting the absence of receptor-mediated desensitization, which can be due to internalization and degradation of adrenergic receptors (Svoboda et al., 2004). A sustained effect of activated SNS on adipose tissue can thus be expected in our model.

Activation of the SNS is well known for its beta receptor-mediated lipolytic effect on the adipose tissue (Lafontan & Langin, 2009). We demonstrated for the first time that IH induces the expression of key lipolysis genes such as HSL and ATGL, accompanied by a tendency of adipocytes to shrink. It also increases the expression of ACC2, which in turn inhibits degradation of FFAs by inhibiting CPT1, a key enzyme of beta-oxidation (Wang et al., 2022). These results are consistent with studies in cellular and animal models showing that IH induced lipolysis and FFA release, both on acute (Jun et al., 2012; Jun et al., 2014) and long-term exposures up to several weeks (Briançon-Marjollet et al., 2016a; Khalyfa et al., 2020; Musutova et al., 2020; Poulain et al., 2014; Weiszenstein et al., 2016). Patients with OSA also have elevated FFA levels that could be due to

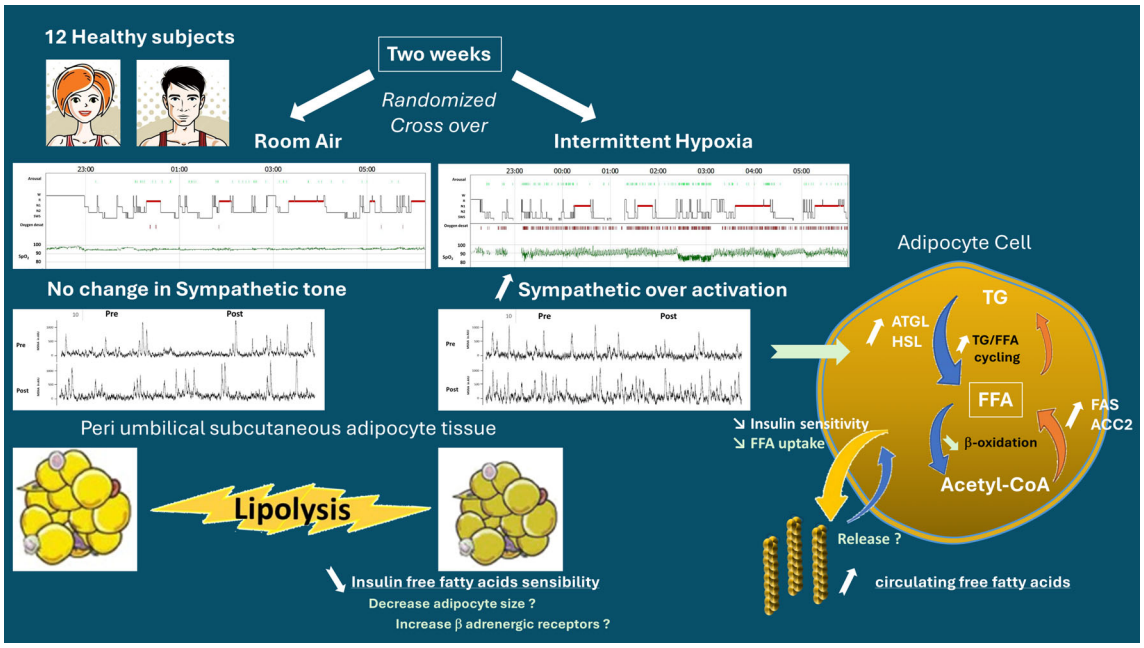

FIGURE 7 This scheme illustrates the main results of the present study showing an increase in circulating FFAs that are likely to be related to several mechanisms, for example, decrease in FFA insulin sensitivity and increase in FFA production. Significant changes are labelled in white, and non-significant or hypothesis are labelled in pale green. FFA, free fatty acid.

SNS-mediated lipolysis, and lipolysis could be one of the mechanisms causing metabolic dysfunction in patients with OSA (Barceló et al., 2011; Chopra et al., 2017; Gu et al., 2017; Meszaros & Bikov, 2022; Trinh et al., 2021).

Lipolysis can be regulated by SNS as well as by inflammatory cytokines or insulin signalling. Interestingly, investigation of adipose tissue showed that in our study, inflammatory cytokines were not modified by IH. We thus suggest that in our model, activated SNS may be the main mediator leading to lipolysis of the adipose tissue induced by IH. Moreover, reduced sensibility of adipose tissue to insulin-induced FFA uptake (as assessed by the decrease of FFA-ISI) could accelerate this SNS-induced lipolysis. We cannot exclude that both SNS-dependent and SNS-independent mechanisms are simultaneously activated by IH, both leading to lipolysis.

In parallel with lipolysis, we observed an increase in the lipogenesis genes FAS and ACC2 that are involved in FFA synthesis from acetyl-CoA. These concomitant lipolytic and lipogenesis pathways could both result in an increase in FFAs in adipocytes, as summarized in the graphical abstract (Figure 7). This suggests an increased turnover of lipids in our IH model, with lipogenesis possibly partially compensating for the activated lipolysis.

Our study has strengths and limitations. Although our study included a small number of participants, robustness of the data was strengthened by its randomized crossover design versus AA, and inclusion of healthy subjects without any confounding factors. Moreover, the present study was powered based on anticipated sympathetic response, and may be underpowered for some of the secondary objectives including changes in glucose metabolism and inflammatory state both at the adipocyte tissue and blood levels. Therefore, our study may be underpowered, which would have to be considered for interpretation. This lack of statistical power may also explain the carryover effect that we observed for some parameters. However, this carryover effect could also be explained by acclimatization of subjects to their environment (the hypoxic tent).

We exposed healthy humans only for 2 weeks, which was a short acute exposure compared with what patients with OSA are exposed across life. Moreover, blood samples were only taken in the morning at the end of AA/IH exposure, and not during the night. However, dynamic metabolic regulation was reported during the night in patients with OSA, suggesting that morning samples may not completely reflect what occurred during night (Chopra et al., 2017). Finally, for accessibility reasons, we analysed only biopsies from subcutaneous adipose tissue. It is known that visceral rather than subcutaneous adipose tissue depots play a crucial role in inflammation, even though subcutaneous adipose tissue is also known to be associated with dyslipidaemia and metabolic syndrome (Henning, 2021). However, this may explain why we did not observe a proinflammatory effect of IH in our subcutaneous adipose tissue biopsies. Despite these limitations, the main strength of the study is the unique design allowing to study the direct effect of IH in human-integrated physiology.

In conclusion, 2 weeks of IH in healthy humans triggers an increased lipolysis possibly mediated through sympathetic activation. This sympathetic overactivity could imbalance the state between lipogenesis and lipolysis in the adipose tissue. The overproduction of FFAs and their lower response to insulin may favour the development of systemic insulin resistance.

AUTHOR CONTRIBUTIONS

Anne Briançon-Marjollet: Conceptualization; writing – original draft; methodology; validation; supervision. **Marie Netchitaïlo:** Investigation; writing – review and editing. **Fanny Fabre:** Investigation; writing – review and editing. **Elise Belaidi:** Conceptualization; methodology; writing – review and editing; supervision. **Claire Arnaud:** Conceptualization; supervision; writing – review and editing. **Anne-Laure Borel:** Conceptualization; writing – review and editing; methodology; formal analysis. **Patrick Levy:** Conceptualization; writing – review and editing; funding acquisition. **Jean-Louis Pépin:** Conceptualization; funding acquisition; writing – review and editing. **Renaud Tamisier:** Conceptualization; funding acquisition; writing – original draft; methodology; validation; supervision.

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CONFLICT OF INTEREST STATEMENT

Renaud Tamisier reports receiving lecture fees from ResMed, and grant support through his institution from ResMed, Agiradom and Philips, and travel grants form Agiradom. Jean-Louis Pépin reports grant support through his institution from ResMed, Agiradom Vitalaire and Philips, and travel grants from Agiradom. Patrick Lévy reports no conflict of interest. Anne Briançon-Marjollet reports no conflict of interest. Marie Netchitaïlo reports travel grants from Asten Santé. Fanny Fabre reports no conflict of interest. Elise Belaidi reports no conflict of interest. Claire Arnaud reports no conflict of interest. Anne-Laure Borel reports no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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