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# Murine double minute-2 mediates exercise-induced angiogenesis in adipose tissue of diet-induced obese mice

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## ABSTRACT

**Objective:** This study aimed to determine the effects of physical exercise on the angio-adaptive response in adipose tissue following weight loss in a mouse model of diet-induced obesity. We hypothesized that physical exercise stimulates angiogenesis through the regulation of *Vascular endothelial growth factor-A* (VEGF-A) pro-/*Thrombospondin-1* (TSP-1) anti-angiogenic signal under the control of the *Murine double-minute 2/Forkhead box Os* (Mdm2/FoxOs) axis, as reported in skeletal muscle.

**Methods:** We studied the effects of 7 weeks-voluntary exercise (Ex) in C57Bl/6 control or diet-induced obese (HFS) mice on vascularization of white adipose tissue (AT).

**Results:** Diet-induced obese sedentary (HFSsed) mice presented a powerful angiostatic control in all adipose tissues, under FoxOs protein regulation, leading to capillary rarefaction. Exercise increased expression of Mdm2, repressing the angiostatic control in favor of adipose vascular regrowth in normal chow (NCex) and HFSex mice. This phenomenon was associated with adipocytes microenvironment improvement, such as decreased adipocytes hypertrophy and adipose tissue inflammation. In addition, adipose angiogenesis stimulation by exercise through Mdm2 pro-angiogenic action, improved visceral adipose insulin sensitivity, activated *browning* process within subcutaneous adipose tissue (ScWAT) and decreased ectopic fat deposition (muscle, heart and liver) in obese HFSex mice. The overall result of this approach of therapy by physical exercise is an improvement of all systemic cardiometabolic parameters.

**Conclusions:** These data demonstrated the therapeutic efficacy of physical exercise against obesity-associated pathologies, and also offer new prospects for molecular therapies targeting the adipose angio-adaptation in obese humans.

## 1. Introduction

Obesity has become in recent decades, a major public health problem whose prevalence is constantly increasing worldwide. Sedentary lifestyle coupled to high fat and carbohydrate rich diet may explain this phenomenon. Adipose tissue (AT) expansion, occurring during obesity, is associated with insulin resistance development (Haffner, 2007; Longo et al., 2019), AT metabolic and endocrine dysfunctions (Berg and Scherer, 2005). Regular practice of physical exercise is well established as a therapeutic approach for

many chronic metabolic and cardiovascular diseases (Lavie et al., 2015; Myers et al., 2019), notably by activating AT browning process (Aldiss et al., 2018; Otero-Díaz et al., 2018; Stanford et al., 2015). AT homeostasis is very dependent on the microcirculation. However, capillary growth remains limited in comparison to the magnitude of AT expansion occurring in case of obesity (Pasarica et al., 2009; Voros et al., 2005). Therefore, understanding the molecular events that could regulate the vascularization process in AT is of great interest.

The biological process of capillary network remodeling, so-called tissue

**Abbreviations:** AKT, Protein kinase B; AT, Adipose tissue; BAT, brown adipose tissue; EWAT, epididymal white adipose tissue; Ex, exercised; FoxO1/3 $\alpha$ , Forkhead box O1/3  $\alpha$ ; HFD, high fat diet; HFS, high fat and high sucrose diet; IL-6, Interleukin-6; ISO, Isoproterenol; MBP, Mean arterial blood pressure; MCP-1, monocyte chemoattractant protein 1; Mdm-2, Murine double-minute 2; NC, normal chow; PGC-1 $\alpha$ , peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$ ; PPAR $\alpha$ , peroxisome proliferator-activated receptor alpha; SEM, standard error of the mean; ScWAT, subcutaneous white adipose tissue; Sirt-3, Sirtuin-3; TGF- $\beta$ 1, transforming growth factor beta 1; TNF $\alpha$ , tumor necrosis factor alpha; TSP-1, Thrombospondin 1; UCP-1, Uncoupling protein 1; VEGF-A, Vascular endothelial cell growth factor-A

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angio-adaptation, has been previously demonstrated in skeletal muscle with the capillary network being dependent on the balance between pro and anti-angiogenic factors (Olfert and Birot, 2011), but its regulation within AT remains largely unknown. To regulate the balance between the pro-angiogenic factor *Vascular endothelial cell growth factor-A* (VEGF-A) (Tang et al., 2004), and the anti-angiogenic factor *Thrombospondin 1* (TSP-1) (Malek and Olfert, 2009), *Murine double-minute 2/Forkhead box O1* (*mdm2/FoxO1*) axis has been shown to play a key role in skeletal muscle angio-adaptive response to exercise (Roudier et al., 2012). In fact, the E3 ubiquitin ligase *Mdm2* has been known for its oncogenic role of the major negative regulator of p53 tumor suppressor protein (Wade et al., 2013). Thus, the inhibition of p53 did increase ischemia-induced arteriogenesis (Pfaff et al., 2018). Furthermore, an increase in *FoxO1* level expression was observed in skeletal muscle of diet-induced obese mice, and a direct interaction between *Mdm2* and *FoxO1* was demonstrated (Milkiewicz et al., 2011). Aside, *FoxO1* has been reported to a transcriptional regulator of TSP1 within vascular endothelial cells (Roudier et al., 2013; Wilhelm et al., 2016), and interestingly, an up-regulation of TSP-1 has been also observed within the muscle and adipose tissue of obese and diabetic mice (Kivelä et al., 2008; Kong et al., 2013).

Therefore, in the present study we investigated the *Mdm2-FoxO1* axis in adipose angiogenesis. We hypothesized that voluntary exercise increased white AT capillary density in diet-induced obese C57/Bl6 mice, then, molecular regulation of angiogenic factors including *Mdm2*, *FoxO1*, *3α* and their downstream effectors, VEGF-A and TSP-1 was investigated.

## 2. Materials and methods

### 2.1. Animals and diets

All investigations were done in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US NIH (National Academies Press US, 8th edition, 2011) and the agreement of European and French Ministry of Agriculture, for the care and the use of laboratory animals, and the local research ethics committee (Comité Régional d'Ethique, n°: 84.004). Male C57BL/6 Inbred mice ( $n = 85$ ) were purchased from Janvier Labs, Saint-Berthevin, France, housed in cages with enriched environment and maintained in controlled environment conditions (20–23 °C; 40% humidity), and on 12 h light/dark cycle. Mice had access to food and water *ad libitum*.

Animals (12-week of age and weighing  $27.4 \pm 1.2$  g) were pair-fed with the standard/normal chow (NC) (A04, 3.1% Fat, caloric value  $3.339 \text{ kcal}\cdot\text{kg}^{-1}$ , Safe, France) or high-fat/high sucrose diet (HFS) (230HF, 60% kcal as fat with caloric value  $5.317 \text{ kcal}\cdot\text{kg}^{-1}$ , Safe, France) completed with 10% sucrose in drinking water over 16 weeks. After 9 weeks of the diet, the animals were randomly assigned into 3 groups as follows: sedentary NC diet group (NCsed,  $n = 15$ ), sedentary HFS (HFSsed,  $n = 30$ ), physical exercise NC group (NCex,  $n = 15$ ) or physical exercise HFS group (HFsex,  $n = 25$ ) for the 7 remaining weeks. Voluntary exercise consisted in the introduction of a wheel in cages (3 mice/cage) in free access. Wheels were connected to a sensor for a daily record of the number of laps being executed in each cage. The animals ran throughout the 7-weeks intervention and achieved a maximum distance of 5 and 3.5 and km/day for NCex and HFsex respectively (Fig suppl 1). The effects of exercise on NCex and HFsex mice were tested in tissue samples collected after 1, 2, 3, 4, and 7 weeks of voluntary exercise (NCsed  $n = 5$ ; NCex  $n = 5$ ; HFS  $n = 5$  and HFsex  $n = 5$  for each period). Tissues were also sampled before starting the exercise protocol, representing time 0 period (NCsed  $n = 4$ ; HFSsed mice  $n = 4$ ). Sacrifice was realized within 1 h after wheel removal. Immediately after sacrifice, adipose tissues (epididymal, EWAT; subcutaneous, ScWAT; perirenal, mesenteric, interscapular brown, BAT), liver, gastrocnemius, soleus, and heart were collected, fixed in formalin or Tissue Tek resin (Sakura) for histological analysis or frozen in liquid nitrogen and stored at  $-80$  °C for biochemical analysis. Aside, two small pieces of EWAT were incubated 30 min at 37 °C with a PBS  $1\times$  solution containing or not insulin ( $20 \text{ mg}\cdot\text{mL}^{-1}$ ) to test tissue insulino-resistance, then tissues were frozen in

liquid nitrogen and stored at 80 °C.

### 2.2. Fasting blood glucose and glucose tolerance tests

The week preceding sacrifice, blood was collected (5  $\mu\text{L}$ ) using the tail-clip method and fasting blood glucose level was assessed (CarensR N, DinnoSante™) according to the manufacturer's instructions. Then, Intra-Peritoneal Glucose Tolerance Tests (IP-GTT) were performed after an i.p. injection of glucose solution ( $1 \text{ g}\cdot\text{kg}^{-1}$ ), and blood glucose levels were measured at 20, 40, 60, 90 and 120 min after injection. For insulin tolerance tests (IP-ITT), insulin was injected *i.p.*, at  $0.75 \text{ U}\cdot\text{kg}^{-1}$  and blood glucose levels were measured at 15, 30, 45, 60, 90 and 120 min after insulin injection.

### 2.3. Blood pressure measurement

Mean arterial blood pressure (MBP) was assessed in conscious mice by tail-cuff method using the CODA tail-cuff system (Kent Scientific, Torrington, CT, USA) two days before sacrifice. The MBP values were calculated as the average of 10 measurements.

### 2.4. Western blotting

Proteins were extracted from EWAT and ScWAT using Tris-HCl buffer (50 mM pH = 7.4; 10% glycerol; 3.5 mM SDS; protease inhibitor cocktail (Sigma Aldrich); phosphatase inhibitor ( $\text{Na}_3\text{VO}_4$ ; Fischer Scientific) at 4 °C. Equivalent amount of denatured protein was separated by electrophoresis on 10% polyacrylamide-SDS gel, transferred onto PVDF membranes (Immobilon, Millipore) and probed with primary antibodies overnight at 4 °C (Annex 1). Immunodetection was carried out using ECL System (Super Signal West Pico Chemiluminescence Substrate, Thermo Scientific) and membranes were then exposed to X-ray films for revelation. Protein content was expressed relative to  $\alpha/\beta$  Tubulin content on the same membrane.

### 2.5. RNA extraction and real-time quantitative PCR

Frozen EWAT and ScWAT were introduced in TRizol reagent for total RNA extraction using a Retsch MM300 tissue lyser. cDNAs were synthesized from 500  $\mu\text{g}$  of total RNA using random primers and Moloney murine leukemia virus reverse transcriptase and the cDNA was used for quantitative real-time PCR in an Mx3005P Real-Time PCR System (Stratagene). Reactions were carried out in duplicate for all conditions using a Sybr Green Master mix (Eurogentec) and expression of the ribosomal protein 18S mRNA was used as endogenous control in the comparative cycle threshold method. Primer sequences were used for qPCR determination (Annex 2).

### 2.6. ELISA assay

Tissues were prepared in lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with a phosphatase inhibitor cocktail (Santa Cruz Biotechnology, CA, USA) and protease inhibitors (Roche). The protein concentration of tissue samples was determined by Bradford assay (BioRad). Monocyte chemoattractant protein 1 (MCP-1) and Interleukin-6 (IL-6) expressions were determined by using the mouse MCP-1 ELISA kit (BMS6005, ThermoFisher Scientific) and IL-6 ELISA kit (KMC0061, ThermoFisher Scientific), respectively according to the manufacturer's instructions. The absorbance of each sample and standard was measured with a plate reader (MultiSkan EX, Thermo).

### 2.7. Tissue histology

Paraffin embedded tissue sections of EWAT and ScWAT were stained with hematoxylin/eosin using standard protocols. To visualize

the vasculature, ABC kit vectastain (Vector Labs), anti-mouse CD-31 (Abcam 28,364) were used for immunostaining of EWAT and ScWAT.

Sections of frozen liver, soleus, gastrocnemius and heart fixed in Tissue Tek resin were stained using standard Oil red-O staining protocol for lipid droplets detection.

## 2.8. Adipose angiogenesis assay

This assay was performed on ScWAT from NC mice (21 weeks old,  $n = 3$ ) according to the protocol described in Rojas-Rodriguez et al. (2014). After removal, AT was cut in  $1\text{mm}^3$  pieces, placed in 24-well plates (20 pieces/well). Then, AT pieces were either treated with Nutlin-3 ( $25\ \mu\text{M}$ ) for 24 h or RNA interference experiment was realized. AT samples were transfected using HiPerfect® transfection reagent (Qiagen) with siRNA targeted against Mdm2 or a nontargeting siRNA according to the manufacturer's instructions (Ambion by Life technologies). Briefly, the AT pieces were transfected overnight using a mixture of  $10\ \mu\text{M}$  siRNA and  $6\ \mu\text{L}$  of lipofectamin reagent per well. The AT pieces were then, separated into 96-well plate (1/well) with EGM-2 MV supplemented EBM-2 medium (Lonza, Switzerland), embedded in Matrigel (Matrigel® Matrix, Corning, European Union) and incubated at  $37\ ^\circ\text{C}$  in 5%  $\text{CO}_2$  for 14 days. Some AT specimens were treated with Isoproterenol for  $\beta$ -adrenergic stimulation ( $10\ \mu\text{M}$ ). The EGM-2 MV supplemented EBM-2 medium was replaced every day.

After 14 days, the amount of capillary growth was evaluated by measuring the area of sprouting in each well on the microscope.

Brightfield images were acquired using  $10\times$  objective, captured Motic® Camera and analyzed through Motic-ImagePlus 3.0 software. For calculation of the growth area, capillary sprout growth area was normalized with the adipose explant area.

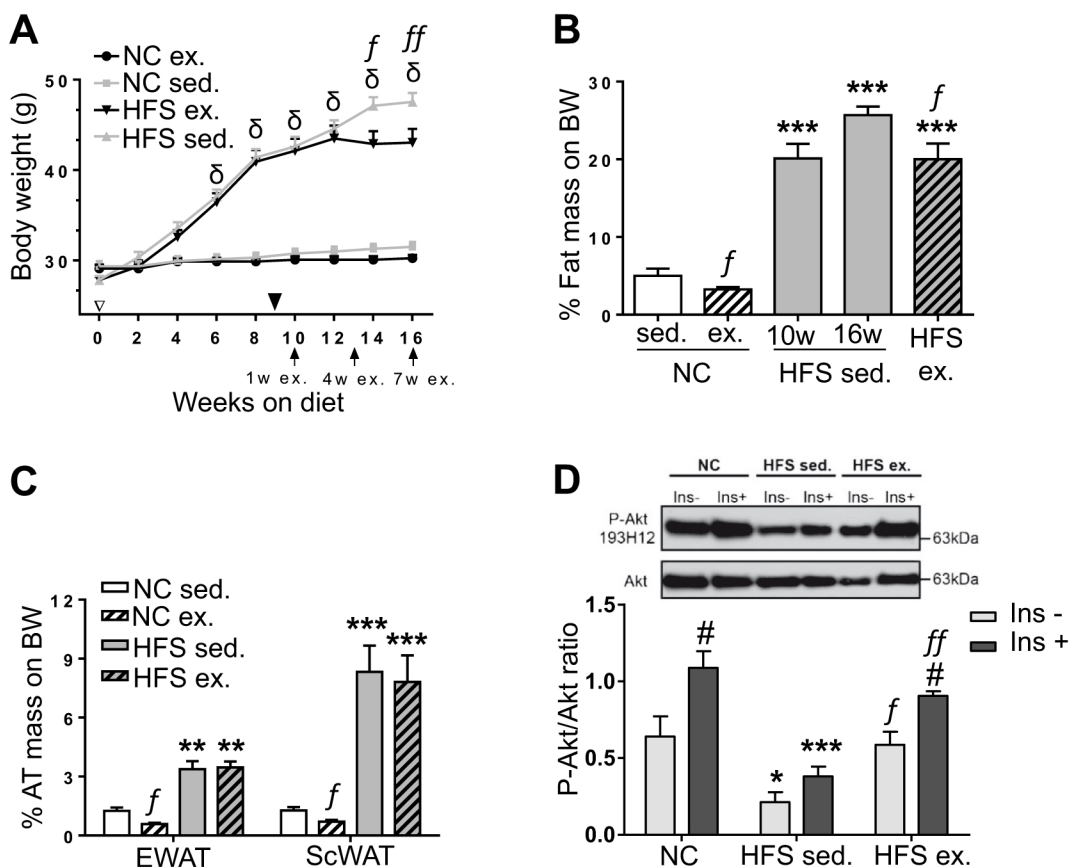
## 2.9. Statistical analysis

For all data, Gaussian distribution was tested by the d'Agostino-Pearson normality test. When data followed a Gaussian distribution, statistical differences were analyzed by one or two-way ANOVA with Tukey post-test. Otherwise, the Kruskal-Wallis test followed by Dunn's post-test were used to verify significance of the observed differences. All statistical analyses were performed using the GraphPad Prism software. Mean  $\pm$  SEM.  $p$  values  $< 0.05$  were considered as statistically significant, \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

## 3. Results

### 3.1. Exercise improved obesity-related metabolic complications induced by HFS diet

After the complete protocol of 16 weeks, enriched fat and sucrose diet feeding significantly increased body mass of HFSSed mice ( $\sim 50\%$ ) compared to their littermate assigned to normal chow diet (NCsed) (Fig. 1A). Sedentary HFS mice had a total fat mass significantly greater



**Fig. 1.** 7 weeks of exercise induced a decreased of body weight, fat mass and increase adipose insulin sensitivity in HFS mice. (A) Body weight curve during HFS diet and voluntary exercise protocols of normal chow (NCsed and NCEx) and HFS mice (HFSSed and HFSEx); HFS diet started on week 0 (V), voluntary exercise started on week 9 for the NCEx and HFSEx mice group (V). (B) Total fat mass at 10 weeks and at the end of the 16 weeks protocols in sedentary normal chow (NCsed), exercised NC (NCEx), sedentary HFS (HFSSed) and exercise-HFS mice (HFSEx); (C) EWAT and ScWAT masses at 16 weeks of diet and physical exercise protocols in NC, NCEx, HFSSed and HFSEx mice and at 10 weeks of protocols in HFSSed mice. (D) Representative immunoblots and densitometry analysis of Phospho-Akt (P-Akt) and Akt on EWAT of sedentary/exercise NC and HFS mice +/- *ex vivo* insulin stimulation (■ Ins-: incubation without insulin; ■ Ins+: incubation with insulin). Data are means  $\pm$  SEM ( $n = 4-5$  per groups and per time point. #  $P < 0.05$  (Ins + relative to Ins-); One (B) or two-way ANOVA (A, C, D) and Tukey post-test; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ ,  $\delta P < 0.001$  (HFS relative to NC);  $f P < 0.05$ ,  $ff P < 0.01$  (ex. relative to sed.).

**Table 1**  
Effect of 16 weeks of HFS diet and voluntary 7 weeks exercise on mean blood pressure, and metabolic parameters.

	NC	NC ex	HFS Sed.	HFS Ex.
Mean blood pressure (mmHg)	110 ± 3.9	111 ± 4.0	140 ± 2.4 ***	128 ± 3.5 * f
Fasting blood glucose (mg.dL <sup>-1</sup> )	211 ± 17	215 ± 26	331 ± 35.7 *	220 ± 17.5 f
IP-GTT (AUC)	1959 ± 75	1699 ± 153	2740 ± 42 ***	2270 ± 139 f
IP-ITT (AUC)	178 ± 10.5	165 ± 20.5	195 ± 27.2	143.7 ± 7.9 f

Results are presented as mean ± SEM.

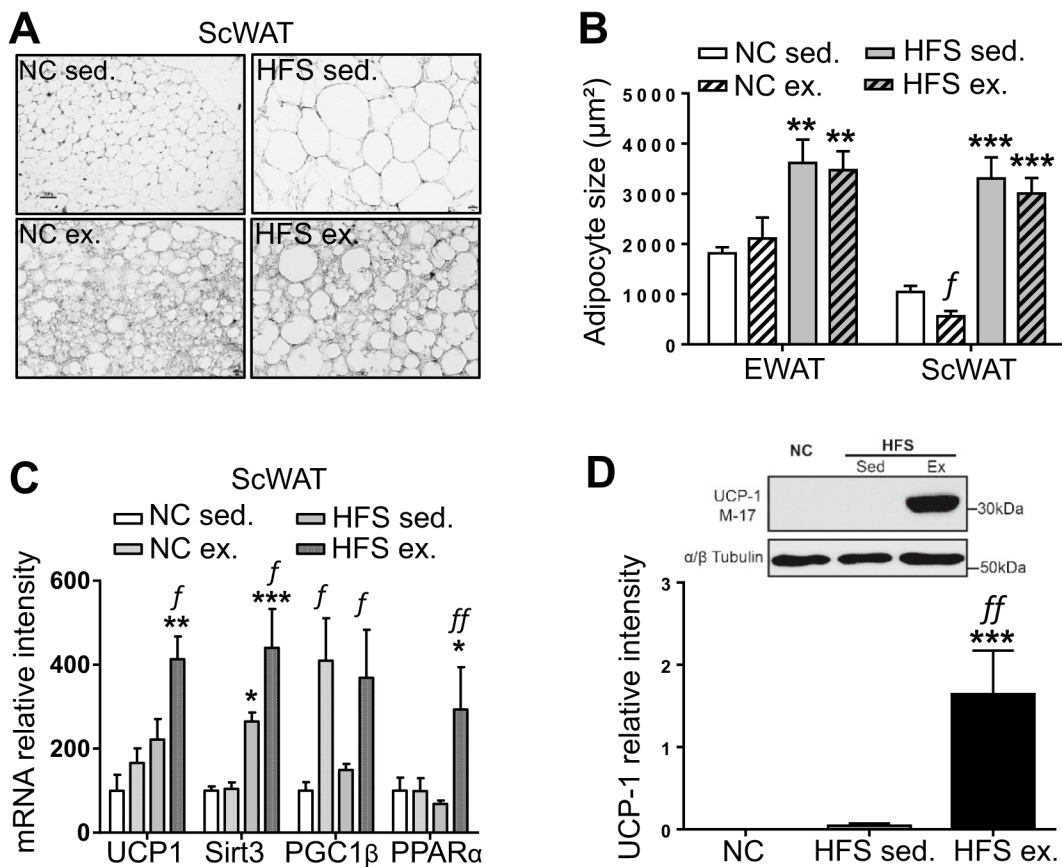
IP-GTT: Glucose tolerance test; IP-ITT: Insulin tolerance test; AUC area under the curve.

\**p* < 0.05 relative to NC; \*\*\**p* < 0.005 relative to NC; *f p* < 0.05 HFS Ex. vs. HFS Sed.

(5 times) than NCsed mice (Fig. 1B). In fact, EWAT, ScWAT and BAT mass (Fig. 1C) were significantly increased in HFSsed mice by a factor 4, 10 and 7 respectively in comparison to NCsed mice. Furthermore, in EWAT and ScWAT of HFSsed mice, adipocyte size significantly augmented by a factor 3 and 4, respectively compared to NCsed mice. After 7 weeks of voluntary exercise, the body mass of HFsex mice was significantly reduced by ~10% (Fig. 1A), concomitantly with a significant reduction of fat mass percentage (Fig. 1B) of 27% and 44% for EWAT and ScWAT respectively in comparison with HFSsed mice. To assess the direct effects of exercise, fat mass of HFsex group at 7 weeks was compared to weight-match HFSsed group (10 weeks of diet) and no difference was found (Fig. 1B). Aside, 7 weeks of exercise did not affect significantly body mass of NCsed mice group but reduced body fat mass (Fig. 1B) with a lower but significant decrease of fat mass percentage

(Fig. 1C) for EWAT and ScWAT in NCex in comparison with NCsed group.

Mean blood pressure (MBP) was higher in HFSsed in comparison with NCsed mice. In HFsex mice, MBP was significantly lowered in comparison with HFSsed mice group, while no change of mean blood pressure was observed in NCex in comparison with NCsed mice. On the other hand, HFSsed mice also presented significantly elevated fasting blood glucose and enhanced elevation of blood glucose during glucose tolerance test (IP-GTT) in comparison with NCsed mice (Table 1), and the HFsex group, the glucose homeostasis was improved as suggested by the reduced fasting glucose and a faster normalization of baseline blood glucose after glucose injection as compared to HFSsed mice (Table 1). Furthermore, HFSsed fed mice insulin-sensitivity (IP-ITT) was lower than NCsed mice but without significance. However HFsex



**Fig. 2.** Voluntary exercise induced thermogenesis in adipose tissue of HFS obese mice. (A) Representative image of ScWAT from sedentary/exercise NC and HFS mice. ScWAT from HFsex mice presented brown-like adipocytes in the middle of white adipocytes. Scale bar = 20  $\mu\text{m}$ . (B) EWAT and ScWAT adipocyte size in sedentary/exercise NC and HFS mice at the end of the 16-weeks of protocol. (C) Thermogenic activity markers mRNA expression (UCP-1, SIRT-3, PGC-1 $\beta$ , PPAR $\delta$  and PPAR $\alpha$ ) from ScWAT of NC, NCex, HFSsed and HFsex mice were analyzed by real-time qPCR, with values normalized to ribosomal protein 18S mRNA. The mRNA data are expressed as relative expression ratios to NCsed mice. (D) UCP-1 protein level analyzed by Western Blot in the ScWAT of NC, NCex, HFSsed and HFsex mice. Blots were stripped and reprobred for  $\alpha/\beta$ -tubulin as a loading control. Data are means  $\pm$  SEM (*n* = 5 per groups). One (D) or two-way ANOVA (B, C) and Tukey post-test \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.005 (HFS relative to NC), *f p* < 0.05 (ex. relative to sed.). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

showed a significant improvement of insulin response (Table 1), while in NCex mice no change was observed. Aside, insulin tissue response and insulin-sensitivity status in EWAT was assessed by measurement of phospho-protein kinase B (P-Akt) protein basal expression or after *in vitro* insulin stimulation. Basal P-Akt/Akt expression ratio was significantly lower in EWAT of HFSSed mice compared to NCsed mice. After insulin stimulation, P-Akt/Akt ratio was significantly increased by 70% in NCsed mice, while no significant increase was noticed in HFSSed mice (Fig. 1D). Interestingly, the P-Akt/Akt ratio was significantly increased by 55% in insulin stimulated EWAT of HFSex mice.

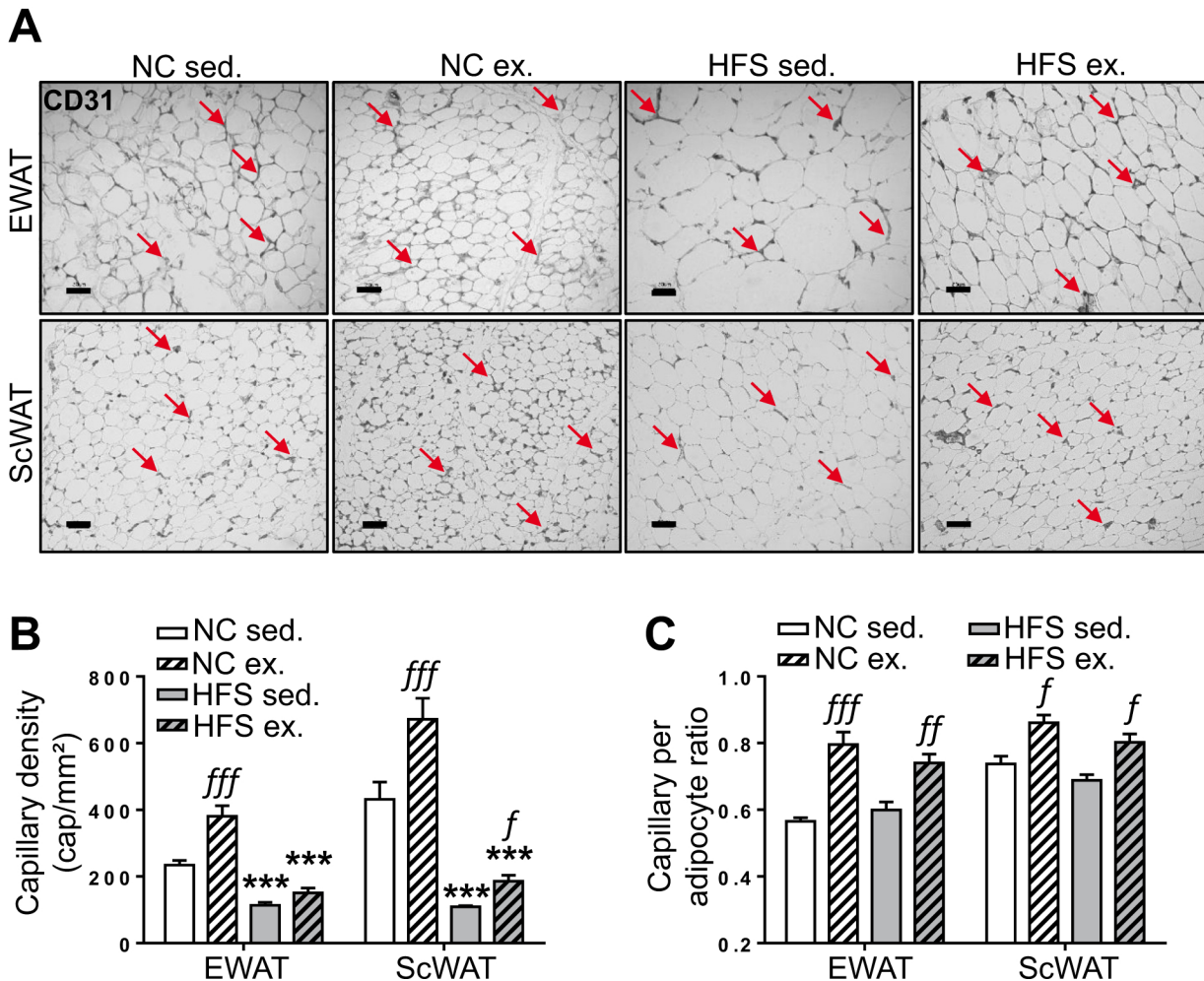
### 3.2. Exercise influenced adipose tissue biology

High fat/high sucrose diet induced severe visceral obesity, with a significant increased adipocyte size in EWAT and ScWAT in HFSSed AT in comparison to NCsed AT. After exercise, EWAT morphology did not change while ScWAT presented focal small multilocular brown-like adipocytes (Fig. 2A) characteristic of a “beige” phenotype. Aside, exercise in NC led only to a decreased adipocyte size of 40% in ScWAT, in comparison to NCsed group (Fig. 2B). To confirm the phenotype changes occurring in AT after exercise, thermogenic activity was assessed in ScWAT by the measurement of specific gene expression markers such as UCP-1, Sirt-3, PGC1 $\alpha$ , PPAR $\alpha$  and  $\delta$  (Fig. 2C). In

HFSSed mice, while UCP-1 mRNA expression and protein level were not changed in ScWAT (Fig. 2C–D), Sirt-3 and PPAR $\delta$  mRNAs were over-expressed in ScWAT in comparison with NCsed mice. Furthermore, after exercise, a significant increased expression of thermogenic markers was retrieved in ScWAT of HFSex mice, such as UCP-1, Sirt3, PGC1 $\beta$  and PPAR $\alpha$  in comparison with HFSSed, while only PGC1 $\beta$  was increased in ScWAT of NCex compared to NCsed mice.

### 3.3. Exercise-induced angiogenesis in adipose tissue of obese mice mediated by *Mdm2* upregulation

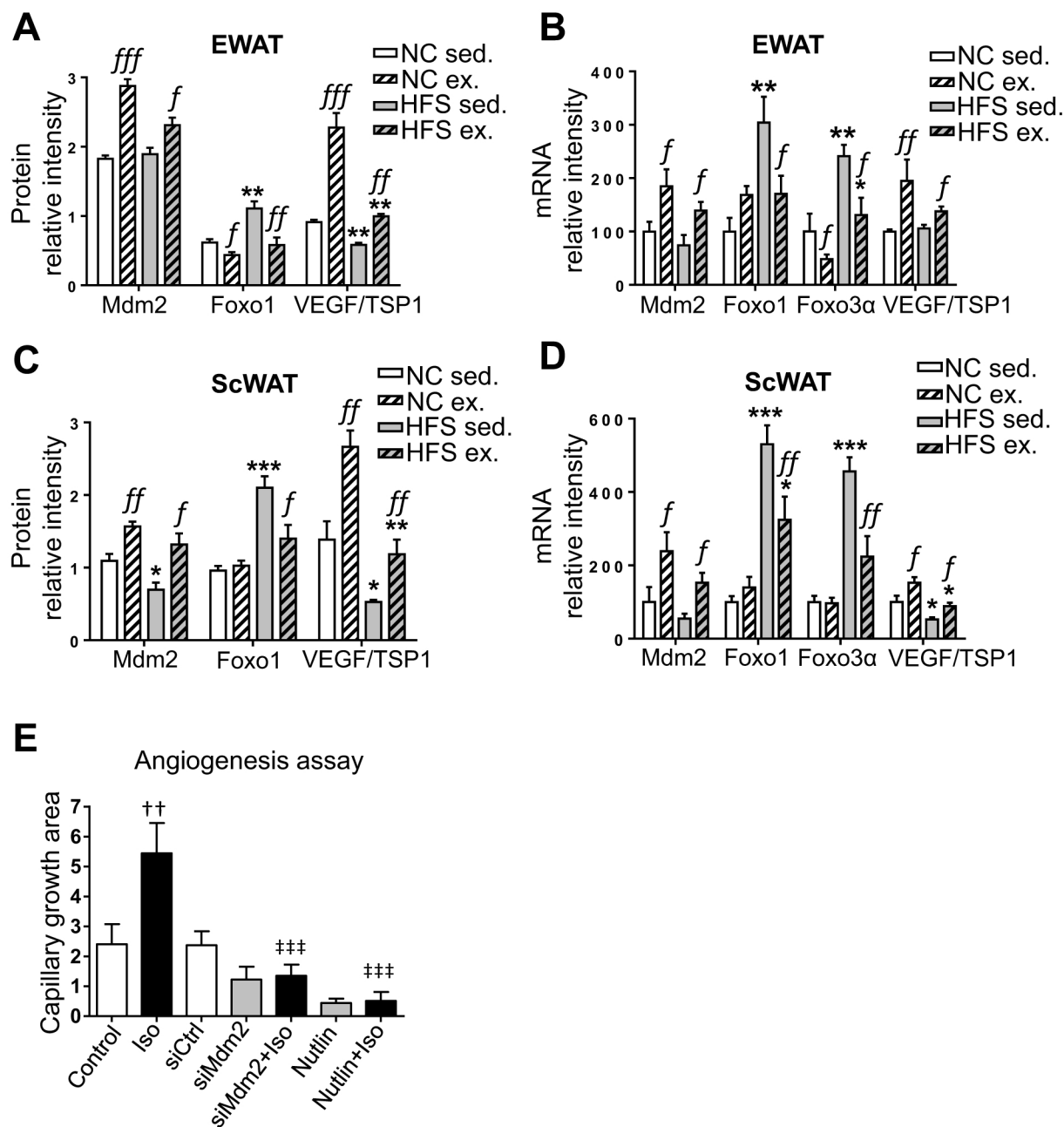
In HFSSed mice, capillary density in EWAT and ScWAT was significantly reduced by 45 and 76%, respectively compared to NCsed mice (Fig. 3A and B). Yet, capillary/adipocyte ratio remained comparable in both groups (Fig. 3B). After 7 weeks-exercise overall capillary density (+66%) and capillary/adipocyte ratio (+14%) were significantly increased in ScWAT of HFSex mice in comparison with HFSSed mice, while physical exercise did not produce any significant effect on overall capillary density in EWAT (Fig. 3A to C), but raised significantly the capillary/adipocyte ratio for both AT (+17%) in comparison with HFSSed mice (Fig. 3C). In addition, exercise increased very significantly capillary density and capillary per adipocyte ratio in NCex group compared to NCsed group (Fig. 3A to C).



**Fig. 3.** High fat diet resulted in capillary rarefaction in adipose tissue while voluntary exercise induced angiogenesis in adipose tissue of HFSex mice. (A) Representative images of capillaries detection after CD31 staining on EWAT and ScWAT cross sections. Scale bar = 50  $\mu$ m. Determination of (B) capillary density, (C) capillary/adipocyte ratio on ATs of NCsed, NC-ex, HFS ex and HFSex mice. Angiogenesis parameters were calculated from 5 to 7 independent fields of view per mouse (n = 4–5). Data are means  $\pm$  SEM (n = 5 per groups). Two-way ANOVA and Tukey post-test; \*\*\*P < 0.005 (HFS relative to NC), f P < 0.05, fff P < 0.005 (ex. relative to sed.).

The detection and quantification of mRNAs and regulating proteins of angiogenesis were assessed by real-time qPCR and western blotting (Fig. 4A–D and Fig. S2A–H). Firstly, significant elevated expression of FoxO1 protein in EWAT and ScWAT of HFSsed mice (Fig. 4A and C) was observed in comparison with NCsed mice. Furthermore, FoxO1 and FoxO3 $\alpha$  mRNAs were significantly overexpressed in EWAT and ScWAT of HFSsed mice. On the other hand, the VEGF-A/TSP-1 ratio, downstream effectors of FoxO1, was significantly decreased in EWAT of HFSsed mice only at the protein level, by 36% compared to NCsed mice (Fig. 4C and D), while in ScWAT of HFSsed mice, both protein and

mRNA VEGF-A/TSP-1 ratios were reduced by 62 and 48% respectively compared to NCsed mice (Fig. 4C and D). Aside, the E3 ubiquitin ligase Mdm2 was expressed differentially among the AT in the HFSsed mice. While in HFSsed mice EWAT, Mdm2 protein and mRNA expression were not different from the NCsed mice (Fig. 4A and B), in HFSsed mice ScWAT, a significant lower protein and messenger Mdm2 expression levels (by 36% and 45% respectively) were observed in comparison with NCsed mice (Fig. 4C and D). Aside, Mdm2 expression was largely increased after exercise in EWAT and ScWAT at the protein and mRNA levels (Fig. 4B to D).



**Fig. 4.** High fat diet and voluntary exercise elicit changes in the angio-adaptative target genes and proteins. Protein or RNA was extracted from EWAT and ScWAT from sedentary/exercise NC and HFS mice. High fat diet and voluntary exercise induced changes in FoxO1 and FoxO3 $\alpha$  protein and mRNA level. Mdm2, FoxO1, VEGF-A and TSP-1 protein levels from EWAT (A) and ScWAT (C) of NCsed, NCex, HFSsed and HFS-x mice) were analyzed by Western blot. The mRNA levels of Mdm2, FoxO1, FoxO3 $\alpha$  and VEGF-A/TSP-1 from EWAT (B) and ScWAT (D) of NCsed, NCex, HFSsed and HFSex mice. (E) Capillary growth areas measured from the *in vitro* angiogenesis assay of NCsed mice adipose explants. These parameters were evaluated within the presence/absence of isoproterenol (ISO), Mdm2 inhibitors (siMdm2 and Nutlin). Data are means  $\pm$  SEM (n = 5 per groups). Two-way ANOVA and Tukey post-test (A, B, C, D) or Kruskal-Wallis test and Dunn's multiple comparisons test (E). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.005 (HFS relative to NC), f P < 0.05, ff P < 0.01, fff P < 0.005 (ex. relative to sed.), †† P < 0.01 (relative to control condition), ‡‡‡ P < 0.005 (relative to ISO condition).

Exercise induced a significant repressing effect on FoxO1 in HFsex group. In fact, in EWAT of HFsex mice, a decreased FoxO1 protein expression (-26%) (Fig. 4A), FoxO1 mRNA level (44%) and FoxO3 $\alpha$  mRNA level (46%) (Fig. 4B) were observed compared to HFssed mice. In HFsex mice, similar effects were observed in ScWAT such as significant decrease in FoxO1 and FoxO3 $\alpha$  protein and mRNA levels (Fig. 4C and D). On the other hand, after exercise, in NCex mice, no modification of FoxO1 expression in EWAT nor ScWAT was noted in comparison with NCsed mice. Furthermore, exercise resulted in a strong and significant increase of VEGF-A/TSP-1 ratio at the protein and mRNA levels in EWAT (Fig. 4A and B) and ScWAT (Fig. 4C and D) of HFsex compared to HFssed mice. Interestingly, Mdm2 protein and mRNA expression were significantly increased in both group NCex and HFsex after 7 weeks of exercise in EWAT and ScWAT (Fig. 4A to D) compared to sedentary NCsed and HFssed groups.

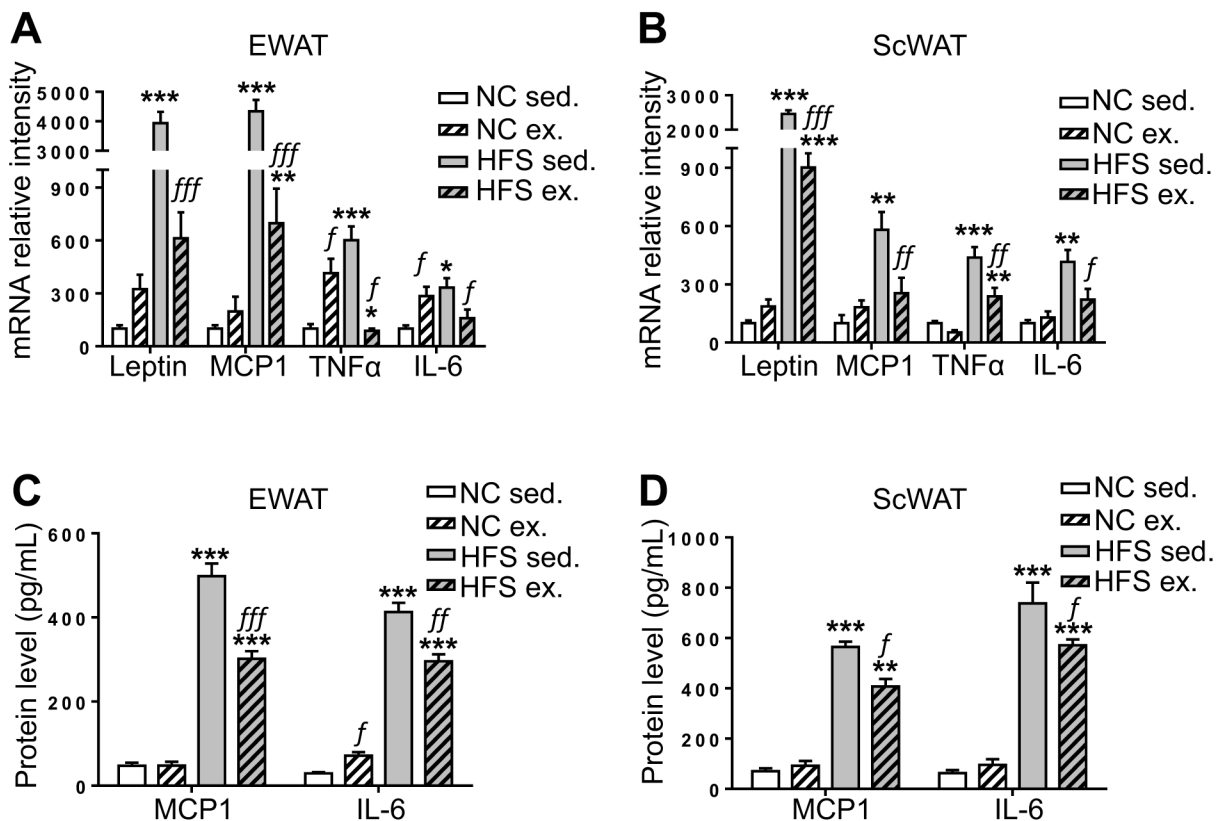
To confirm whether Mdm2 is a key component for adipose angiogenesis in the response to exercise, since its expression was increased in all groups in all adipose tissues by exercise, an *in vitro* angiogenesis assay from control NCsed mice adipose explants using silencing RNAs was realized (Fig. S2I). Isoproterenol (ISO) was used as a nonselective-adrenergic receptor agonist capable to mimic the effect of physical exercise *in vitro* (Youtz et al., 2014). Stimulation with ISO strongly increased capillary growth in adipose explants (+126%) compared to control experiment condition (Fig. 4E). Downregulation of Mdm2 through silencing transfection process drastically reduced capillary growth in ISO-stimulated AT (-75%). Inhibition of Mdm2 activity through addition in the culture medium of the Mdm2-antagonist Nutlin-3 (Secchiero et al., 2007) also induced a strong decrease of adipose angiogenesis in isoproterenol stimulation condition (-91%) (Fig. 4E, Fig. S2I).

### 3.4. Exercise improved the inflammatory status of adipose tissue

Adipokine mRNAs analysis in AT (Fig. 5A and B) revealed that obesity was accompanied by a drastic increase of leptin expression in all ATs of HFssed mice in comparison with NCsed mice. Furthermore, pro-inflammatory chemokines and cytokines mRNAs and protein levels such as Tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), MCP-1, IL-6 were also significantly increased in HFssed (Fig. 5A to D). On the other hand, physical exercise resulted in a significant decrease in both EWAT and ScWAT of leptin mRNA expression as well as the inflammatory MCP-1, TNF $\alpha$  and IL-6 mRNA and protein expression in HFsex mice (Fig. 5A to D). On the contrary, a very mild effect of exercise was noted in NCex mice, with only a significant decrease of TNF $\alpha$  and IL-6 mRNAs and protein expression was shown in EWAT of NCex in comparison with NCsed mice (Fig. 5A and C).

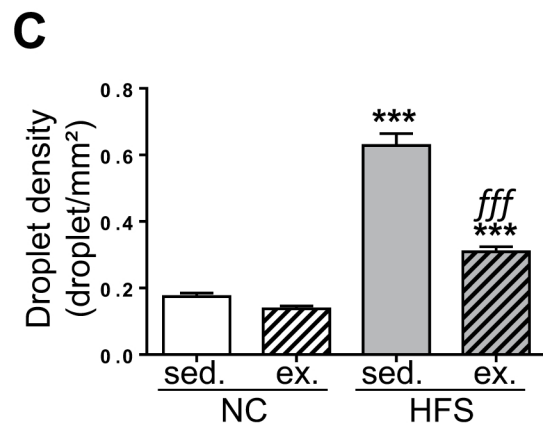
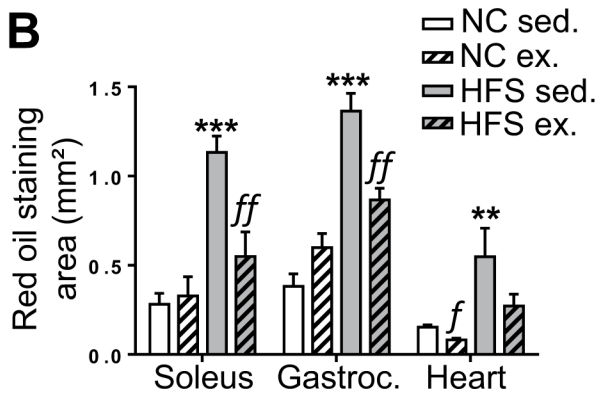
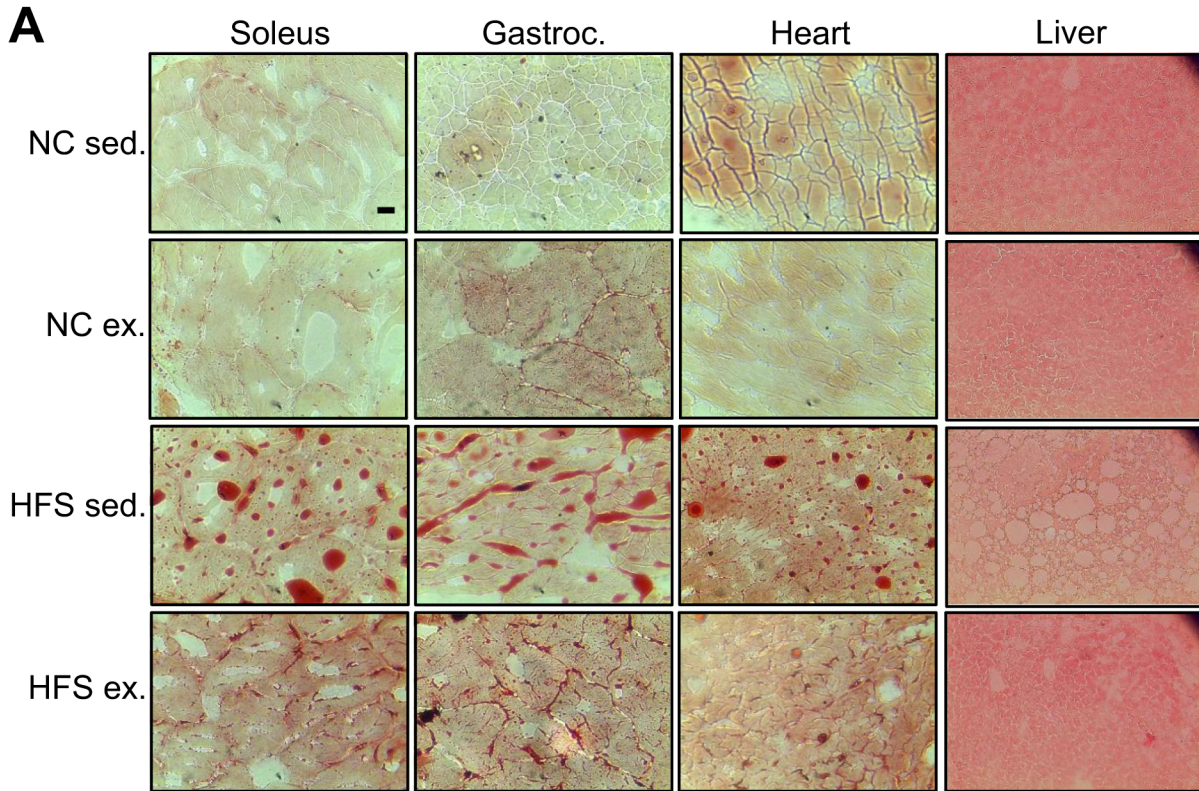
### 3.5. Exercise decreased ectopic fat deposition

Presence of ectopic fat evaluated by red oil staining (Fig. 6A) revealed that lipid storage was enhanced in muscles such as soleus, gastrocnemius and heart of HFssed mice by a factor 4, 3.6 and 3.5 respectively in comparison with NCsed mice (Fig. 6B). Furthermore, liver lipid droplets density was significantly elevated ( $\times 4.4$ ) in HFssed mice liver (Fig. 6C). Physical exercise reduced the red oil staining area in soleus (51%), gastrocnemius (36%) and heart (50%) (Fig. 6B) and lipid droplets density was significantly diminished in HFsex liver by 59% compared to HFssed mice, while no change was observed after exercise in NCex mice compared to NCsed mice (Fig. 6A to C).



**Fig. 5.** Voluntary exercise reduced the pathological adipose microenvironment accompanied inflammation developed after a high fat diet. The mRNA levels of leptin, MCP-1, TNF $\alpha$  and IL-6 on (A) EWAT and (B) ScWAT from sedentary/exercise NC and HFS mice, were analyzed by real-time qPCR, with values normalized to ribosomal protein 18S mRNA. The mRNA data are expressed as relative expression ratios to normal chow mice. The protein levels of MCP-1 and IL-6 on EWAT (C) and SWAT (D) ScWAT from sedentary/exercise NC and HFS mice. Data are means  $\pm$  SEM (n = 5 per groups). Two-way ANOVA and Tukey post-test; \*P < 0.05, \*\*p < 0.01, \*\*\*P < 0.005 (HFS relative to NC), f P < 0.05, ff P < 0.01, fff P < 0.005 (ex. relative to sed.).



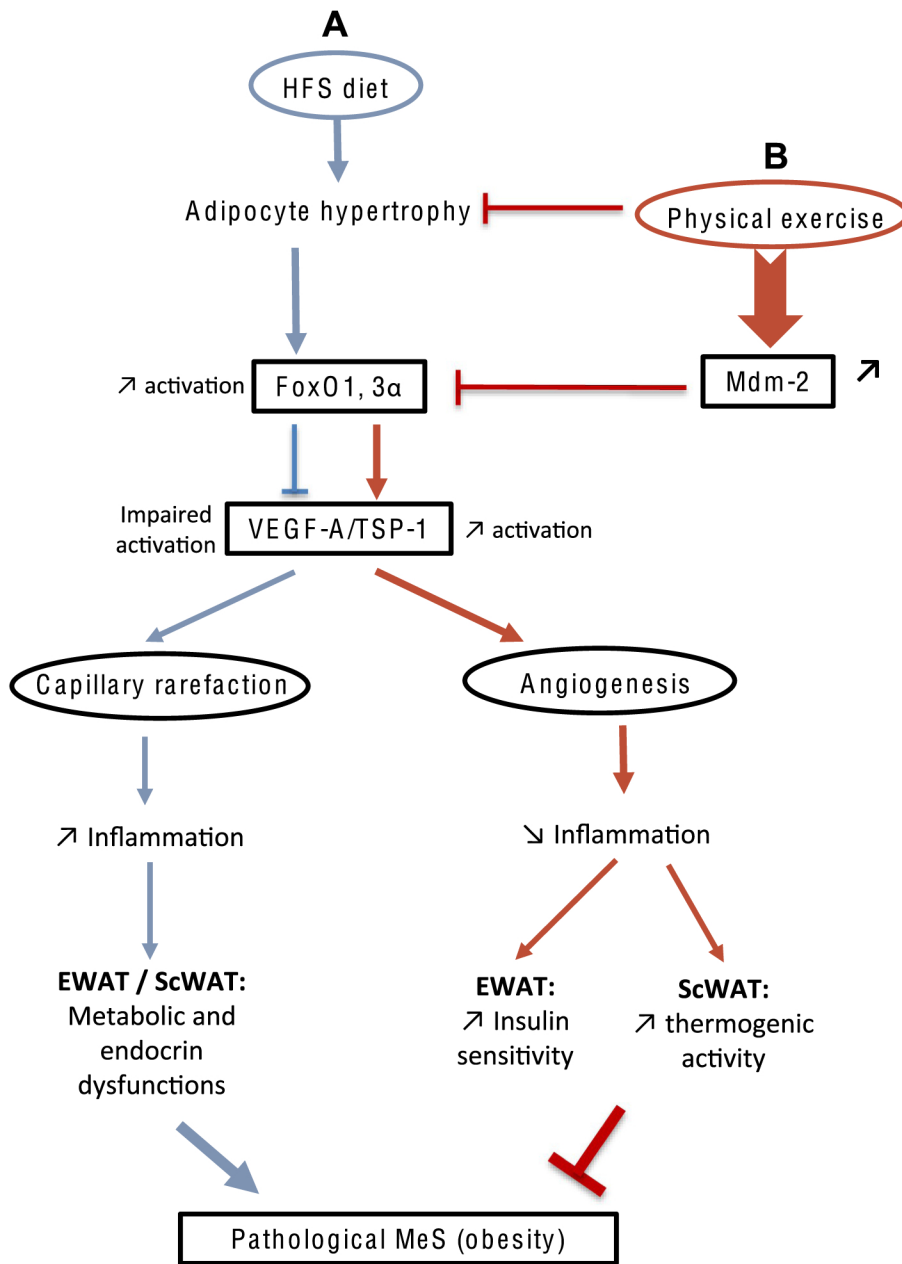


**Fig. 6.** Voluntary exercise reduced the extent of ectopic fat and hepatic steatosis caused by high fat diet. (A) Representative images of lipid droplets detection after Red oil staining on soleus, gastrocnemius, heart and liver cross sections from NCsed, NCex, HFSSed and HFSex mice. Scale bar = 50  $\mu$ m. (B) Determination of ectopic fat by lipid accumulation measurement in soleus, gastrocnemius and heart, by Red oil staining. Results are expressed as positive Red oil staining area ( $\text{mm}^2$ ). (C) Determination of hepatic steatosis by lipid droplets density expressed as droplets/ $\text{mm}^2$ . Positive staining areas were calculated from 3 to 6 independent fields of view per mouse ( $n = 5-9$ ). Data are means  $\pm$  SEM ( $n = 5$  per groups). Two-way ANOVA and Tukey post-test; \* $P < 0.05$ , \*\*\* $P < 0.005$  (HFS relative to NC), ff  $P < 0.01$ , fff  $P < 0.005$  (ex. relative to sed.).

#### 4. Discussion

This study showing that 7 week-voluntary exercise is an effective therapeutic approach to reverse obesity-related disorders and is the first to report that voluntary exercise is able to stimulate angiogenesis in white AT of obese C57/Bl6 mice. This angio-adaptative process was 1/regulated by Mdm-2, FoxO1,3 $\alpha$  and VEGF-A/TSP-1 signaling pathways; 2/associated to AT microenvironment changes with reduced adipocytes hypertrophy and inflammation, and 3/combined with insulin sensitivity improvement in visceral AT and decreased hepatic steatosis and skeletal muscles and heart ectopic fat mass.

First of all, the HFSex mice presented a marked reduction in the total and local fat mass in comparison with weight-matched pair sedentary HFSSed mice. Reduction of adipose mass induced by exercise was accompanied by a decrease in adipocyte hypertrophy in the EWAT and ScWAT. Interestingly, these changes were coupled with a vascular growth process as indicated by the increase of capillary density and capillary per adipocyte ratio in ATs. While already known in white AT of healthy animals (De Matteis et al., 2013; Hatano et al., 2011), our observations extend this finding to obese mice which presented adipose capillary rarefaction as reported in human (Gealekman et al., 2011; Pasarica et al., 2009; Shimizu et al., 2014). At the molecular level, this



**Fig. 7.** Proposed diagram of Mdm2, FoxO<sub>1,3</sub>, VEGF-A/TSP signaling in adipose tissue.

A) In pathological conditions, HFS diet induced adipocyte hypertrophy, activated FoxO-1,3α and blunted VEGF-A/TSP-1 activation inducing an angiostatic process and capillary rarefaction accompanied with increased inflammation ultimately leading to adipose dysfunctions and metabolic disorders obesity-related. B) After physical exercise, Mdm-2 stabilization inactivated FoxO-1,3 abolishing the inhibitory effect of FoxO-1,3 on VEGF-A/TSP-1 and producing a pro-angiogenic stimulus associated with decreased inflammation *in fine* allowing restoration/improvement of adipose and systemic metabolic functions.

phenomenon was related to the activation of a pro-angiogenic signal, such as the increased VEGF-A/TSP-1 ratio observed in white AT after 7-weeks of voluntary exercise in HFsex mice. This change was due to the decrease in TSP-1 in all ATs and the concomitant increase of VEGF-A, as already described in obese rats and trained healthy mice (Hatano et al., 2011).

Beside changes in the VEGF/TSP-1 ratio, other factors such as FoxOs could affect the expression on these proteins as reported previously in skeletal muscles (Roudier et al., 2013; Shikatani et al., 2012). As it was reported before (Paik et al., 2007; Wilhelm et al., 2016), in vascular endothelial cells, deletion of FoxO1 and 3α induced an intense proliferation whereas FoxO1 overexpression was responsible for a sharp decrease in angiogenic process. In addition, the same FoxO deletion was associated with an increase in vascular density in skeletal muscle of HFD mouse model (Nwadozi et al., 2016). Therefore, in our study, the reduction of FoxO proteins observed in HFsex mice is very likely linked to the restoration of angiogenesis. Interestingly, Mdm2 expression was increased in the white AT of the HFS-ex mice and concomitantly to FoxOs decreased expression. Such a role of Mdm2 has been already

demonstrated in skeletal muscle where its deletion was associated with an increase in FoxO1, and a decrease in VEGF-A and angiogenesis in response to physical activity (Roudier et al., 2012). In addition, Mdm2 is able to directly bind and inhibit FoxO1 by ubiquitylation (Milkiewicz et al., 2011), and positively regulates the angio-adaptive balance by directly stimulating the expression of VEGF-A (Zhou et al., 2011). However, this is the first demonstration of the role of Mdm2 in adipose angiogenesis. The biological link between exercised-induced adipose angiogenesis and Mdm2 expression was clearly demonstrated by the present results obtained *in vitro* angiogenesis on AT explants. In fact, our findings showed that isoproterenol-induced vascular growth that mimics exercise stimulus, was completely abolished when Mdm2 was downregulated or inhibited.

Since complex contributions exist between of angiogenesis, inflammation, in healthy and unhealthy AT expansion as reviewed by Crewe et al. (2017), we tempted to understand also the effect of exercise on adipose tissue inflammation. And we found that exercise led to a decreased AT inflammation, promoted by Mdm2 activation and local angiogenesis through a reduced TSP-1 expression as previously

reported (Varma et al., 2008). Furthermore, the decreased pro-inflammatory cytokines and chemokines released (TNF $\alpha$ , IL-6, MCP-1), allowed also the improvement of insulin sensitivity and AKT phosphorylation in EWAT of our HFSex mice as previously reported (Christiansen et al., 2010; Lira et al., 2010; Vieira et al., 2009).

Aside, reduction of ScWAT adipocyte size was accompanied by the appearance of beige adipocytes whose presence was increased in ScWAT of exercised animals during a “browning process” (Cao et al., 2011; Stanford et al., 2015). Beige adipocytes have more intense mitochondrial biogenesis, are metabolically more active, than white adipocytes (Trevellin et al., 2014). This confers them a greater thermogenic activity, which could be explained by the increased UCP-1 expression as well as other thermogenic markers (PGC1 $\beta$ , PPAR $\alpha$ , SIRT-3) that we found in ScWAT of our HFSex mice. The increased expression of VEGF-A in the ScWAT, possibly resulting from the Mdm2-FoxOs axis activation in our model, would participate in the activation of the browning process (During et al., 2015). Taken together, these improvements in white adipose functions may improve systemic lipid content with reduced ectopic fat storages in the muscle, heart and liver.

In summary, our results demonstrated the role of the Mdm2-FoxOs axis in the regulation of the adipose angio-adaptive response to obesity and exercise. FoxO1 appeared to act as an angiostatic break in ATs of HFSexed mice. Then, voluntary exercise was able to stimulate adipose angiogenesis and that stimulus was dependent of Mdm2 expression and activation. Development of a pro-angiogenic microenvironment in AT led to an improvement of AT vascular deficit in HFSex mice and thereby, to a reduction of tissue inflammation and metabolic disorders obesity-related. A potential schematic summary cartoon of AT angio-adaptive processes is proposed in Fig. 7. These findings clarify the

mechanism by which voluntary exercise training could improve AT vascularization and functions. It is then suggested that this non-pharmacological maneuver can be used as a therapeutic approach to treat obesity and its related metabolic disorders.

## Credit author statement

T.L. researched data/wrote/edited manuscript, E.C. and E.K. researched data, J.F.L. and B.J. reviewed manuscript, C.R. researched data/wrote/edited manuscript and is the guarantor of this work and, as such, had full access to all the data in the study.

## Declaration of competing interest

The authors have no potential conflicts of interest.

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## Annex 1. Primary antibodies used for western blotting

- Anti-mouse VEGF-A (C-1) (1:700; Santa Cruz)
- Anti-mouse TSP-1 (1:400; Thermo Scientific)
- Anti-mouse Mdm2 (2A10) (1:100; Millipore)
- Anti-rabbit FoxO1 (C-29) (1:1000; Cell Signaling)
- Anti-rabbit HIF-1 $\alpha$  (H-206) (1:500; Santa Cruz)
- Anti-rabbit phospho-Akt (193H12) (1:500; cell signaling)
- Anti-rabbit Akt (1:800; cell signaling)
- Anti-rabbit UCP-1 (M-17) (1:800; Santa Cruz)
- Anti-rabbit  $\alpha/\beta$  Tubulin (1:3000; Cell Signaling).

## Annex 2. Sequence of the primers used for qPCR

Gene symbol	Primer name	5' → 3' primer sequence
Mdm2	mMdm2_F mMdm2_R	aatgtcctgaattgatgtcaagatt Catagaaccactcacatcgatcttt
Foxo1	mFoxo1_F mFoxo1_R	cttcaagataaggcgaca gacagatttggcgaattga
Foxo3a	mFoxo3a_F mFoxo3a_R	gctaagcaggcctcatctca ttccgtcagtttggggct
VEGFA	mVEGFA_F mVEGFA_R	aaaaacgaaagcgaagaaa tttccgctctgaacaagg
TSP1	mTSP1_F mTSP1_R	gttctgatggtgaatgctg cacgttgcgaattccattg
Leptin	mLeptin_F mLeptin_R	gggtgtgaaagaacctgagctgagg cagtgatgctaattgcccctg
MCP1	mMCP1_F mMCP1_R	catccacgtgttgct gatcatttctggtgaaatga
Ym1	mYm1_F mYm1_R	gaacactgagctaaaaactctctg gagaccatggcactgaacg
RorC	mRorC_F mRorC_R	acctcttttcacggaggga tccacatctcccaattg
Foxp3	mFoxp3_F mFoxp3_R	agaagctgggagctatgcag gctacgatgcagcaagagc
PPAR $\alpha$	mPPAR $\alpha$ _F mPPAR $\alpha$ _R	ctgagacctcggggaac aaactcagttcacagggaag

Pgc1a	mPgc1a_F mPgc1a_R	ttccaaaagaagtcaccatacaca gataaagtgtgtgttggctga
Sirt3	mSirt3_F mSirt3_R	tgctactcattctgggaacctc gggcactgattctgtactgc
TNF $\alpha$	mTNF $\alpha$ _F mTNF $\alpha$ _R	catcttctcaaaatcgatgacaa tgggagtagacaaggtacaacc
IL6	mIL6_F mIL6_R	acaagteggaggcttaattacacat ttgccattgcacaactttttc
IL10	mIL10_F mIL10_R	cacaagcagccttcgagaa agagcaggcagcatagcagtg
CD11c	mCD11c_F mCD11c_R	atggagcctcaagacaggac ggatctgggatgctgaaact
CD3	mCD3_F mCD3_R	tgctcttggttatctcattgc aacagagtctgctgtctgaagc
PPAR $\delta$	mPPAR $\delta$ _F mPPAR $\delta$ _R	tggggataatgggaaaagtt ataccacaccgctgcac
Pgc1 $\beta$	mPgc1 $\beta$ _F mPgc1 $\beta$ _R	gacgtggcagagcttcaact gagcgtcagagctgtgtgt
Cidea	mCidea_F mCidea_R	tgctcttctgtatgccagat gcccgttaaggaatcgtctg
18S	18S_F 18S_R	cgccgctagaggtgaaattct cattcttggcaaatgcttctg

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