Diabetologia (2017) 60:915–926
DOI 10.1007/s00125-017-4228-0

ARTICLE

HMOX1 as a marker of iron excess-induced adipose tissue dysfunction, affecting glucose uptake and respiratory capacity in human adipocytes

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Received: 26 October 2016 / Accepted: 1 February 2017 / Published online: 27 February 2017

Abstract

Aims/hypothesis Iron excess in adipose tissue is known to promote adipose tissue dysfunction. Here, we aimed to investigate the possible role of haem oxygenase 1 (HMOX1) in iron excess-induced adipose tissue dysfunction.

Methods Cross-sectionally, *HMOX1* gene expression in subcutaneous and visceral adipose tissue was analysed in two independent cohorts ($n = 234$ and 40) in relation to obesity. We also evaluated the impact of weight loss ($n = 21$), weight gain (in rats, $n = 20$) on *HMOX1* mRNA; *HMOX1* mRNA levels during human adipocyte differentiation; the effects of inflammation and iron on adipocyte HMOX1; and the effects of HMOX1-induced activity on adipocyte mitochondrial respiratory function, glucose uptake and adipogenesis.

Results Adipose tissue *HMOX1* was increased in obese participants ($p = 0.01$) and positively associated with obesity-related metabolic disturbances, and markers of iron accumulation, inflammation and oxidative stress ($p < 0.01$). *HMOX1* was negatively correlated with mRNAs related to mitochondrial biogenesis, the insulin signalling pathway and adipogenesis ($p < 0.01$). These associations were replicated in an independent cohort. Bariatric surgery-induced weight loss led to reduced *HMOX1* (0.024 ± 0.010 vs 0.010 ± 0.004 RU, $p < 0.0001$), whereas in rats, high-fat diet-induced weight gain resulted in increased *Hmox1* mRNA levels (0.22 ± 0.15 vs 0.54 ± 0.22 RU, $p = 0.005$). These changes were in parallel with changes in BMI and adipose tissue markers of iron excess, adipogenesis and inflammation. In human adipocytes, iron excess and inflammation led to increased *HMOX1* mRNA levels. HMOX1 induction (by haem arginate [hemin] administration), resulted in a significant reduction of mitochondrial respiratory capacity (including basal respiration and spare respiratory capacity), glucose uptake and adipogenesis in parallel with increased expression of inflammatory- and iron excess-related genes.

Conclusions/interpretation *HMOX1* is an important marker of iron excess-induced adipose tissue dysfunction and metabolic disturbances in human obesity.

Keywords Adipogenesis · Adipose tissue · HMOX1 · Iron · Obesity

José María Moreno-Navarrete and José Manuel Fernández-Real contributed equally to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-017-4228-0) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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Abbreviations

DFO	Deferoxamine
eWAT	Epididymal white adipose tissue
HFD	High-fat diet
HMOX1	Haem oxygenase 1

MCM	Macrophage-conditioned medium
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue
SVF	Stromal vascular fraction
VAT	Visceral adipose tissue

Introduction

Adipose tissue dysfunction is a hallmark of obesity-associated metabolic disturbances. It is characterised by a significant reduction in its capacity to store excess fuel [1, 2] and to generate new adipocytes (adipogenesis) [3, 4] in parallel with increased levels of inflammatory markers, as a consequence of macrophage infiltration [5] and adipocyte inflammation [6].

Recent studies demonstrated that iron excess in adipose tissue promotes adipose tissue dysfunction, leading to decreased adipogenesis and enhanced adipocyte inflammation and adipose tissue macrophage M1 polarisation [7–12]. Intracellular mediators of iron homeostasis contribute to changes in adipose tissue physiology and adipogenesis, including SLC40A1 or ferroportin [9], transferrin [11], cytosolic aconitase [13] and mitoNEET [14, 15], but less is known about the source of iron excess.

Haem oxygenase 1 (HMOX1, also known as HO-1) is an inducible enzyme required for haem degradation. Since haem degradation leads to the production of biliverdin, ferrous iron and carbon monoxide, haem oxygenase activity is an important source of intracellular iron. Taking into account that high dietary intake of haem iron, which is more easily absorbed than non-haem iron [16], is considered a risk factor in the progression of type 2 diabetes and obesity-associated metabolic disturbances [16–19], and the importance of intracellular haem levels in adipogenesis [20], we hypothesised that increased adipose tissue HMOX1 activity (an extra source of iron) might contribute to obesity-associated adipose tissue iron excess and, in consequence, promote adipose tissue dysfunction. In line with this hypothesis, a recent study demonstrated that macrophage HMOX1 is required to promote adipose tissue inflammation in mice [21]. Specifically, macrophage HMOX1 deletion improved adipose tissue dysfunction, inhibiting the development of crown-like structures and macrophage infiltration, reducing adipocyte hypertrophy and increasing the expression of adipogenic genes and the number of protective M2 macrophages. Furthermore, this study also showed that the inhibition of HMOX1 activity in liver and in macrophages improved glucose clearance and insulin sensitivity, and decreased high-fat diet (HFD)-induced inflammation [21]. In contrast to this study, two previous studies in murine models demonstrated that systemic induction of HMOX1 activity by intraperitoneal cobalt protoporphyrin administration resulted in reduced adiposity, increased circulating adiponectin and improved insulin sensitivity and glucose tolerance [22, 23].

In humans, a few studies have examined *HMOX1* gene expression in adipose tissue. Specifically, Shakeri-Manesch et al reported the first study showing increased subcutaneous (SAT) and visceral (VAT) adipose tissue *HMOX1* mRNA and protein levels in obese participants (morbidly obese [$n = 20$] vs non-obese [$n = 20$]) [24]. VAT *HMOX1* expression was negatively correlated with HOMA-IR [24]. Two additional studies confirmed increased SAT ($n = 42$ [25]) and VAT ($n = 50$ [21]) *HMOX1* gene expression in obese participants, but in positive association with insulin resistance. In one of these studies the significance of the correlation between SAT *HMOX1* mRNA and insulin resistance was lost after controlling for BMI [25]. The studies indicate a clear association between adipose tissue *HMOX1* expression and adiposity. However, the association with insulin resistance remains controversial.

To the best of our knowledge the possible role of HMOX1 in the relationship between iron metabolism and adipogenesis in human adipose tissue and adipocytes has not been explored. In the current study, we aimed to investigate the possible role of HMOX1 in human adipose tissue, adipogenesis and iron metabolism. To this end, we evaluated (1) SAT and VAT *HMOX1* gene expression in two independent cohorts in relation with obesity; (2) the impact of weight loss and weight gain (in rats) on adipose tissue HMOX1; (3) *HMOX1* mRNA levels during human adipocyte differentiation; (4) the effects of inflammation, iron and haem arginate (hemin) on adipocyte HMOX1; and (5) the effects of induction of HMOX1 activity on adipocyte mitochondrial respiratory function, glucose uptake and adipogenesis.

Methods

Patient recruitment Adipose tissue samples were obtained from three independent cohorts. In cohort 1, a group of 234 adipose tissue samples (120 VAT and 114 SAT) from participants with normal body weight and different degrees of obesity (BMI ranging from 20 to 68 kg/m^2) were analysed. In cohort 2, which comprised morbidly obese (BMI >35 kg/m^2) participants with different degrees of insulin action (measured using hyperinsulinaemic–euglycaemic clamp [9] as detailed in the electronic supplementary material [ESM] Methods), 40 paired SAT and VAT samples were studied. All these participants were recruited at the Endocrinology Service of the Hospital of Girona ‘Dr Josep Trueta’. All participants gave written informed consent, validated and approved by the Ethics Committee of the Hospital of Girona ‘Dr Josep Trueta’, after the purpose of the study was explained to them.

In cohort 3, 21 obese (47 ± 9 years [mean ± SD]) participants of European descent, who underwent bariatric surgery via Roux-en-Y gastric bypass (RYGB) at the Hospital of Girona ‘Dr Josep Trueta’ were part of an ongoing study [9, 14]. Adipose tissue samples from the SAT depot were

obtained during bariatric surgery. Postoperative samples of SAT were obtained by subcutaneous biopsy at the mesogastric level 2 years after surgery. All participants gave written informed consent, validated and approved by the Ethics Committee of the Hospital of Girona 'Dr Josep Trueta', after the purpose of the study was explained to them.
Additional information is detailed in ESM Methods.

Adipose tissue handling Adipose tissue samples were obtained from SAT and VAT (omentum) depots during elective surgical procedures (cholecystectomy, abdominal hernia surgery and gastric bypass surgery). Samples of adipose tissue were immediately transported to the laboratory (5–10 min). The handling of tissue was carried out under strictly aseptic conditions. Adipose tissue samples were washed in PBS, cut with forceps and scalpel into small pieces (100 mg) and immediately flash-frozen in liquid nitrogen before being stored at –80°C. The isolation of adipocyte and stromal vascular fraction (SVF) cells was performed from nine non-frozen SAT samples as described in the ESM Methods.

Effects of weight gain in rats Four-week-old male Wistar rats ($n = 20$) were randomised into two groups fed ad libitum during an average of 6 months either a normal chow diet ($n = 10$) for comparative purposes or an HFD ($n = 10$) to induce obesity. These experiments are detailed in the ESM Methods. All experimental procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 2010/63/EU) and were approved by the Ethics Committee for Animal Experimentation of the University of Navarra (049/10). No data from these experiments has been excluded.

Differentiation of human pre-adipocytes Differentiation of human pre-adipocytes is described in the ESM Methods. For the time course experiment, cells were harvested and stored at –80°C for RNA extraction at day 0, 2, 5, 7, 9, 12 and 14. The effect of administration of (1) macrophage-conditioned medium (MCM, 2.5%) from THP-1 cells previously treated with lipopolysaccharide (10 ng/ml for 24 h), (2) hemin (60 μmol/l), (3) $FeSO_4$ (3 and 30 μg/ml) or (4) deferoxamine (DFO, 20 μmol/l) during subcutaneous adipocyte differentiation was studied. Otherwise, before and after adipocyte differentiation (at day 0 and day 14), subcutaneous pre-adipocytes and fully differentiated adipocytes were incubated with fresh medium (control) or fresh medium containing several treatments. These treatments included hemin (60 μmol/l), $FeSO_4$ (3 and 30 μg/ml) and DFO (20 μmol/l). After 48 and 72 h, cells were harvested, and pellets and supernatants were stored at –80°C. The concentrations of these treatments were selected and optimised according to previous reports [10, 20]. All in vitro experiments were randomised and performed in triplicate. Experimenters were blind to group assignment and outcome assessment. No data from these experiments has been excluded.

Glucose uptake Adipocyte glucose uptake was measured using the Glucose Uptake Assay Kit (ab136955, Abcam, Cambridge, UK) as described in the ESM Methods.

Mitochondrial respiratory function Mitochondrial respiratory function was assessed using the Seahorse XFp Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies, Santa Clara, CA, USA) using the Seahorse XFp Cell Mito Stress Test Kit according to the manufacturer's instructions.

RNA expression RNA purification, gene expression procedures and analyses were carried out as previously described [9, 14]. Briefly, RNA purification was performed using an RNeasy Lipid Tissue Mini Kit (QIAgen, Izasa, Barcelona, Spain) and the integrity was checked using an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Gene expression was assessed by real-time PCR using a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Barcelona, Spain), using TaqMan and SYBR green technology suitable for relative genetic expression quantification. The commercially available and pre-validated TaqMan primer/probe sets used are detailed in the ESM Methods.

Adipose tissue protein extraction VAT ($n = 40$) and SAT ($n = 26$) protein were extracted as described in the ESM Methods. HMOX1 protein levels were measured using Human HO 1 ELISA kit (ab133064, Abcam) in adipose tissue extracts following manufacturer's instructions. Total iron was measured using the Iron-FerroZine colorimetric method following the manufacturer's protocol (11509, Biosystems, Barcelona, Spain). The same amount of total protein (100 μg) was used to perform these measurements.

Analytical methods Serum glucose, insulin, homeostasis model assessment of insulin resistance (HOMA-IR), HDL-cholesterol, fasting triacylglycerols and C-reactive protein concentration were measured as previously described [9, 14].

Statistical analyses Statistical analyses were performed using SPSS 12.0 software. Unless otherwise stated, descriptive results of continuous variables are expressed as mean and SD for Gaussian variables or median and interquartile range. Variables that did not fulfill normal distribution criteria were Log_{10} transformed to improve symmetry for subsequent analyses. The relation between variables was analysed by simple correlation (Pearson's test and Spearman's test) and multiple regression analyses in a stepwise manner. One-factor ANOVA with post hoc Bonferroni test, paired t test and unpaired Student's t test were used to compare *HMOX1* gene expression according to obesity and type 2 diabetes. A nonparametric test (Mann–Whitney U test) was used to analyse in vitro experiments. Levels of statistical significance were set at $p < 0.05$.

Results

HMOX1 mRNA in human adipose tissue Anthropometric and clinical variables of participants in cohort 1 are shown in Table 1. No significant differences in SAT and VAT *HMOX1* expression were found in non-obese (0.019 ± 0.01 vs 0.020 ± 0.02, $p = 0.7$) and obese participants (0.030 ± 0.01 vs 0.031 ± 0.01, $p = 0.6$). In both SAT and VAT, *HMOX1* gene expression was significantly increased in obese participants (Table 1). Both SAT and VAT *HMOX1* gene expression was associated with BMI, but not with fasting glucose (Table 2). In this cohort, the increased SD of fasting glucose in obese compared with non-obese participants (6.2 ± 2.5 vs 5.0 ± 0.5 mmol/l [mean ± SD]) might explain the lack of association between adipose tissue *HMOX1* and fasting glucose in these participants. In obese participants, both SAT and VAT *HMOX1* gene expression values above the first quartile were significantly associated with increased fasting glucose (ESM Table 1, Fig. 1). VAT *HMOX1* gene expression was also positively correlated with fasting triacylglycerols (Table 2). However, this correlation was lost ($r = 0.06$, $p = 0.5$) after controlling for BMI.

HMOX1 gene expression was negatively correlated with adipogenic (*PPARG*, *FASN*, *ACACA*, *SLC2A4*), insulin pathway-related (*IRS1*) and mitochondrial biogenesis-related (*PPARGC1A* [only in SAT], *MTCO3*) genes, and positively correlated with markers of inflammation (*TNF*, *IL10*, *IL6*, *IL8*), iron accumulation (*FTL*, *SLC40A1*) and oxidative stress (*CYBA*, *NFE2L2*) in both SAT and VAT (Table 2). *HMOX1* gene expression was negatively correlated with *CD206* (also known as *MRC*1)/*CD68* ratio (a specific marker of M2 macrophages) in VAT, but not with expression of the non-specific classical macrophage marker, *CD68* (Table 2). In multivariate

Table 2 Bivariate correlation among clinical variables, expression of *HMOX1* gene and expression of adipose tissue-related genes in VAT and SAT from cohort 1

Variable	VAT		SAT	
	r	*p* value	*r*	*p* value
Age (years)	–0.09	0.3	–0.15	0.1
BMI (kg/m^2)	0.32	0.002	0.27	0.01
Fasting glucose (mmol/l)	0.12	0.2	0.12	0.2
Fasting triacylglycerol (mmol/l)	0.22	0.04	0.18	0.08
HDL-cholesterol (mmol/l)	–0.09	0.3	–0.015	0.8
PPARG (RU)	–0.31	0.003	–0.51	<0.0001
SLC2A4 (RU)	–0.42	<0.0001	–0.55	<0.0001
IRS1 (RU)	–0.44	<0.0001	–0.31	0.003
FASN (RU)	–0.27	0.005	–0.36	<0.0001
ACACA (RU)	–0.43	<0.0001	–0.29	0.003
PPARGC1A (RU)	0.04	0.6	–0.33	0.002
MTCO3 (RU)	–0.29	0.003	–0.39	<0.0001
TNF (RU)	0.25	0.02	0.24	0.03
IL10 (RU)	0.51	<0.0001	0.43	<0.0001
IL6 (RU)	0.37	0.001	0.31	0.003
IL8 (RU)	0.42	<0.0001	0.38	<0.0001
FTL (RU)	0.49	<0.0001	0.26	0.02
CYBA (RU)	0.38	0.001	0.23	0.04
SLC40A1 (RU)	0.33	0.003	0.39	<0.0001
NFE2L2 (RU)	0.48	<0.0001	0.34	0.002
CD68 (RU)	0.09	0.4	0.17	0.1
CD206/*CD68*	–0.26	0.01	–0.21	0.06

The data are expressed as mean ± SD or median (interquartile range)

RU, relative units of gene expression relative to *PPIA* as endogenous control

Table 1 Anthropometric and clinical variables of study participants from cohort 1 and cohort 2

Variable	Cohort 1			Cohort 2
	Non-obese	Obese	*p* value	Morbidly obese (BMI >35 kg/m^2)
N	22	98		40
Sex (men/women)	10/12	21/77		9/31
Age (years)	51.3 ± 14.3	45.6 ± 11.2	0.1	47.6 ± 7.9
BMI (kg/m^2)	26.6 ± 2.7	44.7 ± 6.1	<0.0001	45.8 ± 6.7
Fasting glucose (mmol/l)	4.9 (4.4,5.5)	5.4 (4.9,6.4)	0.03	5.4 (4.9,5.9)
HOMA-IR	–	–	–	3.91 (2.31,5.84)
M value (μmol kg^{-1} min^{-1})	–	–	–	14.9 (10.1,24.1)
Fasting triacylglycerol (mmol/l)	1.11 (0.79,1.77)	1.15 (0.90,1.64)	0.7	1.3 (0.96,1.69)
HDL-cholesterol (mmol/l)	1.52 (1.20,1.92)	1.35 (1.13,1.63)	0.6	1.12 (1.03,1.28)
VAT *HMOX1* mRNA (RU)	0.020 ± 0.017	0.031 ± 0.015	0.01	0.032 ± 0.018
SAT *HMOX1* mRNA (RU)	0.019 ± 0.012	0.030 ± 0.013	0.001	0.041 ± 0.019

The data are expressed as mean ± SD or median (interquartile range)

RU, relative units of gene expression relative to *PPIA* as endogenous control

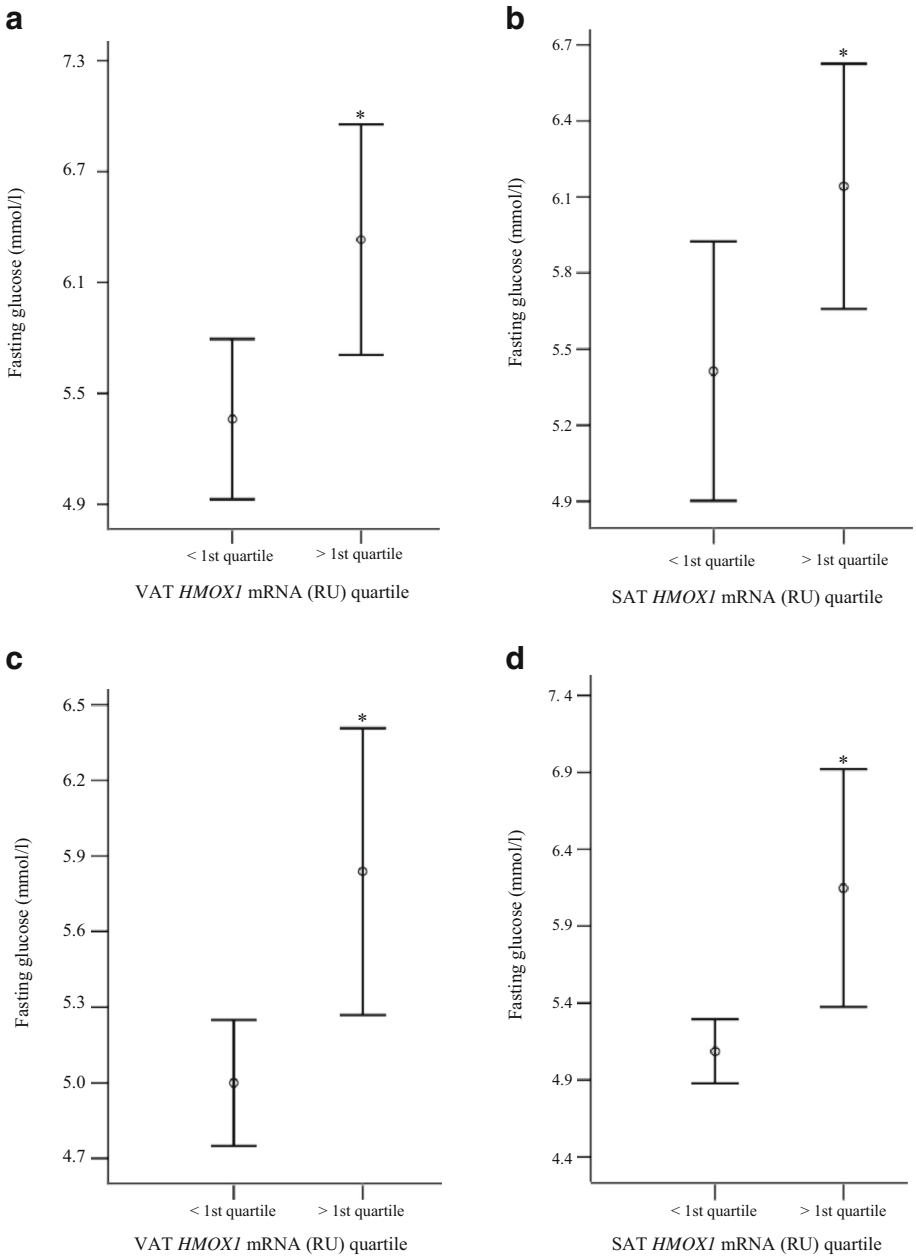

Fig. 1 (**a**, **b**) Fasting glucose according to VAT (**a**) and SAT (**b**) *HMOX1* gene expression first quartile in cohort 1. (**c**, **d**) Fasting glucose according to VAT (**c**) and SAT (**d**) *HMOX1* gene expression first quartile in cohort 2. *$p < 0.05$ compared with *HMOX1* mRNA (RU) <1st quartile. Error bars show 95% CI. In cohort 1, *HMOX1* gene expression values were 0.028 (0.021–0.038) RU in SAT and 0.027 (0.019–0.036) RU in VAT (median and interquartile range) and the first quartiles were 0.021 RU in SAT and 0.019 RU in VAT. In cohort 2, *HMOX1* gene expression values were 0.033 (0.027–0.048) RU in SAT and 0.031 (0.020–0.042) RU in VAT (median and interquartile range) and the first quartiles were 0.027 RU in SAT and 0.020 RU in VAT

regression analysis, all these gene expression associations (except *IRS1* in SAT and *CD206/CD68* in VAT) remained significant after controlling for age and BMI in both VAT (ESM Table 2) and SAT (ESM Table 3).

In a subgroup of participants, HMOX1 protein and total iron levels were measured. Significant associations among *HMOX1* mRNA, HMOX1 protein and total iron levels in both VAT (Fig. 2a–c) and SAT (Fig. 2d–f) were found. *HMOX1* mRNA, HMOX1 protein and total iron levels were increased in those participants with BMI over 35 kg/m^2 (ESM Fig. 1).

A second independent cohort (cohort 2) composed of morbidly obese patients confirmed the association between *HMOX1* gene expression and markers of intracellular iron excess (*SLC40A1* [$r = 0.41$, $p = 0.03$], *FTL* [$r = 0.46$, $p = 0.005$] and *CYBA* [$r = 0.61$, $p < 0.0001$]) and inflammation (*TNF* [$r = 0.58$, $p < 0.0001$], *LBP* [$r = 0.53$, $p = 0.001$]). The association between both SAT and VAT *HMOX1* gene expression values above the first quartile and fasting glucose were also replicated in cohort 2 (ESM Table 1, Fig. 1). SAT *HMOX1* gene expression values above the first quartile were also associated with HOMA-IR (ESM Table 1). In this cohort, *HMOX1* gene expression was non-significantly increased in SAT vs VAT (0.041 ± 0.019 vs 0.032 ± 0.018 $p = 0.06$).

Effects of bariatric surgery-induced weight loss In cohort 3, bariatric surgery-induced weight loss resulted in decreased SAT *HMOX1* mRNA levels (Table 3). The per cent change in *HMOX1* mRNA levels was positively correlated with the

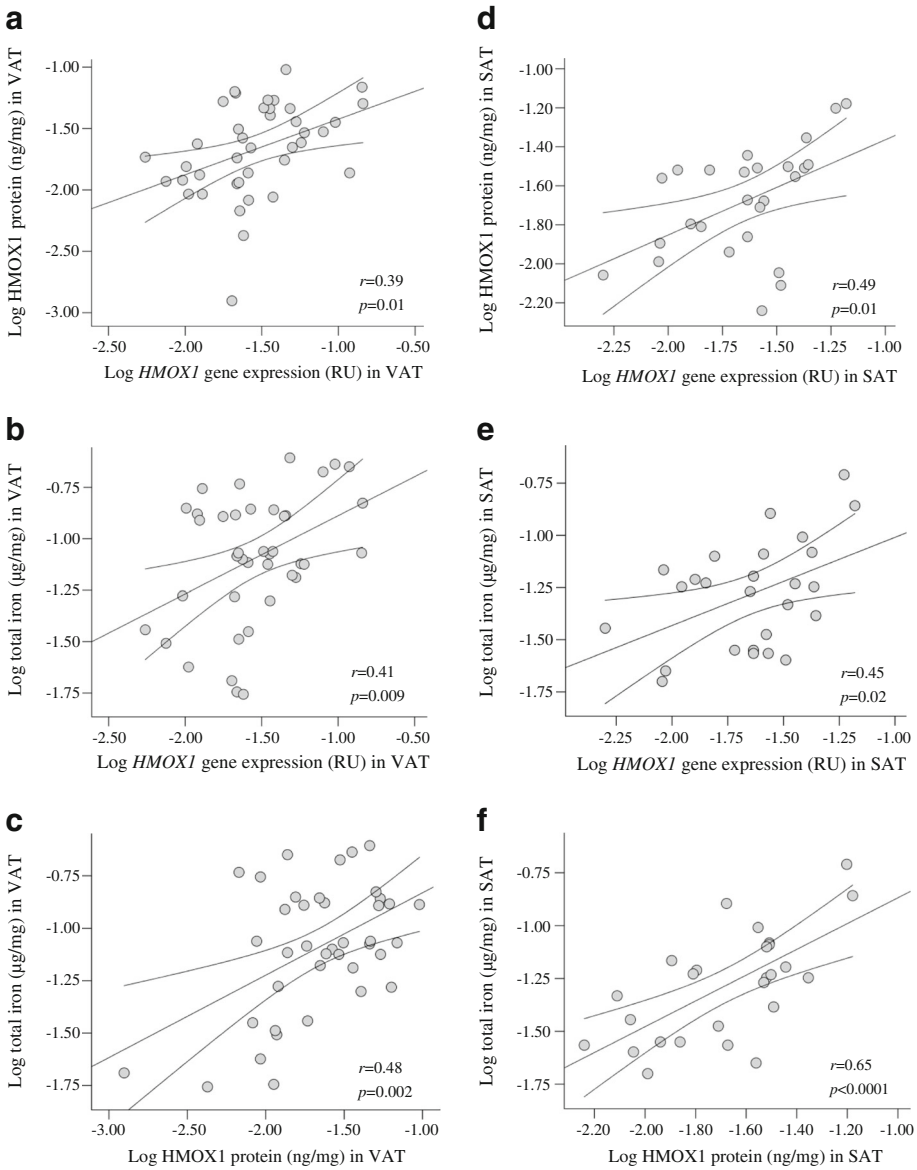

Fig. 2 Bivariate correlations among *HMOX1* gene expression, HMOX1 protein and total iron levels in VAT (**a**–**c**) and SAT (**d**–**f**). Log indicates Log_{10} transformation

per cent change in weight ($r = 0.51$, $p = 0.01$), BMI ($r = 0.60$, $p = 0.004$) and fat mass ($r = 0.66$, $p = 0.001$), but not with the per cent change in fasting glucose, HDL-cholesterol, fasting triacylglycerols, C-reactive protein or insulin sensitivity (estimated using *M* value) (Table 3). In addition, the per cent change in *HMOX1* mRNA levels was negatively correlated with the per cent change in adipogenic (*ADIPOQ*, *SLC2A4*) gene expression and positively correlated with the per cent change in intracellular iron excess-related (*SLC40A1*, *FTL*) gene expression (Table 3). No correlation was found between the per cent change in *HMOX1* and inflammatory-related (*TNF*, *IL6*, *IL8*, *CD14*) gene expression (Table 3).

Effects of HFD-induced weight gain in rats HFD-induced weight gain resulted in increased *Hmox1* mRNA levels in epididymal white adipose tissue (eWAT), positively correlated with weight, eWAT weight, HOMA-IR, circulating leptin and eWAT *Il6*, and negatively correlated with *Adipoq* mRNA levels (Fig. 3a–i).

HMOX1 in human adipogenesis *HMOX1* mRNA levels progressively decreased during differentiation of human adipocytes (Fig. 4a). The analysis of adipose tissue subfractions showed that *HMOX1* mRNA levels were significantly reduced in adipocytes compared with SVF cells (including $CD14^+$ and $CD14^-$ cells) (Fig. 4b), with the highest expression being shown in $CD14^+$ cells (enriched with adipose tissue macrophages) (Fig. 4b). Inflammatory conditions (MCM, 2.5%; Fig. 4c) and iron (Fig. 4d) administration during adipocyte differentiation induced *HMOX1* mRNA levels. Iron administration in pre-adipocytes (Fig. 4e) and fully differentiated adipocytes (Fig. 4f) also resulted in increased *HMOX1*

Table 3 Effects of bariatric-surgery-induced weight loss on *HMOX1* gene expression, anthropometric and clinical variables and other gene expression in SAT in cohort 3

	Pre-surgery ($n=21$)	Post-surgery ($n=21$)	p value	r^a	p value
Weight (kg)	116.6 ± 17.7	78.9 ± 17.9	<0.0001	0.51	0.01
BMI (kg/m^2)	43.5 ± 5.1	29.6 ± 5.6	<0.0001	0.60	0.004
Fat mass (%)	56.8 ± 7.6	40.8 ± 7.4	<0.0001	0.66	0.001
Fasting glucose (mmol/l)	5.3 ± 0.7	4.9 ± 0.7	0.03	0.25	0.3
M value (μmol kg^{-1} min^{-1})	21.9 ± 12.8	30.5 ± 14.9	0.02	0.27	0.3
HDL-cholesterol (mmol/l)	1.45 ± 0.35	1.89 ± 0.58	0.001	-0.10	0.6
Triacylglycerol (mmol/l)	1.14 (0.73,1.67)	0.80 (0.64,1.03)	0.005	0.04	0.8
C-reactive protein (nmol/l)	42.8 (28.6,104.7)	4.7 (2.8,9.5)	0.003	0.31	0.2
ADIPOQ (RU)	2.92 ± 1.3	4.17 ± 1.9	0.02	-0.45	0.04
SLC2A4 (RU)	0.035 ± 0.02	0.093 ± 0.05	<0.0001	-0.54	0.01
FTL (RU)	17.1 ± 6.6	10.5 ± 7.8	0.005	0.49	0.03
SLC40A1 (RU)	0.17 ± 0.10	0.10 ± 0.036	0.008	0.59	0.009
TNF (RU)	0.0028 ± 0.0019	0.00078 ± 0.0003	<0.0001	-0.36	0.1
IL6 (RU)	0.0013 ± 0.0009	0.0005 ± 0.0003	0.003	-0.39	0.07
IL8 (RU)	0.018 ± 0.01	0.0009 ± 0.0006	0.005	0.01	0.9
CD14 (RU)	0.16 ± 0.08	0.06 ± 0.02	<0.0001	0.11	0.6
HMOX1 (RU)	0.024 ± 0.01	0.010 ± 0.004	<0.0001	–	–

The data are expressed as mean ± SD or median (interquartile range)

[a] Bivariate correlation between the change of *HMOX1* gene expression and the change of clinical variables and expression of adipose tissue- related genes

RU, relative units of gene expression relative to *PPIA* as endogenous control

mRNA levels, whereas iron chelation using DFO (20 μmol/l) exerted opposite effects. As expected, the major inductor of *HMOX1* mRNA levels was hemin (60 μmol/l), both in fully differentiated adipocytes (Fig. 4g) and during adipocyte differentiation (Fig. 5a). A significant reduction in intracellular lipid accumulation (Fig. 5b) and reduced adipogenic (*ADIPOQ*, *FABP4*, *SLC2A4* and *PLIN1*) gene expression (Fig. 5c–f) was observed after hemin administration. Increased inflammatory (*IL8*, *TNF*) mRNAs (Fig. 5g–h), increased markers of intracellular iron accumulation (*FTL*, *CYBA* and *SLC40A1*) and reduced *TFRC1* mRNA levels were also observed (Fig. 5i–l). Functionally, hemin led to a significant decrease in glucose uptake (Fig. 5m), basal respiration (Fig. 5n) and spare respiratory capacity (Fig. 5o).

Discussion

In contrast to haem oxygenase 2 (HMOX2), which is constitutively expressed in many tissues, HMOX1 is a facultative gene and is expressed at a relatively low level in most tissues. HMOX1 expression is regulated at transcriptional level being mainly induced by its substrate (haem), iron, oxidative stress and inflammation [26]. Facultative genes need precise regulation and their mRNA levels generally correlated with protein levels. In fact, a large number of studies have demonstrated that HMOX1 activity is proportional to *HMOX1* mRNA and protein levels [27, 28]. Thus, we investigated *HMOX1* mRNA levels as the main outcome in the current study; the association between *HMOX1* mRNA and HMOX1 protein levels was also demonstrated in a subgroup of participants.

To the best of our knowledge this is the first study examining the possible role of HMOX1 in iron accumulation, mitochondrial respiratory function and adipogenesis in human adipocytes and adipose tissue in obese and non-obese participants.

HMOX1 and markers of iron accumulation in adipose tissue Iron accumulation in adipose tissue in obesity is detrimental for adipogenesis [7, 9, 10, 13–15]. In the current study, hemin-induced HMOX1 activity resulted in raised intracellular iron accumulation-related (*FTL*, *SLC40A1* and *CYBA*) markers and concordantly decreased *TFRC* mRNA levels. In agreement with these data, the association between tissue iron and *HMOX1* gene expression and activity has been demonstrated in brain [29, 30], aorta [31] and liver [32]. Furthermore, a positive association between total body iron and HMOX1 activity has been reported [33]. In addition, intracellular free-iron-produced reactive oxygen species (ROS) are important inductors of HMOX1 activity [27, 28]. For this reason, a bidirectional regulation between HMOX1 activity and intracellular iron levels should be considered. In fact, in this study (Fig. 4d–f) iron administration in preadipocytes and adipocytes and during adipocyte differentiation resulted in a significant upregulation of *HMOX1* gene expression, which was attenuated by DFO (an iron chelator) administration. *HMOX1* gene expression was positively correlated with gene expression markers of adipose tissue iron accumulation

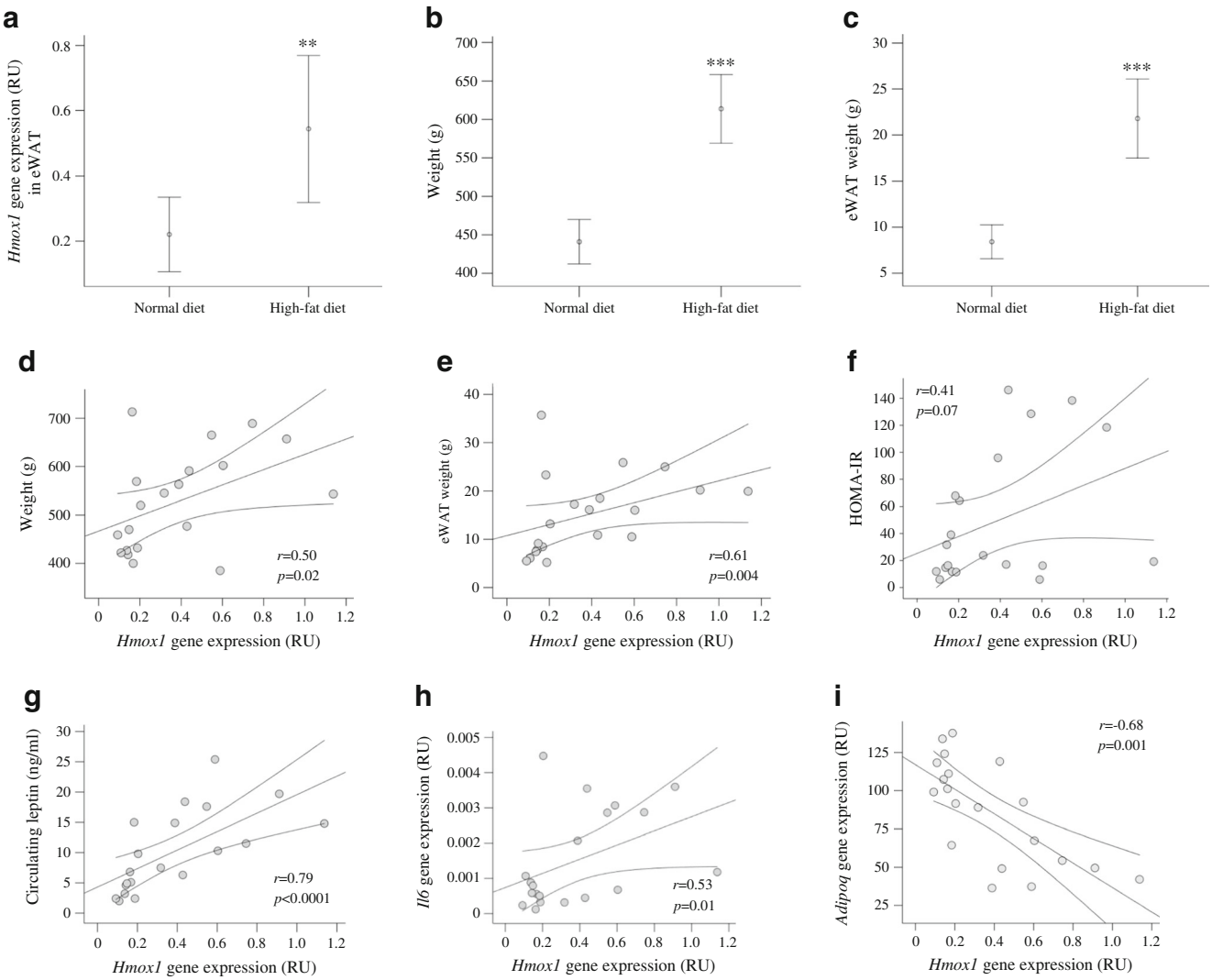

Fig. 3 (**a**–**c**) Effects of HFD in *Hmox1* mRNA (**a**), weight (**b**) and eWAT weight (**c**) in rats. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with normal diet. Error bars show 95% CI. (**d**–**i**) Bivariate correlation between eWAT *Hmox1* mRNA and weight (**d**), eWAT weight (**e**), HOMA-IR (**f**), circulating leptin (**g**), and eWAT *Il6* (**h**) and *Adipoq* (**i**) mRNA

(*FTL*, *SLC40A1* and *CYBA* [9]) in association with obesity in two cross-sectional independent cohorts. *HMOX1* gene expression and HMOX1 protein were also associated with adipose tissue iron levels. The reduction of *HMOX1* gene expression after bariatric surgery weight loss was positively correlated with the reduction of *SLC40A1* and *FTL*. Considering that HMOX1 activity is an important source of intracellular iron, through the conversion of haem into ferrous iron [21], these findings suggest that adipose tissue HMOX1 is a marker of adipose tissue iron accumulation. In addition, the association between *HMOX1* and markers of oxidative stress (*CYBA*, *NFE2L2*) in both SAT and VAT does not exclude a compensatory effect of HMOX1 through its anti-oxidative properties [26, 27].

HMOX1 and adipocyte mitochondrial function Hemin-induced HMOX1 activity during adipocyte differentiation resulted in a significant alteration in mitochondrial respiratory function, decreasing basal respiration and spare respiratory capacity, two functional measures of mitochondrial function [21, 34]. Supporting these data, in this study *HMOX1* gene expression was negatively correlated with gene expression markers of mitochondrial biogenesis (*PPARGC1A*, *TFAM*) even after controlling for age and BMI. Previous studies demonstrated the negative effects of HMOX1 on mitochondrial function in macrophages [21] and hepatocytes [35]. Since iron overload impairs mitochondrial function [36–38], it seems plausible that HMOX1 activity-associated adipocyte iron accumulation might contribute to attenuated mitochondrial respiratory function. The importance of mitochondrial function in adipocyte differentiation [39–41], adipocyte physiology [34], and the possible role of iron homeostasis in mitochondrial function and adipogenesis [10, 15] is well known.

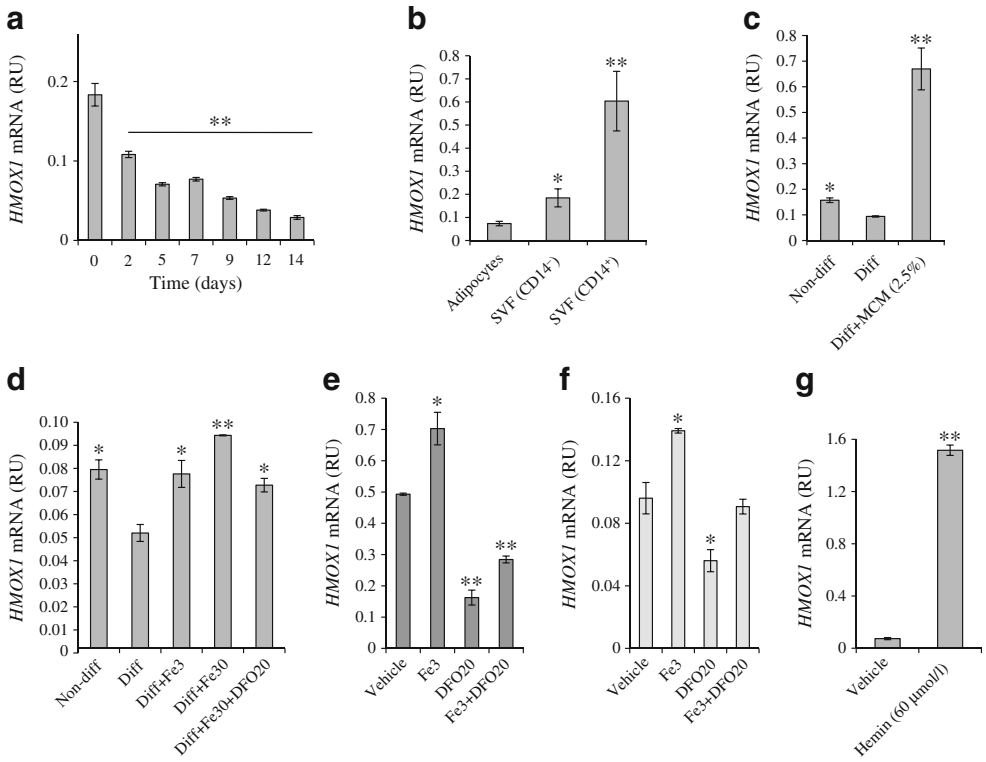

Fig. 4 *HMOX1* gene expression in time course of adipocyte differentiation (**a**) and in adipose tissue subfractions (**b**). *$p < 0.05$ compared with adipocytes; **$p < 0.01$ compared with adipocytes or day 0. Effects of MCM (2.5%) (**c**), $FeSO_4$ (Fe, 3 and 30 μg/ml) and DFO (20 μmol/l) (**d**) on *HMOX1* gene expression during adipocyte differentiation at day 14. *$p < 0.05$, **$p < 0.01$ compared with differentiated adipocytes. Effects of $FeSO_4$ (Fe, 3 and 30 μg/ml) and DFO (20 μmol/l) on *HMOX1* gene expression in pre-adipocytes (**e**) and in adipocytes (**f**) after 72 h. Effects of hemin (60 μmol/l) on *HMOX1* gene expression in adipocytes after 48 h (**g**). *$p < 0.05$, **$p < 0.01$ compared with vehicle. Diff, differentiated; non-diff, non-differentiated

HMOX1 and human adipogenesis Hemin-induced HMOX1 expression resulted in decreased lipid droplet development in parallel with a significant reduction in adipogenic gene expression. A recent study also found anti-adipogenic effects of HMOX1 in pigs [42]. Specifically, this study demonstrated that overexpression of human HMOX1 in pigs resulted in decreased subcutaneous fat tissue development and attenuated capacity of isolated adipose-derived stem cells to differentiate to adipocytes [42].

Interestingly, HMOX1 induction led to a significant reduction of basal and insulin-induced glucose uptake in concordance with the decrease in *SLC2A4* (*GLUT4*) mRNA levels. Since glucose uptake is required for the generation of new adipocytes and adipogenesis [43–45], the negative effects of hemin administration on glucose uptake might explain in part these anti-adipogenic effects. In agreement with these experiments, *HMOX1* gene expression decreased significantly during human adipocyte differentiation and was negatively correlated with gene expression markers of adipogenesis (*PPARG*, *FASN*, *ACACA*) and adipocyte insulin action (*SLC2A4*, *IRS1*) in both VAT and SAT. Furthermore, in contrast with the expression of adipogenic genes, increased *HMOX1* mRNA levels were found in parallel with hyperglycaemia and HOMA-IR in obese participants. Weight gain led to increased *Hmox1* in mice [21] and rats (current results). Consistently, bariatric surgery-induced weight loss resulted in decreased adipose tissue *HMOX1* mRNA. The change in *HMOX1* mRNA levels correlated with changes in BMI, fat mass and adipose tissue markers of adipogenesis. However, no correlations were found between adipose tissue *HMOX1* gene expression and systemic insulin sensitivity. As mentioned above, the association between HMOX1 expression and insulin resistance is not clear [21–23]. Biliverdin and carbon monoxide are two additional products derived from HMOX1 activity that have been associated with improved glucose tolerance and insulin sensitivity [46, 47]. Possibly, these putative positive effects of biliverdin and carbon monoxide might partly counteract the negative effects of HMOX1-associated adipose tissue dysfunction on systemic insulin sensitivity. The systemic upregulation of the haem oxygenase system by hemin or by cobalt protoporphyrin improves some metabolic features in Zucker diabetic fatty rats [48] and in obese Wistar rats [49]. Specifically, in Zucker diabetic fatty rats, hemin induction of the haem oxygenase system normalised glucose levels and reduced perirenal adiposity and inflammation [48]. However, these positive effects were attenuated in Zucker lean controls [48]. In obese Wistar rats, cobalt protoporphyrin-induced HMOX1 exerted positive effects on kidneys improving endothelial dysfunction in association with increased circulating adiponectin levels [49]. These studies investigated the systemic effects of HMOX1 upregulation in rats, whereas the current study focused specifically in adipose tissue *HMOX1* expression in response to HFD-induced weight gain (in Wistar rats) or bariatric surgery-induced weight loss (in humans). For instance, current results demonstrated increased eWAT *Hmox1* in

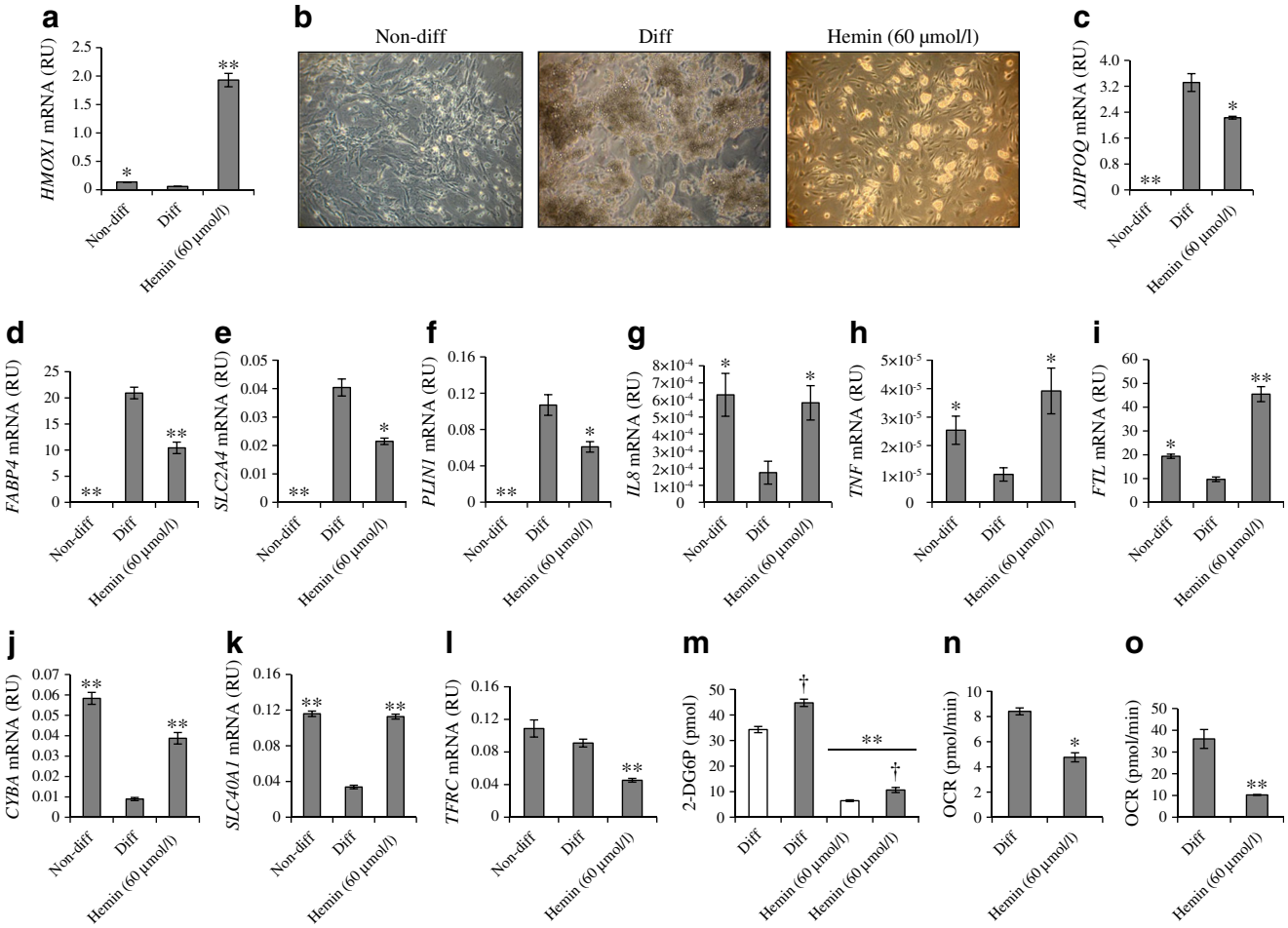

Fig. 5 Effects of hemin (60 µmol/l) administration during adipocyte differentiation on *HMOX1* gene expression (**a**), intracellular lipid accumulation (×10 magnification) (**b**), adipogenic-related (*ADIPOQ*, *FABP4*, *SLC2A4*, *PLIN1*) (**c**–**f**), inflammatory-related (*IL8*, *TNF*) (**g**–**h**), iron accumulation-related (*FTL*, *CYBA*, *SLC40A1*, *TFRC*) (**i**–**l**) gene expression markers, intracellular 2-DG6P in basal (white bars) and insulin stimulated (grey bars) conditions (**m**) and on mitochondrial respiratory function (basal respiration [**n**] and spare respiratory capacity [**o**]) at day 14. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with differentiated adipocytes. $^{\dagger}p < 0.05$ compared with basal. Diff, differentiated; non-diff, non-differentiated; OCR, oxygen consumption rate

association with total weight, circulating leptin and decreased *Adipoq* gene expression in rats. For this reason, our current findings were not comparable with those obtained in the earlier studies. Nevertheless, a possible protective role of adipose tissue HMOX1 in humans cannot be excluded. Further studies are required to investigate specifically the effects of adipose tissue HMOX1 induction on obesity-associated metabolic disturbances and insulin resistance.

HMOX1 and adipose tissue inflammation As expected, we observed bidirectional influences between HMOX1 and inflammatory factors [21]. MCM (enriched with a mixture of inflammatory cytokines) induced adipocyte *HMOX1* mRNA levels, while hemin-induced HMOX1 activity increased adipocyte inflammatory cytokines (*TNF*, *IL8*). *HMOX1* mRNA levels were associated with gene expression of inflammatory cytokines in human adipose tissue and negatively correlated with *CD206*/*CD68* ratio (an M2 macrophage marker). In rats, HFD-induced weight gain resulted in increased *Hmox1* mRNA levels in association with *Il6* in eWAT. Some products derived from the HMOX1 catalytic activity also promoted adipose tissue macrophage infiltration and inflammation in mice fed an HFD [50].

Current findings regarding the importance of haem in adipogenesis [20], the precise regulation in intracellular haem levels through HMOX1 activity required to avoid ROS-induced cellular damage [26, 27], and the importance of iron and its fine-tuned regulation in adipogenesis [7, 10] envision a possible scenario, in which a progressive increase of fat accretion (parallel to obesity) leads to increased intracellular haem levels and, in consequence, to induced *HMOX1* gene expression and activity. Since HMOX1 is an inducible enzyme required for haem degradation, increased HMOX1 activity enhances the conversion of haem into ferrous iron [21], resulting in obesity-associated adipose tissue iron accumulation, which promotes adipose tissue dysfunction [7, 12].

In conclusion, *HMOX1* is proposed as an important marker of iron excess-induced adipose tissue dysfunction in human obesity.

Acknowledgements We acknowledge the technical assistance of E. Loshuertos and O. Rovira (both from Endocrinology, IdIBGi, Spain). We are indebted to the IDIBGI Biobank, integrated in the Spanish National Biobank Network, for the sample and data procurement.

Data availability statement Data sharing is not applicable to this article because of patient confidentiality; all relevant data has been included in the paper and ESM.

Funding This work was partially supported by research grants FIS PI12/02631 and PI15/01934 from the Instituto de Salud Carlos III from Spain and was also supported by Fondo Europeo de Desarrollo Regional (FEDER) funds. CIBEROBN Fisiopatología de la Obesidad y Nutrición is an initiative from the Instituto de Salud Carlos III from Spain.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement JMM-N and JMF-R participated in study design and analysis of data. FO, AR, JL, SB and MS-M participated in acquisition of data. WR and GF participated in interpretation of data. JMM-N and JMF-R wrote and edited the manuscript. FO, AR, JL, SB, MS-M, WR and GF revised the manuscript critically for important intellectual content. All authors participated in final approval of the version to be published. JMM-N and JMF-R are the guarantors of this work.

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