Diabetologia (2014) 57:1957–1967
DOI 10.1007/s00125-014-3298-5

ARTICLE

Fine-tuned iron availability is essential to achieve optimal adipocyte differentiation and mitochondrial biogenesis

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Received: 19 March 2014 / Accepted: 27 May 2014 / Published online: 29 June 2014

Abstract
Aims/hypothesis Adipose tissue from obese and insulin-resistant individuals showed altered expression of several iron-related genes in a recent study, suggesting that iron might have an important role in adipogenesis. To investigate this possible role, we aimed to characterise the effects of iron on adipocyte differentiation.
Methods Intracellular iron deficiency was achieved using two independent approaches: deferoxamine administration (20 and 100 μmol/l) and transferrin knockdown (TF KD). The effects of added $FeSO_4$, holo-transferrin and palmitate were studied during human and 3T3-L1 adipocyte differentiation. Finally, the relationship between iron-related and mitochondrial-related genes was investigated in human adipose tissue.
Results Most adipose tissue iron-related genes were predominantly expressed in adipocytes compared with stromal vascular cells. Of note, transferrin gene and protein expression increased significantly during adipocyte differentiation. Both deferoxamine and TF KD severely blunted adipocyte differentiation in parallel with increased inflammatory mRNAs. These effects were reversed in a dose-dependent manner after iron supplementation. Palmitate administration also led to a state of functional intracellular iron deficiency, with decreased *Tf* gene expression and iron uptake during adipocyte differentiation, which was reversed with transferrin co-treatment. On the other hand, iron in excess impaired differentiation, but this antiadipogenic effect was less pronounced than under iron chelation. Of interest, expression of several genes involved in mitochondrial biogenesis occurred in parallel with expression of iron-related genes both during adipogenesis and in human adipose tissue.
Conclusions/interpretation Precise and fine-tuned iron availability is essential to achieve optimal adipocyte differentiation, possibly modulating adipocyte mitochondrial biogenesis.

Keywords 3T3-L1 · Adipocytes · Adipogenesis · Adipose tissue · Ferritin · Iron · Knockdown · Mitochondrial biogenesis · Transferrin

Abbreviations

FTL	Light ferritin
KD	Knockdown
SAT	Subcutaneous adipose tissue
shRNA	Short hairpin RNA
SVF	Stromal vascular cell fraction
TF KD	Transferrin knockdown
VAT	Visceral adipose tissue

Introduction

Iron seems to play a direct and causal role in the pathophysiology of type 2 diabetes mediated by both beta cell failure and

Electronic supplementary material The online version of this article (doi:10.1007/s00125-014-3298-5) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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insulin resistance [1–6]. Iron in excess impacts on systemic metabolism across the entire spectrum of iron stores. The association of iron overload with increased risk of type 2 diabetes mellitus has been extensively demonstrated in recent systematic reviews and meta-analysis [5–8]. Circulating markers of iron overload are also positively associated with visceral and subcutaneous fat depots [9] and with adipocyte insulin resistance [10]. A previous study using the 3T3-L1 mouse cell line suggested the importance of iron-related genes in adipocyte physiology [11]. Interestingly, recent studies in mice revealed that an iron-enriched diet induced iron accumulation and insulin resistance in visceral adipose tissue (VAT) [12] and that adipocyte iron overload reduced adiponectin biosynthesis in parallel with induction of insulin resistance [13]. Another recent study in mice showed impaired macrophage iron handling in the setting of obesity-associated adipose tissue dysfunction, which resulted in adipocyte iron uptake and overload [14]. Despite these associations, little attention has been paid to the molecular mechanisms regulating iron homeostasis in human adipose tissue.

In a recent study, we found that obese and insulin-resistant individuals showed increased light ferritin (FTL) and SLC40A1, an iron exporter, in their subcutaneous adipose tissue (SAT) and VAT, in parallel with decreased transferrin (*TF*) gene expression. These results suggest that iron accumulates in adipose tissue in humans with increased body fatness and impaired insulin action [15], as previously demonstrated in mice [12–14].

At the cellular level, iron has been recently identified as a key regulator of mitochondrial biogenesis [16]. In fact, intracellular iron is used in the build-up of Fe-S clusters, which are necessary for the regulation of mitochondrial oxidative processes [17].

Interestingly, the mitochondrial biogenesis programme is a key metabolic process in adipocyte differentiation [18] even though the role of mitochondria in regulating adipocyte function has received relatively little attention. Reduced mitochondrial mass and function has been found in adipose tissue of *ob/ob* and *db/db* mice and in adipose tissue from obese individuals with type 2 diabetes in association with increased activity of several inflammatory pathways [18–24]. Given previous findings in whole adipose tissue, we hypothesised that iron might have an important role in adipogenesis, and we decided to explore in greater depth which cells are responsible for the altered expression of genes involved in iron metabolism and to investigate the precise role of iron in adipocyte differentiation. For this reason, the effects of iron depletion and excess during silencing of target genes during adipocyte differentiation were evaluated. Furthermore, to elucidate the effect of metabolic perturbation on iron metabolism during adipocyte differentiation, the effects of saturated fatty acids (palmitate) on these genes were also tested. Since iron is essential for mitochondrial biogenesis [16], and optimal mitochondrial biogenesis and function is a key component in adipocyte differentiation [18], we also analysed genes related to mitochondrial biogenesis in these experiments.

Methods

Recruitment of participants for adipose tissue samples A group of 43 paired VAT and SAT samples from obese non-diabetic participants recruited by the Endocrinology Service of the Hospital of Girona 'Dr Josep Trueta' (Girona, Spain) were analysed. All participants were white and reported that their body weight had been stable for at least 3 months before the study. All 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 had been explained to them. Samples were obtained from SAT and VAT depots during elective surgical procedures (cholecystectomy, surgery for abdominal hernia, and gastric bypass surgery) and transported immediately to the laboratory (5–10 min). Tissue samples were handled under strictly aseptic conditions. They were washed in PBS, cut up with forceps and scalpel into small pieces (100 mg), and immediately flash-frozen in liquid nitrogen before being stored at −80°C.

Adipocytes and stromal vascular cell fraction (SVF) were isolated from eight SAT and eight VAT non-frozen samples as described previously [25] and in electronic supplementary material (ESM) Methods.

Differentiation of human pre-adipocytes Isolated human subcutaneous pre-adipocytes (Zen-Bio, Research Triangle Park, NC, USA) were cultured and differentiated as described previously [25] and in ESM Methods.

Differentiation of mouse cell line, 3T3-L1 The embryonic fibroblast mouse cell line, 3T3-L1 (American Type Culture Collection), was cultured and differentiated as described previously [25] and in ESM Methods. Palmitate (100 and 250 μmol/l), palmitate (100 and 250 μmol/l)+$FeSO_4$ (3 and 30 μg/ml), and palmitate (100 and 250 μmol/l)+holo-transferrin (0.1 and 1 μg/ml) were administered throughout the whole 3T3-L1 adipocyte differentiation process. Palmitate doses were selected according to the physiological range of palmitate concentration and other in vitro studies [26, 27].

Iron addition and chelation experiment Human and 3T3-L1 pre-adipocytes and adipocytes were incubated with fresh medium (control), fresh medium containing $FeSO_4$ (3 and 30 μg/ml), deferoxamine (20 and 100 μmol/l), and the following combinations: $FeSO_4$ (3 μg/ml)+deferoxamine (20 μmol/l), $FeSO_4$ (30 μg/ml)+deferoxamine (20 μmol/l)

and $FeSO_4$ (30 μg/ml)+deferoxamine (100 μmol/l). These experiments were performed during human and 3T3-L1 adipocyte differentiation (14 days for human pre-adipocytes and 7 days for the 3T3-L1 cell line, and adding the appropriate amount in each medium change). At the end of the experiment, the supernatant fractions were centrifuged at 400 *g* for 5 min, the cells were harvested, and pellets and supernatant fractions were stored at −80°C for RNA analysis. All these treatments were performed in four independent replicates.

Short hairpin RNA-mediated knockdown of TF and FTL Permanent silencing (knockdown; KD) was performed using *Tf*- and *Ftl*-targeted and control short hairpin (sh)RNA lentiviral particles (sc-37177-V, sc-40578-V and sc-108080; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and following the manufacturer's instructions. Positive 3T3-L1 pre-adipocytes harbouring the shRNA cassette for *Tf* or *Ftl* were selected with puromycin (3 μg/ml) 60 h after infection.

Oil red staining Intracellular lipid accumulation was measured by Oil red staining as described in ESM Methods.

Iron measurement Iron concentration in conditioned medium and levels of intracellular iron (from cell lysates) were measured using a specific colorimetric commercial assay (Iron-Ferrozine, ref. 11509; BioSystems, Barcelona, Spain) according to the manufacturer's instructions.

RNA expression RNA purification, gene expression procedures and analyses, and primer/probe sets used were as previously reported [25] and briefly described in ESM Methods.

Statistical analysis Statistical analyses were performed using SPSS 12.0 software. Descriptive results of continuous variables are expressed as mean ± SEM. Paired Student's *t* test and non-parametric tests (Mann–Whitney *U* test) were used to evaluate in vitro experiments. For human cross-sectional studies, descriptive results of continuous variables are expressed as mean ± SD, and the relation between variables was analysed by simple correlation (using Spearman's test). Levels of statistical significance were set at $p<0.05$.

Results

Iron-related gene expression in human adipose tissue fractions and during adipocyte differentiation *TF*, *FTL* and *FTH1* were predominantly expressed in adipocytes compared with the SVF (Fig. 1). *TFRC* and *SLC40A1* were mainly expressed in SVF cells (Fig. 1). *TF* gene expression and transferrin protein secretion increased significantly during adipocyte differentiation, attaining the highest levels at the end of the process in parallel with the gene expression pattern of other adipogenic genes (*ADIPOQ*, *FASN*) (Fig. 2). *TF* expression and the rate of transferrin biosynthesis in subcutaneous adipocytes was significantly higher than in visceral adipocytes (4.11±0.18 vs 0.19±0.03 pg 10^3 cells^{-1} h^{-1}; $p<0.0001$). *FTL*, *FTH1* and *SLC40A1* increased significantly in the first 2 days of adipocyte differentiation, and decreased thereafter (Fig. 2). No significant changes were found in *TFRC* gene expression during adipocyte differentiation.

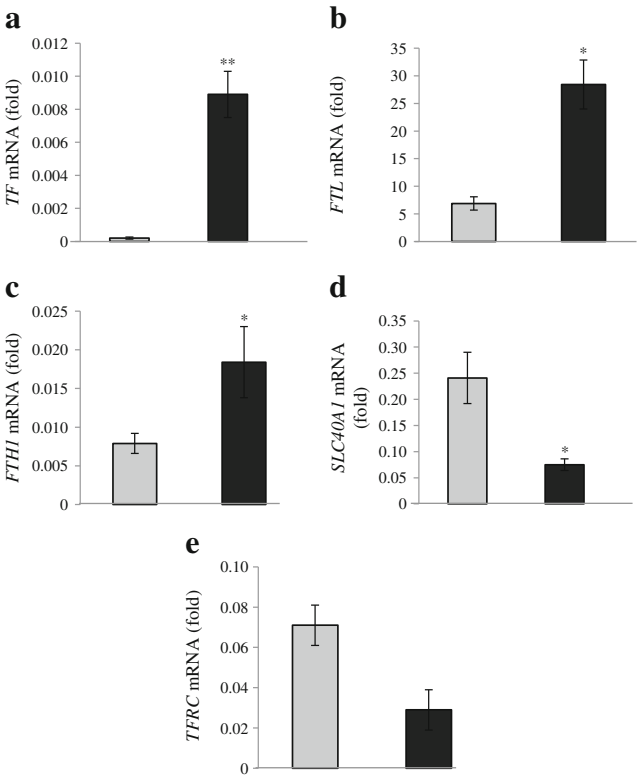

Fig. 1 *TF* (**a**), *FTL* (**b**), *FTH1* (**c**), *SLC40A1* (**d**) and *TFRC* (**e**) gene expression in adipose tissue fractions (SVF [grey bars] vs mature adipocytes [black bars]). *$p<0.05$, **$p<0.01$ compared with SVF. These data are expressed as mean ± SEM. Relative gene expression is expressed as fold change (fold)

These results were replicated in 3T3-L1 cells and are described in ESM Results and ESM Fig. 1.

Effects of TF and FTL KD on adipogenesis *Tf* and *Ftl* gene expression were decreased significantly in shRNA *Tf*- (95%, $p<0.0001$) and shRNA *Ftl*- (75%, $p<0.0001$) silenced 3T3-L1 cells (Fig. 3). Iron concentration in the medium decreased significantly during adipocyte differentiation, this decrease being significantly attenuated in TF KD cells (Fig. 3). Furthermore, increased *Tfrc* and decreased *Fth1* gene expression was found in TF KD cells compared with control cells (ESM Fig. 2), suggesting a possible intracellular iron deficiency. During adipocyte differentiation, TF KD led to a significant decrease in the intracellular lipid accumulation at day 7

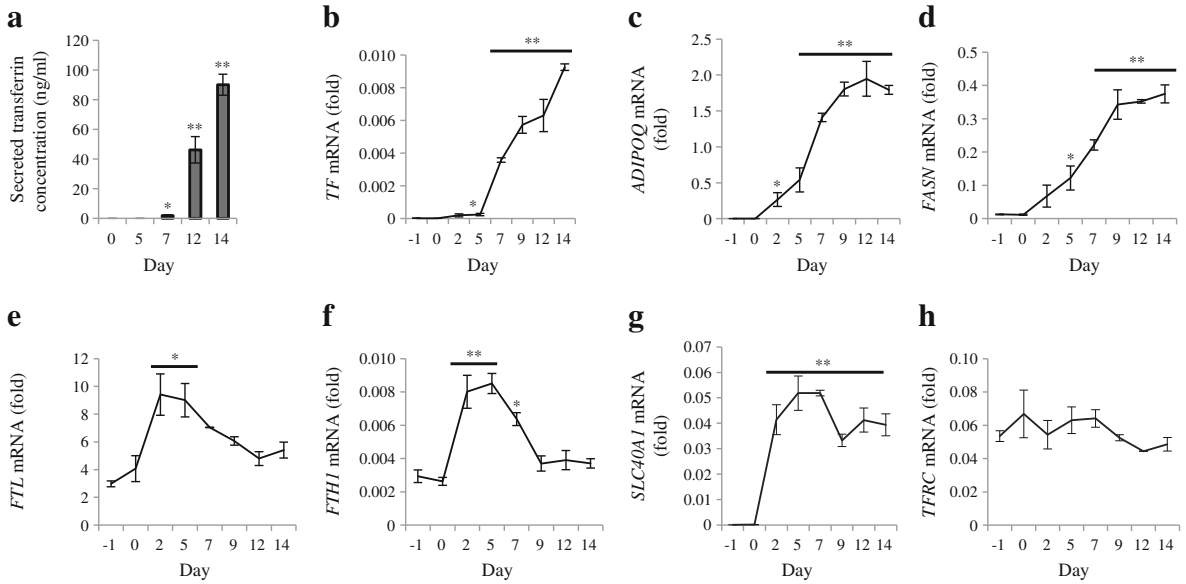

Fig. 2 Secreted transferrin concentration in the medium (**a**) and *TF* (**b**), *ADIPOQ* (**c**), *FASN* (**d**), *FTL* (**e**), *FTH1* (**f**), *SLC40A1* (**g**) and *TFRC* (**h**) gene expression in a time course experiment during human subcutaneous adipocyte differentiation. *$p<0.05$, **$p<0.01$ compared with day 0. These data are expressed as mean ± SEM. Relative gene expression is expressed as fold change (fold)

(Fig. 3) and in adipogenic mRNAs (such as *Pparg*, *Adipoq*, *Glut4*, *Fabp4* and *Cebpa*) (Fig. 3) while increasing inflammatory (*Mcp1* and *Il6*) and *Lep* gene expression (Fig. 3). In contrast, no significant effects were found in FTL KD cells with regard to adipogenic gene expression (Fig. 3). Similarly to *Tf* depletion, *Ftl* depletion also led to increased *Il6* at day 2 and *Mcp1* at day 0 (Fig. 3). Interestingly, expression of *Ppargc1a* and *Tfam* was increased during adipocyte differentiation, and they were significantly decreased in both TF and FTL KD cells (Fig. 3 and ESM Fig. 2).

Effects of iron during adipogenesis Several doses and combinations of $FeSO_4$ (3 and 30 μg/ml) and the iron chelator, deferoxamine (20 and 100 μmol/l), were used in human pre-adipocytes and in the 3T3-L1 cell line during adipocyte differentiation.

As expected, $FeSO_4$ (3 and 30 μg/ml) administration led to increased intracellular iron levels, whereas deferoxamine exerted opposite effects (Fig. 4). Deferoxamine-induced intracellular iron deficiency was reversed with $FeSO_4$ co-administration (Fig. 4). Interestingly, iron excess and iron chelation led to either a slight decrease in adipocyte differentiation or pronounced antiadipogenic effects, respectively, decreasing intracellular lipid accumulation (Fig. 4) in parallel with decreased adipogenic gene expression and increased inflammatory mRNAs (Fig. 5 and ESM Fig. 3). Iron replacement reversed the antiadipogenic effects of deferoxamine (Figs 4 and 5 and ESM Fig. 3). As expected, added iron and iron chelation led to reciprocal changes in *Tfrc* (Fig. 5). Iron chelation also resulted in decreased *Slc40a1* and increased *Fth1* and *Ftl* in these antiadipogenic conditions (Fig. 5). *Tf* followed a similar gene expression pattern to other adipogenic genes with iron chelation (Fig. 5), while *Tf* gene expression decreased with excess iron.

In human pre-adipocytes, similar effects of iron in excess or chelation were found (Fig. 6 and ESM Fig. 4). Similarly to adipogenic gene expression, both iron addition and chelation led to decreased mitochondrial gene expression (*PPARGC1A*, *PPARGC1B* and *MT-CO3*) in human adipocytes, this reduction being more pronounced under iron chelation (Fig. 6 and ESM Fig. 4). The connection between iron metabolism and mitochondrial biogenesis is the Fe-S clusters, which are essential cofactors for the activity of key enzymes of the mitochondrial respiratory chain and of the tricarboxylic acid cycle. Iron–sulphur cluster assembly 2 (ISCA2) is involved in the maturation of mitochondrial iron–sulphur proteins. Strikingly, exogenous iron addition led to decreased *ISCA2* gene expression, whereas iron chelation caused opposite effects (ESM Fig. 4).

Iron chelation, in parallel with the negative effects on adipogenesis and mitochondrial biogenesis, also led to increased expression of both endoplasmic reticulum stress (*HSPA5*) and inflammatory markers (*IL8*, *IL6*) (Fig. 6 and ESM Fig. 4).

In 3T3-L1 pre-adipocytes, similar effects of iron in excess or chelation were found on *Ppargc1a* and *Tfam* and inflammatory (*Il6* and *Mcp1*) gene expression (Fig. 5 and ESM Fig. 3).

Effects of iron in 3T3-L1 TF and FTL KD cells Next, we tested the effects of iron excess and iron chelation during adipogenesis of KD cells. These data are described in ESM Results and ESM Figs 5 and 6.

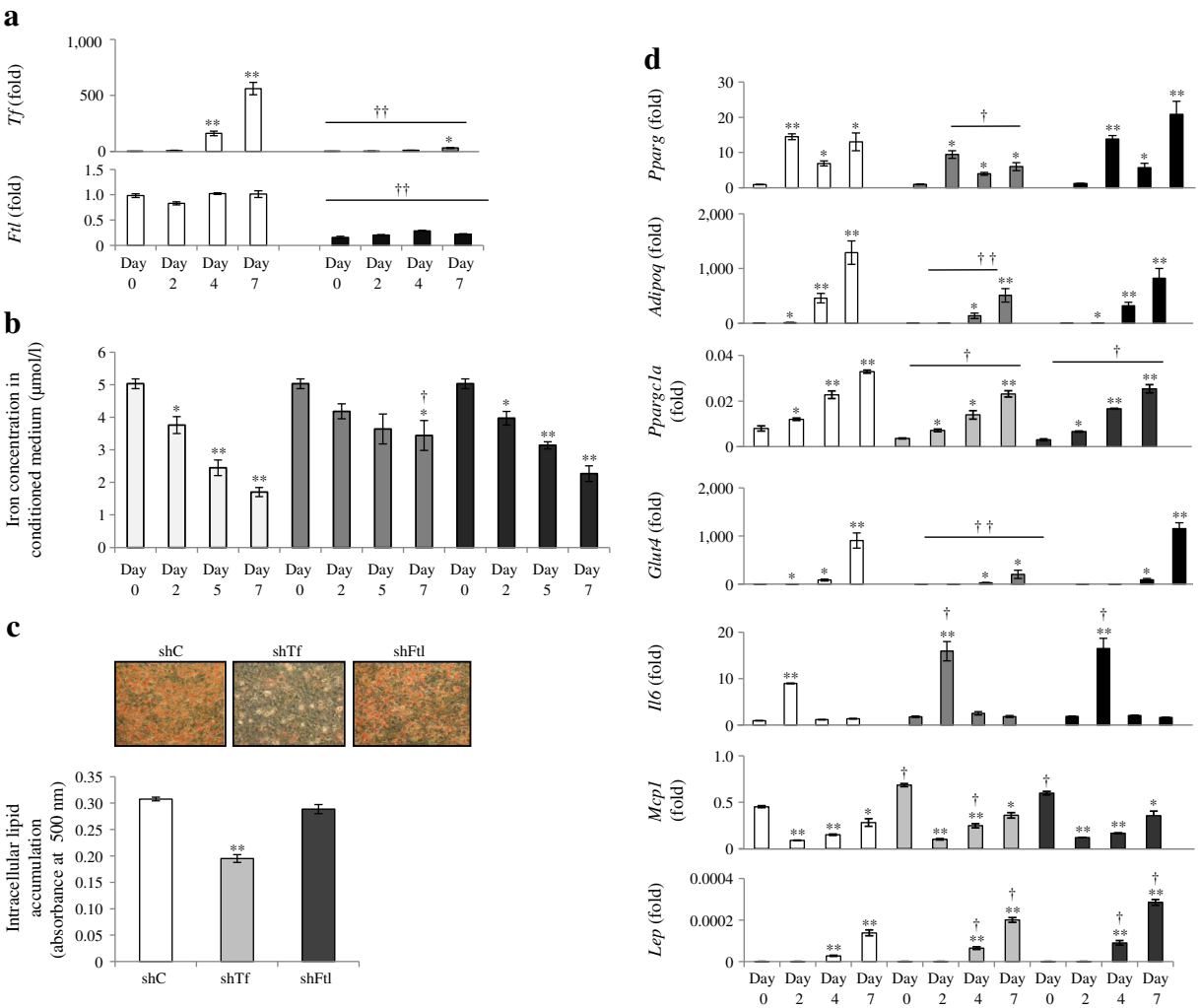

Fig. 3 Effects of TF and FTL KD on *Tf* and *Ftl* expression (**a**), iron concentration in the conditioned medium (an indirect measure of iron uptake) (**b**), intracellular lipid accumulation measured using Oil red staining (**c**), adipogenic, *Ppargc1a*, inflammatory and *Lep* (**d**) gene expression during 3T3-L1 adipocyte differentiation in a time course experiment. shC (white bars), shTf (grey bars) and shFtl (black bars) 3T3-L1 cells. *$p<0.05$, **$p<0.01$ compared with day 0. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ compared with shC. These data are expressed as mean ± SEM. Relative gene expression is expressed as fold change (fold)

Palmitate effect on iron metabolism-related genes during adipocyte differentiation Palmitate (100 and 250 μmol/l) administration significantly decreased transferrin gene expression, similarly to adipogenic genes (Fig. 7), and led to reduced iron uptake, intracellular iron levels being significantly reduced (Fig. 7) and iron concentration increased in the medium (ESM Fig. 7). The maximum dose of palmitate (250 μmol/l) produced a significant increase in *Tfrc* and *Il6* and a significant decrease in *Slc40a1* gene expression. No significant effects were found on *Fth1* and *Ftl* (Fig. 7).

Interestingly, holo-transferrin (0.1 and 1 μg/ml) but not $FeSO_4$ (3 and 30 μg/ml) co-administration reversed the negative effects of palmitate on adipocyte differentiation (Fig. 7).

Genes related to iron metabolism and mitochondrial biogenesis in adipose tissue Given the marked effects of iron on adipocyte differentiation and mitochondrial biogenesis, we decided to explore these relationships in human adipose tissue (n=43). Anthropometric and clinical variables of this cohort are provided in Table 1. These data are described in ESM Results.

Discussion

In a recent study, we found that several gene markers for intracellular iron accumulation (*FTL*, *FTH* and *SLC40A1*) were increased in adipose tissue from individuals with obesity and insulin resistance, while gene expression of transferrin (a mediator of iron uptake) was significantly reduced [15]. To try to understand these findings, we designed the present study

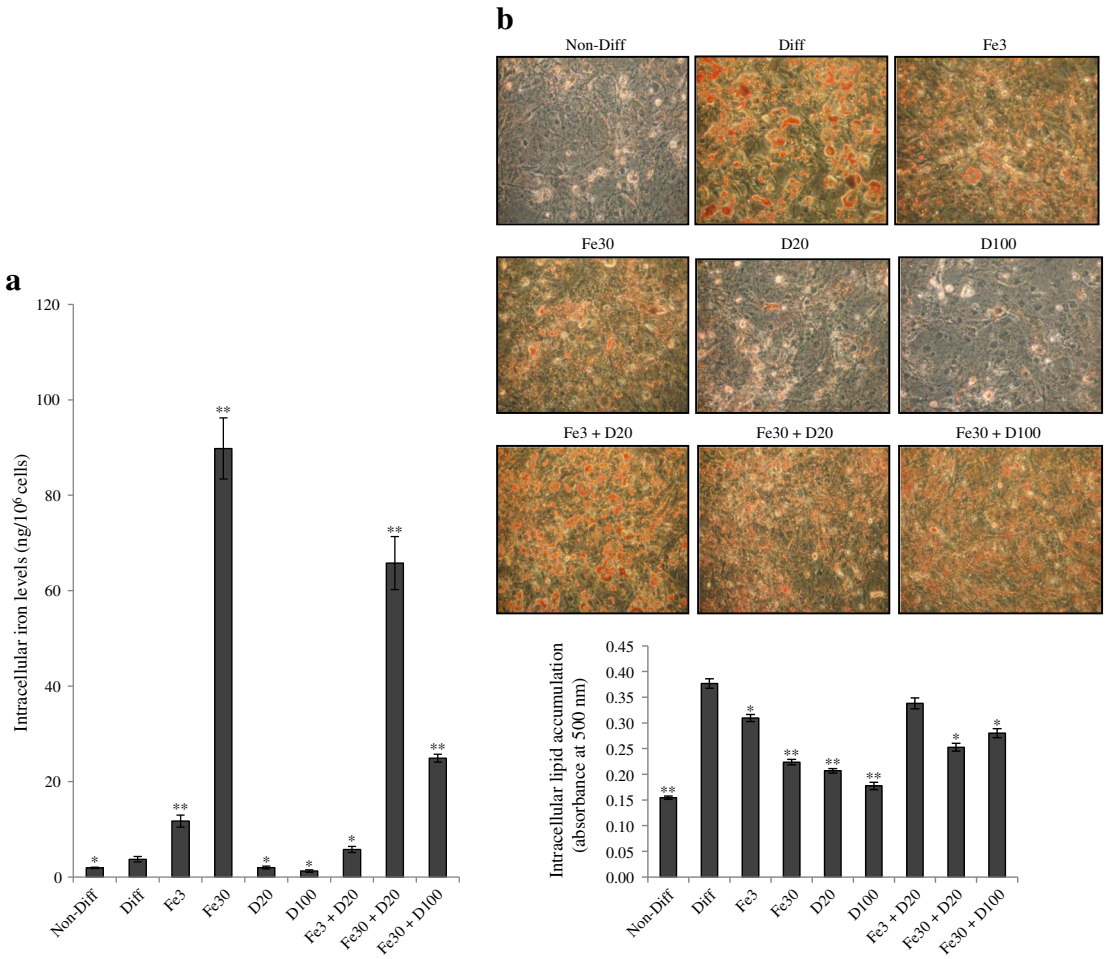

Fig. 4 Effects of FeSO4 (3 and 30 μg/ml) and deferoxamine (20 and 100 μmol/l) on intracellular iron levels (**a**) and intracellular lipid accumulation measured using Oil red staining (**b**) during 3T3-L1 pre-adipocyte differentiation at day 7. Non-Diff, control non-differentiated; Diff, control differentiated; Fe3, FeSO4 (3 μg/ml); Fe30, FeSO4 (30 μg/ml); D20, deferoxamine (20 μmol/l); D100, deferoxamine (100 μmol/l). *$p<0.05$, **$p<0.01$ compared with Diff. These data are expressed as mean ± SEM

in which we show that most of these markers (*TF*, *FTL* and *FTH*) were predominantly expressed in adipocytes (compared with SVF). We here provide new evidence on the importance of iron metabolism in adipocyte differentiation.

Intracellular iron deficiency impairs adipogenesis Iron deficiency severely blunted adipocyte differentiation, which recovered in a dose-dependent manner after iron supplementation, confirming that a minimal iron threshold (intracellular iron availability) is required to achieve optimal adipocyte differentiation. Intracellular iron deficiency was achieved using two independent approaches: transferrin gene silencing and deferoxamine administration.

Interestingly, KD of transferrin affected intracellular iron availability, decreasing adipocyte capacity to take up iron. This KD resulted in impaired adipocyte differentiation, with markedly reduced expression of several adipogenic genes (*Pparg*, *Cebpa*, *Adipoq*, *Glut4* and *Fabp4*) and intracellular lipid accumulation, and increased inflammatory (*Il6*, *Mcp1* and *Lep*) gene expression. In fact, both *Tf* gene expression and the release of transferrin protein increased during adipocyte differentiation in parallel with adipogenic genes (in both 3T3-L1 cells and human adipocytes). Administration of transferrin, as an iron donor, is well known to facilitate adipocyte differentiation [28, 29], but we found no studies of the possible role of endogenous adipocyte transferrin. In fact, human adipose tissue transferrin gene and protein expression was found to be associated positively with adipogenic, and negatively with inflammatory, gene expression [15]. Interestingly, the circulating transferrin concentration correlated significantly with SAT *TF* gene expression in obese individuals [15], suggesting a strong relationship between circulating and adipose tissue transferrin. Establishing a parallelism with transferrin, the gene expression of another iron donor, lactoferrin (a homologue protein), increased during adipocyte differentiation [25], its administration led to enhanced human adipocyte differentiation [30] and its KD also impaired adipocyte differentiation [31].

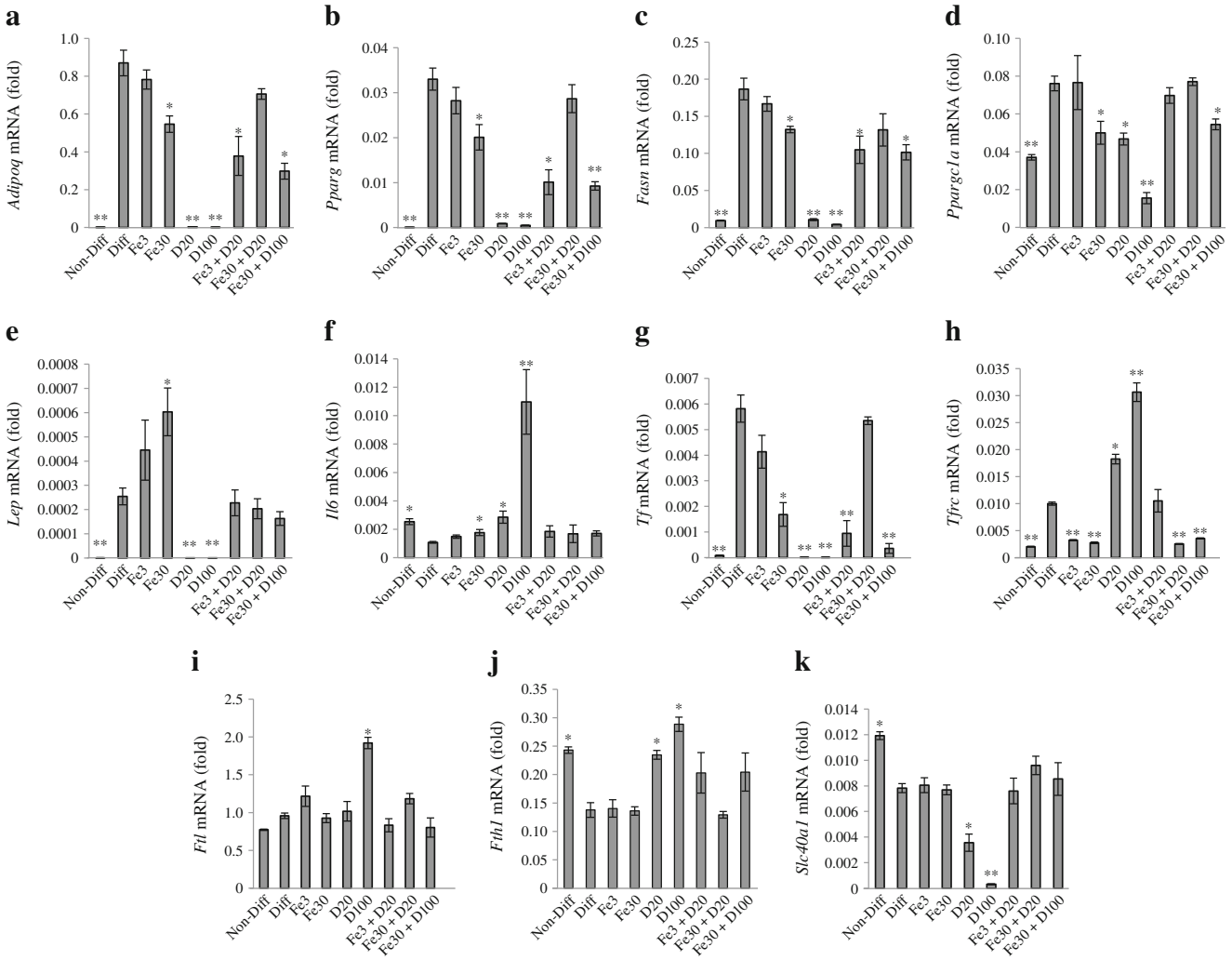

Fig. 5 Effects of FeSO4 (3 and 30 μg/ml) and deferoxamine (20 and 100 μmol/l) on *Adipoq* (**a**), *Pparg* (**b**), *Fasn* (**c**), *Ppargc1a* (**d**), *Lep* (**e**), *Il6* (**f**) *Tf* (**g**), *Tfrc* (**h**), *Ftl* (**i**), *Fth1* (**j**) and *Slc40a1* (**k**) gene expression during 3T3-L1 pre-adipocyte differentiation at day 7. Non-Diff, control non-differentiated; Diff, control differentiated; Fe3, FeSO4 (3 μg/ml); Fe30, FeSO4 (30 μg/ml); D20, deferoxamine (20 μmol/l); D100, deferoxamine (100 μmol/l). *$p<0.05$, **$p<0.01$ compared with Diff. These data are expressed as mean ± SEM. Relative gene expression is expressed as fold change (fold)

Intriguingly, FTL KD did not significantly affect adipocyte differentiation (present report).

On the other hand, iron chelation resulted in increased *TFRC* (an iron importer) and decreased *SLC40A1* (iron exporter), as expected, according to intracellular iron requirements. Strikingly, iron chelation led to increased *FTH1* and *FTL* mRNA levels in a proportion similar to other inflammatory mediators while decreasing *TF*, in parallel with adipogenic genes and intracellular lipid accumulation. Iron chelation is known to markedly increase phosphorylation and activation of proinflammatory kinases, such as stress-activated protein kinases, JNK and p38MAPK, in several cell lines [32]. In human adipose tissue, *FTH1* and *FTL* gene expression were positively associated with expression of inflammatory genes [15], suggesting that *FTH* and *FTL* in adipocytes might be modulated by inflammatory pathways and not merely by the classical regulator of intracellular iron metabolism (iron-related protein 1) [33].

A different gene expression pattern for *FTL* and *SLC40A1* was found during adipocyte differentiation of human and 3T3-L1 cells. In human pre-adipocytes, *FTL* and *SLC40A1* gene expression increased in the first stage of adipocyte differentiation and tended to decrease thereafter, whereas it followed the opposite pattern in 3T3-L1 pre-adipocytes. This finding suggests increased iron requirements for 3T3-L1 adipocyte differentiation compared with human pre-adipocytes. Interestingly, in both TF and FTL KD cells, the highest dose of iron was required to increase the expression of adipogenic genes, in marked contrast with control 3T3-L1 cells, in which differentiation was impaired. These findings reveal that the iron requirements for adipocyte differentiation were in fact increased in TF and FTL KD cells.

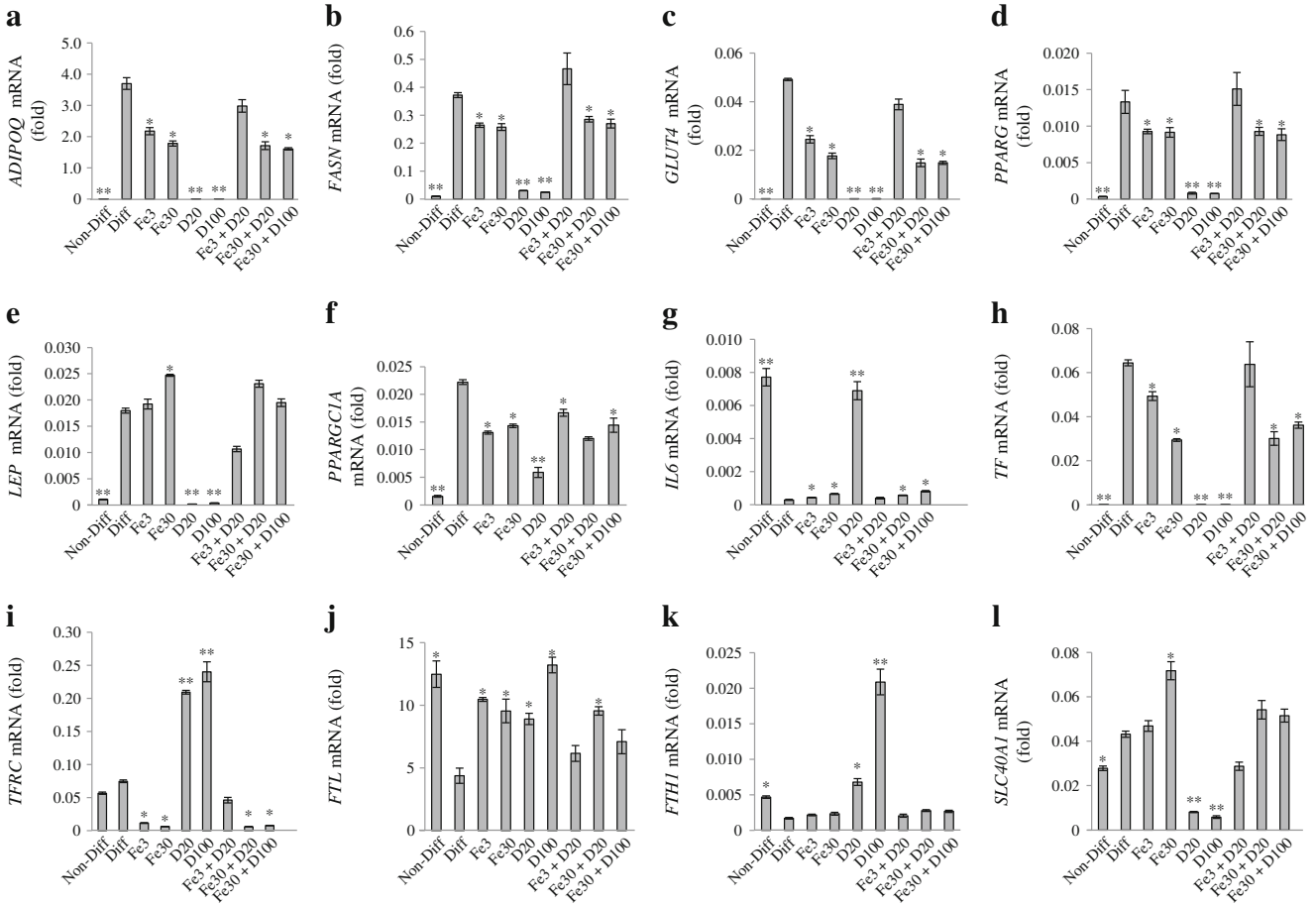

Fig. 6 Effects of FeSO4 (3 and 30 μg/ml) and deferoxamine (20 and 100 μmol/l) on *ADIPOQ* (**a**), *FASN* (**b**), *GLUT4* (**c**), *PPARG* (**d**), *LEP* (**e**), *PPARGC1A* (**f**), *IL6* (**g**), *TF* (**h**), *TFRC* (**i**), *FTL* (**j**), *FTH1* (**k**) and *SLC40A1* (**l**) gene expression during human subcutaneous pre-adipocyte differentiation at day 14. Non-Diff, control non-differentiated; Diff, control differentiated; Fe3, FeSO4 (3 μg/ml); Fe30, FeSO4 (30 μg/ml); D20, deferoxamine (20 μmol/l); D100, deferoxamine (100 μmol/l). *$p<0.05$, **$p<0.01$ compared with Diff. These data are expressed as mean ± SEM. Relative gene expression is expressed as fold change (fold)

Taken together, our present and previous results [15] suggest that obesity might reduce intracellular iron availability, leading to increased expression of inflammatory genes or vice versa. Supporting this hypothesis, palmitate administration led to decreased *Tf* gene expression and iron uptake during adipocyte differentiation. These results are, to some extent, surprising. Endogenous transferrin production and secretion by the adipocytes may result in subsequent transferrin–iron uptake from the medium in normal cells. This uptake would be decreased when the production of endogenous transferrin is limited in TF KD cells, resulting in a smaller uptake in a medium in which no transferrin is produced. Translation of these effects to whole body physiology should be studied in more depth. It is possible that relative extracellular transferrin deficiency in adipose tissue of metabolically compromised individuals influences adipocyte differentiation. Of note, the maximum dose of palmitate displayed similar effects to deferoxamine administration, leading to an iron deficiency phenotype (increased *Tfrc* and decreased *Slc40a1* mRNA and intracellular iron levels) in parallel with increased *Il6*, which were reversed with holo-transferrin co-administration. In agreement with the present data, obesity in rats induced by a high-fat diet led to increased iron requirements in association with greater accretion of body mass and vulnerability to iron deficiency [34]. The negative effects of palmitate in adipogenesis are mediated in part by the induction of inflammatory pathways and concomitant insulin resistance [35, 36]. Moreover, we found that intracellular iron deficiency induced gene expression of inflammatory cytokines, leading us to suggest that iron deficiency might exacerbate the proinflammatory effects of saturated fatty acids. However, additional in vivo studies are needed to explore the effects of high-fat diet and obesity on adipocyte and adipose tissue iron homeostasis.

Iron in excess also blunts adipogenesis Iron in excess also led to impaired differentiation of both human and 3T3-L1 cells. However, this antiadipogenic effect was less pronounced than under iron chelation. Iron overload in mice is known to decrease adiponectin gene expression in association with insulin resistance [13] and to lead to weight gain, adipocyte

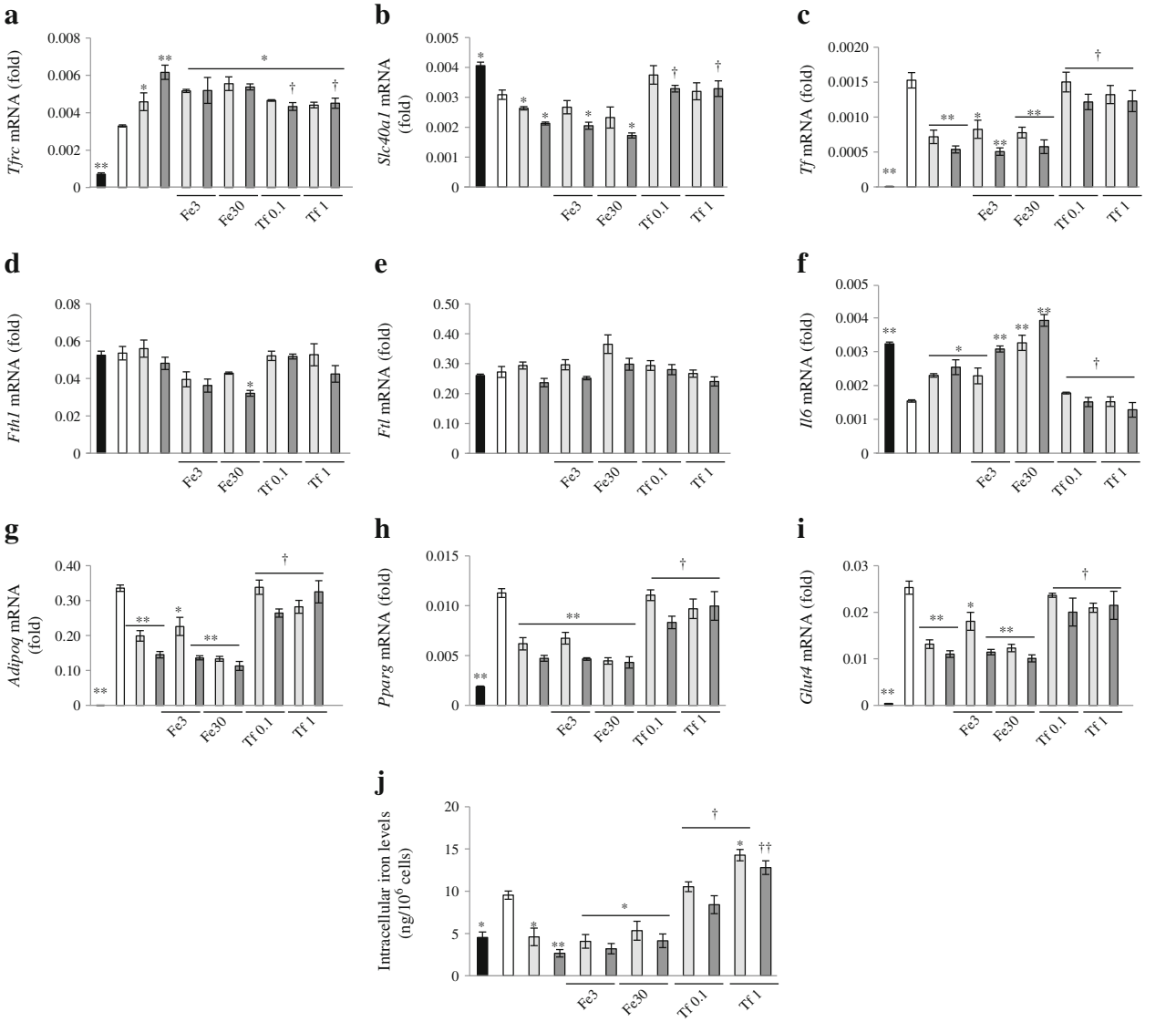

Fig. 7 Effects of palmitate administration (100 and 250 μmol/l) and $FeSO_4$ (3 and 30 μg/ml; Fe3 and Fe30) and holo-transferrin (0.1 and 1 μg/ml; Tf0.1 and Tf1) co-administration on *Tfrc* (**a**), *Slc40a1* (**b**), *Tf* (**c**), *Fth1* (**d**), *Ftl* (**e**), *Il6* (**f**), *Adipoq* (**g**), *Pparg* (**h**) and *Glut4* (**i**) gene expression and on intracellular iron levels (**j**) during 3T3-L1 pre-adipocyte differentiation at day 7. Control non-differentiated (Non-Diff, black bars), control differentiated (Diff, white bars), palmitate (100 μmol/l, light grey bars) and palmitate (250 μmol/l, dark grey bars). Fe3, FeSO4 (3 μg/ml); Fe30, FeSO4 (30 μg/ml). *$p<0.05$, **$p<0.01$ compared with Diff. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ compared with palmitate administration. These data are expressed as mean ± SEM. Relative gene expression is expressed as fold change (fold)

hypertrophy, adipose tissue macrophage infiltration, oxidative stress and metabolic disturbances [37]. In this last model, iron depletion reversed these phenotypes [38]. Adipocyte hypertrophy, which is the 'diabetogenic' adipocyte phenotype, is characterised by increased leptin and reduced adiponectin biosynthesis [39]. In the present study, we found that iron administration exerted inverse effects on *LEP* and *ADIPOQ* gene expression. Exogenous iron administration led to an increase in *LEP* gene expression similar to inflammatory genes, whereas iron also resulted in decreased adiponectin and other adipogenic genes. These results suggest that iron overload induced a diabetogenic adipocyte phenotype. These data are in full agreement with previous studies [13, 37].

Mitochondrial biogenesis: a possible link between iron and adipogenesis The antiadipogenic effect of iron chelation might be caused by disruption of mitochondrial biogenesis. Supporting this hypothesis, findings in human adipose tissue revealed that *ISCA2* (an important mediator in Fe-S cluster biogenesis) and *TF* gene expression were positively associated with expression of *PPARGC1A*, whereas the expression of *CYBA*, which is a marker for intracellular iron accumulation and induced by oxidative stress [15, 28], was negatively linked. *TF* gene expression was positively associated with other genes related to mitochondrial activity and the respiratory chain (such as *PPARGC1B* and *MT-CO3*). As mentioned above, in parallel with the association between transferrin and

Table 1 Anthropometric and clinical variables of the human cohort (n=43)

Variable	Mean ± D
Age (years)	45.3±11.2
BMI (kg/m^2)	43.6±7.7
Fat mass (%)	54.17±11.2
Fasting glucose (mmol/l)	5.31±0.78
HbA_{1c} (%)	4.98±0.49
HbA_{1c} (mmol/mol)	31±1.4
Total cholesterol (mmol/l)	5.01±0.85
HDL-cholesterol (mmol/l)	1.41±0.39
LDL-cholesterol (mmol/l)	2.99±0.83
Fasting triacylglycerol (mmol/l)	1.25±0.49

mitochondrial biogenesis, *TF* gene expression was also significantly associated with adipose tissue functionality (increased adipogenesis and decreased inflammation) [15]. In 3T3-L1 in vitro experiments, iron chelation and TF and FTL KD cells, which had increased iron requirements compared with control cells, showed decreased expression of *Ppargc1α* (the master activator of mitochondrial biogenesis [23]) and *Tfam* (a crucial transcription factor for mitochondrial DNA maintenance and biogenesis [40]) during adipocyte differentiation. In addition, iron chelation decreased expression of mitochondrial-related genes (*PPARGC1A*, *PPARGC1B* and *MT-CO3*) during human adipocyte differentiation. These findings hint at the possibility that iron-associated mitochondrial biogenesis during adipocyte differentiation is a factor linking these processes.

To sum up, all these data emphasise that iron plays an important role in adipogenesis. While iron in excess had detrimental effects on adipocyte differentiation, iron deprivation (TF KD, deferoxamine) prevented this process completely. Precise and fine-tuned iron availability is essential to achieve optimal adipocyte differentiation, possibly modulating adipocyte mitochondrial biogenesis.

Acknowledgements We acknowledge the technical assistance of E. Loshuertos and O. Rovira (both from Endocrinology, IdIBGi, Spain).

Funding This work was partially supported by research grants from the Ministerio de Economía y Competitividad (PI11-00214 and PI12/02631). CIBEROBN Fisiopatología de la Obesidad y Nutrición is an initiative of the Instituto de Salud Carlos III.

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

Contribution statement JMM-N, WR and JMF-R participated in study conception and design. JMM-N, FO and MM participated in data collection and analysis. JMM-N, FO, MM, WR and JMF-R participated in manuscript preparation, drafting the article or revising it critically for important intellectual content, and in final approval of the version to be published. JMM-N and JMF-R are the guarantors of this work.

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