Étude n°1:

L'axe de régulation *Murine double-minute 2 - Forkhead box O1, 3α* contrôle la réponse angio-adaptative à l'exercice du tissu adipeux viscéral chez la souris soumise à un régime induisant l'obésité

Murine double minute-2 and Forkhead Box O-1, 3α mediate physical exercise-induced angio-adaptation in visceral adipose tissue of high fat diet fed mice

Thomas Loustau, Eugénie Coudière, Bernard Jover, Esma Karkeni, Jean-François Landrier, Pascal Laurant, Catherine Riva

Article soumis à International Journal of Obesity

1) But de l'étude :

L'expansion du tissu adipeux blanc viscéral, due à une augmentation du stockage des triglycérides, joue un rôle important dans l'établissement des troubles cardiovasculaires et métaboliques associés à l'obésité (Blüher 2013). Pourtant, dans les premières phases de développement de l'obésité, l'expansion du tissu adipeux viscéral semble être accompagnée d'une hyperplasie des adipocytes et d'une croissance des capillaires sanguins, permettant de conserver l'homéostasie tissulaire. Mais, les capacités hyperplasiques du tissu étant limitées, l'expansion de ce dernier devient par la suite la résultante d'une hypertrophie des adipocytes préexistants, qui va s'accompagner d'une raréfaction capillaire (Fain *et al.* 2004; Drolet *et al.* 2008; Laforest *et al.* 2015). L'hypertrophie adipocytaire et le défaut d'angiogenèse vont alors modifier les fonctions endocrines du tissu et aboutir à la survenue de foyers locaux hypoxiques et fibrotiques avec une inflammation chronique de faible intensité. L'ensemble de ces altérations induit une perte de l'homéostasie glucidique et lipidique du tissu adipeux qui, à moyen terme, se propagera à l'ensemble de l'organisme (Haffner 2007).

La microcirculation du tissu adipeux semble jouer un rôle majeur dans le maintien de l'homéostasie de ce dernier. Pour répondre de façon optimale aux besoins en oxygène et en nutriments du tissu, le réseau capillaire est doté d'une grande plasticité, grâce au processus biologique d'angio-adaptation tissulaire. Les mécanismes moléculaires permettant la croissance, la stabilisation et la régression de ces vaisseaux sanguins demeurent partiellement compris dans le tissu adipeux. En revanche, l'angio-adaptation a été beaucoup plus largement étudiée dans un tissu où d'importants processus d'angiogenèse sont retrouvés à l'exercice, le muscle squelettique. Il a été démontré dans ce tissu que la régulation du processus angio-adaptatif était sous la dépendance d'une balance entre les facteurs pro- et anti-angiogéniques. Parmi toutes les molécules composant ces deux types de facteurs, le VEGF-A et la TSP-1 jouent un rôle essentiel (Tang *et al.* 2004; Malek & Olfert 2009). L'expression de ces molécules est sous le contrôle du facteur de transcription FoxO1, lui-même sous l'influence de l'E3 ubiquitine ligase Mdm2 (Milkiewicz *et al.* 2011; Shikatani *et al.* 2012; Roudier *et al.* 2013a). Lors d'un exercice physique, l'augmentation de la stabilisation de Mdm2 est à l'origine d'une régulation positive du VEGF-A et négative de la TSP-1 qui aboutit à une angiogenèse accrue au sein du muscle squelettique (Roudier *et al.* 2012).

À la vue de ces données, et de l'importance que semble avoir la défaillance angio-adaptative du tissu adipeux lors de l'expansion excessive de celui-ci, nous avons cherché à

savoir si l'axe de régulation Mdm2-FoxO1 joue un rôle dans l'angio-adaptation du tissu adipeux similaire à celui attribué au niveau musculaire. Nous avons observé chez des souris rendues obèses, grâce à un régime riche en graisse et en sucrose, le comportement de la microcirculation du tissu adipeux épididymal, l'expression de Mdm2 et FoxO1, ainsi que la balance angio-adaptative avec ces effecteurs VEGF-A et TSP-1. Notre étude avait également pour but de savoir si l'exercice physique constitue, comme cela a été observé dans le muscle, un stimulus pro-angiogénique capable d'induire l'expression de Mdm2 et de faire pencher la balance angio-adaptative en faveur de l'angiogenèse du tissu adipeux épididymal. Nous voulions également déterminer si, ultimement, cette éventuelle angiogenèse permet de réduire les troubles endocriniens et métaboliques du tissu adipeux viscéral et ses répercussions pathologiques au sein de l'organisme. Pour ce faire, nous avons soumis des souris en cours de régime induisant l'obésité à un protocole d'exercice volontaire.

2) Article n°1 :

Murine double minute-2 and Forkhead Box O-1,3 mediate physical exercise-induced angio-adaptation in visceral adipose tissue of high fat diet fed mice

Thomas Loustau[1], Eugénie Coudière[1], Bernard Jover[2], Esma Karkeni[3,4,5], Jean-François Landrier[3,4,5], Pascal Laurant[1], Catherine Riva[1]

1. Avignon University, LaPEC EA4278, F-84000, Avignon, France
2. EA7288, Faculté de Pharmacie, Montpellier, France
3. INRA, UMR 1260, F-13385, Marseille, France.
4. INSERM, UMR 1062, Nutrition, Obésité et Risque Thrombotique F-13385, Marseille France.
5. Aix-Marseille Université, Faculté de Médecine, F-13385, Marseille, France.

Running title: Mdm2/FoxO1 regulation of adipose tissue angio-adaptation

Corresponding author:

Name: Catherine RIVA
Address: Avignon University, LAPEC EA4278, 74 rue Louis Pasteur, 84000 Avignon, France
Telephone number: +33 (0) 490162933 / Fax number +33 (0) 490162901
E-mail: catherine.riva@univ-avignon.fr

Keywords: Obesity, physical exercise, Mdm-2, FoxO-1, VEGFA, TSP-1, angiogenesis

Words count: 4739 without abstract (234 words)
Total number of figures and tables: 8 figures, 1 table and 2 annexes

Author Contributions: T.L. researched data/wrote/edited manuscript, E.C. research data, E.K. research data, B.J., P.L. and JF.L. 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 and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Acknowledgement: The authors want to thank Sandrine Gayrard for her technical assistance.
Sources of Funding: This work was supported by a personal grant for TL from Bergonier Foundation from Avignon University, France.
Disclosure: The authors have no potential conflicts of interest.

ABSTRACT:

Background: Adipose tissue homeostasis is dependent on the microcirculation, which displays a remarkable plasticity, a process called angio-adaptation. It has been demonstrated that Mdm2/FoxO1 axis plays a key role in muscle angio-adaptive response to exercise affecting pro- (VEGF-A) and anti-angiogenic (TSP-1) factors expression. The regulation of this process occurring during physical exercise and/or obesity in adipose tissue remains unknown.

Methods and Results: We studied the effects of 7 weeks voluntary exercise in mice fed with a high fat/high sucrose diet (HFS) on adipose tissue vascularization and microenvironment. Exercised-HFS mice displayed a lower body fat mass (P=0.0028), reduced epididymal white adipose tissue (EWAT) mass (P=0.0202) and improved metabolic parameters compared to sedentary-HFS mice. Exercise training stimulated an angiogenic process with significant increase of capillaries/adipocyte ratio (P=0.0031) in EWAT associated to increased VEGF-A/TSP-1 expression ratio (P=0.0006) and Mdm2 protein and messenger expression (respectively P=0.041 and P=0.0234) while FoxO1 protein and messenger were decreased (respectively P=0.0063 and P=0.0290). In addition, EWAT insulin sensitivity, hypoxia, fibrosis, inflammatory status were improved by exercise in comparison to sedentary HFS mice.

Conclusions: We reported for the first time that physical exercise acts as an adipose pro-angiogenic stimulus, where Mdm2 is essential in activating Mdm2-FoxO1/FoxO3α axis and the rise of VEGF-A/TSP-1 ratio ultimately promoting AT microenvironment improvement. These findings clarify the mechanism by which physical exercise could improve AT vascularization and functions and have implications for drug targets that could lead to similar benefits in sedentary overweight humans.

ABBREVIATION LIST:

AKT: *Protein kinase B*
AT: *Adipose tissue*
CD 3/4/11c: *Cluster of differentiation 3/4/11c*
EWAT: *Epipidymal white adipose tissue*
Ex: *Exercised*
FoxO1/3α: *Forkhead box O1/ 3 alpha*
Foxp3: *Forkhead box P3*
HFD: *High Fat diet*
HFS: *High Fat and high sucrose diet*
HIF-1α: *Hypoxia Inducible Factor 1 alpha*
IL-6/10: *Interleukin 6/10*
IP-GTT: *Intraperiotoneal glucose tolerance test*
IP-ITT: *Intraperiotoneal insulin tolerance test*
M1/M2: *Macrophage type 1/type 2*
MBP: *Mean arterial blood pressure*
MCP-1: *Monocyte chimioattractant protein 1*
Mdm-2: *Murine double-minute 2*
MeS: *Metabolic syndrome*
NC: *Normal chow*
PBS: *Phosphate-buffered saline*
PGC-1α: *Peroxisome proliferator-activated receptor gamma coactivator 1-alpha*
PPARα: *Peroxisome proliferator-activated receptor alpha*
RorC: *Related Orphan Receptor C*
SDS: *Sodium dodecyl sulfate*
Sed: *Sedentary*
SEM: *Standard error of the mean*
Sirt-3: *Sirtuin-3*
TGF-β1: *Transforming growth factor beta 1*
Th17: *Lymphocyte T helper 17*
TNFα: *Tumor necrosis factor alpha*
Treg: *Lymphocyte T regulator*
TSP-1: *Thrombospondin 1*
UCP-1: *Uncoupling protein 1*
VEGF-A: *Vascular endothelial cell growth factor-A*
YM1: *Chitinase-like 3 (Chil3)*

INTRODUCTION:

Adipose tissue (AT) expansion is associated with obesity, insulin resistance development (1), and AT endocrine dysfunction (2). AT homeostasis is very dependent on the microcirculation, to respond to metabolic and oxygen variation demands in the tissue. Regular practice of physical exercise is well established as a therapeutic approach for many chronic metabolic and cardiovascular diseases (3). However, capillary growth remains limited in comparison to the magnitude of AT expansion occurring in case of obesity (4,5). Therefore understanding the molecular events that could regulate the vascularization process in AT is of great interest.

The biological process of capillary network remodeling, called tissue angio-adaptation, has been widely demonstrated in skeletal muscle where maintenance of capillary network is dependent on the balance between pro and anti-angiogenic factors (6), but its regulation within AT remains largely unknown. To regulate the balance between the pro-angiogenic Vascular endothelial cell growth factor-A (VEGF-A) (7), and the anti-angiogenic factor Thrombospondin 1 (TSP-1) (8), Mdm2-FoxO1 axis has been shown to play a key role in skeletal muscle angio-adaptive response to exercise (9). The E3 ubiquitin ligase Murine double-minute 2 (Mdm2), known for its oncogenic role of the major negative regulator of p53 tumor suppressor protein (10), has also been reported in transgenic Mdm2-deficient mice a key role played by Mdm2 in skeletal muscle capillary maintenance and regulation of exercise muscle angiogenic response (9). While in skeletal muscle of these mice, an increase in Forkhead box O1 (FoxO1) level expression was observed, a direct interaction between Mdm2 and FoxO1 was demonstrated (11). FoxO1 is a transcription factor and a significant regulator of endothelial cell phenotype and metabolic activity (12,13). Aside, an up-regulation of TSP-1 has been reported within the muscle and adipose tissue of obese and diabetic mice (14–16).

In the present study, we hypothesized that Mdm2-FoxO1 axis is involved in adipose angio-adaption leading to capillary rarefaction occurring in AT in obesity. Then, we speculated that physical exercise is able to induce as in skeletal muscle, a pro-angiogenic signal for capillary

restoration in AT of obese subject and that vascular deficit improvement could reduce metabolic disorders obesity-related. To investigate this, we studied temporal effects of voluntary exercise in C57/Bl6 mice fed with a high fat/ high sucrose diet (HFS) on the capillarity of epididymal AT (EWAT), angiogenic factors: Mdm2, FoxO1,3□ and their downstream effectors: VEGF-A and TSP-1, as well as AT and global metabolic and inflammation homeostasis.

MATERIALS AND METHODS

Animals:

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).

Trials were performed on 70 C57BL/6 Inbred male mice (Janvier Labs, Saint-Berthevin, France) of 12-weeks-old (27.4 g ± 1.2g) kept in living quarters with controlled temperature (20-23°C), light (12:12 hours light-dark cycle), and humidity. Mice had free access to food and water ad libitum.

Experimental protocol:

Mice were randomly divided into 2 groups. A normal chow (NC) group (n=15), submitted to a standard diet (3.1% Fat, Safe, Augy, France), and a high fat/ high sucrose (HFS) group (n=55) subjected to fat-enriched food (60% Fat, Safe, Augy) and drink water containing sucrose (10%

D-Saccharose, Fischer Scientific, England). Diet protocols started at 13 weeks of age for a total of 16 weeks.

After 9 weeks of the HFS diet, the mice were randomly allocated to sedentary (n=30) or exercise group (n=25) up to 7-weeks of voluntary exercise which consisted in the introduction of a wheel in cages (3 mice/cage). Wheels were connected to a sensor for a daily record of the number of laps being executed in each cage.

The effects of exercise on HFS mice were tested in tissue samples collected after 1, 2, 3, 4, and 7 weeks of voluntary exercise (T1, T2, T3, T4, and T7; n=5 for each period). Tissues were also sampled before starting the exercise protocol, representing time 0 period (T0, HFS mice n=4). At the same time, sedentary mice were also sacrificed (at T1, NC mice n= 3, HFS mice n=3; at T2, NC mice n= 3, HFS mice n=3; at T3, NC mice n= 3, HFS mice n=5; at T4, NC mice n= 3, HFS mice n=6; and at T7, NC mice n= 3, HFS mice n=9). Immediately after sacrifice, adipose depots (epididymal, subcutaneous, perirenal, mesenteric, interscapular brown) and liver, gastrocnemius, soleus, heart were collected, fixed in formalin for histological analysis or frozen in liquid nitrogen and stored at -80°C for biochemical analysis. During the sampling, two small pieces of EWAT were incubated 30min at 37°C with a PBS solution containing or not insulin ($20mg.mL^{-1}$) to test tissue insulino-resistance. After incubation, tissues were also frozen in liquid nitrogen and stored at 80°C.

Metabolic parameters

The week preceding sacrifice, blood was collected (5µl) using the tail-clip method and fasting blood glucose level was assessed (CarensR N, DinnoSanteTM) according to the manufacturer's instructions.

For glucose tolerance test (IP-GTT), glucose solution was injected i.p at $1g.kg^{-1}$ and for insulin tolerance test (IP-ITT), insulin was injected i.p, at $0.75U.kg^{-1}$ to NC, sedentary-HFS and

exercise-HFS mice and blood glucose levels were measured at 20, 40, 60, 90 and 120 min after glucose injection for GTT and 15, 30, 45, 60, 90 and 120 min after insulin injection for ITT.

Blood pressure measurement:

Mean arterial blood pressure (MBP) was measured in conscious mice using the CODA tail-cuff system (Kent Scientific, Torrigton, CT, USA) at week 7 of the training protocol, two days before sacrifice. The MBP values were calculated as the average of 10 measurements.

Western Blotting:

Proteins were extracted from EWAT using Tris-HCl buffer (50mM pH=7.4; 10% glycerol; 3.5 mM SDS; protease inhibitor cocktail (Sigma Aldrich); phosphatase inhibitor (Na_3VO_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 (Annexe 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 α/β Tubulin content on the same membrane.

RNA extraction and real-time quantitative PCR:

Frozen EWAT was introduced in TRizol reagent for total RNA extraction using a Retsch MM300 tissue lyser. cDNAs were synthesized from 500µg 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 (Annexe 2).

Adipose histology:

Paraffin embedded tissue sections of EWAT were stained with hematoxylin/eosin and Massons's trichrome using standard protocols. To visualize the vasculature and hypoxic area, ABC kit vectastain (Vector Labs), anti-mouse CD-31 (Abcam 28364) and anti-HIF1α (Santa Cruz H-206) were used for immunostaining.

Liver/muscle and heart histology:

Portions of frozen liver, soleus, gastrocnemius and heart were fixed in Tissue Tek resin (O.C.T. Compound, Sakura) and sections performed for lipid droplets staining using standard red oil staining protocol.

Statistical analysis:

Data are expressed as mean ± SEM. Statistical analysis was performed using 1 or 2-way ANOVA with Prism6 (GraphPad Software Inc., San Diego, CA, USA). For 2-way ANOVA analysis, the Turkey post-test was used. A Newman-Keuls post-test was performed after 1-way ANOVA analysis. The results were considered to be statistically significant at values of $P \leq 0.05$ (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

RESULTS

HFS diet induces obesity and metabolic syndrome in sedentary mice:

The analysis of animal weight curve after the 16 weeks complete HFS diet showed a significant increase of body weight in HFS mice group (+51%) compared with NC mice group. The total fat mass (epididymal, subcutaneous, perirenal, mesenteric, interscapular brown fat pads) over body mass percentage was significantly increased (5 times) in HFS mice compared to NC (figure 1A and B). In particular, epididymal fat mass (EWAT) (figure 1C) and adipocytes size of EWAT (figure 1D) were significantly increased in HFS mice by a factor 3.8 and 2.8 respectively in comparison to NC mice. Moreover, HFS mice displayed a metabolic syndrome. Firstly, fasting glucose levels of HFS mice were significantly higher than of NC mice and remained higher throughout the 2-h IP-GTT, as indicated by the higher area under the curve (Table 1). The insulin tolerance test (IP-ITT) did not however reveal any difference between that HFS mice and NC mice. Phospho-protein kinase B (P-Akt) protein expression has been measured in EWAT after in vitro insulin stimulation in order to evaluate effects of obesity on insulin tissue response and insulin-sensitivity status in visceral adipose tissue. Basal P-AktT/Akt expression ratio was significantly lower in EWAT of HFS mice compared to NC mice. After insulin stimulation, P-Akt/Akt ratio was significantly increased by 70% in NC mice compared to basal ratio level, while no significant increase was noticed in HFS mice (Figure 1E). On the other hand, the measurement of a high mean blood pressure (MBP) indicates the occurrence of a hypertension in HFS mice population in comparison to NC mice (Table 1).

Exercise improved Obesity-related metabolic complications:

After 3 weeks of voluntary exercise, the body weight of HFS mice started to decline. At 4 and 7 weeks-exercise, body weight of HFS mice was significantly reduced by about 9.1% and 9.5% respectively in comparison to sedentary-HFS mice (Figure 1A). This weight difference was

very likely explained by a lower percentage of adipose tissue in exercise-HFS mice (Figure 1B) and in particular a significant reduction of 27% of EWAT mass, with a significant decrease of 34% of EWAT adipocyte size observed in exercised-HFS mice compared to sedentary-HFS mice (Figure 1D).

Aside, gene expression of thermogenic activity markers has been measured in EWAT by qPCR (Figure 1F). Compared to NC, HFS sedentary mice exhibited an up-regulation of UCP-1 expression (x2.1) a down-regulation of PGC1α (-25%), while PPARα and SIRT-3 were not affected by the high fat diet. Seven weeks of voluntary exercise in HFS mice group allowed the down-regulation of UCP-1 (- 48% vs HFS mice) and the up-regulation of PPARα (+90% vs. HFS mice), PGC1α (+62% vs. HFS mice) and SIRT-3 (+56% vs. HFS mice).

Voluntary exercise also reduced the difference between NC and HFS mice concerning fasting blood glucose with statistical differences obtained between HFS and exercise-HFS mice as reported in Table 1. To explore whether the improvement in insulin tolerance in the exercise-HFS mice is a function of changes to the adipose microvascular network, we compared insulin-induced phosphorylation of Akt in EWAT of exercise-HFS mice after 7 weeks of voluntary exercise. At this time point, exercise-HFS mice exhibited significantly reduced insulin sensitivity (as assessed by ITT), whereas sedentary-HFS mice retained an ITT response similar to that of the NC group (Table 1). Furthermore, the improvement by 7 week-exercise of tissue response to insulin was accompanied with a significant increase of 55% of P-Akt/Akt ratio in insulin stimulated EWAT (Figure 1E). In vivo and ex vivo insulin stimulation of adipose tissue were compared as a tool to segregate the contributions of vascular delivery of insulin vs. adipocyte receptor activation to the impaired insulin responsiveness of HFS mice compared to NC mice. In addition, in vivo delivery of insulin resulted in substantially greater phosphorylation of Akt (at Ser473) within adipose tissue of exercise-HFS mice compared to

HFS mice. These findings support the hypothesis that improvements in adipose microvascular density underlie the enhanced insulin sensitivity detected in exercise-HFS mice.

Finally, in exercised-HFS mice group the high mean blood pressure was significantly lowered in comparison to sedentary-HFS mice group (Table 1).

FoxO-1, FoxO-3, VEGA/TSP-1 control of capillary-deficit in obese adipose tissue:

The effect of high fat diet and voluntary exercise on adipose tissue capillarisation was assessed on EWAT cross-sections (Figure 2A), in order to measure capillary density and capillaries number per adipocyte ratio. In EWAT, the capillaries per adipocyte ratio was higher (12%) in HFS mice compared to NC mice even though the capillary density was significantly decreased (less than 45%) by high fat diet (Figure 2B). We tested the hypothesis that high fat diet induces the expression of anti-angiogenic factors within adipose tissue, which could be under the control of FoxO proteins. FoxO1 protein levels determined by western blotting and real-time PCR analysis of FoxO1 mRNA levels revealed significant increased expressions by 54% and 304% respectively in HFS mice (Figure 2D and E), as well as FoxO3α mRNA expression (+241%). Then, we examined the expression of downstream effectors of FoxO1 involved in angiogenesis. While pro-angiogenic VEGF-A protein and mRNA expression were not significantly affected by HFS diet (Figure 3A and B), anti-angiogenic TSP-1 expression was significantly increased after 16 weeks of HFS diet at the protein (by 76%; Figure 3C) and mRNAs (by 249%; Figure 3D) levels in comparison to NC mice. When calculating the VEGF-A on TSP-1 expression ratio, it was significantly decreased only at protein level by 36% in HFS mice in comparison to NC mice (Figure 3E).

Akt activation is associated with enhanced proteasomal degradation via ubiquitin ligase pathways. The E3 ubiquitin ligase Mdm2 is a substrate for Akt and was reported to mediate

FoxO ubiquitination. Therefore we examined the expression of Mdm2 in adipose tissue, but we found that protein and mRNA level expression of Mdm2 were no affected by HFS diet in HFS mice (Figure 3G and H).

Murine-double minute-2 induced exercise enhanced angiogenesis:

Voluntary exercise did not produce any significant effect on overall capillary density in adipose tissue after 1, 4 and 7 weeks-exercise (Figure 2B). Yet, after 4 and 7 weeks-exercise the capillaries per adipocyte ratio did raise significantly in EWAT of HFS mice by 11% and 17% respectively in comparison to sedentary-HFS mice (Figure 2C). Aside, after 7 weeks of exercise, a significant reduction of FoxO1 protein expression by 26% (Figure 2D), FoxO1 mRNA level (44%) and FoxO3α mRNA level (46%) (Figure 2E) in EWAT were observed in exercised-HFS mice in comparison to sedentary-HFS mice. Furthermore, a significant increase of pro-angiogenic VEGF-A protein expression (29%) (Figure 3A) and VEGF-A mRNA level (85%) (Figure 3B) in exercise-HFS mice compared to sedentary-HFS mice. The angiostatic protein TSP-1 showed significant reduced protein level expression (by 22%) (Figure 3C) and mRNA levels (by 35%) (Figure 3D) in exercise-HFS mice in comparison to sedentary-HFS mice. Then, the VEGF-A/TSP-1 ratio was calculated to highlight the angiogenic process and the protein ratio (Figure 3E) was significantly increased by 67% as well as the mRNA ratio (Figure 3F) by 31% in exercised-HFS mice compared to sedentary-HFS mice. Finally, E3 ubiquitin ligase Mdm2 protein and mRNA expression were significantly increased after 7 weeks of exercise in EWAT of exercised-HFS mice by respectively 30% (Figure 3G) and 87% (Figure 3H) in comparison to sedentary-HFS mice.

Exercise restored a healthy visceral adipose microenvironment:

Immunostaining of HIF-1α on EWAT cross-sections (Figure 4A) and western blot analysis were assessed to evaluate the effect of high fat diet and exercise on the hypoxia status in visceral adipose tissue. We observed a sustained and higher expression of hypoxia sensitive HIF-1α within visceral adipose tissue in HFS mice compared to NC mice (Figure 4B and C). In exercised-HFS mice group, HIF-1α staining was significantly reduced by 59% in comparison to sedentary-HFS mice (Figure 4B and C).

Trichrome Masson staining of EWAT extracellular matrix (Figure 4D) was performed to estimate adipose tissue fibrosis and revealed that the fibrosis/adipocyte ratio significantly increased in EWAT of HFS mice by a factor 3.2 compared to NC mice. This ratio was significantly reduced in exercised-HFS mice by 45% vs. sedentary-HFS mice adipose tissue (Figure 4E).

Exercise reduced obesity-induced adipose inflammation:

Since chronic low-grade inflammation has been reported to be a contributing factor in the development of insulin resistance in obese individuals, measurement of adipokines and cytokines expression and inflammatory cell infiltrates were performed to evaluate the impact of high fat diet and exercise protocol on adipose tissue inflammation. While leptin mRNA expression was drastically increased (x245 relative intensity; Figure 5A) by high fat diet in HFS mice adipose tissue in comparison to NC mice, 7 weeks of exercise induced a significant decrease (by 55%) of leptin expression in visceral adipose tissue. On the other hand, while high fat diet increased slightly mRNA adiponectin expression (Figure 5A) in EWAT of sedentary and exercised HFS mice compared to NC mice, adiponectin protein expression analysis (Figure 5B) revealed a strong decreased protein expression by 67% in sedentary-HFS mice vs. NC

mice, that was partially restored in exercised-HFS mice (+ 224% compared to sedentary-HFS mice).

The analysis of infiltrating immune cell markers mRNA (Figure 5C and D) and cytokines levels (Figure 5E) in EWAT revealed that obesity induced by high fat diet was accompanied with a steep increase of M1 macrophages (CD11c; x185 relative intensity), and mild increase of M2 macrophages (YM1; x5), T lymphocytes (CD3; x4.4) and T helper 17 (RorC; x2.5) markers expression in HFS mice compared to NC mice with a concomitant decrease of Treg (Foxp3; -41%) level. Pro-inflammatory cytokines levels such as TNFα (x4.4), MCP-1 (x4.2), IL-6 (x4.1) and the anti-inflammatory TGFβ (x4.2) were also increased in HFS mice compared to NC mice while anti-inflammatory IL-10 level was not modified. Exercise training resulted in a significant decrease of mRNAs levels of M1 macrophages (-65%), T lymphocytes (-35%) and T helper 17 (-44%) markers, as well as TNFα (-45%), MCP-1 (-40%) and IL-6 (-47%) genes expression, concomitantly accompanied by an increased expression of M2 macrophages markers (+85%), Treg markers (+83%), TGFβ (+5%) and IL-10 (+29%) in EWAT of exercise-HFS mice of in comparison to sedentary-HFS mice.

Exercise improves systemic lipid homeostasis:

To estimate the effect of high fat diet on lipid storage and homeostasis in mice with or without physical exercise activity, presence of ectopic fat on soleus, gastrocnemius and heart has been evaluated with a red oil staining (Figure 6A). Significant red oil staining area was observed in soleus, gastrocnemius and heart as a consequence of high fat diet in HFS mice. Lipid storage was increased in soleus, gastrocnemius and heart by a factor 4, 3.6 and 3.5 respectively, in comparison to NC mice (Figure 6B). On the other hand, 7 weeks exercise induced a significant decrease of the red oil staining area by 51% in soleus, and 36% in gastrocnemius but a non-

significant reduction by 50% in heart of exercise-HFS mice (Figure 6B) in comparison to sedentary-HFS mice.

In addition, the measurement of liver lipid droplets size and density were estimated on liver cross sections to estimate hepatic steatosis. High fat diet resulted in the accumulation of lipid droplets in liver of HFS mice. In fact, lipid droplet density was drastically raised (x4.4) as well as the lipid droplets size (x4.8) in the liver of HFS mice compared to NC mice (figure 6C and D). After 7 weeks exercise, lipid droplets density and size were significantly reduced in exercise-HFS mice liver by 43 and 59% respectively in comparison to sedentary-HFS mice liver.

DISCUSSION:

This is the first report showing that 7 weeks voluntary exercise is able to stimulate angiogenesis in visceral AT of obese C57/Bl6 mice. This angio-adaptative process was 1/ regulated by Mdm-2, FoxO1,3α and VEGF-A/TSP-1 signaling pathways; 2/ associated to AT microenvironment changes with reduced hypoxia, fibrosis, adipocytes hypertrophy and inflammation, and 3/ combined with insulin sensitivity amelioration in visceral AT and systemic metabolic homeostasis improvement with hepatic steatosis and skeletal muscles and heart ectopic fat reduction.

In the present study, HFS feeding for 16 weeks induced obesity that was accompanied with metabolic syndrome in mice. The body weight of C57/Bl6 mice fed with HFS was significantly increased, and the mice developed an insulin resistance in visceral AT and hypertension. Voluntary exercise training suppressed these effects. The significant lower body weight in exercise-HFS mice was probably due to the reduction of 30% of total fat mass and in particular of 27% of visceral fat mass. Therefore we clearly demonstrated that a 7 week-voluntary exercise

protocol is beneficial to reverse obesity. We show for the first time that physical exercise is able to stimulate angiogenesis in visceral AT, and that is associated to the fat mass reduction. The capillary/adipocyte ratio significantly increased in exercise-HFS mice suggesting an endothelial cell proliferation and the expansion of the adipose capillary network. Indeed, in the present exercise-HFS mice, while a neo-angiogenesis process and enhanced vascularization were observed in visceral AT, we have also found significant reduction of AT hypertrophy, hypoxia through HIF-1α reduced protein expression and fibrosis. On the other hand, insulin-resistance was reduced after physical exercise with an increased P-Akt/Akt ratio before and after insulin stimulation. It is also shown in our exercise-HFS mice an increase expression of PPARα, PGC1α and SIRT-3, three genes commonly associated with thermogenic activities within the AT especially after exercise (17). In the current experiments, we demonstrated that exercise training was an effective therapeutic approach to restore the microvascular deficiencies that accompany chronic diseases such obesity. Indeed, in obesity, AT vascularization does not accompany adipocyte expansion rate since we and other have reported a low capillary density (4,18), resulting in a reduced adipose blood flow (19). Therefore, hypoxia appears to be concomitant with this adipose microvasculature deficit as shown in the sedentary-HFS mice EWAT. Even if pathological events chronology occurring in AT of obese individuals remains unclear; hypoxia seems to be a major factor of numerous obesity-related events within the AT. Previous reports have shown that AT hypoxia failed to increase VEGF-A expression (5,20) and activated pro-inflammatory pathways, modifying adipokines secretion by promoting leptin synthesis and altering the expression of anti-inflammatory adiponectin (19,21,22). In addition, HIF-1α overexpression promotes apoptotic and pro-fibrotic genes expression (23,24). The overproduction of leptin and pro-inflammatory factors from adipocytes activate resident macrophages and T cells recruitment (25). In particular, pro-inflammatory M1 macrophages, producing TNFα, IL-6 and MCP-1 cytokines, which contribute to the onset of obesity-

dependent insulin resistance (26–28), and the M2 macrophages which are considered more anti-inflammatory but are also pro-fibrotic though the production of IL-10 and TGFβ (29–31). TGFβ has been reported to have a controversial role by exacerbating the obesity-related metabolic dysfunction (32) but on the other hand by modulating positively T cell activity and differentiation of both immune tolerance lymphocyte T regulator (Treg) and pro-inflammatory Th17 cells from CD4+ T cells (33). Interestingly, our model of sedentary-HFS obese mice presented all obesity-induced AT dysfunctions and altered microenvironment described herein, as well as skeletal and cardiac muscles ectopic fat development and hepatic steatosis (34).

This is the first report suggesting that the adipose angio-adaptive response to exercise is meditated through the Mdm2-FoxO1 axis regulation. In fact, HFS diet resulted in the increased expression of FoxO3α mRNA, and FoxO1 at the mRNA and protein levels in visceral AT. The FoxO1,3α expression associated with a decreased VEGF-A/TSP-1 ratio in EWAT, suggested the emergence of an anti-angiogenic microenvironment within the AT, which may explain the capillary rarefaction found in this tissue in sedentary-HFS mice. Angiostatic microenvironment mainly due to a sharp increase of TSP-1 expression in AT has already been described in mice (5,15,16) and humans (35) suggesting that angiostatic pathways may overcome pro-angiogenic signals. Our results showing that increased TSP-1 protein expression found in AT of sedentary-HFS mice suggest that under these conditions TSP-1 may be under the control of FoxO1 transcription factor, as reported before in endothelial cells (12). A recent study seems to confirm this hypothesis, showing that a very high FoxO1 expression level in muscle endothelial cells of HFD mice was associated to an anti-angiogenic microenvironment and capillary regression (36). Moreover, our study showed that HFS diet didn't affect Mdm-2 expression in AT and that only physical exercise did modulate directly Mdm-2 protein and mRNA expression in AT, making a link in Mdm2-FoxO1 regulation axis in response to exercise stimulus as in reported previously in skeletal muscle (9). In fact, physical exercise induced a significant mRNA and

protein increased Mdm2 expression, which resulted in the repression of FoxO1 mRNA and protein and a consequent increase of their angiogenic effectors VEGF-A/TSP-1 ratio. These changes suggested the occurrence of a pro-angiogenic microenvironment within the AT in exercise-HFS mice. As previously demonstrated in skeletal muscle, Mdm2 appeared to be a central regulator of the angio-adaptive process with physical activity in AT, by enhancing VEGF-A expression and down-regulating TSP-1 through negative regulation of FoxO1 (11,37). Interestingly, the onset of this AT pro-angiogenic microenvironment has been linked in literature with the same improvement of obesity-related metabolic disorders found in our exercise-HFS mice. Indeed, down-regulation of FoxO1 expression in murine models of obesity or human has beneficial effects on insulin sensitivity, inflammatory status and adipose metabolism as we and others have reported (36,38–40) Furthermore, TSP-1 loss prevented EWAT hypertrophy and inflammation in mice subjected to a HFD (41) whereas VEGF-A overexpression was correlated to a reduced weight gain, hypoxia, macrophages infiltration, insulin resistance and increased adipose capillarity (42,43). Finally, beneficial effects of exercise training on inflammation found in our model have been partially reported on exercised mice subjected to a standard diet with pharmacologically-induced inflammation (44,45) and on AT hypoxia, global insulin sensitivity and liver steatosis (46). In fact, 7 week-voluntary exercise ameliorated AT inflammation through 1/ the reduction of pro-inflammatory actors such leptin, T lymphocytes, T helper 17 cells and M1 macrophages populations and their pro-inflammatory cytokines secretion (TNFα, MCP-1 and IL-6) concomitantly with increased adiponectin production, and 2/ the increase of anti-inflammatory cells such as Treg and M2 macrophages populations and their anti-inflammatory factors (TGFβ1 and IL-10). Improvement of adipose function by physical exercise was also combined with global metabolic homeostasis improvement represented by hepatic steatosis reduction, and by the disappearance of ectopic fat in skeletal muscles and heart.

In summary, our results showed that voluntary physical exercise was able to stimulate visceral adipose angiogenesis, increasing the number of capillary/adipocyte. This process seemed be dependent of the Mdm2-FoxO1 axis and their effectors VEGF-A and TSP-1 to induce the development of a pro-angiogenic microenvironment in EWAT, improving the AT vascular deficit of sedentary-HFS obese mice thereby to reduce tissue hypoxia and metabolic disorders obesity-related. A proposed schematic summary diagram of Mdm2, FoxO1, VEGF-A/TSP-1 signaling in visceral obesity is displayed in figure 7. These findings clarify the mechanism by which voluntary exercise training could improve AT vascularization and functions and can be used as a therapeutic approach to treat obesity and its related metabolic disorders.

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

	NC	HFS Sed.	HFS Ex.
Mean blood pressure (mmHg)	110 ± 3.9	140 ± 2.4 ***	128 ± 3.5 * *f*
Fasting blood glucose (mg.dL-1)	211 ± 17	331 ± 35.7 *	220 ± 17.5 *f*
IP-GTT (AUC)	1959 ± 75	2740 ± 42 ***	2270 ± 139 *f*
IP-ITT (AUC)	178 ± 10.4	209 ± 28.9	134 ± 6.5

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.

Legends to figures

Figure 1: 7 weeks of exercise induced a decreased of body weight, fat mass and adipocyte size in HFS mice. Body weight curve (A) during HFS diet and voluntary exercise protocols of normal chow (N) and sedentary-HFS mice (HFS-sed) and exercise-HFS mice (HFS-ex); HFS diet started on week 0 (▽), voluntary exercise started on week 9 for the exercise-HFS mice group (▼). Total fat mass (B) and EWAT mass (C) at the end of the 16 weeks protocols in normal chow (NC), sedentary-HFS mice (HFS-sed) and exercise-HFS mice (HFS-ex); EWAT adipocytes size (D) in normal chow (NC), sedentary-HFS mice (HFS-sed) and exercise-HFS mice (HFS-ex) at the weeks 10, 13 and 16 (corresponding to 1, 4 and 7 weeks of voluntary exercise for exercise-HFS mice). (E) Representative immunoblots and densitometry analysis of Phospho-Akt (P-Akt) and Akt on EWAT of normal chow (NC), sedentary-HFS mice (HFS-sed) and exercise-HFS mice (HFS-ex) +/- ex vivo insulin stimulation (❑ Ins-: incubation without insulin; ■ Ins+: incubation with insulin). (F) Thermogenic activity markers mRNA expression (UCP-1, PPARα, PGC-1α and SIRT-3) from EWAT of normal chow (❑), sedentary-HFS mice (❑) and exercise-HFS mice (■) at the week 16 was 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 (NC). Data are means ± SEM (n=3-5 per groups and per time point). δ P<0.001, * P<0.05, ** P<0.01, *** P<0.005 (HFS-sed and HFS-ex relative to NC); *f* P<0.05, *ff* P<0.01 (HFS-ex relative to sed-HFS); σ P<0.05 (Ins+ relative to corresponding Ins -); One-way ANOVA and post hoc comparison.

Figure 1

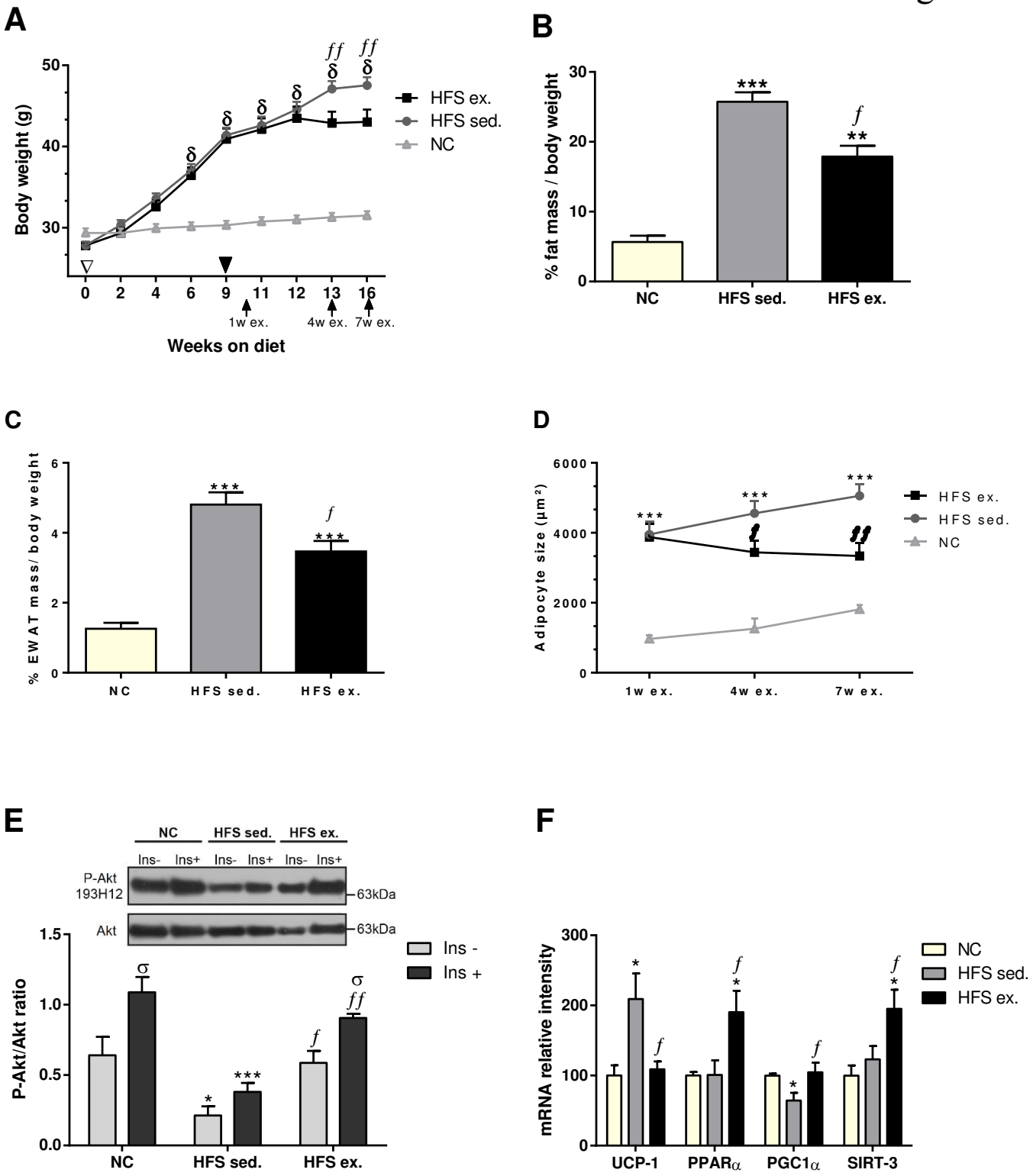

A
Body weight (g)
Weeks on diet
1w ex.
4w ex.
7w ex.
HFS ex.
HFS sed.
NC
B
% fat mass / body weight
NC
HFS sed.
HFS ex.
C
% EWAT mass/ body weight
D
Adipocyte size (µm²)
1w ex.
4w ex.
7w ex.
E
NC
HFS sed.
HFS ex.
Ins-
Ins+
P-Akt 193H12
Akt
63kDa
P-Akt/Akt ratio
Ins -
Ins +
F
mRNA relative intensity
UCP-1
PPARα
PGC1α
SIRT-3

Figure 2: High fat diet resulted in capillary rarefaction in visceral adipose tissue while voluntary exercise induced angiogenesis in visceral adipose tissue of HFS obese mice. (A) Representative images of capillaries detection after CD31 (endothelial marker) staining on EWAT cross sections from normal chow (NC), sedentary-HFS mice (HFS-sed) and exercise-HFS mice (HFS-ex) at 16 weeks; scale bar = 50µm; Arrows point to CD31 positive stained capillaries. Determination of (B) capillary density and (C) capillary/adipocyte ratio on EWAT of normal chow (NC), sedentary-HFS mice (HFS-sed) and exercise-HFS mice (HFS-ex) at the weeks 10, 13 and 16 (corresponding to 1, 4 and 7 weeks of voluntary exercise for exercise-HFS mice). Data are means ± SEM (n=5 per groups and per time point). High fat diet and voluntary exercise elicit changes in FoxO1 and FoxO3a protein and mRNA. (D) Total FoxO1 protein level from EWAT of normal chow (NC), sedentary-HFS mice (HFS-sed) and exercise-HFS mice (HFS-ex) at the week 16, was analyzed by Western blot. Blots were stripped and reprobed for α/β Tubulin as a loading control. Total FoxO1 protein levels were normalized to α/β Tubulin. (E) The mRNA levels of FoxO1 and FoxO3α from EWAT of normal chow (❑), sedentary-HFS mice (❑) and exercise-HFS mice (■) at the week 16 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 (NC). Data are means ± SEM (n=5 per groups). * $P<0.05$, ** $P<0.01$, *** $P<0.005$ (HFS-sed and HFS-ex relative to NC), *ƒ* $P<0.05$ (HFS-ex relative to HFS sed); One-way ANOVA and post hoc comparison.

Figure 2

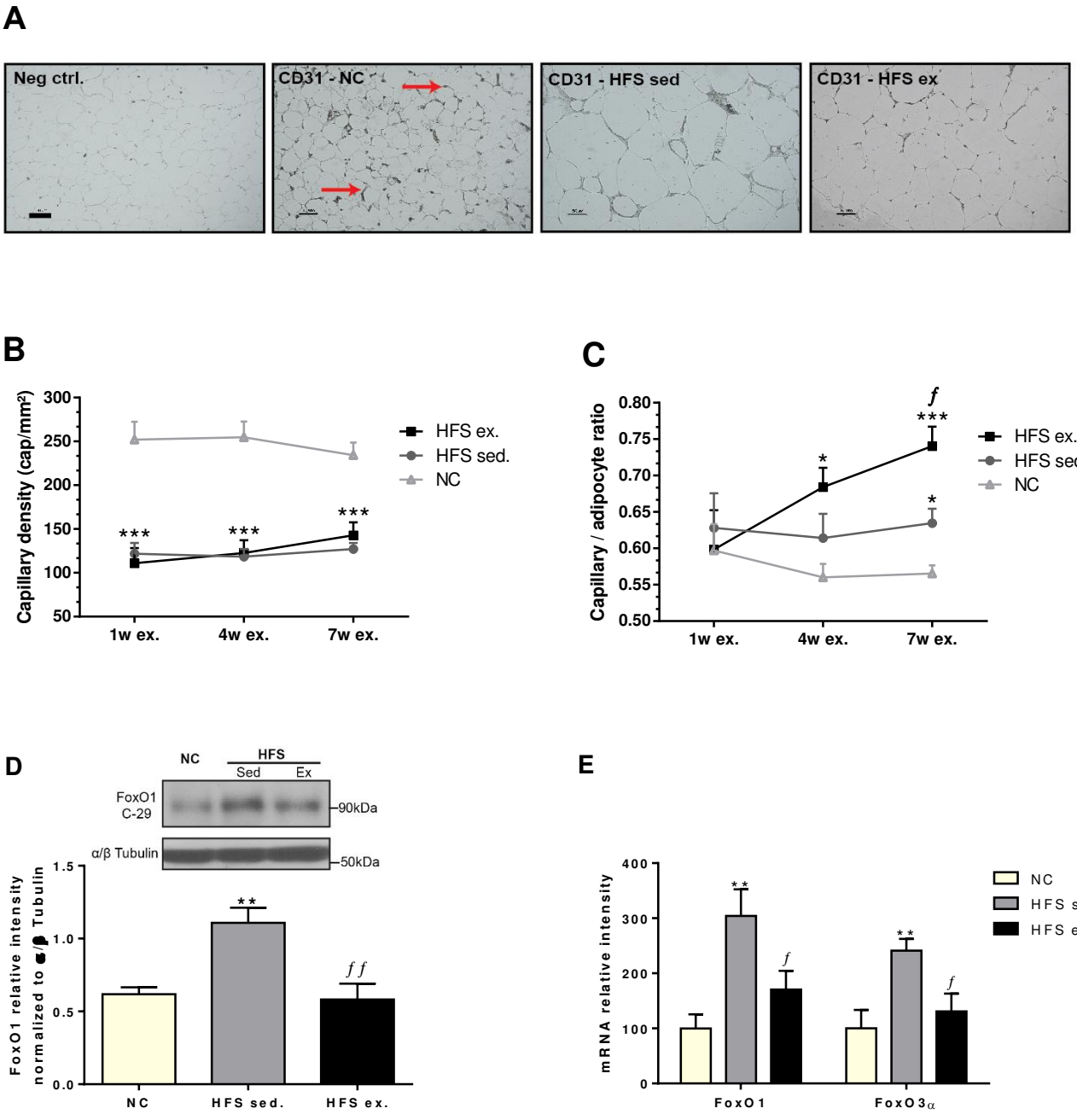

A
Neg ctrl.
CD31 - NC
CD31 - HFS sed
CD31 - HFS ex
B
Capillary density (cap/mm²)
300
250
200
150
100
50
1w ex.
4w ex.
7w ex.
***
***
***
HFS ex.
HFS sed.
NC
C
Capillary / adipocyte ratio
0.80
0.75
0.70
0.65
0.60
0.55
0.50
1w ex.
4w ex.
7w ex.
f
***
*
*
HFS ex.
HFS sed.
NC
D
NC
HFS
Sed
Ex
FoxO1
C-29
90kDa
α/β Tubulin
50kDa
FoxO1 relative intensity
normalized to α/β Tubulin
1.5
1.0
0.5
0.0
**
f f
NC
HFS sed.
HFS ex.
E
mRNA relative intensity
400
300
200
100
0
**
f
**
f
NC
HFS sed.
HFS ex.
FoxO1
FoxO3α

Figure 3: Responsiveness of angio-adaptative target genes. Protein or RNA was extracted from EWAT of normal chow (NC), sedentary-HFS mice (HFS-sed) and exercise-HFS mice (HFS-ex) at the week 16. (A) pro-angiogenic VEGF-A and (C) anti-angiogenic TSP-1 protein levels were analyzed by Western blot. Blots were stripped and reprobed for α/β-tubulin as a loading control; (B) The mRNA levels of (B) VEGF-A and (D) TSP-1 were analyzed by real-time qPCR, with values normalized to ribosomal protein 18S mRNA. Then VEGF-A/TSP-1 protein level ratio (E) and VEGF-A/TSP-1 mRNA level (F) ratio were calculated. (G) Mdm-2 protein level was analyzed by Western blot. Blots were stripped and reprobed for α/β-tubulin as a loading control; (H) The mRNA levels of Mdm-2 was 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 (NC). Data are means ± SEM (n=5 per groups). * P<0.05, ** P<0.01, *** P<0.005 (HFS-sed and HFS-ex relative to NC), *ƒ* P<0.05, *ƒƒ* P<0.01, *ƒƒƒ* P<0.005 (HFS-ex relative to HFS sed); One-way ANOVA and post hoc comparison.

Figure 3

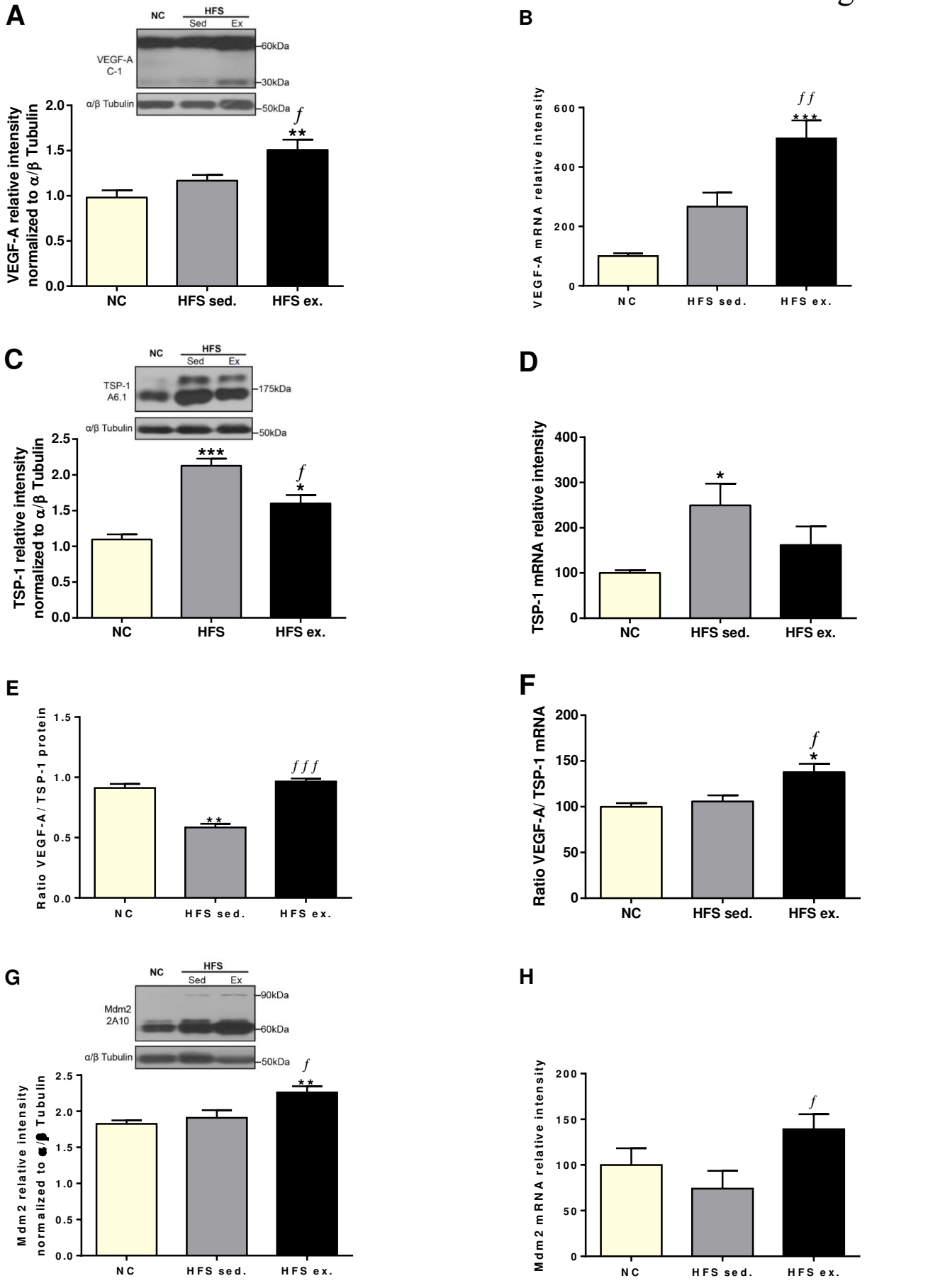

A
NC
HFS
Sed
Ex
VEGF-A
C-1
60kDa
30kDa
α/β Tubulin
50kDa
VEGF-A relative intensity normalized to α/β Tubulin
NC
HFS sed.
HFS ex.
B
VEGF-A mRNA relative intensity
C
TSP-1
A6.1
175kDa
TSP-1 relative intensity normalized to α/β Tubulin
HFS
D
TSP-1 mRNA relative intensity
E
Ratio VEGF-A/TSP-1 protein
F
Ratio VEGF-A/TSP-1 mRNA
G
Mdm2
2A10
90kDa
Mdm2 relative intensity normalized to α/β Tubulin
H
Mdm2 mRNA relative intensity

Figure 4: Voluntary exercise reduced the pathological visceral adipose microenvironment accompanied with hypoxia and fibrosis developed after a high fat diet. (A) Representative images of hypoxia detection after HIF-1α staining on EWAT cross sections from normal chow (NC), sedentary-HFS mice (HFS-sed) and exercise-HFS mice (HFS-ex) at 16 weeks; scale bar = 20µm. Arrows point to HIF-1α positive staining area; (B) Representation of HIF-1α positive staining areas were calculated from 3–6 independent fields of view per mouse (n = 5–9). Results are expressed as positive HIF-1α staining area (mm^2). (C) HIF-1α protein level was analyzed by Western blot. Blots were stripped and reprobed for α/β-tubulin as a loading control; Representative images of fibrosis detection after trichrome masson staining (D) on EWAT cross sections from normal chow (NC), sedentary-HFS mice (HFS-sed) and exercise-HFS mice (HFS-ex) at 16 weeks; scale bar = 50µm. (E) Determination of the fibrosis area /adipocyte ratio was calculated from 3–6 independent fields of view per mouse (n = 5–9). Results are expressed as positive fibrosis area/ adipocyte ($mm^{2)}$. ** P<0.01, *** P<0.005 (HFS-sed and HFS-ex relative to NC), *ff* P<0.01, *fff* P<0.005 (HFS-ex relative to HFS sed); One-way ANOVA and post hoc comparison.

Figure 4

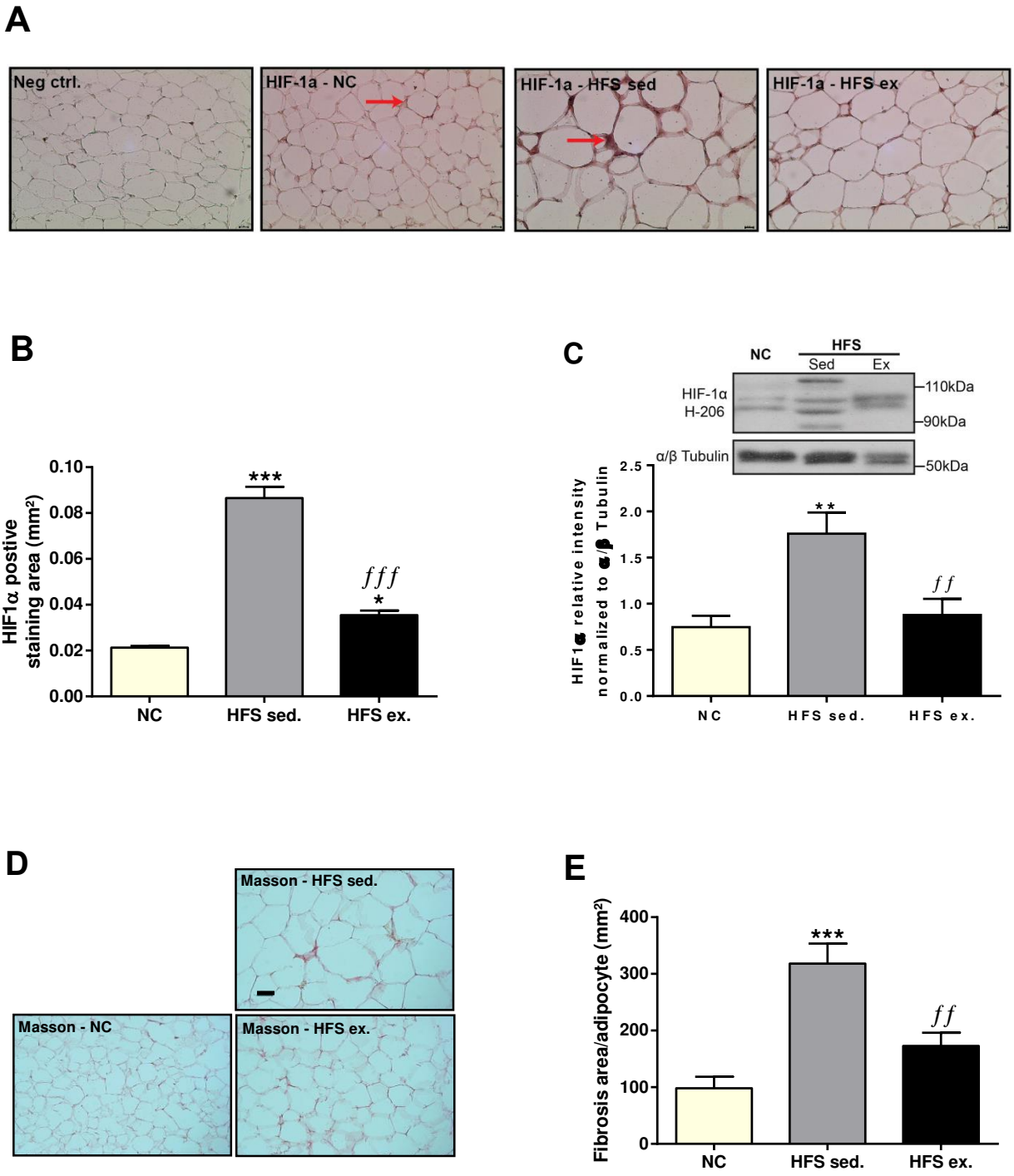

A
Neg ctrl.
HIF-1a - NC
HIF-1a - HFS sed
HIF-1a - HFS ex
B
HIF1α postive staining area (mm²)
0.10
0.08
0.06
0.04
0.02
0.00
***
fff
*
NC
HFS sed.
HFS ex.
C
NC
HFS
Sed
Ex
HIF-1α H-206
110kDa
90kDa
α/β Tubulin
50kDa
HIF1α relative intensity normalized to α/β Tubulin
2.5
2.0
1.5
1.0
0.5
0.0
**
ff
NC
HFS sed.
HFS ex.
D
Masson - HFS sed.
Masson - NC
Masson - HFS ex.
E
Fibrosis area/adipocyte (mm²)
400
300
200
100
0
***
ff
NC
HFS sed.
HFS ex.

Figure 5: Voluntary exercise reduced visceral adipose inflammation caused by high fat diet. RNA or protein was extracted from EWAT of normal chow (NC), sedentary-HFS mice (HFS-sed) and exercise-HFS mice (HFS-ex) at the week 16. The mRNA levels of (A) leptin, adiponectin, (C) CD11c, YM1, CD3, RorC, Foxp3, (D) TNFα, MCP-1, IL-6, TGFβ1 and IL-10 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 (NC). (B) Adiponectin protein level was analyzed by Western blot. Blots were stripped and reprobed for α/β-tubulin as a loading control; Data are means ± SEM (n=5 per groups). * P<0.05, ** P<0.01, *** P<0.005 (HFS-sed and HFS-ex relative to NC), *ƒ* P<0.05, *ƒƒ* P<0.01, *ƒƒƒ* P<0.005 (HFS-ex relative to HFS sed); One-way ANOVA and post hoc comparison.

Figure 5

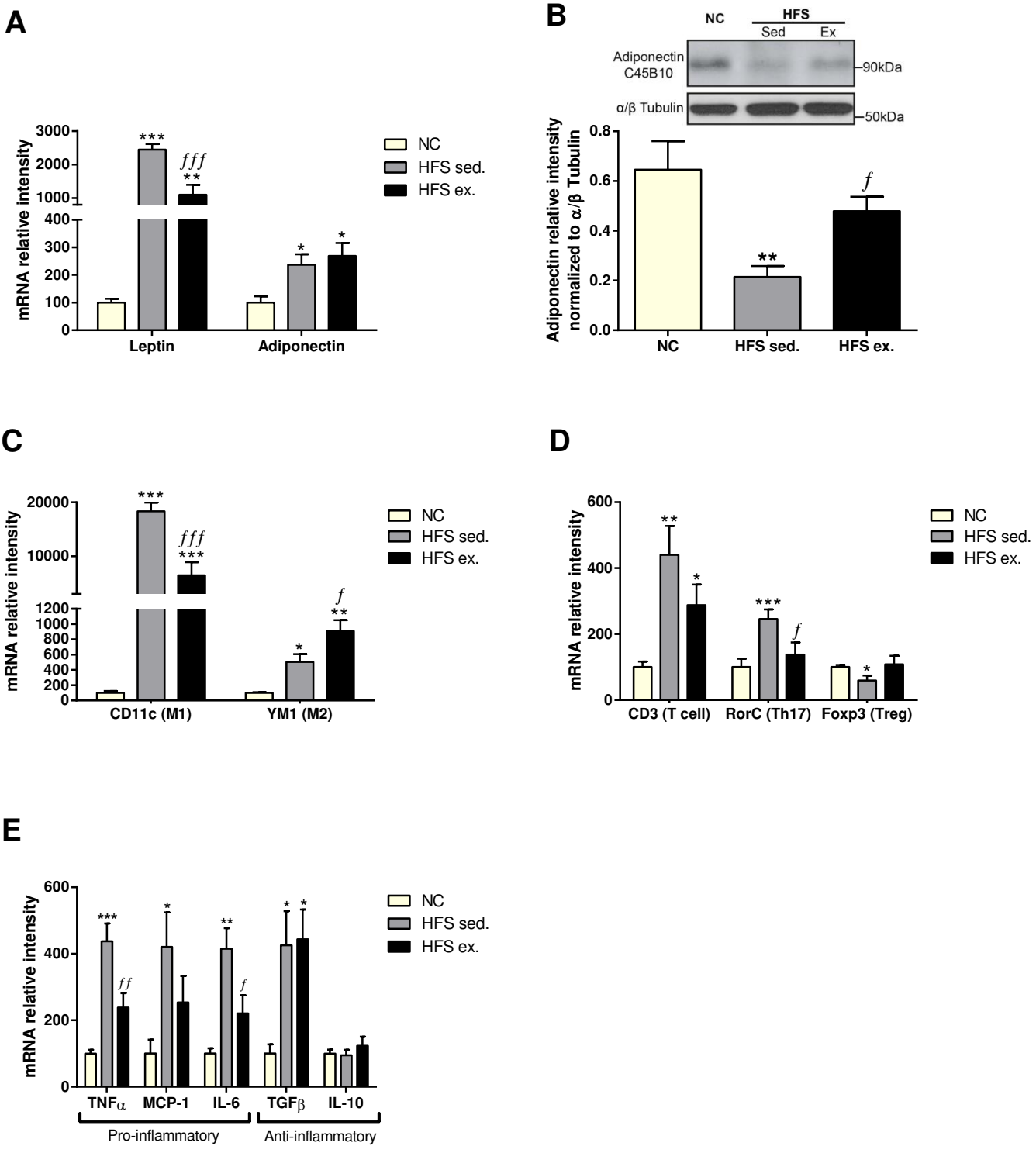

A
mRNA relative intensity
3000
2000
1000
400
300
200
100
0
***
fff
**
*
*
Leptin
Adiponectin
NC
HFS sed.
HFS ex.
B
NC
HFS
Sed
Ex
Adiponectin
C45B10
90kDa
α/β Tubulin
50kDa
Adiponectin relative intensity
normalized to α/β Tubulin
0.8
0.6
0.4
0.2
0.0
**
f
NC
HFS sed.
HFS ex.
C
mRNA relative intensity
20000
10000
1200
1000
800
600
400
200
0
***
fff
***
*
f
**
CD11c (M1)
YM1 (M2)
NC
HFS sed.
HFS ex.
D
mRNA relative intensity
600
400
200
0
**
*
***
f
*
CD3 (T cell)
RorC (Th17)
Foxp3 (Treg)
NC
HFS sed.
HFS ex.
E
mRNA relative intensity
600
400
200
0
***
ff
*
**
f
*
*
TNFα
MCP-1
IL-6
TGFβ
IL-10
Pro-inflammatory
Anti-inflammatory
NC
HFS sed.
HFS ex.

Figure 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 normal chow (NC), sedentary-HFS mice (HFS-sed) and exercise-HFS mice (HFS-ex) at 16 weeks; scale bar = 50µm. (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 (mm^2). (C) Determination of hepatic steatosis by lipid droplets density (C) expressed as droplets/mm^2 and size ($\mu m^{2)}$ (D). Positive staining areas were calculated from 3–6 independent fields of view per mouse (n = 5–9). Data are means ± SEM (n=5 per groups). * P<0.05, ** P<0.01, *** P<0.005 (HFS-sed and HFS-ex relative to NC), *ff* P<0.01, *fff* P<0.005 (HFS-ex relative to HFS sed); One-way ANOVA and post hoc comparison.

Figure 6

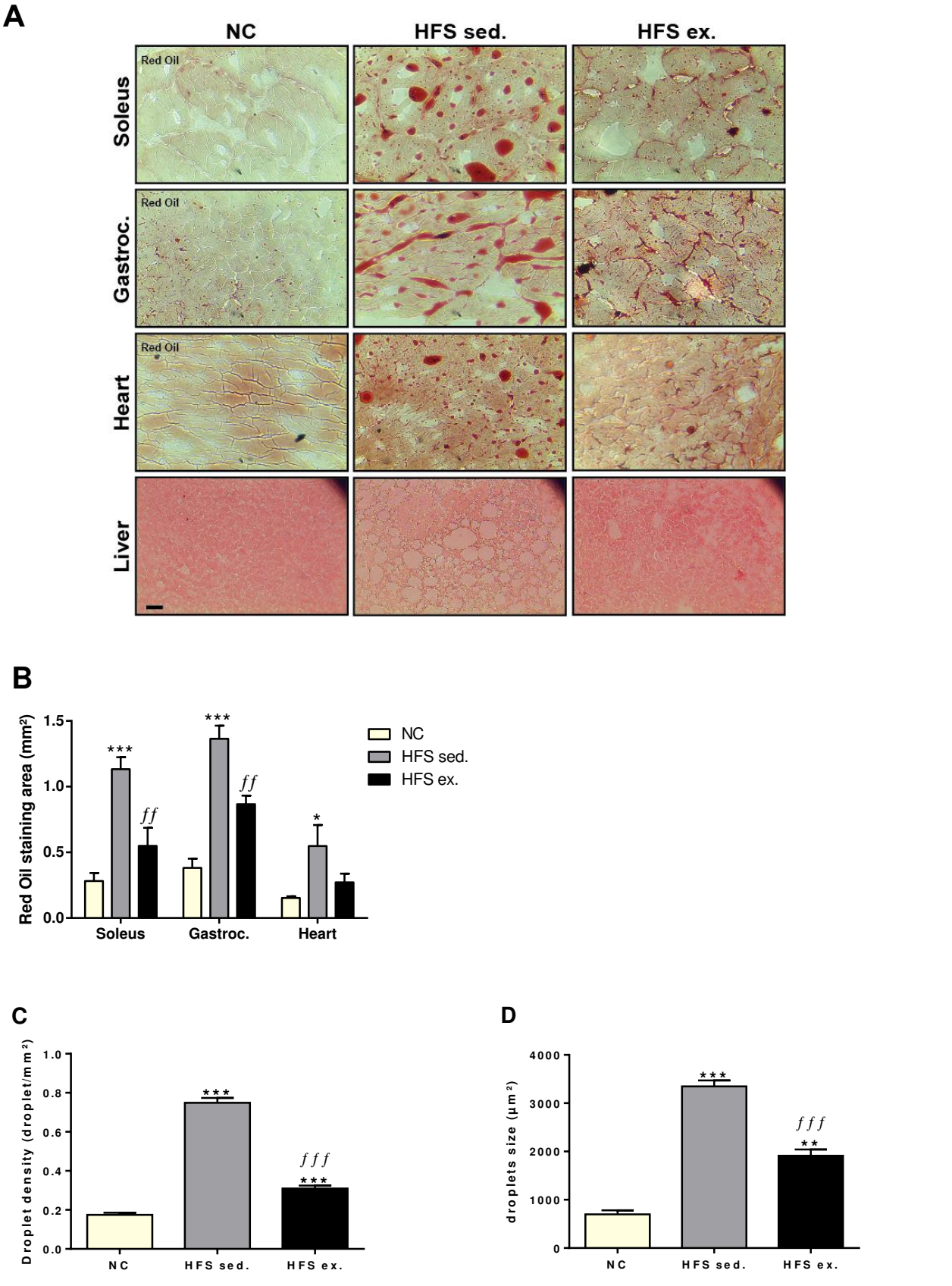
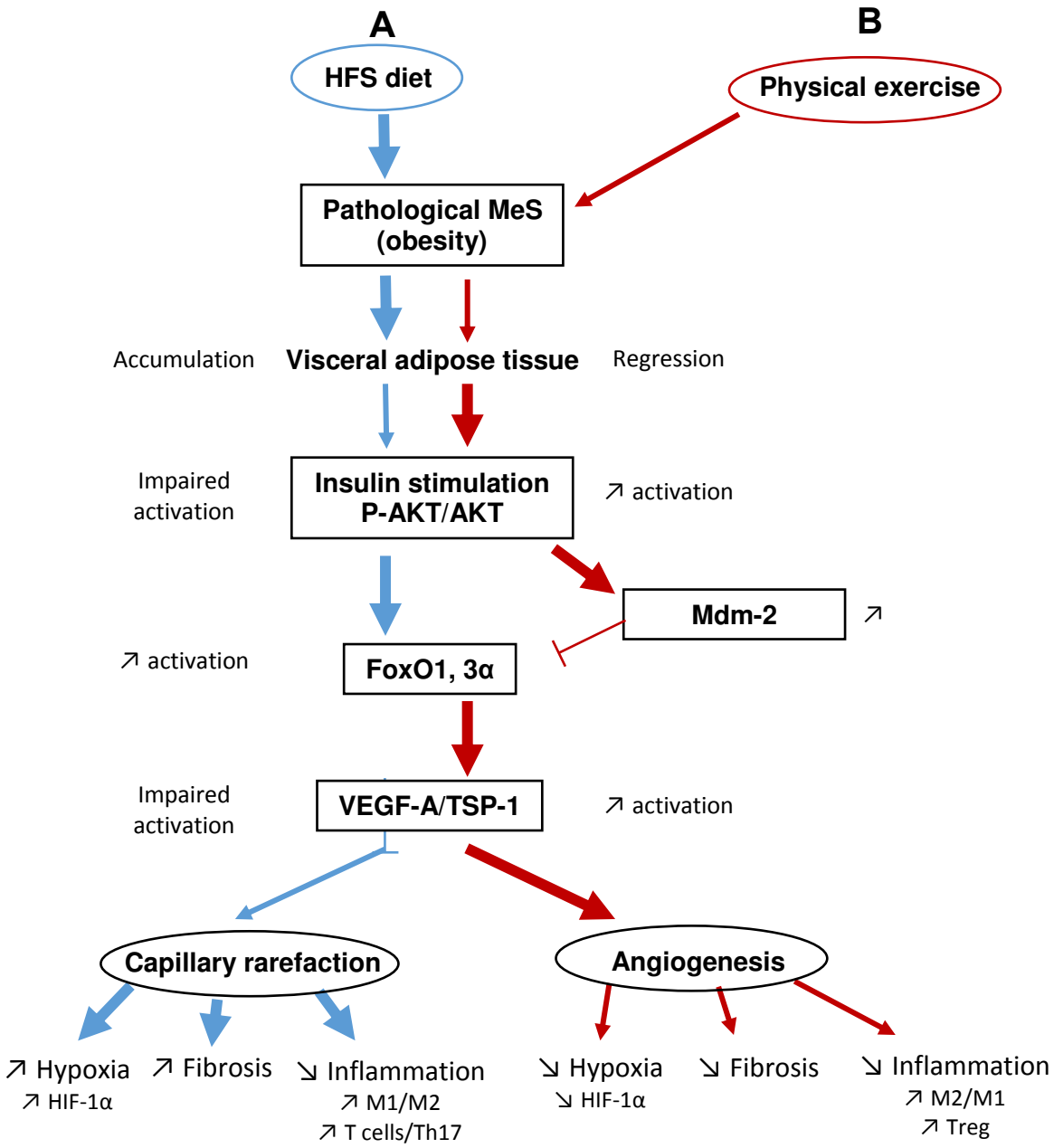

A
NC
HFS sed.
HFS ex.
Red Oil
Soleus
Gastroc.
Heart
Liver
B
Red Oil staining area (mm²)
1.5
1.0
0.5
0.0
***
ff
*
NC
HFS sed.
HFS ex.
Soleus
Gastroc.
Heart
C
Droplet density (droplet/mm²)
1.0
0.8
0.6
0.4
0.2
0.0
***
fff
NC
HFS sed.
HFS ex.
D
droplets size (µm²)
4000
3000
2000
1000
0
***
fff
**
NC
HFS sed.
HFS ex.

Figure 7: Proposed diagram of Mdm2, FoxO1,3α, VEGA/TSP signaling in EWAT

A) In pathological conditions, HFS diet impaired insulin AKT activation, activates FoxO-1,3α and blunted VEGFA/TSP-1 activation inducing an angiostatic process and capillary rarefaction accompanied with increased hypoxia (increased HIF-1α), fibrosis and inflammation (increased M1/M2, T cell and Th17). B) After physical exercise, insulin stimulates AKT activation which in turn activates Mdm-2 which then inactivates FoxO-1,3α lifting the inhibitory effect of FoxO-1,3α on VEGFA/TSP-1 producing an pro-angiogenic stimulus associated with decreased hypoxia (HIF-1α), fibrosis and inflammation (increased M2/M1, Treg).

Figure 7


A
B
HFS diet
Physical exercise
Pathological MeS (obesity)
Accumulation
Visceral adipose tissue
Regression
Impaired activation
Insulin stimulation P-AKT/AKT
↗ activation
Mdm-2
↗
↗ activation
FoxO1, 3α
Impaired activation
VEGF-A/TSP-1
↗ activation
Capillary rarefaction
Angiogenesis
↗ Hypoxia
↗ HIF-1α
↗ Fibrosis
↘ Inflammation
↗ M1/M2
↗ T cells/Th17
↘ Hypoxia
↘ HIF-1α
↘ Fibrosis
↘ Inflammation
↗ M2/M1
↗ Treg

References:

1. Haffner SM (2007) Abdominal adiposity and cardiometabolic risk: do we have all the answers? Am J Med 120:S10–16; discussion S16–17. doi: 10.1016/j.amjmed.2007.06.006

2. Berg AH, Scherer PE (2005) Adipose tissue, inflammation, and cardiovascular disease. Circ Res 96:939–949. doi: 10.1161/01.RES.0000163635.62927.34

3. Lavie CJ, Arena R, Swift DL, et al (2015) Exercise and the cardiovascular system: clinical science and cardiovascular outcomes. Circ Res 117:207–219. doi: 10.1161/CIRCRESAHA.117.305205

4. Pasarica M, Sereda OR, Redman LM, et al (2009) Reduced Adipose Tissue Oxygenation in Human Obesity Evidence for Rarefaction, Macrophage Chemotaxis, and Inflammation Without an Angiogenic Response. Diabetes 58:718–725. doi: 10.2337/db08-1098

5. Voros G, Maquoi E, Demeulemeester D, et al (2005) Modulation of angiogenesis during adipose tissue development in murine models of obesity. Endocrinology 146:4545–4554. doi: 10.1210/en.2005-0532

6. Sun K, Kusminski CM, Scherer PE (2011) Adipose tissue remodeling and obesity. J Clin Invest 121:2094–2101. doi: 10.1172/JCI45887

7. Olfert IM, Birot O (2011) Importance of Anti-angiogenic Factors in the Regulation of Skeletal Muscle Angiogenesis. Microcirculation 18:316–330. doi: 10.1111/j.1549-8719.2011.00092.x

8. Tang K, Breen EC, Gerber H-P, et al (2004) Capillary regression in vascular endothelial growth factor-deficient skeletal muscle. Physiol Genomics 18:63–69. doi: 10.1152/physiolgenomics.00023.2004

9. Malek MH, Olfert IM (2009) Global deletion of thrombospondin-1 increases cardiac and skeletal muscle capillarity and exercise capacity in mice. Exp Physiol 94:749–760. doi: 10.1113/expphysiol.2008.045989

10. Roudier E, Forn P, Perry ME, Birot O (2012) Murine double minute-2 expression is required for capillary maintenance and exercise-induced angiogenesis in skeletal muscle. FASEB J 26:4530–4539. doi: 10.1096/fj.12-212720

11. Wade M, Li Y-C, Wahl GM (2013) MDM2, MDMX and p53 in oncogenesis and cancer therapy. Nat Rev Cancer 13:83–96. doi: 10.1038/nrc3430

12. Milkiewicz M, Roudier E, Doyle JL, et al (2011) Identification of a Mechanism Underlying Regulation of the Anti-Angiogenic Forkhead Transcription Factor FoxO1 in Cultured Endothelial Cells and Ischemic Muscle. Am J Pathol 178:935–944. doi: 10.1016/j.ajpath.2010.10.042

13. Salih DA, Brunet A (2008) FoxO transcription factors in the maintenance of cellular homeostasis during aging. Curr Opin Cell Biol 20:126–136. doi: 10.1016/j.ceb.2008.02.005

14. Roudier E, Milkiewicz M, Birot O, et al (2013) Endothelial FoxO1 is an intrinsic regulator of thrombospondin 1 expression that restrains angiogenesis in ischemic muscle. Angiogenesis 16:759–772. doi: 10.1007/s10456-013-9353-x

15. Wilhelm K, Happel K, Eelen G, et al (2016) FOXO1 couples metabolic activity and growth state in the vascular endothelium. Nature 529:216–220. doi: 10.1038/nature16498

16. Shikatani EA, Trifonova A, Mandel ER, et al (2012) Inhibition of Proliferation, Migration and Proteolysis Contribute to Corticosterone-Mediated Inhibition of Angiogenesis. PLoS ONE. doi: 10.1371/journal.pone.0046625

17. Kivelä R, Silvennoinen M, Lehti M, et al (2008) Exercise-induced expression of angiogenic growth factors in skeletal muscle and in capillaries of healthy and diabetic mice. Cardiovasc Diabetol 7:13. doi: 10.1186/1475-2840-7-13

18. Kong P, Gonzalez-Quesada C, Li N, et al (2013) Thrombospondin-1 regulates adiposity and metabolic dysfunction in diet-induced obesity enhancing adipose inflammation and stimulating adipocyte proliferation. AJP Endocrinol Metab 305:E439–E450. doi: 10.1152/ajpendo.00006.2013

19. Moraes RC, Blondet A, Birkenkamp-Demtroeder K, et al (2003) Study of the Alteration of Gene Expression in Adipose Tissue of Diet-Induced Obese Mice by Microarray and Reverse Transcription-Polymerase Chain Reaction Analyses. Endocrinology 144:4773–4782. doi: 10.1210/en.2003-0456

20. Ringholm S, Grunnet Knudsen J, Leick L, et al (2013) PGC-1α Is Required for Exercise- and Exercise Training-Induced UCP1 Up-Regulation in Mouse White Adipose Tissue. PLoS ONE. doi: 10.1371/journal.pone.0064123

21. Higa TS, Spinola AV, Fonseca-Alaniz MH, Evangelista FS (2014) Remodeling of white adipose tissue metabolism by physical training prevents insulin resistance. Life Sci 103:41–48. doi: 10.1016/j.lfs.2014.02.039

22. Goossens GH, Bizzarri A, Venteclef N, et al (2011) Increased Adipose Tissue Oxygen Tension in Obese Compared With Lean Men Is Accompanied by Insulin Resistance, Impaired Adipose Tissue Capillarization, and Inflammation. Circulation 124:67–76. doi: 10.1161/CIRCULATIONAHA.111.027813

23. Hosogai N, Fukuhara A, Oshima K, et al (2007) Adipose Tissue Hypoxia in Obesity and Its Impact on Adipocytokine Dysregulation. Diabetes 56:901–911. doi: 10.2337/db06-0911

24. Corvera S, Gealekman O (2014) Adipose Tissue Angiogenesis: Impact on Obesity and Type-2 Diabetes. Biochim Biophys Acta 1842:463–472. doi: 10.1016/j.bbadis.2013.06.003

25. Jiang C, Kim J-H, Li F, et al (2013) Hypoxia-inducible Factor 1α Regulates a SOCS3-STAT3-Adiponectin Signal Transduction Pathway in Adipocytes. J Biol Chem 288:3844–3857. doi: 10.1074/jbc.M112.426338

26. Halberg N, Khan T, Trujillo ME, et al (2009) Hypoxia-Inducible Factor 1α Induces Fibrosis and Insulin Resistance in White Adipose Tissue. Mol Cell Biol 29:4467–4483. doi: 10.1128/MCB.00192-09

27. Trayhurn P (2014) Hypoxia and Adipocyte Physiology: Implications for Adipose Tissue Dysfunction in Obesity. Annu Rev Nutr 34:207–236. doi: 10.1146/annurev-nutr-071812-161156

28. Dalmas E, Clément K, Guerre-Millo M (2011) Defining macrophage phenotype and function in adipose tissue. Trends Immunol 32:307–314. doi: 10.1016/j.it.2011.04.008

29. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS (1997) Protection from obesity-induced insulin resistance in mice lacking TNF-α function. Nature 389:610–614. doi: 10.1038/39335

30. Taeye BMD, Novitskaya T, McGuinness OP, et al (2007) Macrophage TNF-α contributes to insulin resistance and hepatic steatosis in diet-induced obesity. Am J Physiol - Endocrinol Metab 293:E713–E725. doi: 10.1152/ajpendo.00194.2007

31. Kanda H, Tateya S, Tamori Y, et al (2006) MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J Clin Invest 116:1494–1505. doi: 10.1172/JCI26498

32. Spencer M, Yao-Borengasser A, Unal R, et al (2010) Adipose tissue macrophages in insulin-resistant subjects are associated with collagen VI and fibrosis and demonstrate alternative activation. Am J Physiol - Endocrinol Metab 299:E1016–E1027. doi: 10.1152/ajpendo.00329.2010

33. Verrecchia F, Mauviel A (2002) Transforming Growth Factor-β Signaling Through the Smad Pathway: Role in Extracellular Matrix Gene Expression and Regulation. J Invest Dermatol 118:211–215. doi: 10.1046/j.1523-1747.2002.01641.x

34. Zeyda M, Farmer D, Todoric J, et al (2007) Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. Int J Obes 31:1420–1428. doi: 10.1038/sj.ijo.0803632

35. Yadav H, Quijano C, Kamaraju AK, et al (2011) Protection from obesity and diabetes by blockade of TGF-β/Smad3 signaling. Cell Metab 14:67–79. doi: 10.1016/j.cmet.2011.04.013

36. Sanjabi S, Zenewicz LA, Kamanaka M, Flavell RA (2009) Anti- and Pro-inflammatory Roles of TGF-β, IL-10, and IL-22 In Immunity and Autoimmunity. Curr Opin Pharmacol 9:447–453. doi: 10.1016/j.coph.2009.04.008

37. Qureshi K, Abrams GA (2007) Metabolic liver disease of obesity and role of adipose tissue in the pathogenesis of nonalcoholic fatty liver disease. World J Gastroenterol WJG 13:3540–3553. doi: 10.3748/wjg.v13.i26.3540

38. Varma V, Yao-Borengasser A, Bodles AM, et al (2008) Thrombospondin-1 Is an Adipokine Associated With Obesity, Adipose Inflammation, and Insulin Resistance. Diabetes 57:432–439. doi: 10.2337/db07-0840

39. Nwadozi E, Roudier E, Rullman E, et al (2016) Endothelial FoxO proteins impair insulin sensitivity and restrain muscle angiogenesis in response to a high-fat diet. FASEB J Off Publ Fed Am Soc Exp Biol. doi: 10.1096/fj.201600245R

40. Fu W, Ma Q, Chen L, et al (2009) MDM2 Acts Downstream of p53 as an E3 Ligase to Promote FOXO Ubiquitination and Degradation. J Biol Chem 284:13987–14000. doi: 10.1074/jbc.M901758200

41. Zhou S, Gu L, He J, et al (2011) MDM2 Regulates Vascular Endothelial Growth Factor mRNA Stabilization in Hypoxia ▿. Mol Cell Biol 31:4928–4937. doi: 10.1128/MCB.06085-11

42. Kim JJ, Li P, Huntley J, et al (2009) FoxO1 Haploinsufficiency Protects Against High-Fat Diet–Induced Insulin Resistance With Enhanced Peroxisome Proliferator–Activated Receptor γ Activation in Adipose Tissue. Diabetes 58:1275–1282. doi: 10.2337/db08-1001

43. Karki S, Farb MG, Ngo DT, et al (2015) Forkhead Box O-1 Modulation Improves Endothelial Insulin Resistance in Human Obesity. Arterioscler Thromb Vasc Biol 35:1498–1506.

44. Nakae J, Cao Y, Oki M, et al (2008) Forkhead Transcription Factor FoxO1 in Adipose Tissue Regulates Energy Storage and Expenditure. Diabetes 57:563–576. doi: 10.2337/db07-0698

45. Inoue M, Jiang Y, Barnes RH, et al (2013) Thrombospondin 1 Mediates High-Fat Diet-Induced Muscle Fibrosis and Insulin Resistance in Male Mice. Endocrinology 154:4548–4559. doi: 10.1210/en.2013-1587

46. Elias I, Franckhauser S, Ferré T, et al (2012) Adipose Tissue Overexpression of Vascular Endothelial Growth Factor Protects Against Diet-Induced Obesity and Insulin Resistance. Diabetes 61:1801–1813. doi: 10.2337/db11-0832

47. Sung H-K, Doh K-O, Son JE, et al (2013) Adipose Vascular Endothelial Growth Factor Regulates Metabolic Homeostasis through Angiogenesis. Cell Metab 17:61–72. doi: 10.1016/j.cmet.2012.12.010

48. Kawanishi N, Niihara H, Mizokami T, et al (2015) Exercise training attenuates neutrophil infiltration and elastase expression in adipose tissue of high-fat-diet-induced obese mice. Physiol Rep. doi: 10.14814/phy2.12534

49. Castellani L, Root-Mccaig J, Frendo-Cumbo S, et al (2014) Exercise training protects against an acute inflammatory insult in mouse epididymal adipose tissue. J Appl Physiol 116:1272–1280. doi: 10.1152/japplphysiol.00074.2014

50. Haczeyni F, Barn V, Mridha AR, et al (2015) Exercise improves adipose function and inflammation and ameliorates fatty liver disease in obese diabetic mice: Exercise Improves Adipose Dysfunction in Obesity. Obesity 23:1845–1855. doi: 10.1002/oby.21170

Highlights section:

1- FoxO1,3α proteins impair insulin sensitivity and restrain adipose angiogenesis in response to high fat/high sucrose diet
2- Physical exercise promotes adipose angiogenesis via Mdm-2 activation and FoxO1,3α proteins repression
3- Exercise-activated adipose angiogenesis is accompanied with reduced hypoxia, fibrosis, adipocytes hypertrophy and inflammation
4- Physical exercise leads to a reduction of hepatic steatosis and of skeletal muscles and heart ectopic fat.

Annexe 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-1α (H-206) (1:500; Santa Cruz)
- Anti-rabbit phospho-Akt (193H12) (1:500; cell signaling)
- Anti-rabbit Akt (1:800; cell signaling)
- Anti-rabbit adiponectin (C45B10) (1:1000; cell signaling)
- Anti-rabbit α/β Tubulin (1:3000; Cell Signaling).

Annexe 2. Sequence of the primers used for qPCR.

Gene symbol	Primer name	5'→3' primer sequence
Mdm2	mMdm2_F	aatgtcctgaattgatgtcaagatt
	mMdm2_R	Catagaaccactcacatcgatcttt
Foxo1	mFoxo1_F	cttcaaggataagggcgaca
	mFoxo1_R	gacagattgtggcgaattga
Foxo3a	mFoxo3a_F	gctaagcaggcctcatctca
	mFoxo3a_R	ttccgtcagtttgagggtct
VEGFA	mVEGFA_F	aaaaacgaaagcgcaagaaa
	mVEGFA_R	tttctccgctctgaacaagg
TSP1	mTSP1_F	gttcctgatggtgaatgctg
	mTSP1_R	cacgttgctgaattccattg
Leptin	mLeptin_F	ggtgtgaaagaacctgagctgagg
	mLeptin_R	cagtggatgctaatgtgccctg
Adiponectin	mAdiponectin_F	tcctggagagaagggagagaaag
	mAdiponectin_R	tcagctcctgtcattccaaca

MCP1	mMCP1_F	catccacgtgttggct
	mMCP1_R	gatcatcttgctggtgaatga
TGFb1	mTGFb1_F	tggagcaacatgtggaac
	mTGFb1_R	gtcagcagccggttacc
Ym1	mYm1_F	gaacactgagctaaaaactctcctg
	mYm1_R	gagaccatggcactgaacg
RorC	mRorC_F	acctcttttcacgggagga
	mRorC_R	tcccacatctcccacattg
Foxp3	mFoxp3_F	agaagctgggagctatgcag
	mFoxp3_R	gctacgatgcagcaagagc
PPARα	mPPARα_F	ctgagaccctcggggaac
	mPPARα_R	aaacgtcagttcacagggaag
Pgc1a	mPgc1a_F	ttccaaaaagaagtcccatacaca
	mPgc1a_R	gataaagttgttggtttggcttga
Sirt3	mSirt3_F	tgctactcattcttgggacctc
	mSirt3_R	gggcactgatttctgtactgc
TNFα	mTNFα_F	catcttctcaaaattcgagtgacaa
	mTNFα_R	tgggagtagacaaggtacaaccc
IL6	mIL6_F	acaagtcggaggcttaattacacat
	mIL6_R	ttgccattgcacaactcttttc
IL10	mIL10_F	cacaaagcagccttgcagaa
	mIL10_R	agagcaggcagcatagcagtg
CD11c	mCD11c_F	atggagcctcaagacaggac
	mCD11c_R	ggatctgggatgctgaaatc
CD3	mCD3_F	tgctcttggtgtatatctcattgc
	mCD3_R	aacagagtctgcttgtctgaagc
18S	18S_F	cgccgctagaggtgaaattct
	18S_R	cattcttggcaaatgctttcg

3) Résultats / conclusions :

Les 16 semaines de régime HFS ont conduit au développement d'une obésité viscérale chez nos souris. Celle-ci était accompagnée d'une accumulation de lipides ectopiques au niveau musculaire et hépatique, ainsi que d'une augmentation de la glycémie et d'une intolérance au glucose. Au sein du tissu adipeux épididymal, l'expansion tissulaire se traduit par une hypertrophie adipocytaire et une réduction de la densité capillaire, associée à une augmentation de l'hypoxie, de la fibrose, de l'inflammation et de l'insulino-résistance tissulaire. L'augmentation de l'expression de FoxO1 retrouvée au niveau messager et protéique pourrait être à l'origine du phénomène de raréfaction capillaire. En effet, celle-ci est concomitante avec l'apparition d'un microenvironnement adipeux angiostatique représenté par la réduction du ratio VEGF-A/TSP-1.

Alors que l'expression de Mdm2 ne semble pas affectée par l'obésité, les 7 semaines d'exercice physique volontaire aboutissent à une augmentation du niveau protéique et messager de Mdm2. Celle-ci est corrélée à une réduction de l'expression de FoxO1 et 3α et une augmentation du ratio VEGF-A/TSP-1. Il en résulte la survenue d'un microenvironnement pro-angiogénique qui se traduit au niveau du tissu adipeux épididymal par un processus d'angiogenèse et une augmentation du nombre de capillaires par adipocyte. Ce processus, couplé à la réduction de la taille adipocytaire engendre une réduction de l'hypoxie et de la fibrose tissulaire. La sécrétion des adipokines et des cytokines pro-inflammatoires tend à être normalisée, tout comme l'homéostasie lipidique et glucidique au niveau systémique.

L'exercice semble donc intervenir comme un stimulus pro-angiogénique au sein du tissu adipeux viscéral à l'obésité. L'angio-adaptation de ce tissu à l'exercice semble être médiée par Mdm2, dont la surexpression est en mesure de lever le frein angiostatique médié par FoxO1 au sein du microenvironnement adipeux. L'angiogenèse qui en découle permet de restaurer l'homéostasie du tissu adipeux viscéral et de réduire les troubles cardiovasculaires et métaboliques associés à l'obésité.

Cette angio-adaptation à l'exercice physique est-elle retrouvée dans les autres types de dépôts adipeux ? Est-elle également médiée par l'axe de régulation Mdm2-FoxOs ? Au vu de l'importance physiologique de la raréfaction capillaire mise en place au cours de l'obésité dans les tissus adipeux sous-cutané et brun, ces questions méritent d'être posées.