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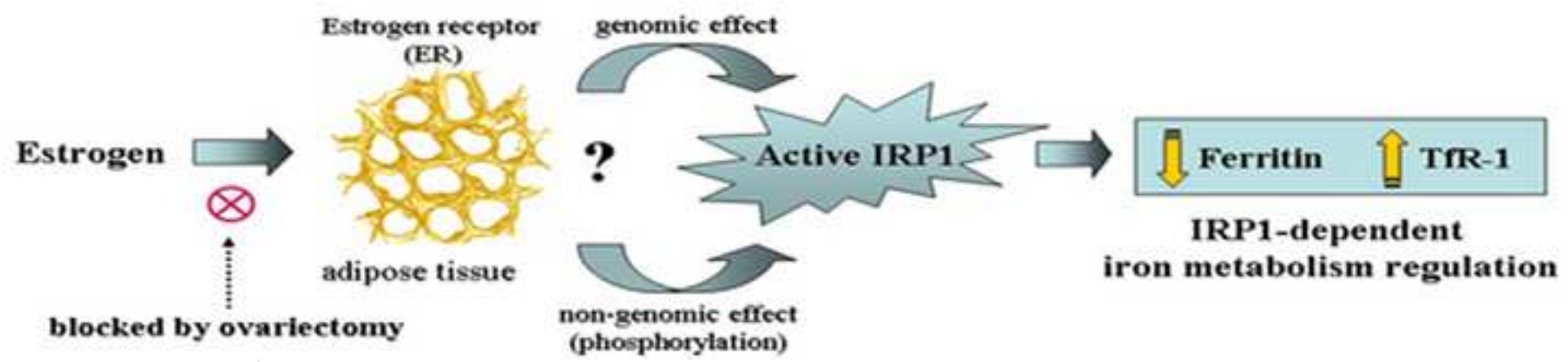
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Manuscript

**Estrogen regulates the RNA-binding activity of the IRP1 and consequently the expression of the ferritin and transferrin receptor in adipose tissue**



1 **Ovariectomy and estrogen treatment modulate iron metabolism in rat adipose**  
2 **tissue**  
3

4  
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**ABSTRACT**

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2 Iron is essential for many biological processes and its deficiency or excess is involved in  
3  
4 pathological conditions. At cellular level, the maintenance of iron homeostasis is largely  
5  
6 accomplished by the transferrin receptor (TfR)-1 and by ferritin, whose expression is mainly  
7  
8 regulated post-transcriptionally by Iron Regulatory Proteins (IRPs).  
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11 This study examines the hypothesis that modification of serum estrogen levels by ovariectomy and  
12  
13  $17\beta$ -estradiol ( $E_2$ ) treatment in rats modulate serum iron-status parameters and iron metabolism in  
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15 adipose tissue. In particular, we evaluated the RNA binding of IRP1 by electrophoretic mobility  
16  
17 shift assay and IRP1, ferritin, and TfR-1 expression in adipose tissue by Western blot analysis.  
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21 Ovariectomy, besides a lowered serum iron and transferrin iron binding capacity, remarkably  
22  
23 decreased the binding activity of IRP1 in peritoneal and subcutaneous adipose tissues, and these  
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25 effects were reversed by  $E_2$  treatment. Moreover, ovariectomy determined a decrease of IRP1  
26  
27 expression, which was significant in subcutaneous adipose tissue. Consistent with IRP1 regulation,  
28  
29 an increase of ferritin and a decrease of TfR-1 expression were observed in peritoneal adipose tissue  
30  
31 from ovariectomized animals, while the treatment with  $E_2$  reconstituted TfR-1 level. A similar  
32  
33 expression profile of TfR-1 was observed in subcutaneous adipose tissue, where ferritin level did  
34  
35 not change in ovariectomized animals, and was increased after  $E_2$  treatment.  
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39 Our results indicate that estrogen level changes can regulate the binding activity of the IRP1, and  
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41 consequently ferritin and TfR-1 expression in adipose tissue, suggesting a relationship among  
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43 serum and tissue iron parameters, estrogen status and adiposity.  
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56 Key words: estrogen; ovariectomy; adipose tissue; iron metabolism; iron regulatory proteins.  
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## 1. Introduction

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2 Iron requirement in vertebrates arises since it is an essential component of proteins that perform  
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4 redox or non-redox roles in many critical cellular functions, including respiration and cell division.  
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7 Its deficiency or excess is involved in pathological conditions, i.e. iron-deficient anemia or iron-  
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9 overload, such as hemochromatosis [1]. In addition to anemia, iron deficiency impairs muscle,  
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11 immune and cognitive functions [2]. Dysregulation of iron metabolism associated with  
12  
13 hemochromatosis and other iron overload disorders is also a significant health concern [3]. Iron  
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15 cytotoxicity is due to the ability by which free iron, as  $Fe^{2+}$  ions, participates in redox reactions,  
16  
17 leading to the production of harmful oxygen radicals (ROS) that can damage cellular structures [4].  
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20 Consequently, to suppress the potential deleterious effects of iron, cells have evolved homeostatic  
21  
22 mechanisms that regulate transport, storage and mobilization of this essential element.  
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26 In mammals, iron is mostly bound to various cellular components, such as haemoglobin, heme,  
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28 various enzymes and ferritin, the ubiquitous intracellular iron storage protein [5]. Extracellular iron  
29  
30 is bound to transferrin (Tf), which shuttles iron through the blood to target tissues. Mono- and  
31  
32 diferric-transferrin bind to the Tf-receptors (TfR-1 and TfR-2), that transport iron into the cell via  
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34 clathrin-coated pits [6]. At the cellular level, maintenance of iron homeostasis is largely  
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36 accomplished by the TfR-1, that allows iron uptake, and by ferritin, which is crucial to sequester  
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38 this metal in a non-toxic form. The levels of these and other proteins involved in iron metabolism  
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40 are mainly regulated post-transcriptionally by interaction between the Iron Regulatory Proteins  
41  
42 (IRP1 and IRP2) and stem-loop structures, termed IREs (Iron Responsive Elements), located in the  
43  
44 5' untranslated region (UTR) of ferritin mRNAs and in the 3' UTR of TfR mRNA [7, 8].  
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51 The IRPs provide a central role in the regulation of cellular iron homeostasis. IRP1, the cytosolic  
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53 counterpart of mitochondrial aconitase [9], is a bifunctional protein that, through [4Fe-4S] cluster  
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55 assembly/disassembly, switches from the aconitase to the IRP1 form mainly in response to the  
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57 intracellular iron level. IRP2 is homologous to IRP1 but lacks the [4Fe-4S] cluster and its activity  
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59 increases in iron-depleted cells by protein stabilization [10]. Intracellular free iron regulates IRP1,  
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1 affecting its RNA-binding affinity, and IRP2, inducing its proteasomic degradation [11, 12].

2 Specifically, when intracellular iron content decreases, IRPs interact with multiple IREs within the  
3 3'-UTR of TfR-1 mRNA, strongly increasing mRNA half-life and TfR-1 protein expression [13].

4  
5 Simultaneously, the binding of IRPs to IRE *cis*-element in the 5'-UTR of ferritin mRNA prevents  
6 its synthesis [14, 15]. On the other hand, high iron levels reduce IRPs affinity to IREs (i.d. IRP1 is  
7 no longer able to bind IRE and IRP2 is degraded), resulting in rapid TfR-1 mRNA degradation and  
8 in efficient translation of ferritin [16].

9  
10 The IRPs RNA-binding activity is also regulated by iron-independent factors, such as oxidative  
11 stress [17], nitric oxide signalling [18], protein phosphorylation [19], hypoxia [20, 21], as well as  
12 oxalomalic acid, an inhibitor of aconitase/IRP1 [22, 23], and virus infection [24]. Among hormones,  
13 thyroid hormones regulate post-transcriptionally the synthesis of proteins involved in iron  
14 metabolism by affecting IRPs ability to bind to IRE [25]. Insulin and IGF1 have also been  
15 implicated in the regulation of ferritin at the mRNA level [26]. Estrogen hormones might also  
16 influence the expression of iron-related proteins, but the only relevant data are related to systemic  
17 iron metabolism. In fact, Haouari et al. [27, 28] described the effects of 17 $\beta$ -estradiol (E<sub>2</sub>) on iron  
18 absorption and uptake in ovariectomized rats. In particular, they found a significant rise in iron  
19 uptake by the small intestine and a subsequent increase in serum iron level, accompanied by a  
20 decrease in total iron binding capacity (TIBC) in E<sub>2</sub>-treated ovariectomized rats. Furthermore, a  
21 correlation between E<sub>2</sub> administration and an increase of splenic iron stores has been demonstrated  
22 in ovariectomized rats subjected to hormone therapy, showing that the amount of iron stored  
23 depends directly on circulating estrogens [29].

24  
25 Besides its function as storing energy, adipose tissue secretes a variety of peptides, such as leptin  
26 and adiponectin which act in autocrine/paracrine and endocrine manner [30] and is involved in the  
27 metabolism of sex steroids, expressing enzymes for their activation, interconversion, and  
28 inactivation [31]. Although adipose tissue is not considered the main target of estrogens, its excess  
29 or obesity, particularly in the visceral compartment, is associated with insulin resistance,  
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1 hyperglycemia, dyslipidemia, hypertension, and inflammation [32], as well as with estrogen  
2 deficiency. In turn, overweight and obesity are often related with systemic iron status: in fact iron  
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4 may also be involved in the maintenance of body weight and composition [33-34].  
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7 Very little attention has been paid to the molecular mechanisms regulating iron homeostasis in  
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9 adipose tissues. It has been previously reported a specific regulation of iron homeostasis, with a  
10  
11 consistent accumulation of ferritin, in mature adipocytes [35].  
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14 Here, we evaluated the iron-status parameters, and the activity and expression of the main proteins  
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16 of iron metabolism in adipose tissues from estrogen deficient rats (7 weeks after ovariectomy) and  
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18 after estrogen treatment by E<sub>2</sub>. Seven-week ovariectomy induced in rats a mild obesity that mimics  
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20 estrogen insufficiency in human [36] and creates a useful *in vivo* experimental model of  
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22 hypoenestrogenism.  
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## 2. Material and methods

### 2.1. Animals and treatments

Female Sprague Dawley rats (~170 g) were purchased from Harlan Italy (San Pietro al Natisone, Udine, Italy) and housed under a 12-h light:12-h dark cycle. The animals were acclimated to their environment for 1 week and had ad lib. access to tap water and rodent standard diet. All animal experiments complied with the Italian (D.L. no.116 of January 27, 1992) and associated guidelines in the European Communities Council (Directive of November 24, 1986, 86/609/ECC). All efforts were made to minimize animal suffering, and to reduce the number of animals used. The rats were divided into the three following groups of 6 animals: 1) sham-operated controls (SHAM); 2) ovariectomized animals (OVX); and 3) OVX treated with E<sub>2</sub> (OVX+E<sub>2</sub>). At the onset of the study, OVX rats were bilaterally ovariectomized under anesthesia (ketamine, 100 mg/kg; xylazine, 5 mg/kg i.p.). The SHAM animals were subjected to the same general surgical procedure as OVX groups, except that the ovaries were not excised. E<sub>2</sub> treatment (25 µg/kg s.c. twice a week; Sigma, St. Louis, MO) began 2 days after surgery and continued for 7 weeks. SHAM and OVX rats received vehicle (sterile sesame oil). Last administration was performed 2 h before sacrifice.

### 2.2. Body weight, food intake, and body gain and fat mass

Throughout the treatment period, body weight and food intake were monitored once a week. At the end of 7<sup>th</sup> week, food intakes were calculated and expressed as area under the curve obtained plotting the grams of food versus time (average of g/7 weeks/animal). Before sacrifice, bioelectrical impedance analysis (BIA) was applied to body composition assessment using a BIA 101 analyzer, modified for the rat (Akern, Florence, Italy). Fat-free mass (FFM) was calculated using the bioelectrical impedance analysis (50 kHz) prediction equation of Ilagan et al. [37], and fat mass content was determined as the difference between body weight and fat-free mass.

### 2.3. Tissue collection and serum iron-status parameters

SHAM rats at random stages of the estrous cycle, OVX, and E<sub>2</sub>-treated rats were sacrificed at 7 weeks. Blood collected by cardiac puncture was centrifuged at 1 500×g at 4°C for 15 min and sera were stored at -80°C for later biochemical and hormonal measurements. Iron-status analyses included ferritin, iron, TIBC (Total Iron Binding Capacity) and UIBC (Unsaturated Iron-Binding Capacity), measured by the Roche/Hitachi Modular Analytics P clinical chemistry analyzer (Roche Diagnostics GmbH, Mannheim, Germany), using manufacturer's reagents. Serum E<sub>2</sub> was determined by an ELISA kit (Abbott Laboratories, Abbott Park, IL). Peritoneal and subcutaneous white adipose tissues were excised and immediately frozen in liquid nitrogen.

### 2.4. Preparation of tissue extracts

To obtain cytosolic extracts for electrophoretic mobility-shift assay (EMSA) and Western blot analysis tissue samples (0.3 g) were disrupted by homogenization on ice in lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaF, 150 mM NaCl, 1 % Nonidet P-40 (NP-40), 1 mM phenylmethylsulphonyl fluoride, 1 mM Na<sub>3</sub>VO<sub>4</sub>, leupeptin and trypsin inhibitor 10 µg/ml). After 30 min the supernatant fraction was obtained by centrifugation at 21 000x g for 15 min at 4°C and then stored at -80°C. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, Milan, Italy).

### 2.5. Electrophoretic mobility-shift assay (EMSA)

Plasmid pSPT-fer containing the sequence corresponding to the IRE of the H-chain of human ferritin, linearized at the *Bam* HI site, was transcribed *in vitro* as previously described [21, 22]. For RNA-protein band-shift analysis, cytosolic extracts (5 µg) were incubated for 30 min at room temperature with 0.2 ng of *in vitro*-transcribed <sup>32</sup>P-labelled IRE RNA. The reaction was performed

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2 in buffer containing 10 mM HEPES, pH 7.5, 3 mM MgCl<sub>2</sub>, 40 mM KCl, 5 % (v/v) glycerol, 1 mM  
3 DTT and 0.07 % (v/v) NP-40, in a final volume of 20 µl. To recover total IRP1 binding activity,  
4 cytosolic extracts were pre-incubated for 10min with 2-mercaptoethanol (2-ME) at a 2 % (v/v) final  
5 concentration, before the addition of <sup>32</sup>P-labelled IRE RNA. Unbound RNA was digested for 10  
6 min with 1 U RNase T<sub>1</sub> (Roche, Mannheim, Germany), and non-specific RNA–protein interactions  
7 were displaced by the addition of 5 mg/ml heparin for 10 min. RNA–protein complexes were  
8 separated on 6 % non-denaturing polyacrylamide gel for 2 h at 200 V. After electrophoresis, the gel  
9 was dried and autoradiographed at –80°C. IRPs-RNA complexes were then quantified by GS-800  
10 imaging densitometer (Bio-Rad). The results are expressed as percent of IRP1 binding activity of  
11 the control treated with 2-ME (100 % of IRP1 RNA-binding activity) and are the average ± S.E.M.  
12 values of four independent experiments.  
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## 29 2.6. Western blot analysis

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34 Samples containing 50-100 µg of proteins were denatured, separated on a 12 % (for ferritin) or 8 %  
35 (for IRP1 and TfR-1) SDS–polyacrylamide gel and electro-transferred onto a nitrocellulose  
36 membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) using a Bio-Rad  
37 Transblot (Bio-Rad). Proteins were visualized on the filters by reversible staining with Ponceau-S  
38 solution and destained in PBS. Membranes were blocked at room temperature in milk buffer [1x  
39 PBS, 10 % (w/v) non-fat dry milk, 0.1 % (v/v) Tween-20] and then incubated at 4°C overnight with  
40 1:1 000 rabbit polyclonal antibody to human ferritin cross-reactive with rat protein (Dako  
41 Cytomation, Glostrup, Denmark), or with 1:1 000 mouse antibody to human transferrin receptor-1  
42 cross-reactive with rat TfR-1 (Zymed Laboratories Inc., CA, USA), or with 1:250 goat antibody to  
43 human IRP1 cross-reactive with rat IRP1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).  
44 Subsequently, the membranes were incubated for 90 min at room temperature with peroxidase-  
45 conjugated goat anti-mouse IgG+IgM, or peroxidase-conjugated rabbit anti-goat IgG, or  
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1 peroxidase-conjugated goat anti-rabbit IgG (all the secondary antibodies were purchased from  
2 Jackson ImmunoResearch Laboratories, Baltimore Pike, West Grove, PA). The resulting complexes  
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4 were visualized using chemoluminescence Western blotting detection reagents (ECL, Amersham  
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6 Biosciences, Little Chalfont, Buckinghamshire, UK). The optical density of the bands was  
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8 determined by a GS-800 imaging densitometer (Bio-Rad). Normalisation of results was ensured by  
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10 incubating the nitrocellulose membranes in parallel with the  $\beta$ -actin antibody (Sigma).  
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### 17 *2.7. Statistical analysis*

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22 All data were presented as mean  $\pm$  S.E.M. Statistical analysis was performed by one-way ANOVA  
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24 test followed by Bonferroni's test. Statistical significance was set at  $P < 0.05$ .  
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### 3. Results

#### 3.1. *Body weight gain, food intake, and body fat in ovariectomized and E<sub>2</sub>-treated rats*

As evident in table 1, ovariectomy leads to a significant increase of body weight, and this change in body weight paralleled to the increase in food intake. Moreover, the change in body weight was related to the variation of fat mass, which was significantly increased in OVX animals. All these modifications were significantly reverted by E<sub>2</sub> treatment.

#### 3.2. *Serum parameters*

E<sub>2</sub> levels were evaluated in all animal groups to confirm the hypoestrogenism induced by OVX and its level in E<sub>2</sub>-treated animals. As reported in table 2, after 7 weeks, OVX significantly reduced the E<sub>2</sub> level (P<0.001) and as expected, E<sub>2</sub> treatment increased the hormone level as compared to either OVX (P<0.001) or SHAM (P<0.05) group.

Moreover, in OVX animals a significant decrease in serum iron was evidenced. This reduction in serum iron level was accompanied with a very significant decrease in TIBC. Conversely, no significant modification of ferritin and UIBC was evidenced in OVX rats. Interestingly, when OVX rats were treated with E<sub>2</sub> (25 µg/kg, s.c.), increased iron, ferritin, and TIBC were observed. In E<sub>2</sub>-treated OVX rats, UIBC value did not significantly change, even though a decreasing trend was observed.

#### 3.3. *IRP1 activity and expression in adipose tissues from ovariectomized and E<sub>2</sub>-treated rats*

As shown in Fig. 1a, ovariectomy caused a remarkable decrease in the RNA-binding activity of IRP1 in peritoneal adipose tissue. A similar regulation of IRP1 activity, albeit with a lesser but

1 significant extent, was observed in subcutaneous adipose tissue after ovariectomy (Fig. 2a). In  
2 both types of adipose tissue, IRP1 RNA-binding activity was efficiently restored by hormone  
3 therapy (E<sub>2</sub>). All samples were incubated with mercaptoethanol (2-ME) to ensure full activation of  
4 IRP1. Concerning IRP1 protein expression, Western blot analysis showed an overall decreasing  
5 trend in ovariectomized rats, which was more pronounced and significant in subcutaneous adipose  
6 tissue (fig. 2b). Differently from IRP1 activity modulation, E<sub>2</sub> treatment was not able to reverse  
7 the decrease of IRP1 expression induced by ovariectomy.  
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### 19 *3.4. Ferritin and TfR-1 expression in adipose tissues from ovariectomized and E<sub>2</sub>-treated rats*

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24 Ovariectomy increased ferritin expression in peritoneal adipose tissue, whereas hormone therapy  
25 had no significant effect on OVX rats (fig. 3a). Conversely, no significant variation of ferritin  
26 content was observed in subcutaneous adipose tissue of OVX animals, whereas a marked up-  
27 regulation of protein expression was revealed after E<sub>2</sub> treatment (fig. 4a).  
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34 As depicted in fig. 3b and 4b, TfR-1 expression was significantly lowered by ovariectomy in both  
35 peritoneal and subcutaneous adipose tissues, when compared with SHAM animals. In this  
36 circumstances, E<sub>2</sub> treatment caused an overall increasing trend of TfR-1 expression in OVX  
37 animals, which was statistically significant in peritoneal adipose tissue.  
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#### 4. Discussion

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2 Iron is essential for many biological processes and its deficiency or excess is involved in  
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4 pathological conditions [1]. A number of studies have reported a correlation between obesity and  
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6 poor iron status [33-35], although the molecular basis underlying this phenomenon is still obscure.  
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9 In the present study we have evaluated the binding activity of IRP1 and the expression of ferritin  
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11 and TfR-1 in adipose tissue from rats having a mild obesity induced by seven-week ovariectomy, a  
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13 model that mimics estrogen insufficiency [36]. We demonstrate for the first time, to our knowledge,  
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15 that E<sub>2</sub> modulates the RNA binding activity of IRP1 and affect the ferritin and TfR-1 expression in  
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17 adipose tissue. Moreover, we observed that ovariectomy induces a significant decrease of serum  
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19 iron, accompanied with a trend toward a decreased serum ferritin.  
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23 Surprisingly, we found that the mild obesity present in ovariectomized rats was associated with a  
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25 decrease in TIBC, an indirect value of hepatic transferrin synthesis, and that the E<sub>2</sub> therapy reverted  
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27 this effect. The low level of TIBC found in ovariectomized rats is unusual because when serum iron  
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29 decreases and cellular iron demand is high, the TIBC level usually increases. The atypical scenario  
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31 that we have found is compatible with a chronic inflammatory condition, in which iron release from  
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33 cells decreases, lowering the level of TIBC [38], as may occur in certain type of iron deficiency  
34  
35 related to inflammation and /or liver disease, two common patho-physiological conditions  
36  
37 associated with menopause or ovariectomy [39]. A linkage between E<sub>2</sub> serum level and iron  
38  
39 metabolism disturbances has been described not only in reference to hypoestrogenism, such as,  
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41 occurs following ovariectomy or in menopause, but also during pregnancy, a condition in which E<sub>2</sub>  
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43 is elevated [40].  
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51 In our study E<sub>2</sub> treatment increases serum iron and ferritin, showing that these parameters of iron  
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53 status depend on circulating estrogen levels. Iron deficiency detected in ovariectomized rats could  
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55 be considered a compensatory mechanism by which the excess of adipose tissue tries to protect  
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57 itself against the damaging effects of iron-induced oxidative stress. Serum iron decrease in  
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59 overweight status may be due to larger blood volume and higher basal iron losses associated to  
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1 higher body weight [34]. On the basis of previous findings, the reduction of serum iron level after  
2 ovariectomy could also be related to a lower iron uptake. In fact, it was demonstrated that the  
3 increase of serum iron in OVX rats treated with E<sub>2</sub> was due to a direct stimulating effect of the  
4 hormone in iron by duodenum and its transfer into the blood and liver [27-28].  
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7 Evidence suggests that seven-week ovariectomy increases fat mass and leptin serum content in rats,  
8 both features of estrogen loss [36]. Leptin might play a role in regulating iron metabolism in  
9 overweight status because it shares a number of common biological features with interleukin (IL)-6,  
10 a cytokine released in response to inflammation, a condition often associated with obesity [41]. IL-6  
11 induces an increase of hepcidin secretion from liver cells [42], that in turn negatively regulates  
12 systemic iron metabolism by directly interacting with ferroportin (FPN1), a protein devoted to  
13 control the cellular iron efflux [43]. Therefore, the iron deficiency, that we have found in OVX rats,  
14 could be indirectly related to leptin via increasing the expression of hepcidin.  
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17 Although adipose tissue is considered a non classical target of estrogen stimulation, in vivo and in  
18 vitro studies have shown that estrogen is involved in the modulation and distribution of body fat  
19 mass [44]. In fact, the increased subcutaneous fat in women develops pubertally, indicating that  
20 estrogen may preferentially promote subcutaneous adipose deposition. In contrast, the accretion of  
21 abdominal fat in premenopausal women appears to be inhibited by estrogen, whereas men tend to  
22 depot abdominal fat [45]. Gender differences in body fat distribution implicate sex steroids in the  
23 regulation of adiposity. In particular, both ER $\alpha$  and ER $\beta$  are expressed in adipose tissue and  
24 evidence for the involvement of ER $\alpha$  in white adipose tissue distribution and in the suppression of  
25 fat accumulation and hyperlipidemia was provided by analyzing ER $\alpha$  knockout mice [46].  
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28 We previously demonstrated in differentiated 3T3-L1 adipocytes an accumulation of ferritin mRNA  
29 with a consequent increased content of H-rich isoform that may limit the toxicity of iron in  
30 adipose tissue, thus exerting an antioxidant function [35]. In the present study we found that the  
31 systemic iron deficiency in ovariectomized rats is linked to an opposite condition in adipose tissue,  
32 similar to that observed in iron-rich cells in which ferritin content enhanced and TfR-1 expression  
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1 decreased. In particular, this condition seems to be more effective in peritoneal adipose tissue,  
2 where the molecular mechanism governing the post-transcriptional regulation of ferritin and TfR-1  
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4 gene expression by estrogen level (OVX and E<sub>2</sub> treatment), is based on regulated changes in the  
5  
6 IRE binding activity of the IRPs. In fact, in peritoneal adipose tissue of OVX rats the reduced IRP1  
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8 binding capacity is consistent with the increase in ferritin expression and the decrease of TfR-1  
9  
10 expression. Furthermore, the recovery of IRP1 binding activity by E<sub>2</sub> treatment in ovariectomized  
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12 rats reinforces the association between post-transcriptional machinery regulation and estrogen  
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14 levels. . In fact, it has been reported that hormonal signals may regulate post-transcriptionally the  
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16 synthesis of proteins involved in iron metabolism by affecting IRPs ability to bind to IRE, possibly  
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18 through induction of signal transduction cascades that result in IRPs phosphorylation [25, 47]. In  
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20 adipose tissue estrogen may also have rapid, non-genomic biological effects, believed to be  
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22 mediated through a small fraction of estrogen receptors localized at or near the cell membrane [45].  
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24 By these receptors, estrogen could trigger the protein kinase C-dependent phosphorylation of IRP1  
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26 which increases its RNA binding activity [8], resulting in an increased TfR-1 expression and in a  
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28 reduced ferritin content.  
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32 A similar profile of IRP1-dependent post-transcriptional regulations occurs also in subcutaneous  
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34 adipose tissue of OVX animals treated or not with E<sub>2</sub>, albeit the effect is reproduced to a lesser  
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36 extent, in particular for ferritin expression.  
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40 Indeed, the synthesis of TfR-1 and ferritin is under post-transcriptional as well as transcriptional  
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42 control [48], so that both mechanisms could be effective in response to environmental changes in  
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44 iron and/or metabolic signals.  
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51 In conclusion, our results suggest that estrogens can functionally regulate the IRE binding activity  
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53 of the IRP1 and consequently the expression of the ferritin and TfR-1. However, it is difficult to  
54  
55 address if iron homeostasis in ovariectomized and E<sub>2</sub> treated rats is affected directly by E<sub>2</sub> level  
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57 changes or indirectly by the modifications of peritoneal and subcutaneous fat mass content induced  
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59 by the hormone, that might have a different iron demand. Further studies are required to support our  
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1 results and to investigate the nature and significance of the relationship among serum and tissue  
2 iron parameters, estrogen status and adipose derived hormones.  
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Disclosure statement:

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2 GIUSEPPINA MATTACE RASO “no conflicts of interest”  
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4  
5 CARLO IRACE “no conflicts of interest”  
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7  
8 EMANUELA ESPOSITO “no conflicts of interest”  
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10  
11 CARMEN MAFFETTONE “no conflicts of interest”  
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14 ANNA IACONO “no conflicts of interest”  
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17 ANTONIO DI PASCALE “no conflicts of interest”  
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20 RITA SANTAMARIA “no conflicts of interest”  
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23 ALFREDO COLONNA “no conflicts of interest”  
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26 ROSARIA MELI “no conflicts of interest”  
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## Legends to figures

1  
2 Figure 1. a) IRP1 RNA-binding activity in rat peritoneal adipose tissue after ovariectomy (OVX)  
3 and hormone therapy (OVX + E<sub>2</sub>). RNA band-shift assay was performed with 5 µg of cytoplasmic  
4 proteins and an excess of <sup>32</sup>P-labelled IRE probe in absence or presence of 2% 2-ME. RNA–protein  
5 complexes were separated on non-denaturing 6% polyacrylamide gel, revealed by autoradiography  
6 and IRP1-RNA complexes were then quantified by densitometric analysis. The results of  
7 experiments performed without 2-ME were plotted in a bar graph as percent of the control (SHAM)  
8 treated with 2-ME, a condition that reveals total RNA-binding activity of IRP1 (100%). A  
9 representative autoradiogram is shown. b) Western blot analysis showing the effect of ovariectomy  
10 (OVX) and the subsequent hormone therapy (OVX + E<sub>2</sub>) on IRP1 protein levels in cytosolic  
11 extracts from rat peritoneal adipose tissue. Equal amounts of proteins were separated on a 8% SDS–  
12 polyacrylamide gel and subjected to Western blot analysis using 1:250 dilution of IRP1 antiserum.  
13 After chemoluminescence, the corresponding bands were quantified by densitometric analysis and  
14 plotted as relative O.D. in a bar graph. β-Actin (β-act) was used as internal control to standardize  
15 the amounts of proteins in each lane. Shown are the means of relative O.D. ± S.E.M. of all bands  
16 plotted in a bar graph compared to sham animals (SHAM).

17 \*\*\*P<0.001 vs SHAM; °P<0.01 and °°P<0.001 vs OVX

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43 Figure 2. a) IRP1 RNA-binding activity in rat subcutaneous adipose tissue after ovariectomy (OVX)  
44 and hormone therapy (OVX + E<sub>2</sub>). EMSA was performed as described in legend of figure 1. The  
45 results of experiments performed without 2-ME were plotted in a bar graph as percent of the control  
46 (SHAM) treated with 2-ME and are the average ± S.E.M. values of all bands. Control treated with  
47 2-ME represents the 100% of IRP1 RNA-binding activity. A representative autoradiogram is  
48 shown. b) Western blot analysis showing the effect of ovariectomy (OVX) and the subsequent  
49 hormone therapy (OVX + E<sub>2</sub>) on IRP1 protein levels in cytosolic extracts from rat subcutaneous  
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1 adipose tissue. The experiments were performed as illustrated in legend of figure 1. Shown are the  
2 means of relative O.D.  $\pm$  SEM of all bands.  
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4 **\*\*P<0.01 vs SHAM; °P<0.01 vs OVX.**  
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9 Figure 3. Western blot analysis showing the ferritin (Fer) (a) and the transferrin receptor-1 (TfR-1)  
10 levels (b) in cytosolic extracts obtained from peritoneal adipose tissue of sham-operated (SHAM),  
11 ovarietomized (OVX) and hormone treated (OVX + E<sub>2</sub>) rats. For ferritin content analysis, equal  
12 amounts of cytosolic lysates were fractionated by 12% SDS–PAGE and subjected to Western blot  
13 analysis using 1:1 000 dilution of ferritin antiserum. For TfR-1 content analysis, equal amounts of  
14 cytosolic lysates were fractionated by 8% SDS–PAGE and subjected to Western blot analysis using  
15 1:1 000 dilution of TfR-1 antiserum. The Fer and TfR-1 bands were quantified by densitometric  
16 analysis and plotted as relative O.D. in a bar graph. The anti- $\beta$ -actin antibody was used to  
17 standardize the amounts of proteins in each lane. Shown are the average of the relative O.D.  $\pm$  SEM  
18 values of all bands.  
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33 **\*\*P<0.01 vs SHAM; °° P<0.01 vs OVX.**  
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39 Figure 4. Western blot analysis showing the ferritin (Fer) (a) and the transferrin receptor-1 (TfR-1)  
40 levels (b) in cytosolic extracts obtained from subcutaneous adipose tissue of sham-operated  
41 (SHAM), ovarietomized (OVX) and hormone treated (OVX + E<sub>2</sub>) rats. Western blot analysis were  
42 performed as described in legend of figure 3. Shown in a bar graph are the average of the relative  
43 O.D.  $\pm$  SEM values of all bands.  
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50 **\*\*P<0.01 vs SHAM; °°° P<0.001 vs OVX.**  
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**Tab. 1** Changes in body weight, body weight gain, food intake and fat mass in sham operated (SHAM) or ovariectomized rats treated with (OVX+E<sub>2</sub>) or without (OVX) 17  $\beta$ -estradiol for 7 weeks.

	SHAM	OVX	OVX+E <sub>2</sub>
Body weight (g)	239 $\pm$ 3	305 $\pm$ 3 <sup>a</sup>	212 $\pm$ 6 <sup>b,c</sup>
Body weight gain (g)	74 $\pm$ 3	142 $\pm$ 3 <sup>a</sup>	47 $\pm$ 4 <sup>a,c</sup>
Dietary intake (g/7 weeks/animal)	100.3 $\pm$ 3.5	111.5 $\pm$ 1.5 <sup>b</sup>	96.1 $\pm$ 1.9 <sup>c,d</sup>
Fat mass (g)	21.7 $\pm$ 1.1	35.2 $\pm$ 0.7 <sup>a</sup>	17.6 $\pm$ 0.7 <sup>c,d</sup>

Values are means  $\pm$ S.E.M of six animals.

<sup>a</sup>P<0.001 vs SHAM

<sup>b</sup>P<0.01 vs SHAM

<sup>c</sup>P<0.001 vs OVX

<sup>d</sup>P<0.05 vs SHAM

**Tab. 2** Changes in serum parameters in sham operated (SHAM) or ovariectomized rats treated with (OVX+E<sub>2</sub>) or without (OVX) 17  $\beta$ -estradiol for 7 weeks.

	SHAM	OVX	OVX+E <sub>2</sub>
Estradiol (pg/ml)	26.0 $\pm$ 1.3	4.2 $\pm$ 0.4 <sup>a</sup>	30.6 $\pm$ 1.6 <sup>b,c</sup>
Iron ( $\mu$ g/dl)	446.5 $\pm$ 16.8	279.8 $\pm$ 38.23 <sup>c</sup>	570.4 $\pm$ 33.7 <sup>b,a</sup>
Ferritin (ng/ml)	140.8 $\pm$ 14.8	127.6 $\pm$ 2.5	193.0 $\pm$ 19.5 <sup>d</sup>
Transferrin iron binding capacity (TIBC)	574.8 $\pm$ 6.8	385.4 $\pm$ 14.0 <sup>a</sup>	630.8 $\pm$ 13.6 <sup>b,c</sup>
Unsaturated iron binding capacity (UIBC) ( $\mu$ g/dl)	128.2 $\pm$ 16.4	119.4 $\pm$ 23.5	60.4 $\pm$ 21.1

Values are means  $\pm$ S.E.M of five animals.

<sup>a</sup>P<0.001 vs SHAM

<sup>b</sup>P<0.001 vs OVX

<sup>c</sup>P<0.05 vs SHAM

<sup>d</sup>P<0.05 vs OVX

trip

