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## THE OBESE HEALTHY PARADOX: IS INFLAMMATION THE ANSWER?

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**Running title:** Inflammation absence in NIR-MO

**ABSTRACT**

A paradoxical but common finding in the obesity clinic is the identification of individuals who can be considered “inappropriately” healthy for their degree of obesity. We think that studying these obese but metabolically healthy individuals and comparing them with equally obese but insulin resistant individuals could provide important insights on the mechanistic link between adipose tissue expansion and associated metabolic alterations. In this study we investigate whether there are differences in inflammatory and insulin signalling pathways in visceral adipose tissue (VAT) that could account for the metabolic differences exhibited by morbid obese individuals either insulin resistant (IR-MO) or paradoxically insulin sensitive (NIR-MO). Our results indicate that there are pathways common to obesity and unrelated to insulin resistance and others that are discriminative for insulin resistance for a similar degree of obesity. For instance all MO patients, irrespectively of their insulin resistance, showed increased expression of TNF- $\alpha$  and activation of JNK1/2. However the IR-MO group showed significantly elevated expression levels of IL-1 $\beta$  and IL-6 and increased macrophage infiltrates compared with non obese individuals and obese non insulin resistant. I $\kappa$ B- $\alpha$ , the activation of ERK 1/2 kinases and NF- $\kappa$ B were discriminative of the state of insulin resistance and correlated with differential changes in IRS-1 expression and AKT activation between IR-MO and NIR-MO-individuals. Our results support the concept that obese healthy individuals lack the inflammatory response that characterises the obese insulin resistant patient and that IL-6, IL-1 $\beta$ , ERK and NF- $\kappa$ B are important effectors mediating the inflammation effects promoting insulin resistance.

**KEYWORDS:** Obesity, Inflammation, Insulin resistance, Signal transduction

## INTRODUCTION

Obesity is becoming a major health problem worldwide despite medical and political efforts to control its increased prevalence. Despite the recent success in identifying the molecular mechanisms controlling energy homeostasis, the truth is that there are not efficient treatments besides surgical approaches. The failure to prevent/reverse the development of obesity makes it more urgent to devise strategies to contain what would be a more devastating second wave of cardiovascular complications. Thus an immediate challenge is to identify who among these obese individuals will be at high risk for developing metabolic complications. We think the clue to this important information may come from understanding why some obese individuals seem to be protected against the deleterious effects of their increased fat mass.

Accumulating evidence identifies inflammation as the potential link between adipose tissue expansion and cardiometabolic complications. In fact obesity is now considered a condition that facilitates the development of a low grade inflammatory state characterized by increased plasma levels of proinflammatory cytokines such as tumor necrosis factor (TNF- $\alpha$ ), Interleukins (ILs) and cytokine-like proteins known as adipokines [2]. Some of these molecules seem to be secreted by adipocytes, whereas others are predominantly derived from adipose tissue infiltrated macrophages. This inflammatory cascade involves activation of a complex network of signalling pathways, including the activation of various serine/tyrosine kinases and transcription factors such as Jun N-terminal kinase (JNK), protein kinase C (PKC) and nuclear factor  $\kappa$ B (NF- $\kappa$ B) [3].

One of the consequences of this state of inflammation is the development of insulin resistance as well as increased risk of type 2 diabetes development. The specific mechanisms linking inflammation and defects in insulin sensitivity have been partially characterised and have revealed an incomplete picture of a complex cross-talk integrating metabolic, nutritional, and inflammatory signalling pathways, eventually leading to the development of obesity induced insulin resistance [4]. The insulin receptor substrate (IRS) 1 protein is one of the defective molecular effectors associated to obesity related insulin resistance. Under physiological conditions insulin stimulates the tyrosine phosphorylation of IRS-1 leading to activation of downstream signals including phosphatidylinositol-3 kinase and the AKT pathway [5]. However in the context of overnutrition induced insulin resistance, IRS-1 becomes the target substrate for multiple proinflammatory serine kinases [6, 7]. In fact, there is evidence that TNF $\alpha$  phosphorylates and inhibits IRS-1 on serine residues, resulting in impaired insulin action [8, 9, 10]. Particularly, JNK1 and IKK $\alpha$  kinases have been shown to directly phosphorylate ser 307 and inhibit the IRS1 signalling [7, 9]. Other studies performed in 3T3-L1 adipocytes have also implicated activation of other signalling pathways, such as extracellular signal-regulated kinase (ERK) 1/2 [11] and p38 mitogen-activated protein kinase (MAPK), in the development of insulin resistance [12].

The mechanisms involved in obesity-induced insulin resistance are not completely elucidated. Furthermore, whereas the link between obesity and insulin resistance is well established at epidemiological level, there is clinical evidence that there are obese individuals metabolically healthy that do not develop insulin resistance [13]. Thus, we hypothesised that studying this specific group could provide some important insights on the specific mechanisms linking adipose tissue expansion, inflammation and insulin resistance.

Here, we investigate the differences in inflammatory pathways and insulin signalling markers in the visceral adipose tissue of equally obese individuals that differ in their degree of insulin resistance. Our study focused on the visceral adipose depot as the most likely involved in the metabolic disturbances associated to obesity [14], and specifically we have evaluated the existence of differences in macrophage infiltration, mRNA gene expression profiles of proinflammatory cytokines and transcription factors such as TNF $\alpha$ , IL-1 $\beta$ , IL-6, and NF- $\kappa$ B and I $\kappa$ B $\alpha$ . We have also measured the mRNA and protein levels of IRS-1, as well as the activation of downstream pathways, such as JNK, ERK, AKT and the transcription factor NF- $\kappa$ B.

## EXPERIMENTAL

### Subjects

This study included 24 morbidly obese (MO) subjects (body mass index (BMI)  $56 \pm 6.8$  kg/m<sup>2</sup>) (12 women and 12 men) who underwent bariatric surgery with mixed techniques, combining gastric reduction with an intestinal bypass, and 12 non-obese subjects (BMI 18.5-24.9 kg/m<sup>2</sup>) (6 women and 6 men) with no alterations in lipid or glucose metabolism, as controls. All subjects were included after informed consent. The morbidly obese patients were selected in base to similar clinical profile but different insulin resistance degree. Any patients received oral antidiabetic agents or insulin therapy. The weight of all individuals had been stable for at least one month and none had associated renal pathology. The morbidly obese patients were classified according to their insulin sensitivity. Specifically patients having HOMA-IR score <5 were considered non-insulin resistance (NIR-MO) group. This cut point was established from the mean  $\pm$  2SD of a healthy control population. The morbidly obese with a HOMA-IR score >8, was considered as the insulin resistant (IR-MO) group. Clinical details of patients included in the study are indicated in Table I.

Visceral adipose tissue (VAT) biopsies were obtained from MO patients undergoing bariatric surgery procedures or laparoscopic surgery procedures (hiatus hernia repair or cholecystectomies) in lean subjects. Tissue samples were washed in physiological saline, immediately frozen in liquid nitrogen and stored at -80°C for the different assays described below.

### Nuclear and Cytoplasmic extracts

Cytoplasmic and nuclear extracts were prepared from visceral adipose tissue using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Chemical Co. Rockford, IL). 100 mg of tissue were homogenized in CER I Reagent with protease inhibitors (Sigma, St. Louis, MO), 100 mM phenylarsine oxide (PAO) (Sigma) and 1 mM sodium orthovanadate (Sigma) using an ULTRATURRAX T25 basic (IKA Werke GmbH, Staufen, Germany). After 10 min at 4°C, the CER II Reagent was added to the lysate. Samples were pelleted by centrifugation at 15000 g for 10 min at 4°C. The supernatant (cytoplasmic lysates) was recovered and frozen at -80 °C.

The pellets (nuclear lysates) were incubated on ice for 40 min in NER Reagent with protease inhibitors (Sigma), 100 mM PAO (Sigma) and 1 mM sodium orthovanadate (Sigma). Samples were pelleted by centrifugation at 15000 g for 10 min at 4°C. Nuclear lysates were recovered and frozen at -80 °C.

Reagent extraction volume was concentrated using Nanosep Centrifugal Devices (Pall Corporation, NY, USA). Protein concentrations were determined using BCA protein assay reagent (Pierce Chemical Co. Rockford) with bovine serum albumin as a standard. The fractionated proteins were assayed by Western blot and EMSA.

### Western blot

Cytoplasmic cell lysates (25 µg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblottings were incubated with the following antibodies: human anti-phospho Ser472 AKT, anti-phospho-JNK1/2, anti-phospho Tyr IRS-1 (Santa Cruz Biotechnology, CA, USA) and anti-phospho-ERK 1/2 (Cell Signaling Technology, Boston, USA). The immunoblots were reprobated with human anti-JNK1/2, anti-AKT, anti-IRS-1 (Santa Cruz Biotechnology), and anti-ERK 1/2 (Calbiochem, La Jolla, CA). The equal amount of protein was detected by staining with Ponceau S (Sigma). Immunocomplexes were detected with appropriate horseradish peroxidase-conjugated secondary antibodies and detected by enhanced chemiluminescence (ECL) (GE Healthcare, Little Chalfont, UK). Protein levels were quantified using the image analysis software Quantity One, version 4.4.0 (Bio-rad, Barcelona, Spain). Results were calculated as integrated optical density (IOD) and expressed in arbitrary units (AU).

### Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were tested for NF- $\kappa$ B binding activity, employing consensus oligonucleotides (50-AGTTGAGGGGACTTCCAGGC-30 and 30-TCAACTCCCCTGAAAGGGTCCG-50) (Santa Cruz Biotechnology), using the Digoxigenin EMSA kit from Roche Diagnostics (Basel, Switzerland). 25  $\mu$ g nuclear protein was incubated with digoxigenin labelled NF- $\kappa$ B oligonucleotide in binding buffer containing 100 mM HEPES at pH 7.6, 5 mM Na<sub>2</sub>-EDTA, 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5 mM DTT, 1% (w/v) Tween 20, and 150 mM KCl, together with 1  $\mu$ g of poly[d(I-C)], and poly L-lysine to a final volume of 20  $\mu$ l. After 15 min of incubation at room temperature, the protein-DNA complexes were resolved on native 8% polyacrylamide gel in a 0.5X Tris-borate-EDTA buffer system and run at 200 V for 2 h. Gels were transferred to nylon membranes in a semidry transfer System (Bio-Rad) at 10 V and 300 mA for 30 min. The membranes were exposed to UV-light in a transilluminator for 5 min, and incubated with anti-digoxigenin alkaline phosphatase-conjugated antibody. Complexes were detected with CSPD chemiluminescent substrate (Roche Diagnostics) and exposed to Hyperfilm (GE Healthcare) in a film holder for 4 – 16 h at room temperature.

Antibody supershift assays were performed by incubation of the nuclear proteins with 4  $\mu$ g of polyclonal affinity purified antibodies (Santa Cruz Biotechnology) against NF- $\kappa$ B p50 (H-119), NF- $\kappa$ B p65 (C-20), and NF- $\kappa$ B p52 (K-27) subunits for 30 min on ice before adding the labeled probe. Specific competition control of unlabeled oligonucleotides at 125-fold excess was added to the binding reaction mixture.

#### RNA extraction and quantitative real-time polymerase chain reaction

VAT RNA isolation was performed by homogenization with an ULTRATURRAX T25 basic (IKA Werke GmbH) using Trizol reagent (Invitrogen, Barcelona, Spain). Samples were purified using a RNAEasy Mini kit (QIAGEN, Barcelona, Spain) and treated with DNase (RNase-free DNase Set, Qiagen). For first strand cDNA synthesis, 1  $\mu$ g of total RNA were reverse transcribed using random hexamers (Roche Diagnostic) as primers and Transcriptor Reverse Transcriptase (Roche Diagnostic). Gene expression was assessed by real time PCR using an ABI Prism 7000 Sequence Detection System (*Applied Biosystems, Darmstadt, Germany*), using TaqMan® technology suitable for relative genetic FASN expression quantification. The reaction was performed, following the manufacturers protocol, in a final volume of 25  $\mu$ l. Commercially available and pre-validated TaqMan® primer/probe sets were used as follows: Cyclophilin (*4333763, RefSeq. NM\_002046.3, Cyclophilin A*), used as endogenous control for the target gene in each reaction, TNF $\alpha$  (*Hs00174128\_m1, RefSeq. NM\_000594.2, Tumor Necrosis Factor*), IL-6 (*Hs00174131\_m1, RefSeq. NM\_000600.2, Interleukin 6*), IL-1B (*Hs00174097\_m1, RefSeq. NM\_000576.2, Interleukin 1 beta*), CD11b (*Hs01064804\_m1, RefSeq. NM\_000632.3, integrin, alpha M (complement component 3 receptor 3 subunit)*), PLAU (*Hs00959822\_m1, 2 RefSeq. NM\_001005376.1, NM\_002659.2, plasminogen activator, urokinase receptor*), MCP-1 (*Hs00234140\_m1, RefSeq. NM\_002982.3, chemokine (C-C motif) ligand 2*), CSF-3 (*Hs00357085\_g1, 2 RefSeq. NM\_172219.1, NM\_000759.2, colony stimulating factor 3 (granulocyte)*), IRS1 (*Hs00178563\_m1, RefSeq. NM\_005544.2, Insulin receptor substrate 1*), IRS2 (*Hs00275843\_s1, RefSeq. NM\_003749.2, Insulin receptor substrate 2*), RELA (*Hs00153294\_m1, RefSeq. NM\_021975.2, p65*) and I $\kappa$ B $\alpha$  (*Hs00153283\_m1, RefSeq. NM\_020529.2, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha*).

A threshold cycle (Ct value) was obtained for each amplification curve and a  $\Delta$ Ct value was first calculated by subtracting the Ct value for human PPIA cDNA from the Ct value for each sample and transcript. Fold changes compared with the endogenous control were then determined by calculating  $2^{-\Delta C_t}$ , so FASN expression results are expressed as the expression ratio relative to PPIA gene expression according to the manufacturer's guidelines.

#### Immunohistochemical analysis of adipose tissue

VAT biopsies were obtained during gastric bypass. A portion of each biopsy was fixed overnight at 4°C in 4% paraformaldehyde and then processed for standard paraffin embedding. Section of 4  $\mu$ M were dewaxed and rehydrated according to standard protocols. After



washing slides with PBS 0.1M, pH 7.4 for 15 minutes, the sections were treated with PBS containing 10% methanol and 3% hydrogen peroxide to block endogenous peroxidase activity in dark during 20 min. Then, antigen retrieval procedures was performed (Target Retrieval Solution High pH, Dako, Denmark). Following several washes with PBS, they were exposed overnight to monoclonal mouse anti-human CD68 (Dako). Washed sections were then incubated with the appropriate biotinylated secondary antibodies for 1h (Polyclonal Rabbit Anti-Mouse immunoglobulins/Biotinylated (Dako) and Extravidin-peroxidase (Sigma) for 30 min. Staining was visualized using peroxidase substrate 3,3'-diaminobenzidine (Sigma). Sections were counter stained with Mayer's Hematoxylin (Sigma) and mounting solution and coverslips were added. Slides were observed under an Olympus BX41 microscope (Olympus, UK). Digital images were captured by a camera with an Olympus DP70 digital camera (Olympus, UK).

### Cytokine Determination

The quantitative determination of IL-1 $\beta$  and IL-6 were performed by Milliplex High Sensitivity Human Cytokine Immuno assay (Millipore Corporation, Billerica MA) according to the manufacturers' protocols. Multianalyte profiling was performed on the Luminex-100 XMAPTM Technology (Luminex Corporation).

The xMAP technology (Luminex Corp.) combines the principle of a sandwich immunoassay with fluorescent bead-based technology, allowing individual and multiplex analysis in a single microtiter well [15]. Acquired fluorescence data were analyzed by the Luminex 100 xMAP software (version 2.2).

### Statistical analysis

All data presented are expressed as means  $\pm$  SEM. Statistical analyses were carried out with the statistical software package SPSS (version 17.0; SPSS Inc, Chicago, IL). The data were analyzed by analysis of variance one way (ANOVA). Post hoc statistical analysis was completed by using Duncan contrast statistic. Differences were considered statistically significant at  $p < 0.05$ . The Spearman correlation coefficient was calculated to estimate the linear correlations between variables. The rejection level for a null hypothesis was  $p < 0.05$ .

## RESULTS

### Proinflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ and IL-6 in adipose tissue

We compared the mRNA expression levels of TNF $\alpha$ , IL-1 $\beta$  and IL-6 in visceral adipose tissue obtained from non insulin resistant (NIR-MO) and insulin resistant (IR-MO) morbid obese patients. Real time PCR analysis showed that obesity is associated with high levels of TNF $\alpha$  mRNA expression in adipose tissue independently of their degree of insulin resistance ( $p < 0.05$ ) (Figure 1A). Also, we found that NIR-MO patients expressed low levels of both, IL-1 $\beta$  and IL-6 mRNA compared to IR-MO patients. Of interest levels of IL-1 $\beta$  and IL-6 mRNA in IR-MO were not statistically different from the levels observed in the lean control group. However, the most interesting finding was that both IL-1 $\beta$  and IL-6 mRNA expression levels were markedly elevated in IR-MO compared to NIR-MO (IL-1 $\beta$   $p = 0.001$ ; IL-6  $p = 0.004$ ) (Figure 1B and C). Moreover, we analyzed the protein expression of IL-1 $\beta$  and IL-6 in the visceral adipose tissue from the three populations included in this study. The results showed that IL-1 $\beta$  and IL-6 protein expression was increased in all morbid obese compared with control group. In agreement with the results obtained in the mRNA study, the IR-MO individuals expressed higher levels of IL-1 $\beta$  and IL-6 protein compared with NIR-MO subjects (Figure 1D and E).

### Macrophage infiltration in visceral adipose tissue

Given the increased expression of IL-1 $\beta$  and IL-6 in IR-MO adipose tissue, we hypothesised that these changes may be related to the degree of macrophage infiltration in adipose tissue. In agreement with this hypothesis we found that the expression of macrophage markers cluster of differentiation molecule 11B (CD11b), plasminogen activator urokinase receptor (PLAUR), monocyte chemotactic protein-1 (MCP-1) and colony-stimulating factor 3 (CSF-3) was increased in the obese group compared to lean control (Figure 2A, B, C and D, respectively). More importantly our data also showed that the expression of macrophage markers was increased in the adipose tissue of IR-MO compared to NIR-MO patients ( $p < 0.05$ ) further supporting the concept that macrophage infiltration may be an important determinant factor of the metabolic complications associated with obesity.

Further morphological evidence of the involvement of macrophages was obtained using immunoreactivity for the CD68 marker. The number of macrophages was normalized to 100 adipocytes for comparison between patients. Our data confirm a 5 fold increase in the infiltration of macrophages in visceral adipose tissue of IR-MO patients compared to NIR-MO ( $24.5 \pm 6.5$  vs.  $5.5 \pm 2.1\%$ , respectively). As shown in the figure 2G and H, we also observed that VAT from IR-MO characteristically showed macrophages surrounding adipocytes forming the typical crowns. Conversely, crown structures were hardly seen in the VAT from NIR-MO subjects (Figure 2E and F).

#### **Expression and activation of inflammatory mediators: JNK1/2, ERK 1/2 and NF- $\kappa$ B**

After having demonstrated increased inflammatory profile in adipose tissue from IR-MO patients we then set to investigate the signalling effectors that may affect insulin sensitivity. Our first candidate was JNK kinase. Western blot analyses showed that all obese patients independently of their degree of insulin resistance, had significantly elevated levels of JNK 1 and 2 expression and phosphorylation versus control group. Of note we did not observe significant differences between NIR-MO and IR-MO groups (Figure 3A). As shown in the figure 3A, the increase of the phosphorylation of JNK1/2 observed in all MO group was probably due to the increase of its expression.

Our second candidate was ERK as it has been documented elevated activity of ERK in adipocytes of obese rodents and humans [16]. Indeed there is evidence indicating that ERK may be a regulatory node controlling the inflammation signalling network. Thus we next studied the expression and activation of ERK 1/2 in VAT of our three experimental groups. Our results indicated that ERK 1 and 2 isoforms were not phosphorylated in lean individuals. We found that ERK 1/2 was activated in both obese groups (NIR-MO and IR-MO) compared with control subjects (Figure 3C); however, there was a significant increased of ERK phosphorylation in IR-MO group versus NIR-MO subjects.

We next investigated the NF- $\kappa$ B signalling pathway. Our results indicated that obese subjects had similar mRNA expression levels of the p65 subunit (Rel-A) compared with the non obese subjects (Figure 3D). More interestingly the mRNA expression levels of I $\kappa$ B- $\alpha$  subunit were significantly higher in IR-MO patients versus NIR-MO and non obese subjects (Figure 3F). This result was further supported by the confirmation that the DNA binding of the transcriptionally active NF- $\kappa$ B (p65/p50) not only was elevated in VAT of obese subjects compared with lean individuals, but more importantly that NF- $\kappa$ B activity was significantly higher in IR-MO than in insulin sensitive obese patients (Figure 3E). Changes in NF- $\kappa$ B signalling and levels of adipokines seems to be coordinated as suggested by of the correlation between IL-1 $\beta$  and IL-6 with I $\kappa$ B- $\alpha$  expression levels in visceral adipose tissue of both NIR-MO and IR-MO patients (Figure 4). This correlation was found maintained in both experimental groups separately (NIR-MO and IR-MO) and in total MO group. All correlations coefficients (Rs) and probability values for these correlations are stated in the figure 4.



### Effects on markers of insulin signalling

Many studies have demonstrated that the nutritionally induced insulin resistance is associated with inhibition of specific postreceptor signal transduction steps, including inhibition of insulin receptor substrate (IRS) 1 or 2 functions [1, 17, 18]. We studied the mRNA expression of both IRS-1 and IRS-2 in visceral adipose tissue from lean, NIR-MO and IR-MO subjects. Real time PCR analysis revealed elevated levels of mRNA of IRS-1 in NIR-MO individuals compared with lean control and IR-MO subjects (Figure 5A). Gene expression data were associated with appropriate protein changes as indicated by western blot protein analysis of IRS-1 showing increased levels in NIR-MO patients versus controls and IR-MO individuals (Figure 5B). These changes seemed to be specific for IRS1 since no significant changes in IRS-2 mRNA were observed (data not shown). In addition, it was observed an increase in the tyrosine phosphorylation of the IRS-1 in NIR-MO group compared with lean and IR-MO subjects (Figure 5B). This increase might probably be attributed to the changes in the IRS-1 protein expression.

In support for an increased insulin signalling activity in the NIR-MO population, not only compared to IR-MO but also to lean insulin sensitive individuals, we found an increased expression and activation of insulin regulated kinase AKT in our experimental groups. Western blot analyses showed that NIR-MO subjects had significant elevated levels of AKT expression and phosphorylation compared with non obese and IR-MO subjects (Figure 5C). The elevated phosphorylation of AKT might be due to the changes in the expression levels of this kinase.

### DISCUSSION

In this study, we investigated the paradox of the inappropriately healthy morbid obese subject and provide evidence supportive of the concept that a key factor linking the adipose tissue expansion and associated metabolic complications is the degree of inflammation of the adipose tissue. A particularly unique feature of our study is its focus on the visceral adipose depot. In fact many studies focused on adipose tissue biology and metabolic syndrome tend to focus on the subcutaneous depot given its relatively easy accessibility. However accumulating evidence suggests that is the visceral depot the one that may contribute to the pathogenesis of obesity associated insulin resistance. In this regard our data provide direct evidence of differential macrophage infiltration degree, inflammatory state and insulin resistance markers between visceral adipose tissue from insulin sensitive and insulin resistant obese individuals.

Our data also provided evidence that inflammation does not depend on the level of expansion of the adipose tissue. In fact our data indicated that the degree of expansion is per se not the main determinant of associated inflammation. In some way this data supports our hypothesis stating that the main determinant for inflammation may be related to the remaining capacity for adipose expansion (see review, Virtue and Vidal-Puig) [19]. Moreover, our data are consistent with the hypothesis that the effectors linking obesity and insulin resistance are inflammatory. The data supporting that proinflammatory networks are involved in the obesity associated metabolic complications, are supported by the fact that visceral adipose tissue from insulin resistant obese individuals had more macrophages.

To address this issue we have assembled a cohort of extremely well weight/ metabolic state matched individuals and distributed them in three experimental groups, including two obese populations differing in their level of insulin sensitivity. Comparison of the visceral adipose tissue of these experimental groups has revealed that IR-MO subjects exhibit a more proinflammatory profile than the NIR-MO subjects, and this proinflammatory profile is characterised by increased macrophage infiltration, increased levels of IL-1 $\beta$ , IL-6 and activation of specific proinflammatory signalling ERK and NF- $\kappa$ B which can in our opinion account for the increased insulin resistance state that defined the experimental groups.

Our results indicate that TNF $\alpha$  mRNA levels are increased in parallel with adipose tissue mass expansion, regardless of the insulin sensitivity of the individual. In agreement with others [10, 20], we have also found that obesity per se is associated to increased JNK1/2 expression and phosphorylation compared with non obese patients. Nevertheless, levels of both TNF $\alpha$  and JNK1/2 did not allow discriminating insulin sensitive from insulin resistant obese individuals.

Searching for discriminative markers we investigated IL-1 $\beta$  and IL-6. As in previous studies, we observed that expression of IL-1 $\beta$  is increased in the visceral adipose tissue of obese individuals [21]. However, our study indicated that changes in IL-1 $\beta$  are per se discriminative of the state of insulin resistance associated to obesity. In fact our data provide convincing evidence that changes in IL-1 $\beta$  are restricted to the IR-MO group. Furthermore, no significant differences were observed in NIR-MO, which remained at levels comparable to lean individuals.

Another candidate we evaluated was IL-6. Some studies have suggested its involvement in the pathophysiology of insulin resistance. However, it remains controversial its specific role modulating insulin sensitivity in different tissues such as liver, adipose tissue or skeletal muscle [22]. Here we provided evidence that IL-6 mRNA was increased in visceral adipose tissue of IR-MO patients compared with healthy NIR-MO subjects. In this regard IL-6 and IL-1 $\beta$  followed a similar pattern of regulation as indicated by their correlative changes, that were observed in both NIR-MO and IR-MO subjects. Globally considered our data indicate that IL-1 $\beta$  and IL-6 might act as important effectors of the insulin resistance associated to obesity. More importantly we speculate that measurements of these adipokines might be of some use as diagnostic and therapeutic predictors of obesity-associated metabolic complications.

Our direct comparison between equally obese individuals with different degrees of insulin resistance also provided interesting data at molecular level. For instance we confirmed that elevated activity of ERK was observed in adipocytes of obese and insulin-resistant rodent and humans [23]. However our data indicate that these effects on ERK signalling are more directly related to the degree of insulin resistance than to the expansion of fat mass. In fact, similarly to IL-1 $\beta$ , and IL6, ERK was also more activated in IR-MO patients than in NIR-MO subjects.

How these changes may relate to the state of insulin resistance? Recently it has been shown that IL-1 $\beta$  reduces IRS-1 expression and prevents AKT activation through a mechanism that is partly mediated by ERK activation [24]. Our results indicated that IRS-1 expression and AKT activation were increased in visceral adipose tissue from the insulin sensitive healthy obese patients (NIR-MO) whose adipose tissue had low levels of IL-1 $\beta$ , IL-6 and ERK activation. Conversely the insulin resistant obese group (IR-MO), was characterised by high expression of IL-1 $\beta$ , IL-6, and decreased IRS-1 and AKT expression and activation compared with NIR-MO patients. Globally considered, these data suggest an important role of IL-1 $\beta$ , IL-6 and ERK activation as discriminators between insulin sensitive and insulin resistant obese individuals and as important determinants of inhibition of insulin signalling in human visceral adipose tissue.

IRS-1 is a relevant modulator of insulin signalling in adipose tissue [25]. Our data suggest that IRS-2, whose expression was unchanged in our three experimental groups, may not be an important determinant of obesity associated insulin resistance at least in visceral adipose tissue. Interestingly, our data also suggest that our NIR-MO patients may be in a state of facilitated insulin signalling. In fact NIR-MO patients had increased IRS-1 mRNA expression in VAT compared not only with obese insulin resistant individuals but even with lean individuals. This maintained insulin signalling may contribute to the paradox of inappropriately healthy carbohydrate metabolism in these individuals.

Our insulin resistant obese group showed a decrease in the expression of IRS-1. Moreover, in our study, we found an inverse correlation in vivo between the levels of IL-1 $\beta$  and those of IRS-

1 but not with IRS-2, which corroborate recent in vitro studies, showing that IL-1 $\beta$  promotes a decrease in the amount of IRS-1 but not IRS-2 expression in both 3T3-L1 and human adipocytes [24]. Our data outline the relevance of IRS-1 as a potential mode of control in obesity associated insulin resistance.

Our results suggest that obesity-associated insulin resistance is related to inflammation as indicated by nuclear accumulation of transcription nuclear factor  $\kappa$ B. This agrees with the observation that obesity associates with an increase in NF- $\kappa$ B DNA binding activity [26]. In addition, our results indicated that insulin sensitive obese individuals have low levels of NF- $\kappa$ B activation compared to insulin resistant patients. In contrast, expression of mRNA p65 (RELA) subunit levels does not seem to be affected by the degree of obesity and/or insulin resistance, as the three groups (non obese, NIR and IR-MO) exhibited similar levels. These results agree with the recent observation that circulating mononuclear cells of obese individuals presented elevated levels of mRNA p105 NF- $\kappa$ B subunit, but similar levels of p65 (RELA) when compared with non obese subjects [27]. We also observed significantly higher levels of I $\kappa$ B- $\alpha$  in visceral adipose tissue from IR-MO individuals than in NIR-MO and non obese subjects. That data are in accordance with previous observations, indicating that NF- $\kappa$ B activation was positively correlated with an increase of I $\kappa$ B- $\alpha$  mRNA levels in circulating mononuclear cells of obese patients [28, 29]. Altogether these results suggest that NF- $\kappa$ B may be an important effector of the proinflammatory response associated to obesity related metabolic complications.

Globally considered our results supports the concept that the development of insulin resistance associated to obesity depends on the activation of inflammatory cascade and that IL-6 and IL-1 $\beta$ , ERK and NF- $\kappa$ B seems to be important effectors mediating the inflammation effects promoting insulin resistance.

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## FIGURE LEGENDS

**Figure 1 mRNA and protein expression of proinflammatory cytokines in visceral adipose tissue of non obese, NIR-MO and IR-MO subjects.** (A, B and C) mRNA expression of TNF $\alpha$ , IL-1 $\beta$  and IL-6. Total RNAm were extracted from adipose tissue and subjected to PCR real-time amplification using TaqMan® technology suitable for relative genetic FASN expression quantification. (D and E) Protein expression of IL-1 $\beta$  and IL-6. The quantitative determination of IL-1 $\beta$  and IL-6 were performed by Milliplex High Sensitivity Human Cytokine Immuno assay (Millipore Corporation, Billerica MA) according to the manufacturers' protocols. The panels show the mean  $\pm$  SEM from 12 subjects of each group. Significant differences (Duncan;  $p < 0.05$ ) are indicated with different words.

**Figure 2 Presence of infiltrated macrophages in visceral adipose tissue from non obese, NIR-MO and IR-MO subjects.** (A, B, C and D) CD11b, PLAUR, MCP-1 and CSF-3 mRNA expression. Total RNAm were extracted from adipose tissue and subjected to PCR real-time amplification using TaqMan® technology suitable for relative genetic FASN expression quantification. The panels show the mean  $\pm$  SEM from 12 subjects of each group. Significant differences (Duncan;  $p < 0.05$ ) are indicated with different words. (E, F, G and H) Immunohistochemical detection of CD68+ macrophages in visceral adipose. Macrophage infiltration was examined through immunostaining with human anti-CD68. Representative photographs of visceral adipose tissue from 12 IR-MO subjects and 12 NIR-MO patients with similar results.

**Figure 3 Inflammatory signalling in visceral adipose tissue from non obese, NIR-MO and IR-MO individuals.** (A) Expression and phosphorylation of JNK 1/2 isoforms. (B) Equal amount of protein was detected by staining with Ponceau S. (C) Expression and activation of ERK1/2 isoforms. Cell lysates from visceral adipose tissue were prepared and cytoplasmic proteins (25 $\mu$ g/lane) were electrophoresed on a 10% SDS/PAGE gels and then transferred to nitrocellulose membranes. The membranes were probed with human anti-phospho-JNK1/2, anti-phospho-ERK1/2, anti-JNK1/2 and anti-ERK1/2 antibodies. The panels show two representative samples of each group with similar results. Graphs show the relative integrated optical density (IOD) values of the ratio of phosphoprotein to total protein of the respective blots, mean  $\pm$  SEM from 12 subjects of each group. Significant differences (Duncan;  $p < 0.05$ ) are indicated with different words. (D and F) mRNA expression of NF- $\kappa$ B (p65) and I $\kappa$ B, total RNAm were extracted from adipose tissue and subjected to PCR real-time amplification using TaqMan® technology suitable for relative genetic FASN expression quantification. The panels show the mean  $\pm$  SEM of 12 subjects. Significant differences (Duncan;  $p < 0.05$ ) are indicated with different words. (E) Activation of NF- $\kappa$ B in the three groups studied. Nuclear fractions (25  $\mu$ g) were incubated with digoxigenin-labelled oligonucleotides, corresponding to a NF- $\kappa$ B consensus sequence. Complexes were subjected to native PAGE (8 %), transferred into nylon membranes and incubated with anti-digoxigenin alkaline phosphatase-conjugated antibody. The panel shows two representative samples of each group with similar results.

**Figure 4 Positive correlations among IL-1 $\beta$ , IL-6 and I $\kappa$ B- $\alpha$  mRNA expression.** Correlations were assessed by Spearman product-moment correlation. Differences were considered significant if  $p < 0.05$ .

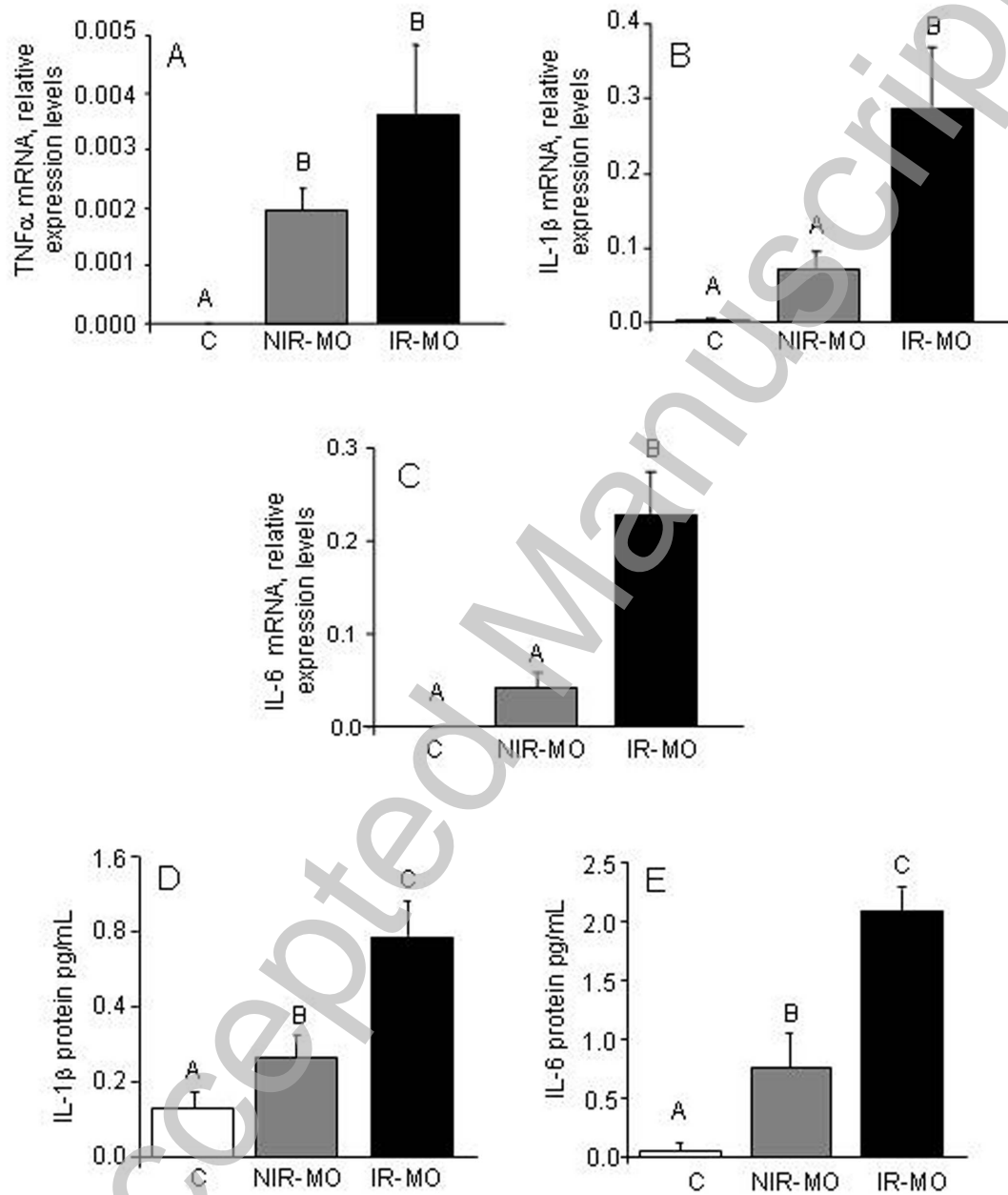


**Figure 5 IRS-1 and AKT activation in visceral adipose tissue from non obese, NIR-MO and IR-MO subjects.** (A) mRNA expression levels of IRS-1. Total mRNA were extracted from adipose tissue and subjected to PCR real-time amplification using TaqMan® technology suitable for relative genetic FASN expression quantification. The panels show the mean  $\pm$  SEM of 12 subjects. Significant differences (Duncan;  $p < 0.05$ ) are indicated with different words. (B) Expression and phosphorylation of IRS-1. Cell lysates from visceral adipose tissue were prepared and cytoplasmic proteins (100  $\mu$ g/lane) were electrophoresed on 6% SDS/PAGE gels and then transferred to nitrocellulose membranes. The membranes were probed with human anti-phosphotyrosine-IRS-1 and anti-IRS-1 antibodies. The upper panel shows two representative samples of each group with similar results. The lower panel shows the relative integrated optical density (IOD) values of the ratio of phosphotyrosine-IRS-1 to IRS-1 of the respective blots, mean  $\pm$  SEM from 12 subjects of each group. Significant differences (Duncan;  $p < 0.05$ ) are indicated with different words. (C) Expression and phosphorylation of AKT. Cytoplasmic proteins (25  $\mu$ g/lane) were electrophoresed on an 8% SDS/PAGE gels and then transferred to nitrocellulose membranes. The membranes were probed with human anti-phospho-Ser472 AKT and anti-AKT antibodies. The panel shows two representative samples of each group with similar results. Graph show the relative integrated optical density (IOD) values of the ratio of phospho-Ser472 AKT to total AKT of the respective blots, mean  $\pm$  SEM from 12 subjects of each group. Significant differences (Duncan;  $p < 0.05$ ) are indicated with different words.

Table 1. Baseline biological characteristics of the healthy persons and morbidly obese patients

	Controls	NIR-MO	MO-IR
Male/female, n/n	6/6	6/6	6/6
Age, years	40.66 ± 3.62	46.63 ± 4.53	37.00 ± 3.13
Weight, Kg	65.00 ± 3.92	144.34 ± 7.89	154.88 ± 5.34
Height, cm	168.83 ± 0.03	161.67 ± 1.94	166.55 ± 3.26
BMI, Kg/m <sup>2</sup>	22.57 ± 0.84	55.00 ± 2.20	55.85 ± 1.32
Waist circumference, cm	80.08 ± 3.18	141.88 ± 6.04	147.80 ± 4.70
Hip circumference, cm	91.54 ± 3.72	156.63 ± 6.7	154.64 ± 2.89
Waist to Hip ratio	0.87 ± 0.02	0.91 ± 0.01	0.94 ± 0.03
Serum insulin, UI/ml	7.19 ± 0.41	14.37 ± 1.73	45.46 ± 2.44
HOMA-IR	1.44 ± 0.11	3.31 ± 0.32	11.48 ± 0.68
Serum glucose, mg/dl	80.25 ± 2.96	98.50 ± 4.18	104.18 ± 2.93
Serum cholesterol, mg/dl	196.00 ± 11.01	199.65 ± 13.78	196.57 ± 10.05
HDL cholesterol, mg/dl	58.75 ± 5.10	49.00 ± 5.91	41.18 ± 4.67
LDL cholesterol, mg/dl	117.75 ± 9.06	125.81 ± 13.13	118.02 ± 12.44
Triglycerides, mg/dl	78.83 ± 9.82	115.62 ± 13.98	159.18 ± 33.50
GOT, U/l	22.97 ± 1.55	20.85 ± 2.59	20.60 ± 1.72
GPT, U/l	39.67 ± 2.84	33.12 ± 4.82	38.20 ± 3.60
GGT, U/l	32.25 ± 4.89	52.12 ± 21.81	34.81 ± 4.06

<sup>1</sup> Values are means ± SEM, n= 12. HDL-c= High density lipoprotein-cholesterol; LDL-c= Low density lipoprotein-cholesterol; GPT = Glutamic pyruvate transaminase; GGT= Gamma glutamil transferase; GOT = Glutamic oxalacetic transaminase



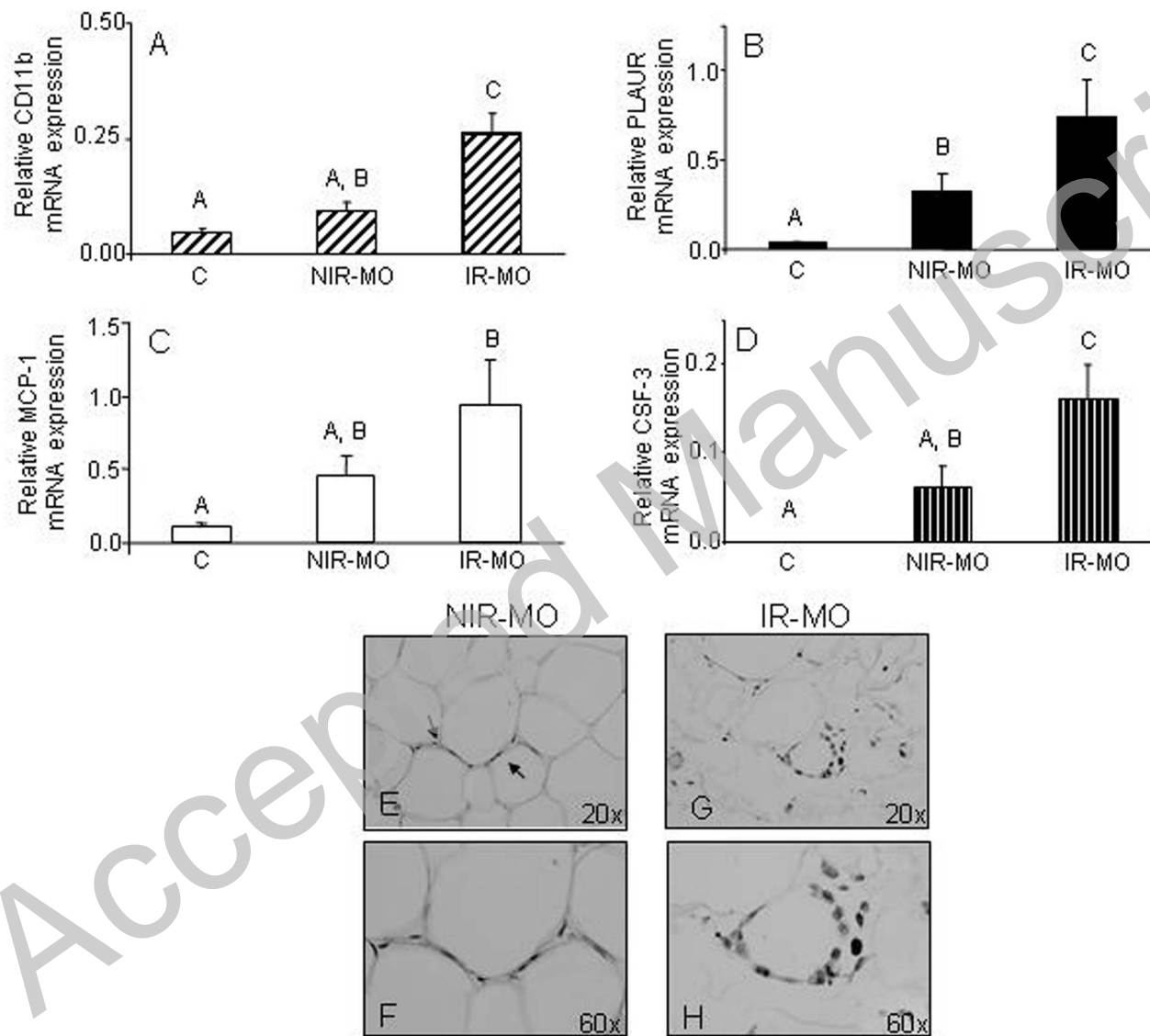
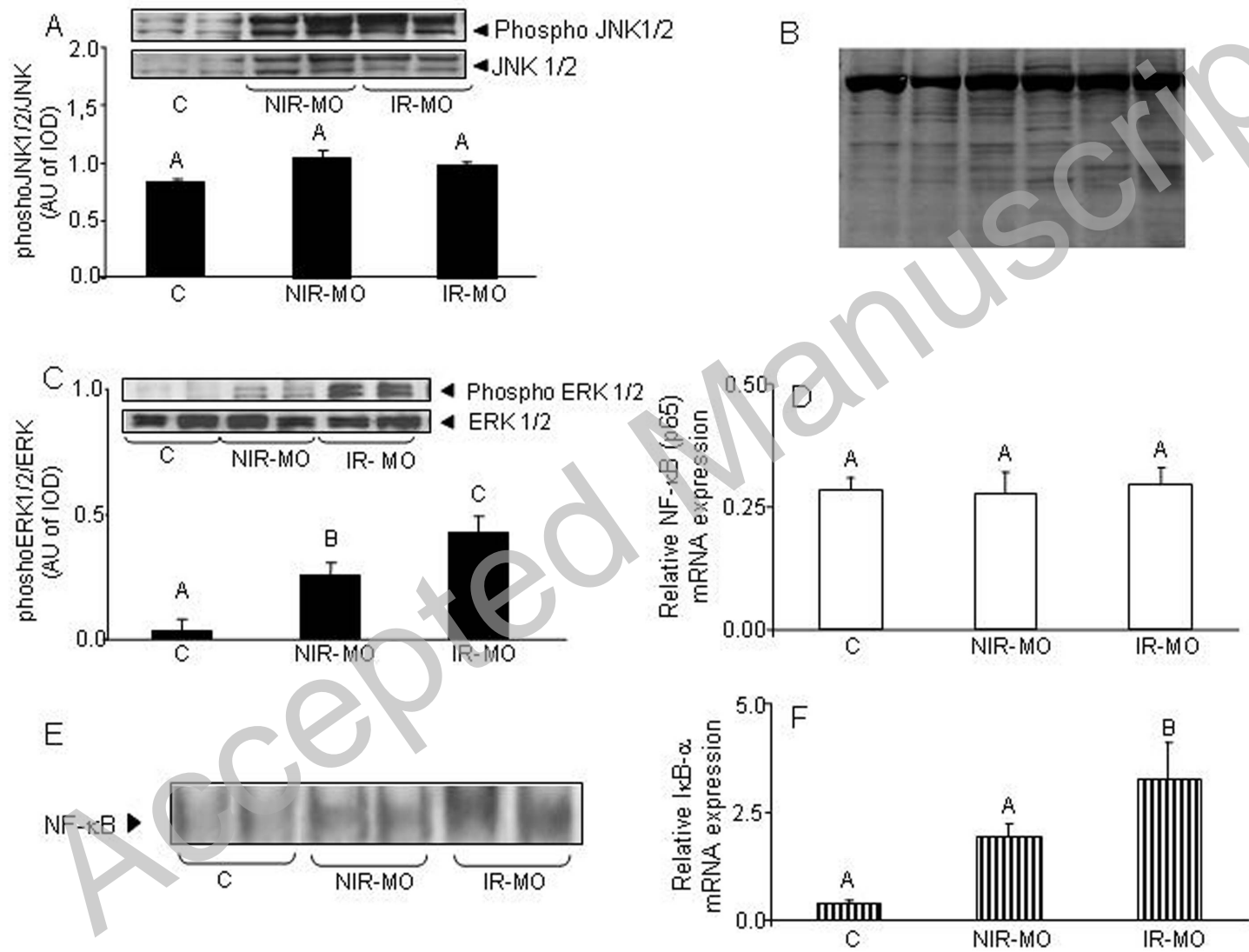
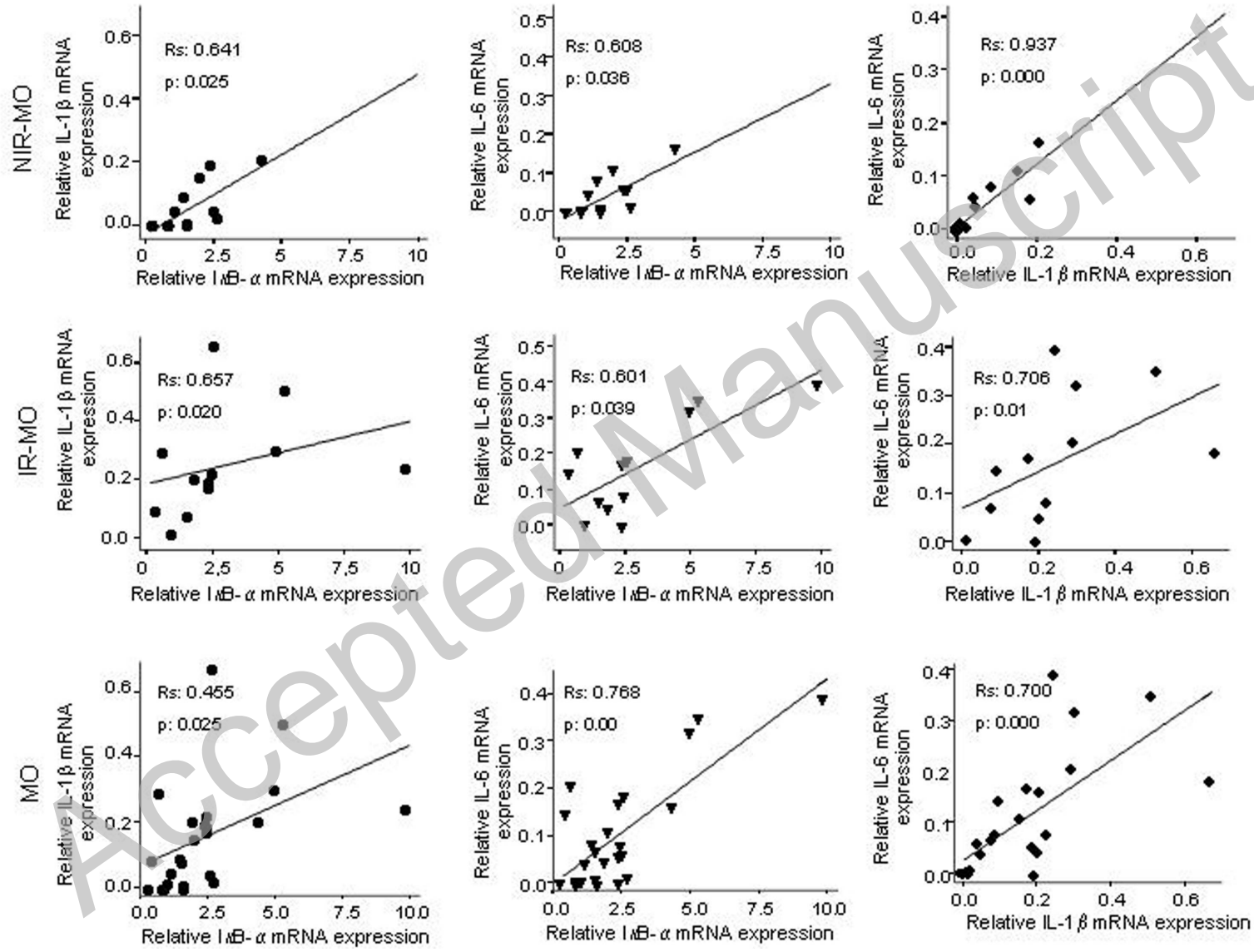


Figure 2







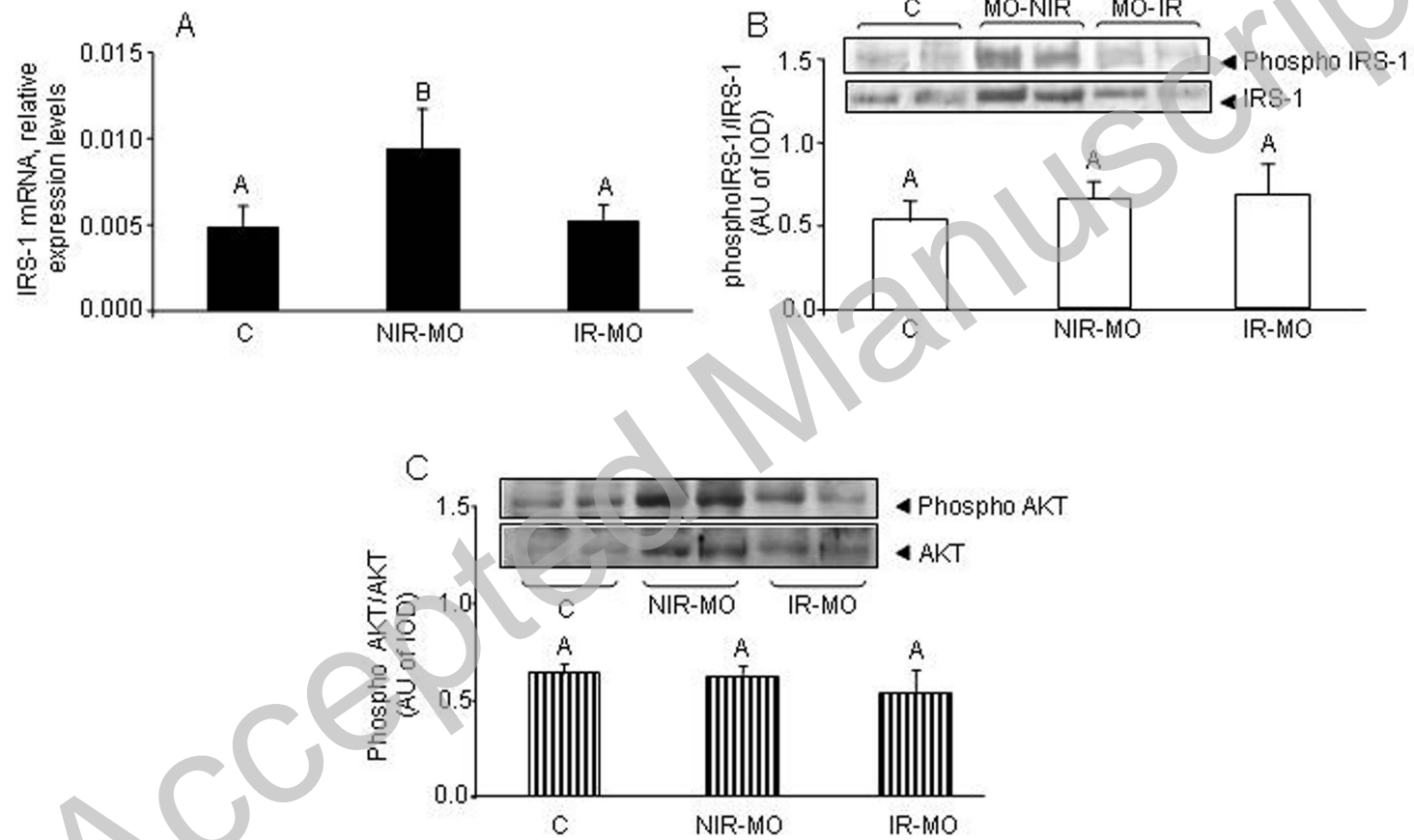


Figure 5