



# Insight into mechanism of in vitro insulin secretion increase induced by antipsychotic clozapine: Role of FOXA1 and mitochondrial citrate carrier

A. Menga<sup>a</sup>, V. Infantino<sup>d</sup>, F. Iacobazzi<sup>a</sup>, P. Convertini<sup>a</sup>,  
F. Palmieri<sup>a,b,c</sup>, V. Iacobazzi<sup>a,b,c,\*</sup>

<sup>a</sup>Department of Biosciences, Biotechnology and Pharmacological Sciences, University of Bari, Via Orabona 4, 70125 Bari, Italy

<sup>b</sup>Center of Excellence in Comparative Genomics, University of Bari, via Orabona 4, 70125 Bari, Italy

<sup>c</sup>CNR Institute of Biomembranes and Bioenergetics, Bari, Italy

<sup>d</sup>Department of Sciences, University of Basilicata, 85100 Potenza, Italy

Received 3 July 2012; received in revised form 3 August 2012; accepted 20 August 2012

## KEYWORDS

Clozapine;  
Gene expression;  
FOXA1;  
Silencing;  
Antipsychotics;  
Citrate carrier;  
Insulin secretion

## Abstract

The use of clozapine and other antipsychotic drugs is known to be associated with a number of adverse metabolic side effects, including diabetes mellitus. These side effects could be, at least in part, the result of impaired islet cell function and abnormal insulin secretion, although the underlying mechanisms are unknown. The aim of this study is the identification of targets for clozapine related to the abnormal insulin secretion. We identify a specific activation of the transcriptional factor FOXA1, but not FOXA2 and FOXA3, by clozapine in HepG2 cells. Clozapine enhances FOXA1 DNA-binding and its transcriptional activity, increasing mitochondrial citrate carrier gene expression, which contains a FOXA1 site in its promoter. Haloperidol, a conventional antipsychotic drug, does not determine any increase of FOXA1 gene expression. We also demonstrate that clozapine upregulates FOXA1 and CIC gene expression in INS-1 cells only at basal glucose concentration.

In addition, we find that abnormal insulin secretion in basal glucose conditions could be completely abolished by FOXA1 silencing in INS-1 cells treated with clozapine.

The identification of FOXA1 as a novel target for clozapine may shed more light to understand molecular mechanism of abnormal insulin secretion during clozapine treatment.

© 2012 Elsevier B.V. and ECNP. All rights reserved.

\*Corresponding author at: Department of Biosciences, Biotechnology and Pharmacological Sciences, University of Bari, Via Orabona 4, 70125 Bari, Italy. Tel.: +39 80 5442322; fax +39 80 5442770.

E-mail address: vito.iacobazzi@uniba.it (V. Iacobazzi).

## 1. Introduction

Clozapine is the prototype of atypical antipsychotic drugs and has been described as a drug of superior efficacy in

otherwise treatment-resistant schizophrenia (Conley et al., 1999; Freedman, 2003). However, subjects treated with clozapine show a high risk of developing different metabolic side effects, such as dyslipidemia, body weight gain, glucose homeostasis disturbance, abnormal insulin secretion and diabetes as compared to the general population (Jin et al., 2004; Raeder et al., 2006; Scheen and De Hert, 2007; Vestri et al., 2007; Kessing et al., 2010). Since insulin regulates lipid and glucose metabolism, the effect of clozapine on insulin secretion and/or on insulin action, at least in part, might explain its capability to induce metabolic disturbances. Regarding the insulin secretion during clozapine treatment, it is unknown whether the insulin levels are increased as secondary effect to drug-induced peripheral resistance or due to a direct effect of the agent on the pancreatic  $\beta$ -cells, or caused by both actions (Yazici et al., 1998; Melkersson et al., 1999). A naturalistic study of clozapine treatment reported that 30–40% of patients received a diagnosis of type II diabetes during the 5- and 10-year follow-ups (Henderson et al., 2000, 2005).

However, despite the number of clinical reports, the molecular mechanism by which clozapine or other antipsychotic drugs, although to a different extent, could cause dysregulation of insulin release and diabetes is still unknown. In general, molecular basis of onset of the metabolic disturbance has been poorly investigated. Only clozapine-induced activation of the sterol regulatory element-binding protein (SREBP) transcription factors related to cellular lipogenesis has been reported (Fernø et al., 2006, 2009). Therefore, it is likely that different genes and related transcriptional factors, important in lipid and carbohydrate homeostasis, in insulin secretion and in  $\beta$ -cells function, might be affected by clozapine treatment. For example, metabolic and secretory features of  $\beta$ -cell are maintained by FOXA1 and FOXA2 (Gao et al., 2010). Members of the winged helix/forkheadbox (FOX) transcription factors subfamily, FOXA1, FOXA2 and FOXA3, play important roles in both metabolism and homeostasis through the regulation of multiple target genes in the liver, pancreas and adipose tissue (Friedman and Kaestner, 2006). FOXA2 mediates fasting responses, including fatty acid oxidation, ketogenesis, gluconeogenesis and increased lipoprotein secretion (Wolfrum et al., 2004; Friedman and Kaestner, 2006; Gao et al., 2010; Convertini et al., 2011). FOXA3 also regulates glucose homeostasis during prolonged fast through maintenance of GLUT2 and gluconeogenic gene expression (Shen et al., 2001). FOXA1 induces glucagon gene expression and insulin secretion in pancreatic cells (Shih et al., 1999; Vatamaniuk et al., 2006). Moreover, FOXA1 plays an important role in regulation of mitochondrial citrate carrier gene expression (CIC) and in insulin secretion (Iacobazzi et al., 2009a).

CIC is an integral inner mitochondrial membrane protein belonging to the mitochondrial carrier protein family SLC25 (Palmieri, 2004, 2012) that catalyzes the export of citrate from the mitochondrial matrix in exchange for cytosolic malate (Bisaccia et al., 1989, 1990). This transporter is essential for fatty acid biosynthesis because citrate in the cytosol is cleaved to acetyl-CoA and oxaloacetate by citrate lyase. Acetyl-CoA is directly used for fatty acid synthesis, and oxaloacetate produces NADPH plus  $H^+$  (also necessary for fatty acid production) via malate dehydrogenase and

malic enzyme (Kaplan, 2001; Palmieri, 2004). Epigenetic mechanisms and various transcriptional factors regulate human CIC gene expression (Infantino et al., 2007, 2011a; Iacobazzi et al., 2008, 2009a, 2009b). Furthermore, CIC has been found to be involved in the control of glucose-stimulated insulin secretion (Joseph et al., 2006).

This study investigates and identifies transcriptional factors and related genes, particularly involved in insulin secretion, affected by clozapine treatment. We demonstrate for the first time that clozapine, but not haloperidol, specifically upregulates FOXA1 and its regulated CIC gene expression and that FOXA1 silencing completely abolishes the abnormal insulin secretion in INS-1 cells.

## 2. Experimental procedures

### 2.1. Construction of plasmids

For heterologous promoter expression, a threefold repeat FOXA site (5'-CCTGGACAATATTATTTTGGCTG-3') was cloned into the pGL3 promoter-LUC vector (Promega) upstream of the SV40 basal promoter (Iacobazzi et al., 2009a). For lentivirus-based RNAi experiments, the rat FOXA1 mRNA sequence CTAACCCCTTGCTTGAAAT from 2098 to 2116 bp (Accession no. NM\_012742.1) was selected as a hairpin-loop structure by using the siRNA design tool (<http://jura.wi.mit.edu/bioc/siRNAext/home.php>). This sequence was cloned in the lentivirus RNAi transfer plasmid pLKO.1 (Sigma) to obtain the pLKO.1-FOXA1 lentivirus silencing construct (Iacobazzi et al., 2009a).

### 2.2. Cell culture, RNA interference, and transient transfection

INS-1 cells (gift from Dr. P. Maechler) were grown in RPMI 1640 medium (Roswell Park Memorial Institute) supplemented with 10 mM Hepes, 5% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50  $\mu$ M  $\beta$ -mercaptoethanol, 100 U penicillin, and 100  $\mu$ g/ml streptomycin at 37 °C in 5% CO<sub>2</sub> (Merglen et al., 2004). HepG2 cells (Sigma) were grown in high glucose DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U penicillin, and 100  $\mu$ g/ml streptomycin at 37 °C in 5% CO<sub>2</sub>. HEK293T cells (Sigma) were grown similarly to the HepG2 cells, except for the L-glutamine concentration (4 mM). Transient transfection was performed as reported (Iacobazzi et al., 2005) using 0.5  $\mu$ g of pGL3 promoter-LUC vector containing the CIC gene FOXA site. The extent of transfection was normalized by  $\beta$ -galactosidase activity (Infantino et al., 2011b). In RNA interference experiments, the specific pre-designed small interfering RNA (siRNA) targeting human FOXA1 (s6689, Ambion), was transfected in HepG2 cells using siPORT<sup>TM</sup> NeoFX<sup>TM</sup> Transfection Agent (Ambion). A siRNA (Cat. no. C6A-0126, Ambion) with no significant similarity to human, mouse, or rat gene sequences was used as negative control. To generate lentivirus, HEK293T cells were transfected with 0.5  $\mu$ g of pLKO.1-FOXA1 (or control pLKO.1 vector) and 10  $\mu$ l of MISSION Lentiviral Packaging Mix (Sigma) by using Eugene HD (Roche). Viruses were harvested 48 and 72 h after transfection. Viral titer was measured by the HIV p24 Antigen enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix). Transduction of INS-1 cells was performed with recombinant pLKO.1-FOXA1 lentivirus at a viral titer of approximately 10<sup>5</sup> transducing units/ml in the presence of 10  $\mu$ g/ml polybrene/10 mM Hepes, pH 7.4. An empty lentivirus construct used at the same titer served as control (Iacobazzi et al., 2009a).

### 2.3. Drug exposure

Clozapine and haloperidol (Sigma Aldrich, St. Louis, USA) were dissolved in DMSO. HepG2 and INS-1 cells were plated and cultured overnight at a concentration of  $10^6$  cells (2 ml) per well (six-well format). Cells were treated with 25  $\mu$ M clozapine (up to 48 h) or 25  $\mu$ M haloperidol (24 h) or vehicle (DMSO) equal to solvent (Vik-Mo et al., 2009). The cells were detached by trypsinization, pelleted by centrifugation (12000 rpm; 5 min) and placed at  $-80^\circ\text{C}$  prior to total RNA extraction. For western blotting experiments cells were grown up to approximately 80% confluence before exposing to the drug for 48 h. The effect of clozapine and haloperidol was investigated in culture medium containing 3 mM or 17.5 mM glucose. Twenty-four hours before drug treatment, cells were pre-incubated in culture medium containing 3 mM glucose instead of 11 mM glucose as in the ordinary medium.

### 2.4. Insulin secretion assays

Insulin secretion assays were performed as reported by Hohmeier et al. (2000). Briefly, INS-1 cells were seeded onto 24-well plates and grown to 100% confluence. Twenty four hours before assay, cells were maintained in culture medium containing 5.5 mM glucose instead of 11 mM glucose as in the ordinary medium and exposed to 25  $\mu$ M clozapine. Then the cells were washed in 0.5 ml Hanks' balanced salt solution (HBSS) glucose-free, followed by 1 h pre-incubation in 0.5 ml HBSS glucose-free buffer. Subsequently, they were detached from the wells by trypsinization, counted by use of a counting-chamber, seeded onto new 24-well plates at a concentration of  $2 \times 10^5$  cells/ml and incubated for 2 h in 0.5 ml HBSS containing 3 or 17.5 mM glucose. For determination of total insulin secretion, samples were analyzed by the High Range Rat Insulin ELISA (EIA-3985, DRG Diagnostics, Germany).

### 2.5. Cell viability

Cell viability was evaluated by the CellTiter 96<sup>®</sup> Non-Radioactive Cell Proliferation Assay (Promega). In brief, HepG2 and INS-1 cells were seeded onto 96-well microtiter plates and treated with 25  $\mu$ M clozapine up to 48 h. After incubation, 15  $\mu$ l of the Dye solution was added to each well and the cells were incubated for 4 h at  $37^\circ\text{C}$ . Subsequently, solubilization solution/stop mix (100  $\mu$ l) was added to each well and the cells were incubated for 1 h at  $37^\circ\text{C}$  to promote the solubilization of formazan crystals. The level of MTT formazan was determined by measuring its absorbance at 570 nm using a 96-well plate reader (Victor<sup>3</sup>, PerkinElmer).

### 2.6. Other methods

Electrophoretic mobility shift assays (EMSA) were performed using biotin 3'-end labeled DNA probe from  $-1104$  to  $-1081$  bp region of the human CIC gene promoter. Probes were incubated with 10  $\mu$ g of HepG2 nuclear extracts (exposed for 24 h to 25  $\mu$ M clozapine) for 20 min at room temperature. The remaining steps follow the Light Shift chemiluminescent EMSA Kit protocol (Pierce). Total RNA was extracted from  $1 \times 10^6$  HepG2 and INS-1 cells, and reverse-transcription was performed as reported (Infantino et al., 2011b). Real-time PCR was conducted as previously described (Infantino et al., 2011c). Assay-on-demand for human and rat CIC (Hs00761590\_m1 and Rn00820906\_g1, respectively), human and rat FOXA1 (Hs00293689\_s1 and Rn00562516\_m1, respectively), human FOXA2 (Hs00232764\_m1), human FOXA3 (Hs00270130\_m1), and human and rat  $\beta$ -actin (4326315E and 4352340E, respectively) were purchased from Applied Biosystems. All transcript levels were normalized against the  $\beta$ -actin expression levels. For western blotting analysis, proteins were electroblotted onto nitrocellulose

membranes (Bio-Rad) subsequently treated with anti-CIC (Infantino et al., 2011a), anti- $\beta$ -actin (Santa Cruz Biotechnology) or anti-FOXA1-2-3 (Aviva Systems Biology) antibodies. The immunoreaction was detected by the Immobilon western ECL system (Millipore).

## 3. Results

### 3.1. Identification of FOXA1 as target for clozapine

The finding that clozapine increased basal (low glucose level) insulin secretion from pancreatic cells (Melkersson, 2004) led us to study this effect at molecular level. Our investigation was focused on FOXA family members, since it is known that FOXA1 and FOXA2 transcription factors play a central role in glucose and lipid homeostasis and maintain the metabolic and secretory features of  $\beta$ -cells. Moreover, inducible ablation of both transcription factors in  $\beta$ -cells leads to impaired glucose homeostasis and insulin secretion (Gao et al., 2010).

First of all we examined the effects of clozapine on FOXA members expression in HepG2 cells. The choice of drug concentration was based on the previous works (Fernø et al., 2006; Raeder et al., 2006; Vik-Mo et al., 2009) that report a concentration of 25  $\mu$ M clozapine used for cell cultures. The concentration takes also into consideration that a higher clinical dosage is used for clozapine compared to other antipsychotic drugs (Vik-Mo et al., 2009). Cells were treated with 25  $\mu$ M clozapine for 24 h and then used to quantify FOXA1, FOXA2 and FOXA3 mRNAs and proteins by real-time PCR and western blotting, respectively. Clozapine induced a remarkably specific increase only of FOXA1 mRNA and protein, whereas no effect was exerted on FOXA2 and FOXA3 expression levels (Figure 1A and B).

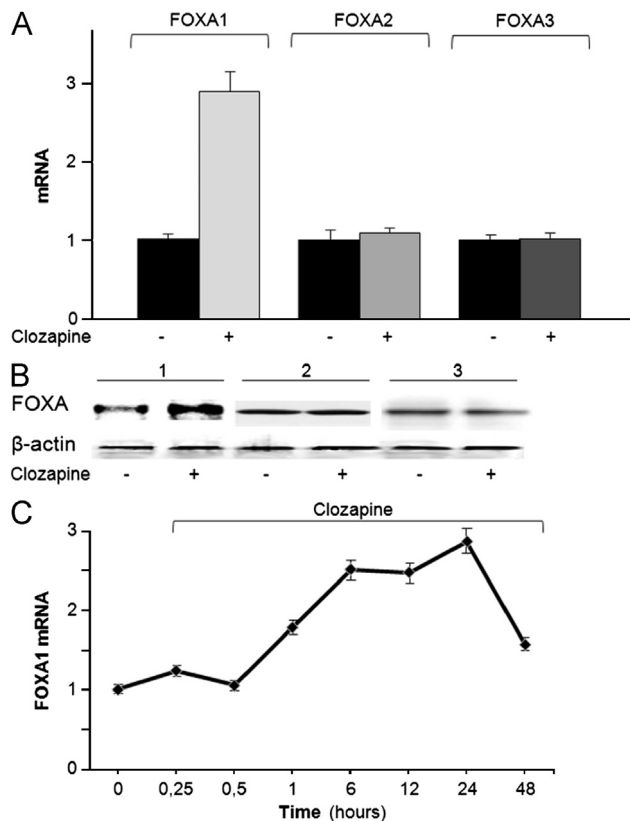
To verify the time-extent of FOXA1 activation, we used real-time PCR to examine the time course of FOXA1 expression in clozapine exposed HepG2 cells up to 48 h. A progressive increase of FOXA1 expression was clearly evident after 30 min up to 6 h. No further increase from 6 to 12 h was observed followed by a slight increase at 24 h. A decrease of FOXA1 expression was evident at 48 h (Figure 1C).

These data clearly indicate that clozapine specifically affects expression of FOXA1 and that this effect is still high up to 24 h.

### 3.2. Clozapine enhances FOXA1 DNA-binding and FOXA1 transcriptional activity

Next we checked whether clozapine affects transcriptional activity of FOXA1. We used the CIC gene as target since its promoter contains a FOXA1 site (Iacobazzi et al., 2009a). By EMSA experiments, we checked whether clozapine affects binding of FOXA1 to its responsive sequence present in the CIC gene promoter. Incubation of biotinylated probe with HepG2 nuclear extracts in the presence of clozapine resulted in a very strong hybridization band (Figure 2A, lane 4) compared to the band intensity when clozapine was absent (Figure 2A, lane 2). Competition experiments with a large excess of not biotinylated probe in the presence or absence





**Figure 1** Effect of clozapine on expression of FOXA members (A and B) and time course of expression (C). (A) HepG2 cells were seeded onto 6-well plates, grown to 50% confluence and then treated with 25  $\mu$ M clozapine or vehicle for 24 h. FOXA1, FOXA2 and FOXA3 were quantified by real-time PCR. mRNAs measured in untreated cells were set at 100%. Data are shown as means  $\pm$  SD of five duplicate independent experiments. Only the differences between FOXA1 mRNA sample in the presence of clozapine (gray bars) and relative controls (black bars) were significant ( $P < 0.05$ , one-way ANOVA). (B) FOXA1, FOXA2 and FOXA3 and  $\beta$ -actin proteins of HepG2 cells treated or untreated with clozapine for 48 h were immunodecorated with specific antibodies. The result is representative of three independent experiments. (C) HepG2 cells were seeded onto 6-well plates, grown to 50% confluence and then treated with 25  $\mu$ M clozapine for 0.25, 0.5, 1, 6, 12, 24 and 48 h. Completed the time of exposure, except for 24 h and 48 h time-points, the cells were suspended in fresh medium up to 24 h. Subsequently, the clozapine-treated cells were lysed and used to quantify FOXA1 mRNA by real-time PCR. The relative level of FOXA1 gene expression was normalized against vehicle exposed culture at each time point. Data are shown as means  $\pm$  SD of five duplicate independent experiments.

of clozapine resulted in disappearance of hybridization band (Figure 2A, lanes 3 and 5, respectively).

Having established that clozapine increases FOXA1 binding to its responsive element, next we determined the clozapine-induced transcriptional activity of FOXA1. A threefold repeat FOXA1 site used in EMSA experiment was cloned into pGL3 promoter-LUC (pGL3 3xFOXA-LUC) vector and used to transfect HepG2 cells. After 24 h clozapine was added or not and

cells were incubated for further 24 h. In a parallel experiment HepG2 cells were transduced with siRNA targeting human FOXA1, before transfection with pGL3 3xFOXA-LUC and clozapine treatment. After these treatments luciferase activity was measured. Clozapine treatment increased luciferase activity by about 100% in unsilenced HepG2 cells, whereas in FOXA1 silenced cells, luciferase activity was unaffected (Figure 2B).

Finally, we examined the effect of FOXA1 upregulation on CIC mRNA and protein by real-time PCR and western blotting, respectively. CIC mRNA extracted from HepG2 cells treated with clozapine showed an increase of about 80% with respect to control cells (Figure 2C). A parallel increase of CIC protein was observed by western blotting experiments performed on cell lysate (Figure 2D).

All these data show that clozapine increases FOXA1 binding to its responsive sequence present in the CIC gene promoter inducing an augment of transcriptional activity.

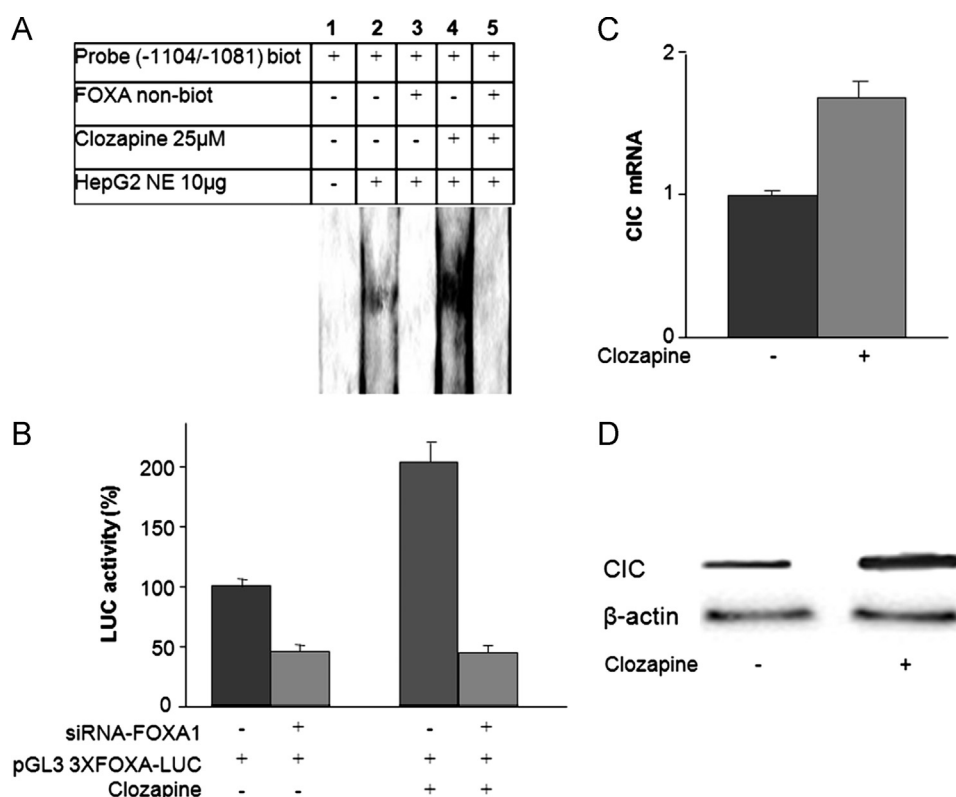
### 3.3. Clozapine increases FOXA1 and CIC expression in basal condition in INS-1 cells

Since FOXA1 expression is upregulated by clozapine, next we investigated whether this effect could be related to the abnormal insulin secretion, a well-known side effect of clozapine treatment. This rationale was based on the previous findings that (i) FOXA1 controls insulin secretion in INS-1 cells (Iacobazzi et al., 2009a); (ii) ablation of FOXA1 in mature  $\beta$ -cells leads to abnormal insulin secretion (Vatamaniuk et al., 2006; Gao et al., 2010).

Experiments were performed on INS-1 cells that retain insulin-secretory responsiveness to glucose within physiological range. First, we verified that INS-1 viability was not affected by 25  $\mu$ M clozapine treatment (data not shown). Then, FOXA1 and CIC expression levels were investigated at low (3 mM) and high (17.5 mM) glucose concentration in the presence or absence of clozapine. Real-time PCR performed on mRNA from INS-1 cells showed an increase of FOXA1 expression of about 120% at 3 mM glucose; whereas a negligible effect was observed at 17.5 mM glucose (Figure 3A). The same result was obtained by western blotting analysis (Figure 3B). In the same conditions as above, CIC mRNA and protein expression levels were determined. As shown in Figure 3C and D, an increase of CIC mRNA (about 100%) and protein was observed at 3 mM glucose in presence of clozapine, whereas no significant effect was observed at 17.5 mM glucose.

Moreover, in INS-1 cells transduced with lentivirus RNAi pLKO.1-FOXA1 vector to silence FOXA1 and incubated with clozapine, the amounts of FOXA1 and CIC mRNA and protein were determined by real-time PCR and western blotting. Both FOXA1 and CIC expression levels showed a markedly reduction of expression in FOXA1-silenced INS-1 cells in the presence or absence of clozapine as compared to control cells (Figure 4A-D). These results indicate that FOXA1 and CIC upregulation induced by clozapine is abolished in FOXA1 silenced cells.

Next we checked whether FOXA1 upregulation by clozapine in INS-1 cells was also exerted by conventional antipsychotic, such as haloperidol (Melkersson, 2004). Real-time PCR and western blotting performed under the same conditions used with clozapine (at low and high glucose



**Figure 2** Effect of clozapine on FOXA1 DNA-binding and transcriptional activity. (A) The biotinylated probe from -1104 to -1081 bp of CIC gene promoter was incubated with 10 μg of HepG2 nuclear extracts (HepG2 NE) in the presence (lane 4) or absence (lane 2) of 25 μM clozapine. Where indicated, 10-fold excess of non-biotinylated probe was added (lanes 3 and 5). (B) HepG2 cells transfected with or without siRNA targeting FOXA1 and with pGL3 promoter-LUC vector containing a threefold repeat FOXA1 site (pGL3 3XFOXA-LUC), were exposed to 25 μM clozapine or vehicle and then assayed for LUC expression activity. Data are shown as means ± SD of five duplicate independent experiments. Differences between FOXA1 silenced samples (gray bars) and unsilenced samples (black bars) were significant ( $P < 0.05$ , one-way ANOVA). Differences between unsilenced samples treated and untreated with clozapine were also significant ( $P < 0.05$ , one-way ANOVA). (C) Total RNA extracted from HepG2 cells treated or not with clozapine was used to quantify CIC mRNA by real-time PCR; data are shown as means ± SD of five duplicate independent experiments. Differences between samples and relative controls were significant ( $P < 0.05$ , one-way ANOVA). (D) CIC and β-actin protein from HepG2 cells in presence or absence of 25 μM clozapine were immunodecorated with specific antibodies. The result is representative of three independent experiments.

concentration) showed no change in FOXA1 mRNA and protein levels (Figure 5A and B).

These results indicate that clozapine affects FOXA1 and CIC gene expression only at low glucose concentration and that this effect is not exerted by conventional antipsychotic haloperidol.

### 3.4. FOXA1 silencing abolishes clozapine-induced abnormal insulin secretion in INS-1 cells

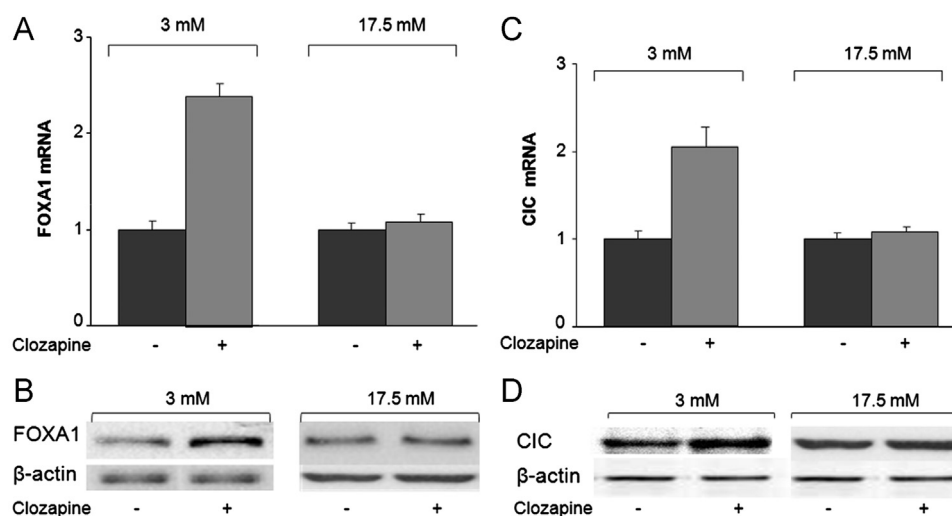
Given that FOXA1 and CIC expression levels are upregulated only in basal glucose conditions (3 mM), we tested the insulin secretion in the presence of clozapine. Insulin secretion in INS-1 cells, transduced with pLKO-FOXA1 lentivirus or empty vector and incubated within HBSS containing 3 mM or 17.5 mM glucose in the presence of clozapine, was quantified by ELISA. As shown in Figure 6A, under basal glucose conditions insulin content was about 60% higher in INS-1 cells treated with clozapine with respect to control cells. Under the same conditions, when FOXA1 was silenced the abnormal insulin secretion was abolished (Figure 6A).

By contrast, no increase in insulin secretion was observed in the presence of clozapine at glucose 17.5 mM (Figure 6B). Unexpectedly insulin level was reduced by about 50% in FOXA1-silenced INS-1 cells and treated with clozapine compared to control cells. The reason of this finding is not known. It could be due to the down-regulation of all FOXA1-dependent genes expression, including genes involved in β-cells proliferation and insulin secretion.

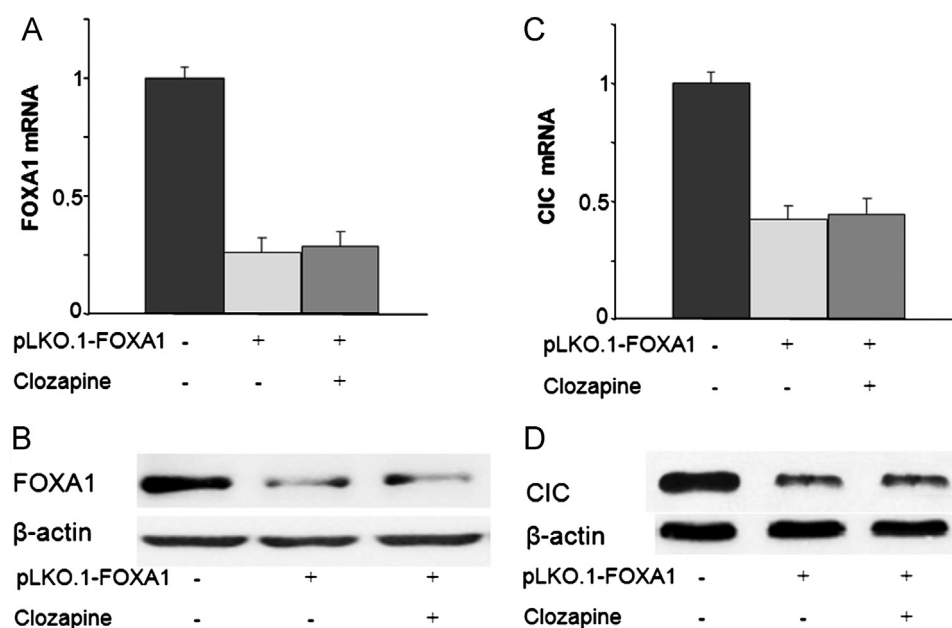
These data highlight the involvement of FOXA1 in regulating basal insulin secretion upon clozapine treatment.

## 4. Discussion

Treatment with the atypical antipsychotic drug clozapine has been associated with increased insulin levels and insulin resistance (Melkersson et al., 1999; Melkersson and Jansson, 2007). Since insulin is a hormone that is involved in glucose regulation and lipid metabolism as well as in the regulation of body weight (Yazici et al., 1998), the ability of clozapine to stimulate insulin secretion directly from beta-cells might explain its capability to induce body weight gain, glucose



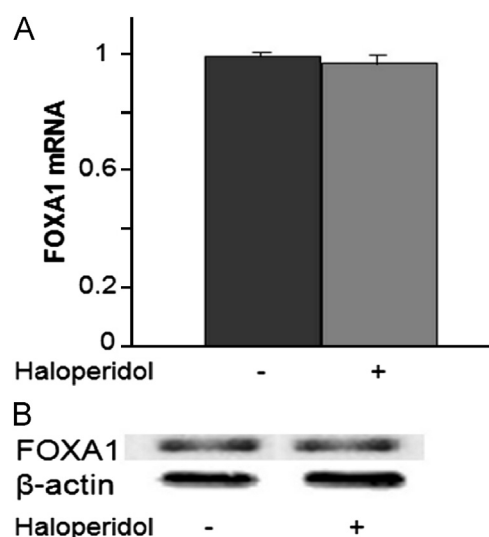
**Figure 3** Effect of clozapine on FOXO1 and CIC gene expression in INS-1 cells. (A) FOXO1, mRNA of INS-1 cells treated or not with 25  $\mu$ M clozapine in presence of 3 mM or 17.5 mM glucose concentration was quantified by real-time PCR. Data are shown as means  $\pm$  SD of five duplicate independent experiments. (B) FOXO1 and  $\beta$ -actin of INS-1 cells, treated as above, were immunodecorated with specific antibodies. (C) CIC mRNA of INS-1 cells, treated as in (A), was quantified by real-time PCR. (D) CIC and  $\beta$ -actin of INS-1 cells, treated as in (B), were immunodecorated with specific antibodies. In (A) and (C), data are shown as means  $\pm$  SD of five duplicate independent experiments. In (A) and (C), only the differences between samples in the presence of 3 mM glucose treated with clozapine (gray bars) and relative controls (black bars) were significant ( $P < 0.05$ , one-way ANOVA). In (B) and (D), the results are representative of three independent experiments.



**Figure 4** Effect of clozapine on FOXO1-silenced INS1 cells. (A) INS-1 cells grown in medium containing 3 mM glucose were transduced with pLKO-FOXO1 lentivirus or empty vector and treated with 25  $\mu$ M clozapine or vehicle. FOXO1 mRNA was quantified by real-time PCR. (B) FOXO1 and  $\beta$ -actin of INS-1 cells, transduced and treated as above, were immunodecorated with specific antibodies. (C) CIC mRNA of INS-1 cells, in the same conditions as in (A), was quantified by real-time PCR. (D) CIC and  $\beta$ -actin of INS-1 cells, in the same conditions as in (A), were immunodecorated with specific antibodies. In (A) and (C), data are shown as means  $\pm$  SD of five duplicate independent experiments. In (A) and (C), differences between samples (gray bars) and relative controls (black bars) were significant ( $P < 0.05$ , one-way ANOVA). In (B) and (D), the results are representative of three independent experiments.

dysregulation, lipid abnormalities and diabetes mellitus (Umbricht et al., 1994; Henderson et al., 2000). Treatment with clozapine will increase insulin secretion, resulting in

stimulated appetite and weight gain, which in turn lead to the development of concomitant obesity-associated insulin resistance, with a compensatory and further increased

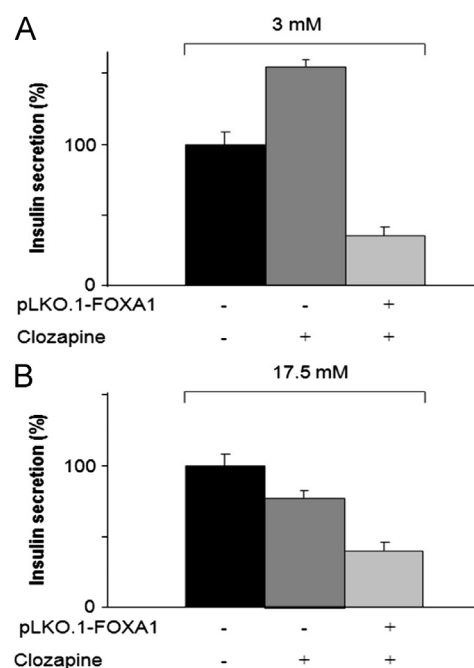


**Figure 5** Effect of haloperidol on FOXA1 gene expression. (A) FOXA1 mRNA of INS-1 cells treated for 24 h with 25  $\mu$ M haloperidol in the presence of 3 mM or 17.5 mM glucose concentration was quantified by real-time PCR. mRNA measured in untreated cells was set at 100%. Data are shown as means  $\pm$  SD of five duplicate independent experiments. Differences between samples (gray bars) and relative controls (black bars) were significant ( $P < 0.05$ , one-way ANOVA). (B) FOXA1 and  $\beta$ -actin proteins of INS-1 cells treated or untreated with haloperidol as above, were immunodecorated with specific antibodies. The result is representative of three independent experiments.

insulin secretion as consequence. Then, when failure of this compensation occurs, hyperglycemia and diabetes will develop (Olefsky, 1997). Most of patients develop diabetes mellitus within 6 months after commencing clozapine therapy, and some recovered after its discontinuation (Koller et al., 2001). Reduction of the dosage of clozapine by switching to other drugs resulted in improved glycemic control (Reinstein et al., 1999). However, the mechanisms by which clozapine (or other atypical antipsychotics) influences glucose-insulin homeostasis and the molecular targets by which it exerts its action are unknown.

In the present study we investigate on the mechanism underlying abnormal insulin secretion observed in patients treated with clozapine. We identify for the first time that (i) the transcription factor FOXA1 is a novel target for clozapine, the prototype of atypical antipsychotic drugs; (ii) clozapine upregulates FOXA1 gene expression and the lipogenic carrier CIC; (iii) FOXA1 silencing completely abolishes abnormal insulin secretion induced by clozapine treatment in INS-1 cells. These outcomes could shed more light in understanding the molecular mechanism of abnormal insulin secretion and diabetogenic effect caused by clozapine treatment.

Clozapine exposure of HepG2 cells greatly affects FOXA1 expression levels compared to FOXA2 and FOXA3, indicating that FOXA1 is a specific target for clozapine, but not for the conventional antipsychotic drug, such as haloperidol. The reason of the specificity for FOXA1 is unknown. It could be related to the role exerted by FOXA1 on insulin secretion. In fact, previous investigations demonstrated that FOXA1



**Figure 6** Effect of FOXA1 silencing on insulin secretion in INS-1 cells. (A) INS-1 cells were transduced with lentivirus RNAi pLKO.1-FOXA1 and grown in culture medium containing 5.5 mM glucose in the presence of clozapine for 24 h. After washing in glucose free buffer, cells were incubated in HBSS buffer containing 3 mM glucose for 2 h. Then basal insulin secretion was assayed by ELISA. (B) Glucose-stimulated insulin secretion in INS-1 cells, transduced as above and incubated with HBSS containing 17.5 mM glucose, was quantified by ELISA. Data are shown as means  $\pm$  SD of five duplicate independent experiments. All differences between samples and relative controls were significant ( $P < 0.05$ , one-way ANOVA).

controls glucose-stimulated insulin secretion in INS-1 cells also through transcriptional regulation of CIC gene (Iacobazzi et al., 2009a). FOXA1<sup>-/-</sup> mice have low plasma insulin levels (Shih et al., 1999). FOXA1-deficient  $\beta$ -cells have severely impaired glucose-stimulated insulin secretion (Vatamaniuk et al., 2006). Moreover, insulin content and insulin gene expression is reduced in islets of FOXA1<sup>-/-</sup> mice (Vatamaniuk et al., 2006).

It is likely that FOXA family might explain some side effects of clozapine treatment. While FOXA1 is involved in insulin secretion, FOXA2 activity is greatly affected by hyperinsulinemia. FOXA2 is a sensor of circulating insulin, that regulates lipid metabolism, ketone bodies and fatty acids  $\beta$ -oxidation. It is phosphorylated in response to insulin signaling, resulting in inhibition of its transcriptional activity and promoting lipid accumulation (Wolfrum et al., 2004). It could be argued that lipid accumulation as consequence of clozapine treatment could be due to FOXA1-induction by clozapine and to inhibition of FOXA2 by hyperinsulinemia.

Clozapine greatly affects insulin secretion: it is suppressed at high glucose concentration, whereas it is stimulated in basal glucose conditions (Melkersson and Jansson, 2007). Suppression of insulin secretion at high glucose concentration in INS-1 cells could be related to impairment of glucose oxidation. The first phase of insulin release is



determined by the activity of the  $K_{ATP}$  channel, which in turn depends on the efficient utilization of glucose and the production of ATP. Sasaki et al. (2006) reported that clozapine impairs insulin secretion via multiple sites including disturbance of glucose oxidation and distal step insulin exocytosis. Reduction of glucose oxidation with unchanged glucose utilization led authors to hypothesize an impairment of TCA cycle in mitochondria through inhibition of clozapine, although the mechanism of inhibition was not investigated (Sasaki et al., 2006). Our results are in agreement with the latter suggestion. In fact, expression of FOXA1 and CIC are not increased at high glucose concentration in the presence of clozapine. Inhibition of TCA causes a reduction of oxidation metabolites, such as citrate, that act as signals/second messengers in insulin secretion. Reduction of citrate production may affect expression of related mitochondrial carrier (CIC) and the upstream transcription factor FOXA1 by a feedback mechanism. Decrease of transcriptional factor FOXA1 expression indicates also that inhibition of insulin secretion is a sequential complex mechanism starting at transcriptional level. It is not excluded that other mechanisms are involved. Pharmacological studies hypothesized that clozapine and other atypical antipsychotic drugs suppress cholinergic-stimulated insulin secretion by a direct action on the pancreas, which involves antagonism of muscarin  $M_3$  receptors on  $\beta$ -cells (Johnson et al., 2005).

Our data evidently demonstrate that clozapine increases binding of FOXA1 to its responsive element (Figure 2A) and increases its transcriptional activity (Figure 2B and C). Therefore, it is conceivable that activation of FOXA1 by clozapine triggers activation of genes encoding proteins involved in exocytosis process. However, since exocytosis is a complex process, it is not excluded that expression of other proteins, such as components of granules, cytosolic proteins, plasma membrane proteins, and the synaptic-like vesicles might be affected by clozapine or FOXA-1 regulated factors.

It should be mentioned that FOXA proteins, by altering chromatin structure, are known to facilitate binding of other transcription factors (Gualdi et al., 1996; Cirillo et al., 2002). In particular, FOXA1 may translate epigenetic signatures, such as distribution of histone H3 lysine 4 dimethylation, into change of chromatin conformation, thus facilitating lineage-specific transcriptional programs (Lupien et al., 2008). Moreover, it is not excluded an auto-regulation of FOXA1, since a FOXA1 binding element is present in the distal promoter region (Peterson et al., 1997).

Although the molecular mechanism underlying the abnormal insulin secretion induced by clozapine needs to be investigated in details, our data clearly indicate that clozapine exerts a direct stimulatory effect on insulin secretion through FOXA1.

In this regard, it is worth mentioning that FOXA1 silencing completely abolishes abnormal insulin secretion at low glucose concentration (Figure 6A). Thus FOXA1 plays a critical role in clozapine treatment opening a new metabolic pathway to understand the molecular mechanism of action of clozapine and atypical antipsychotic drugs.

In conclusion, this study identifies for the first time FOXA1 as new target for clozapine. Clozapine affects expression of

FOXA1 and its regulated gene CIC. It also demonstrates that abnormal basal insulin secretion can be inhibited by FOXA1 silencing. Therefore, FOXA1 or other downstream members of signaling pathway not yet identified could provide novel targets for therapeutic intervention on diabetogenic and other side effects of clozapine treatment.

## Role of funding source

The research has been funded by the by Grants from the Ministero dell' Università e della Ricerca (MIUR), the National Research Council (CNR), the Center of Excellence in Genomics (CEGBA), Apulia Region, the University of Bari; the Italian Human Proteome Net no. RBRN07BMCT\_009 (MIUR).

## Contributors

Menga A., Iacobazzi F. and Convertini P. conducted the experiments. Infantino V. and Iacobazzi V. planned the experiments and wrote the paper. Palmieri F. revised the paper before submission.

## Conflict of interest

The authors have no financial interests to disclose.

## Acknowledgments

This work was supported by Grants from the Ministero dell' Università e della Ricerca (MIUR), the National Research Council (CNR), the Center of Excellence in Genomics (CEGBA), Apulia Region, the University of Bari; the Italian Human Proteome Net no. RBRN07BMCT\_009 (MIUR).

## References

- Bisaccia, F., De Palma, A., Palmieri, F., 1989. Identification and purification of the tricarboxylate carrier from rat liver mitochondria. *Biochim. Biophys. Acta* 977 (2), 171-176.
- Bisaccia, F., De Palma, A., Prezioso, G., Palmieri, F., 1990. Kinetic characterization of the reconstituted tricarboxylate carrier from rat liver mitochondria. *Biochim. Biophys. Acta* 1019 (3), 250-256.
- Cirillo, L.A., Lin, F.R., Cuesta, I., Friedman, D., Jarnik, M., Zaret, K.S., 2002. Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. *Mol. Cell* 9 (2), 279-289.
- Conley, R.R., Tamminga, C.A., Kelly, D.L., Richardson, C.M., 1999. Treatment-resistant schizophrenic patients respond to clozapine after olanzapine non-response. *Biol. Psychiatry* 46, 73-77.
- Convertini, P., Infantino, V., Bisaccia, F., Palmieri, F., Iacobazzi, V., 2011. Role of FOXA and Sp1 in mitochondrial acylcarnitine carrier gene expression in different cell lines. *Biochem. Biophys. Res. Commun.* 404 (1), 376-381.
- Fernø, J., Skrede, S., Vik-Mo, A.O., Håvik, B., Steen, V.M., 2006. Drug-induced activation of SREBP-controlled lipogenic gene expression in CNS-related cell lines: marked differences between various antipsychotic drugs. *BMC Neurosci.* 7, 69.
- Fernø, J., Vik-Mo, A.O., Jassim, G., Håvik, B., Berge, K., Skrede, S., Gudbrandsen, O.A., Waage, J., Lunder, N., Mørk, S., Berge, R.K., Jørgensen, H.A., Steen, V.M., 2009. Acute clozapine exposure in vivo induces lipid accumulation and marked sequential changes in the expression of SREBP, PPAR, and LXR target genes in rat liver. *Psychopharmacology* 203 (1), 73-84.



- Freedman, R., 2003. Schizophrenia. *N. Engl. J. Med.* 349, 1738-1749.
- Friedman, J.R., Kaestner, K.H., 2006. The Foxa family of transcription factors in development and metabolism. *Cell. Mol. Life Sci.* 63, 2317-2328.
- Gao, N., Le Lay, J., Qin, W., Doliba, N., Schug, J., Fox, A.J., Smirnova, O., Matschinsky, F.M., Kaestner, K.H., 2010. Foxa1 and Foxa2 maintain the metabolic and secretory features of the mature beta-cell. *Mol. Endocrinol.* 24 (8), 1594-1604.
- Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J.R., Zaret, K.S., 1996. Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. *Genes Dev.* 10 (13), 1670-1682.
- Henderson, D.C., Cagliero, E., Gray, C., Nasrallah, R.A., Hayden, D.L., Schoenfeld, D.A., Goff, D.C., 2000. Clozapine, diabetes mellitus, weight gain, and lipid abnormalities: a five-year naturalistic study. *Am. J. Psychiatry* 157 (6), 975-981.
- Henderson, D.C., Nguyen, D.D., Copeland, P.M., Hayden, D.L., Borba, C.P., Louie, P.M., Freudenreich, O., Evins, A.E., Cather, C., Goff, D.C., 2005. Clozapine, diabetes mellitus, hyperlipidemia, and cardiovascular risks and mortality: results of a 10-year naturalistic study. *J. Clin. Psychiatry* 66 (9), 1116-1121.
- Hohmeier, H.E., Mulder, H., Chen, G., Henkel-Rieger, R., Prentki, M., Newgard, C.B., 2000. Isolation of INS-1-derived cell lines with robust ATP-sensitive K<sup>+</sup> channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* 49, 424-430.
- Iacobazzi, V., Infantino, V., Costanzo, P., Izzo, P., Palmieri, F., 2005. Functional analysis of the promoter of the mitochondrial phosphate carrier human gene: identification of activator and repressor elements and their transcription factors. *Biochem. J.* 391, 613-621.
- Iacobazzi, V., Infantino, V., Palmieri, F., 2008. Epigenetic mechanisms and Sp1 regulate mitochondrial citrate carrier gene expression. *Biochem. Biophys. Res. Commun.* 376 (1), 15-20.
- Iacobazzi, V., Infantino, V., Bisaccia, F., Castegna, A., Palmieri, F., 2009a. Role of FOXA in mitochondrial citrate carrier gene expression and insulin secretion. *Biochem. Biophys. Res. Commun.* 385 (2), 220-224.
- Iacobazzi, V., Infantino, V., Convertini, P., Voza, A., Agrimi, G., Palmieri, F., 2009b. Transcription of the mitochondrial citrate carrier gene: identification of a silencer and its binding protein ZNF224. *Biochem. Biophys. Res. Commun.* 386 (1), 186-191.
- Infantino, V., Iacobazzi, V., De Santis, F., Mastrapasqua, M., Palmieri, F., 2007. Transcription of the mitochondrial citrate carrier gene: role of SREBP-1, upregulation by insulin and downregulation by PUFA. *Biochem. Biophys. Res. Commun.* 356, 249-254.
- Infantino, V., Convertini, P., Cucci, L., Panaro, M.A., Di Noia, M.A., Calvello, R., Palmieri, F., Iacobazzi, V., 2011a. The mitochondrial citrate carrier: a new player in inflammation. *Biochem. J.* 438 (3), 433-436.
- Infantino, V., Convertini, P., Iacobazzi, F., Pisano, I., Scarcia, P., Iacobazzi, V., 2011b. Identification of a novel Sp1 splice variant as a strong transcriptional activator. *Biochem. Biophys. Res. Commun.* 412 (1), 86-91.
- Infantino, V., Castegna, A., Iacobazzi, F., Spera, I., Scala, I., Andria, G., Iacobazzi, V., 2011c. Impairment of methyl cycle affects mitochondrial methyl availability and glutathione level in Down's syndrome. *Mol. Genet. Metab.* 102 (3), 378-382.
- Jin, H., Meyer, J.M., Jeste, D.V., 2004. Atypical antipsychotics and glucose dysregulation: a systematic review. *Schizophr. Res.* 71, 195-212.
- Johnson, D.E., Yamazaki, H., Ward, K.M., Schmidt, A.W., Lebel, W.S., Treadway, J.L., Gibbs, E.M., Zawulich, W.S., Rollem, H., 2005. Inhibitory effects of antipsychotics on carbachol-enhanced insulin secretion from perfused rat islets: role of muscarinic antagonism in antipsychotic-induced diabetes and hyperglycemia. *Diabetes* 54 (5), 1552-1558.
- Joseph, J.W., Jensen, M.V., Ilkayeva, O., Palmieri, F., Alárcon, C., Rhodes, C.J., Newgard, C.B., 2006. The mitochondrial citrate/isocitrate carrier plays a regulatory role in glucose-stimulated insulin secretion. *J. Biol. Chem.* 281, 35624-35632.
- Kaplan, R.S., 2001. Structure and function of mitochondrial anion transport proteins. *J. Membr. Biol.* 179 (3), 165-183.
- Kessing, L.V., Thomsen, A.F., Mogensen, U.B., Andersen, P.K., 2010. Treatment with antipsychotics and the risk of diabetes in clinical practice. *Br. J. Psychiatry* 197 (4), 266-271.
- Koller, E., Schneider, B., Bennett, K., Dubitsky, G., 2001. Clozapine-associated diabetes. *Am. J. Med.* 111 (9), 716-723.
- Lupien, M., Eeckhoutte, J., Meyer, C.A., Wang, Q., Zhang, Y., Li, W., Carroll, J.S., Liu, X.S., Brown, M., 2008. FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell* 132, 958-970.
- Melkersson, K.I., Hulting, A.L., Brismar, K.E., 1999. Different influences of classical antipsychotics and clozapine on glucose-insulin homeostasis in patients with schizophrenia or related psychoses. *J. Clin. Psychiatry* 60 (11), 783-791.
- Melkersson, K., 2004. Clozapine and olanzapine, but not conventional antipsychotics, increase insulin release in vitro. *Eur. Neuropsychopharmacol.* 14 (2), 115-119.
- Melkersson, K., Jansson, E., 2007. Effects of the atypical antipsychotic clozapine on insulin release in vitro. *Neuroendocrinol. Lett.* 28 (6), 854-860.
- Merglen, A., Theander, S., Rubi, B., Chaffard, G., Wollheim, C.B., Maechler, P., 2004. Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology* 145, 667-678.
- Olefsky, J.M., 1997. Insulin resistance. In: Porte, D., Sherwin, R.S. (Eds.), *Diabetes Mellitus* 5th ed. Appleton & Lange, Stamford (CT), pp. 513-552.
- Palmieri, F., 2004. The mitochondrial transporter family (SLC25): physiological and pathological implications. *Pflugers Arch.* 447, 689-709.
- Palmieri, F., 2012. The mitochondrial transporter family SLC25: identification, properties and physiopathology. *Mol. Aspects Med.* <http://dx.doi.org/10.1016/j.mam.2012.05.005>.
- Peterson, R.S., Clevidence, D.E., Ye, H., Costa, R.H., 1997. Hepatocyte nuclear factor-3 alpha promoter regulation involves recognition by cell-specific factors, thyroid transcription factor-1, and auto activation. *Cell Growth Differ.* 8 (1), 69-82.
- Raeder, M.B., Ferno, J., Vik-Mo, A.O., Steen, V.M., 2006. SREBP activation by antipsychotic- and antidepressant-drugs in cultured human liver cells: relevance for metabolic side-effects? *Mol. Cell. Biochem.* 289, 167-173.
- Reinstein, M.J., Sirovskaya, L.A., Jones, R.E., Mohan, S., Chasanov, M.A., 1999. Effect of clozapine-quetiapine combination therapy on weight and glycemic control. *Clin. Drug Invest.* 18, 99-104.
- Sasaki, N., Iwase, M., Uchizono, Y., Nakamura, U., Imoto, H., Abe, S., Iida, M., 2006. The atypical antipsychotic clozapine impairs insulin secretion by inhibiting glucose metabolism and distal steps in rat pancreatic islets. *Diabetologia* 49 (12), 2930-2938.
- Scheen, A.J., De Hert, M.A., 2007. Abnormal glucose metabolism in patients treated with antipsychotics. *Diabetes Metab.* 33, 169-175.
- Shen, W., Searce, L.M., Brestelli, J.E., Sund, N.J., Kaestner, K.H., 2001. Foxa3 (hepatocyte nuclear factor 3 gamma) is required for the regulation of hepatic GLUT2 expression and the maintenance of glucose homeostasis during a prolonged fast. *J. Biol. Chem.* 276, 42812-42817.
- Shih, D.Q., Navas, M.A., Kuwajima, S., Duncan, S.A., Stoffel, M., 1999. Impaired glucose homeostasis and neonatal mortality in hepatocyte nuclear factor 3alpha-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* 96, 10152-10157.

- Umbricht, D.S., Pollack, S., Kane, J.M., 1994. Clozapine and weight gain. *J. Clin. Psychiatry* 55 (Suppl. B), 157-160.
- Vatamaniuk, M.Z., Gupta, R.K., Lantz, K.A., Doliba, N.M., Matschinsky, F.M., Kaestner, K.H., 2006. Foxa1-deficient mice exhibit impaired insulin secretion due to uncoupled oxidative phosphorylation. *Diabetes* 55, 2730-2736.
- Vestri, H.S., Maianu, L., Moellering, D.R., Garvey, W.T., 2007. Atypical antipsychotic drugs directly impair insulin action in adipocytes: effects on glucose transport, lipogenesis, and antilipolysis. *Neuropsychopharmacology* 32, 765-772.
- Vik-Mo, A.O., Fernø, J., Skrede, S., Steen, V.M., 2009. Psychotropic drugs upregulate the expression of cholesterol transport proteins including ApoE in cultured human CNS- and liver cells. *BMC Pharmacol.* 9, 10.
- Wolfrum, C., Asilmaz, E., Luca, E., Friedman, J.M., Stoffel, M., 2004. Foxa2 regulates lipid metabolism and ketogenesis in the liver during fasting and in diabetes. *Nature* 432 (7020), 1027-1032.
- Yazici, K.M., Erbas, T., Yazici, A.H., 1998. The effect of clozapine on glucose metabolism. *Exp. Clin. Endocrinol. Diabetes* 106, 475-477.