

ORIGINAL ARTICLE

Prednisolone induces the Wnt signalling pathway in 3T3-L1 adipocytes

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Abstract

Synthetic glucocorticoids are potent anti-inflammatory drugs but show dose-dependent metabolic side effects such as the development of insulin resistance and obesity. The precise mechanisms involved in these glucocorticoid-induced side effects, and especially the participation of adipose tissue in this are not completely understood. We used a combination of transcriptomics, antibody arrays and bioinformatics approaches to characterize prednisolone-induced alterations in gene expression and adipokine secretion, which could underlie metabolic dysfunction in 3T3-L1 adipocytes. Several pathways, including cytokine signalling, Akt signalling, and Wnt signalling were found to be regulated at multiple levels, showing that these processes are targeted by prednisolone. These results suggest that mechanisms by which prednisolone induce insulin resistance include dysregulation of wnt signalling and immune response processes. These pathways may provide interesting targets for the development of improved glucocorticoids.

Introduction

Synthetic glucocorticoids (GCs) such as prednisolone and dexamethasone are widely used for the treatment of inflammatory diseases such as rheumatoid arthritis, asthma, inflammatory bowel disease and psoriasis (Del Rosso Do, 2006; Hillier, 2007; Schwartz & Cohen, 2008). Despite their excellent efficacy, GC usage is hampered because of adverse (metabolic) side effects, such as insulin resistance, glucose intolerance, diabetes, central adiposity, dyslipidemia, skeletal muscle wasting and osteoporosis (De Bosscher & Haegeman, 2009; Rhen & Cidlowski, 2005; Rockall *et al.*, 2003; Schacke *et al.*, 2002, 2006). The precise mechanisms involved in these GC-induced side effects are not completely understood and are tissue specific (Chrousos & Kino, 2009; Kino, 2007; Lu & Cidlowski, 2006; van Raalte *et al.*, 2009). One of the key tissues thought to be involved in metabolic side effects is the adipose tissue. Several studies have shown that the side-effects of GCs in adipose tissue include the development of central adiposity (Rockall *et al.*, 2003), dyslipidaemia

Keywords

Gene profiling, glucocorticoids, metabolic dysfunction

History

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(Taskinen *et al.*, 1983; Wajchenberg, 2000) and the inhibition of insulin-stimulated glucose uptake (Bazuine *et al.*, 2004; Buren *et al.*, 2002, 2008; Sakoda *et al.*, 2000).

It is now well established that adipose tissue is not only involved in energy storage, but also functions as an endocrine organ that secretes hundreds of bioactive substances known as adipokines (Kershaw & Flier, 2004; Ouchi *et al.*, 2011; Lehr *et al.*, 2011). Previously, GCs have been found to affect the secretion of several adipokines, including TNF- α , IL-6, Resistin, Adiponectin and Leptin in rodents (Fasshauer & Paschke, 2003). Because recent proteomic approaches have led to the characterization of numerous novel adipokines, we applied a combination of genomics, antibody arrays and bioinformatics approaches to identify prednisolone-induced alterations in (adipokine) gene expression and secretion which could potentially underlie prednisolone-induced metabolic dysfunction of 3T3-L1 adipocytes.

Materials and methods

Set up of the study

In this study, differentiated 3T3-L1 adipocytes were treated with prednisolone or DMSO for 0, 1, 6, 24 and 48 h and the effects of prednisolone were studied at gene expression and protein secretion level.

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Cell culture

3T3-L1 pre-adipocytes obtained from ATCC were cultured and differentiated in adipocytes as previously described (van den Berghe *et al.*, 1994). Cells were used seven days after completion of the differentiation process. Only cultures in which >95% of cells displayed adipocyte morphology were used. Prior to use, adipocytes were serum starved for 16 h with DMEM supplemented with 0.5% foetal bovine serum.

Analysis of insulin signalling

Differentiated 3T3-L1 adipocytes, grown in 12-well plates, were incubated with prednisolone (1 μ M) or DMSO at day 8 for 48 h. After incubation with prednisolone or DMSO, cells were serum-starved for 2 h, and then stimulated with insulin (100 nM) for 10 min. Following insulin stimulation, cells were washed twice with ice-cold PBS, and lysed as described previously (Linssen *et al.*, 2011). Protein expression and phosphorylation were determined by Western blotting as described (Linssen *et al.*, 2011) using the following antibodies: phospho-insulin receptor substrate 1-Tyr1222, phospho-Akt-Thr308, phospho-Akt-Ser473 (all from Cell Signaling Technology, Danvers, MA, USA), total Akt, phospho-Akt substrate of 160-kDa (AS160), total AS160 (all from Millipore, MA, USA), glucose transporter 4 (GLUT4), and β -actin (both from Abcam, Cambridge, UK).

Assay of 2-deoxy-D-glucose (2DOG) uptake

Differentiated 3T3-L1 adipocytes, grown in 12-wells plates, were incubated with prednisolone (1 μ M) or DMSO at day 8 for 6, 24 and 48 h. After incubation with prednisolone or DMSO for the indicated times, the cells were washed once with HEPES buffer, consisting of 50 mM HEPES, 0.14 M NaCl, 1.85 mM CaCl₂, 1.3 mM MgSO₄, and 4.8 mM KCl [pH 7.4]), and then incubated in this buffer for an additional hour at 37°C. Then, cells were stimulated with insulin (100 nM) or kept untreated. After 15 min, 2DOG uptake was initiated by the addition of 2-deoxy-D-[¹⁴C] glucose (0.075 μ Ci per well) in 3 mM 2DOG. After 10 min, the assay was terminated by three quick washes with ice-cold PBS. Cells were lysed in 0.1 M NaOH, 0.2% SDS, whereafter incorporated 2-deoxy-D-[¹⁴C] glucose was determined by liquid scintillation counting.

RNA isolation

Differentiated 3T3-L1 adipocytes grown in 6-well plates were incubated with prednisolone (1 μ M) for 1, 6, 24 and 48 h and used for RNA isolation and whole-genome expression profiling. RNA was isolated using Tripure (Roche), and subsequently purified using the RNeasy mini kit according to the manufacturer's protocol (Qiagen Benelux B.V. Venlo, The Netherlands). To remove residual traces of genomic DNA, the RNA was treated with DNase I (Invitrogen, Leek, The Netherlands) while bound to the RNeasy column. Quality and quantity of the purified RNA was controlled using a NanoDrop spectrophotometer (Nanodrop Technologies, Montchanin, DE, USA). RNA integrity was investigated by using the 2100 Bioanalyser (Agilent technologies, Philadelphia, PA, USA).

Whole-genome expression analysis using Affymetrix GeneChip Mouse Genome 430 2.0 Array

Amplification of 20 ng total RNA was performed with the Two-Cycle Eukaryotic Target Labeling kit (Affymetrix, Santa Clara, CA). Briefly, after the first round of cDNA synthesis, an unlabeled ribonucleotide mix (MEGAscript T7 kit, Ambion) was used to generate unlabelled cRNA according to the protocol of Affymetrix. After cleanup of the cRNA with a GeneChip Sample Cleanup Module IVT Column (Affymetrix, Santa Clara, CA), the unlabelled cRNA concentration was determined and 150 ng was reverse transcribed using random primers. Subsequently, the T7-Oligo(dT) Promoter Primer was used in the second-strand cDNA synthesis to generate double-stranded cDNA containing T7 promoter sequences. The resulting double-stranded cDNA was then amplified and biotin labelled using the IVT Labeling kit (Affymetrix, Santa Clara, CA). Biotin-labelled cRNA was fragmented at 1 μ g/ μ l following the manufacturer's protocol. After fragmentation, cRNA (10 μ g) was hybridized at 45°C for 16 h to the Human Genome U133 2.0 Plus array (Affymetrix, Santa Clara, CA). Following hybridization, the arrays were washed, stained with phycoerythrin-streptavidin conjugate (Molecular Probes, Eugene, OR), and the signals were amplified by staining the array with biotin-labelled anti-streptavidin antibody (Vector Laboratories, Burlingame, CA) followed by phycoerythrin-streptavidin. The arrays were laser scanned with a GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Data was saved as raw image file and quantified using AGCC v 1.0 (Affymetrix, Santa Clara, CA).

Murine adipokine antibody arrays

This array allows simultaneous detection of 308 secreted mouse proteins in cell culture supernatants (AAM-BLM, L-series, Raybiotech, Inc, Norcross, GA). Differentiated 3T3-L1 adipocytes were subjected to 4 h pred/dmsol, followed by two rinsing steps and culture medium containing 0.2% FBS and Prednisolone or vehicle (1 μ M) for 44 h. To prepare samples for the antibody array, samples were subjected to centricon tubes (Millipore amicon ultra-15 ultracel 3 K) and washed three times with PBS, pH 8.0. Finally proteins were recovered in 2 ml PBS. Samples were biotin-labelled according to manufacturer's protocol. Then, membranes were blocked and incubated with the biotinylated samples overnight at 4°C, and finally incubated with HRP conjugated streptavidin. Signals were detected using enhanced chemiluminescence and quantified on a LumiImager system using LumiAnalyst software version 3.1 (Roche Diagnostics, Mannheim, Germany).

Gene expression data

Microarray data were analysed with packages from the BioConductor library. Gene expression data was normalized using *gcRMA*. Identification of regulated probes was done using *limma* package from Bioconductor. For identifying differentially expressed probes after incubation with prednisolone, the following selection criteria were used: fold change >2, intensity >20, and *p* value <0.05 after correction for multiple testing using the Benjamini-Hochberg correction.

All statistical analysis was performed in R. Significance of regulation of the Wnt pathway has been calculated using globaltest.

Secreted protein data obtained from murine adipokine antibody arrays

Regulated proteins as a result of prednisolone treatment have been selected using a *p* value <0.05 (calculated using a standard Student's *t*-test).

Keyword enrichment analysis

Keyword enrichment analysis on the microarray data was performed using CoPub, a text mining algorithm that detects co-occurring biomedical concepts in abstracts from the MedLine literature database (Fleuren et al., 2011), using the following settings for the threshold values: *p* value <0.05, *R*-scaled score >35 and abstract count >3. A heat map of the pathway terms was been generated using a transformation of the *p* value in the following way:

$$Pval_{transform} = \frac{1}{(1 + 6 * \exp(-0.35 * \log 10(p \text{ value})))}$$

Additional gene annotation enrichment analysis was done using the functional annotation module of Database for Annotation, Visualization and Integrated Discovery (DAVID) tool (Huang da et al., 2009a, b) with default settings against the KEGG pathway database.

Results

Effect of prednisolone on insulin action in 3T3-L1 adipocytes

Exposure of differentiated 3T3-L1 adipocytes to 1 μM prednisolone had no effect on the expression of key signalling molecules, like the insulin receptor β-subunit, Akt, AS160 and GLUT4. Prednisolone significantly impaired the phosphorylation of Akt-Thr308 and Akt-Ser473 in response to insulin, whereas no effects were observed on IRS1-Tyr1222 (Figure 1). Intriguingly, but in line with a study on the effects of dexamethasone on insulin action in 3T3-L1 adipocytes (Hoehn et al., 2008), prednisolone did not impact on insulin-induced phosphorylation of AS160-Thr642 (Figure 1). The mechanism(s) underlying this discordance in signalling from Akt to AS160 is not understood. Nevertheless, prednisolone was found to impair insulin-stimulated glucose uptake already after 6 h of incubation, and this inhibition was still present at 48 h after incubation (Figure 1). These findings are in line with earlier observations using dexamethasone in 3T3-L1 adipocytes (Bazuine et al., 2004; Hoehn et al., 2008) and confirm the induction of insulin resistance by prednisolone in our experimental conditions.

Whole genome expression profiling

We next studied prednisolone-induced changes in gene expression following exposure of 3T3-L1 adipocytes to prednisolone or DMSO for 1, 6, 24 and 48 h. Principle Component Analysis (PCA) showed a separation between prednisolone- and DMSO-treated samples after 6, 24 and 48 h

(Supplementary figure 1). At each time-point, the significantly differentially expressed probes were identified between cells treated with prednisolone and DMSO (Supplementary Table 2). Comparison of probes significantly affected by prednisolone at 6 h with probes significantly affected by prednisolone at 48 h showed that probes with a high fold change are found to be regulated at both time points (Figure 2). Probes which are only regulated at 6 h or at 48 h have a lower fold change.

The same trend was observed when comparing prednisolone induced probes at 6 h with prednisolone induced probes at 24 h (Figure 2B). At all time points, 25 genes represented by 34 probes (in fact 26 genes were found, but for probe *1434025_at* we could not find annotation), were found to be significantly regulated by prednisolone (Table 1). Most of these genes were up-regulated and showed a sustained response to prednisolone at all time points, starting after 1 h of incubation, with the highest response after 6 h, and still present after 48 h of incubation.

Biological pathways targeted by prednisolone

To identify biological pathways that were affected by prednisolone, we mapped the regulated genes to biological pathway terms using the text mining algorithm CoPub (Fleuren et al., 2011) (Figure 3). It appeared that the most significant pathway terms could be divided into three categories:

- immune system/inflammation (*eicosanoid metabolism, prostaglandin metabolism, cytokine receptors, cytokine network, immune system, platelet activation*),
- general metabolism (*fatty acid metabolism, glucose metabolism, gluconeogenesis, lipid metabolism, fatty acid metabolism, glycolysis*) and
- signalling (*insulin signalling, TGF-β signalling, Akt signalling, Wnt signalling, MAPK signalling*).

Additional functional analysis of these regulated genes using DAVID, an annotation server based on GeneOntology and KEGG pathways (Huang da et al., 2009b), gave similar results (results not shown).

We are interested in mechanisms and pathways that could underlie prednisolone-induced metabolic dysfunction and more specific prednisolone-induced insulin resistance in adipocytes. Therefore for the remainder of the paper, we focused on pathways that are known to play a role in the disturbance of insulin signalling that eventually could lead to insulin resistance, i.e. Akt/insulin signalling, cytokine signalling, TGF-β signalling and MAPK signalling. Furthermore, we also focused on pathways that play a role in the disturbance of adipocyte differentiation that could lead to dyslipidaemia and obesity, i.e. Wnt signalling and cytokine signalling. In Figure 4 the prednisolone-induced genes have been categorized according to these pathways. Genes that have an effect on insulin signalling, e.g. *growth-factor insulin-like growth factor I (Igf1)*, *insulin receptor substrate 1 (Irs1)* and *platelet derived growth factor receptor, beta polypeptide (Pdgfrb)* and genes from the TGF-β pathway such as *MAD homolog 3 (Drosophila) (Smad3)*, *gremlin 1 (Grem1)*, *transforming growth factor, beta 2 (Tgfb2)* and *transforming growth factor, beta induced (Tgfb1)* were down-regulated.

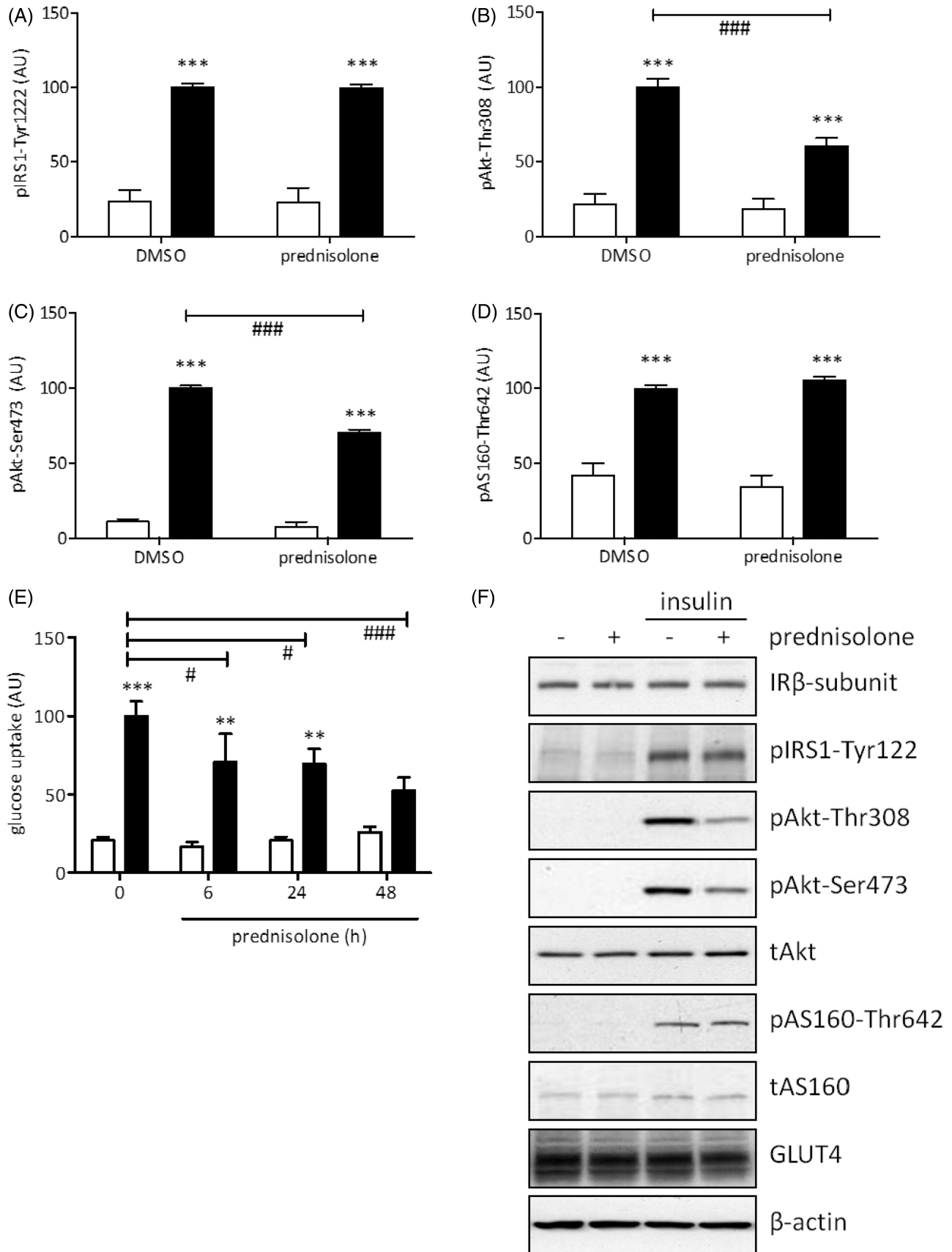


Figure 1. Effect of prednisolone on insulin action in 3T3-L1 adipocyte cells. Effects on protein expression of the insulin receptor (IR) β -subunit, Akt, AS160, and GLUT4, as well as the phosphorylation of insulin receptor substrate 1 (IRS1-Tyr1222), Akt-Thr308, Akt-Ser473, and AS160-Thr642 were determined by Western blotting in cells exposed to 1 μ M prednisolone for 48 h prior to stimulation with insulin (10 min, 100 nM). Data are presented as mean \pm standard error of the mean of >4 independent experiments (A–D), and representative Western blots (F). E. Effect of prednisolone on 2-deoxyglucose uptake in in 3T3-L1 adipocytes after 6, 24 and 48 h of incubation with prednisolone. Data are mean \pm standard error of the mean of >4 independent experiments. In all bar graphs, open columns represent the basal condition, and filled bars depict insulin-stimulated cells. The effects of prednisolone on insulin action were analysed using a two-way ANOVA followed by Bonferonni analysis for multiple comparisons. ***Indicates a $p < 0.001$; ** $p < 0.01$. Effects of insulin versus basal were analysed using a Student's t -test. ### $p < 0.001$; ## $p < 0.01$; # $p < 0.05$.

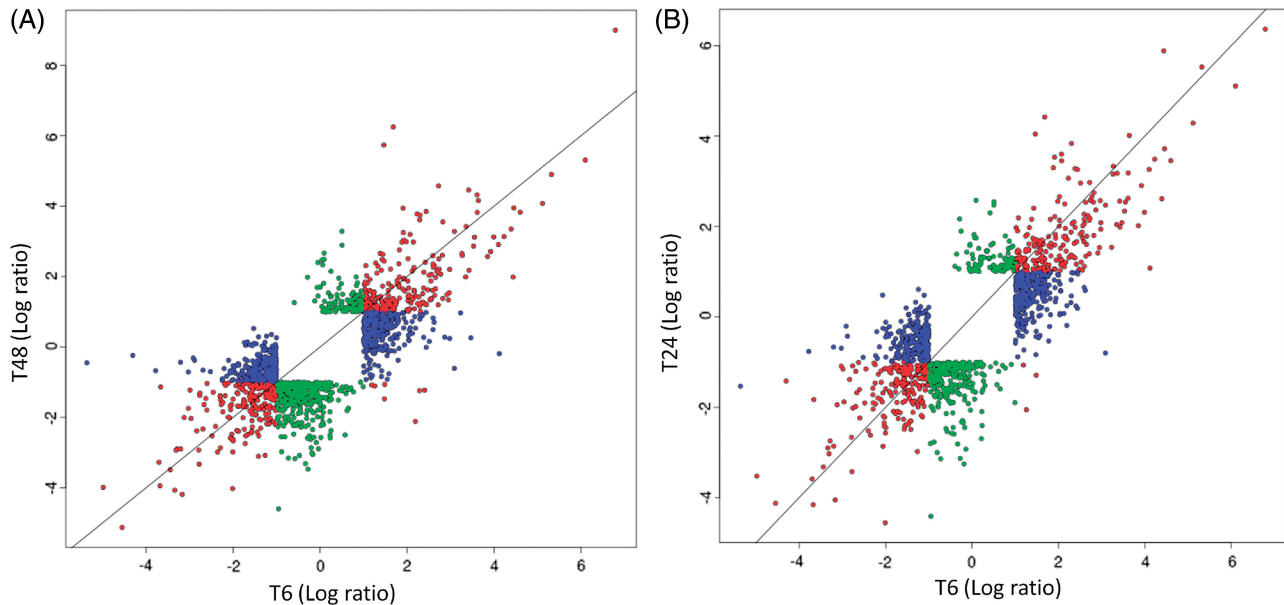


Figure 2. Regulated probes by prednisolone at 6 h versus at respectively 48 h (A) and 24 h (B). Probes represented by red dots are regulated at both time points. Probes represented by blue dots are only regulated by prednisolone at 6 h. Probes represented by green dots are only regulated by prednisolone at 48 h. In B the regulation of the probes by prednisolone after 6 h versus 24 h is shown. The scale on both axes is log₂ fold change.

Phosphatase and tensin homolog (Pten) an important regulator of the Akt pathway was up-regulated.

The majority of the genes coding for cytokines, such as *Interleukin-6 (Il-6)*, *chemokine (C-X-C motif) ligand 5 (Cxcl5)*, *chemokine (C-X-C motif) ligand 10 (Cxcl10)* and *chemokine (C-C motif) ligand 7 (Ccl7)*, were down regulated by prednisolone. Other cytokines such as *interleukin 1 receptor, type I (IL1r1)* and *interleukin 1 receptor accessory protein (IL1rap)* were up regulated.

Many Wnt signalling pathway members were significant down regulated by prednisolone in our study. We observed down regulation of frizzled receptors *Fzd1*, *Fzd2*, *Fzd4*, *Fzd5* together with ligand *wingless-related MMTV integration site 5A (Wnt5A)* and antagonist *secreted frizzled-related protein 2 (Sfrp2)*. Also several down-stream molecules such as *Wisp1*, *Wisp2*, *Id2* were down regulated (Figure 4).

Regulation of adipokines measured after 48 h of incubation with prednisolone

The microarray data showed several transcripts that encode hormones secreted by adipocytes, like cytokines, and regulators of the Wnt, Akt and TGF- β pathway. We therefore decided to study to what extent the altered levels of gene expression would lead to changes in protein expression, by analyzing the effects of prednisolone on adipokine secretion by 3T3-L1 adipocytes treated with DMSO or prednisolone for 48 h. For this purpose, the supernatants of the 3T3-L1 adipocytes that were used in the gene expression study were analysed. Out of the 308 proteins on the array, 25 proteins were significant regulated by prednisolone (Table 2, p value <0.05. For all proteins, see Supplementary Table 1). The majority of these proteins are cytokines involved in inflammatory and immune response related processes (indicated with a purple background in Table 2). Cytokines such as *CXCL2*, *CCL3*, *CCL9*, *IL31* were down regulated by

prednisolone while *CCR6*, *IL12B*, *TNFSF8*, *TNFRSF7* and *TNFRSF17* showed an up regulation.

Also several components of the Wnt signalling pathway were significantly regulated at protein level (Table 2, indicated with a blue background). *DKK4* an inhibitor of the Wnt signalling pathways was up-regulated. Furthermore downstream regulators of the Wnt signalling pathway, *LRP6* and *WISP1* are down regulated. Additionally, initiation of insulin signalling is represented by the down regulation of *IGFBP5*, *IGFBP3* and *Resistin*.

These results indicate that the regulation of the Akt/insulin signalling, cytokine signalling and Wnt signalling pathways by prednisolone is not only found on the transcriptome level, but is also translated into altered proteins levels in these pathways.

Regulation of the Wnt signalling pathway by prednisolone

Individual Wnt signalling components were found to be significantly regulated by prednisolone at both gene and protein levels. Therefore, we next investigated whether the entire Wnt signalling pathway was affected by prednisolone. The KEGG database was used to select genes upstream in the canonical Wnt signalling pathway that are more exclusively linked to the Wnt signalling in comparison with down-stream genes like *Smad3*, *Lef*, *Tak1* and *Ppar δ* , which are also linked to TGF- β signalling, MAPK signalling and Cell cycle (Supplementary Table 3). We used the expression profiles of these 51 Wnt signalling genes to test whether there is a difference in expression for the entire Wnt signalling pathway between prednisolone and DMSO treated samples. In this method, genes that do not meet the FC >2-fold and Pval <0.05 cut offs but may have nevertheless a changed expression level were taken into consideration. A significant difference in expression for the entire Wnt signalling pathway was observed after 6 h (p value = 0.031). In Figure 5, the

Table 1. Regulated probes (genes) by prednisolone after 1, 6, 24 and 48 hours of incubation. For each probe the log fold change at each time point is shown. Probes that are up-regulated are indicated with a red color and probes that are down-regulation are indicated with a green color.

Affymetrix id	Entrezgene ID	Gene Symbol	Gene Name	PRED_T1- DMSO_T1	PRED_T6- DMSO_T6	PRED_T24- DMSO_T24	PRED_T48- DMSO_T48
1451021_a_at	12224	Klf5	Kruppel-like factor 5	1.92E+00	3.61E+00	2.62E+00	4.32E+00
1423233_at	12609	Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	1.97E+00	2.97E+00	2.57E+00	1.54E+00
1448700_at	14373	G0s2	G0/G1 switch gene 2	-1.55E+00	-1.53E+00	-2.38E+00	-1.12E+00
1425281_a_at	14605	Tsc22d3	TSC22 domain family, member 3	1.76E+00	3.27E+00	3.33E+00	2.60E+00
1420772_a_at	14605	Tsc22d3	TSC22 domain family, member 3	1.60E+00	2.74E+00	2.58E+00	2.21E+00
1450297_at	16193	Il6	Interleukin 6	-1.32E+00	-2.06E+00	-2.86E+00	-2.58E+00
1422557_s_at	17748	Mt1	Metallothionein 1	1.13E+00	2.16E+00	1.96E+00	2.26E+00
1428942_at	17750	Mt2	Metallothionein 2	2.27E+00	4.23E+00	3.49E+00	3.13E+00
1421471_at	18166	Npy1r	Neuropeptide Y receptor Y1	1.32E+00	1.02E+00	1.97E+00	1.77E+00
1448830_at	19252	Dusp1	Dual specificity phosphatase 1	1.68E+00	2.71E+00	1.40E+00	1.20E+00
1420380_at	20296	Ccl2	Chemokine (C-C motif) ligand 2	-1.42E+00	-3.12E+00	-1.94E+00	-2.00E+00
1421228_at	20306	Ccl7	Chemokine (C-C motif) ligand 7	-1.50E+00	-2.00E+00	-1.14E+00	-1.48E+00
1416029_at	21847	Klf10	Kruppel-like factor 10	-1.42E+00	-1.71E+00	-1.48E+00	-1.00E+00
1417273_at	27273	Pdk4	Pyruvate dehydrogenase kinase, isoenzyme 4	1.55E+00	2.82E+00	2.75E+00	3.56E+00
1417309_at	57259	Tob2	Transducer of ERBB2, 2	1.48E+00	2.98E+00	2.09E+00	2.21E+00
1448666_s_at	57259	Tob2	Transducer of ERBB2, 2	1.40E+00	2.55E+00	1.87E+00	1.87E+00
1417310_at	57259	Tob2	Transducer of ERBB2, 2	1.40E+00	2.71E+00	2.14E+00	2.11E+00
1448667_x_at	57259	Tob2	Transducer of ERBB2, 2	1.32E+00	2.16E+00	1.73E+00	1.61E+00
1435595_at	69068	181001101Rik	RIKEN cDNA 1810011010 gene	2.38E+00	3.42E+00	1.99E+00	2.18E+00
1451415_at	69068	181001101Rik	RIKEN cDNA 1810011010 gene	2.24E+00	3.35E+00	2.04E+00	1.99E+00
1436839_at	72123	2010109K11Rik	RIKEN cDNA 2010109K11 gene	1.58E+00	2.81E+00	2.54E+00	2.61E+00
1429100_at	72123	2010109K11Rik	RIKEN cDNA 2010109K11 gene	1.42E+00	2.68E+00	2.24E+00	2.16E+00
1419816_s_at	74155	Errf1	ERBB receptor feedback inhibitor 1	3.21E+00	5.12E+00	4.29E+00	4.08E+00
1416129_at	74155	Errf1	ERBB receptor feedback inhibitor 1	2.68E+00	4.60E+00	3.46E+00	3.83E+00
1428306_at	74747	Ddit4	DNA-damage-inducible transcript 4	2.35E+00	2.24E+00	1.31E+00	1.23E+00
1437424_at	214804	Syde2	Synapse defective 1, Rho GTPase, homolog 2 (C. elegans)	1.21E+00	1.62E+00	1.63E+00	1.22E+00
1457035_at	226691	Al607873	Expressed sequence A1607873	2.12E+00	4.44E+00	5.89E+00	1.99E+00
1460011_at	232174	Cyp26b1	Cytochrome P450, family 26, subfamily b, polypeptide 1	1.07E+00	2.29E+00	1.88E+00	3.61E+00
1439293_at	235493	BC031353	cDNA sequence BC031353	1.53E+00	1.93E+00	1.82E+00	3.03E+00
1434202_a_at	268709	Fam107a	Family with sequence similarity 107, member A	1.70E+00	6.79E+00	6.37E+00	9.00E+00
1434228_at	381511	Pdpl	Pyruvate dehydrogenase phosphatase catalytic subunit 1	1.25E+00	1.62E+00	1.70E+00	1.18E+00
1438201_at	381511	Pdpl	Pyruvate dehydrogenase phosphatase catalytic subunit 1	1.22E+00	1.71E+00	1.44E+00	2.07E+00
1457102_at	399638	A030001D16Rik	RIKEN cDNA A030001D16 gene	1.34E+00	3.85E+00	2.02E+00	2.57E+00
1434025_at	NA	NA	NA	1.87E+00	3.42E+00	2.56E+00	4.46E+00

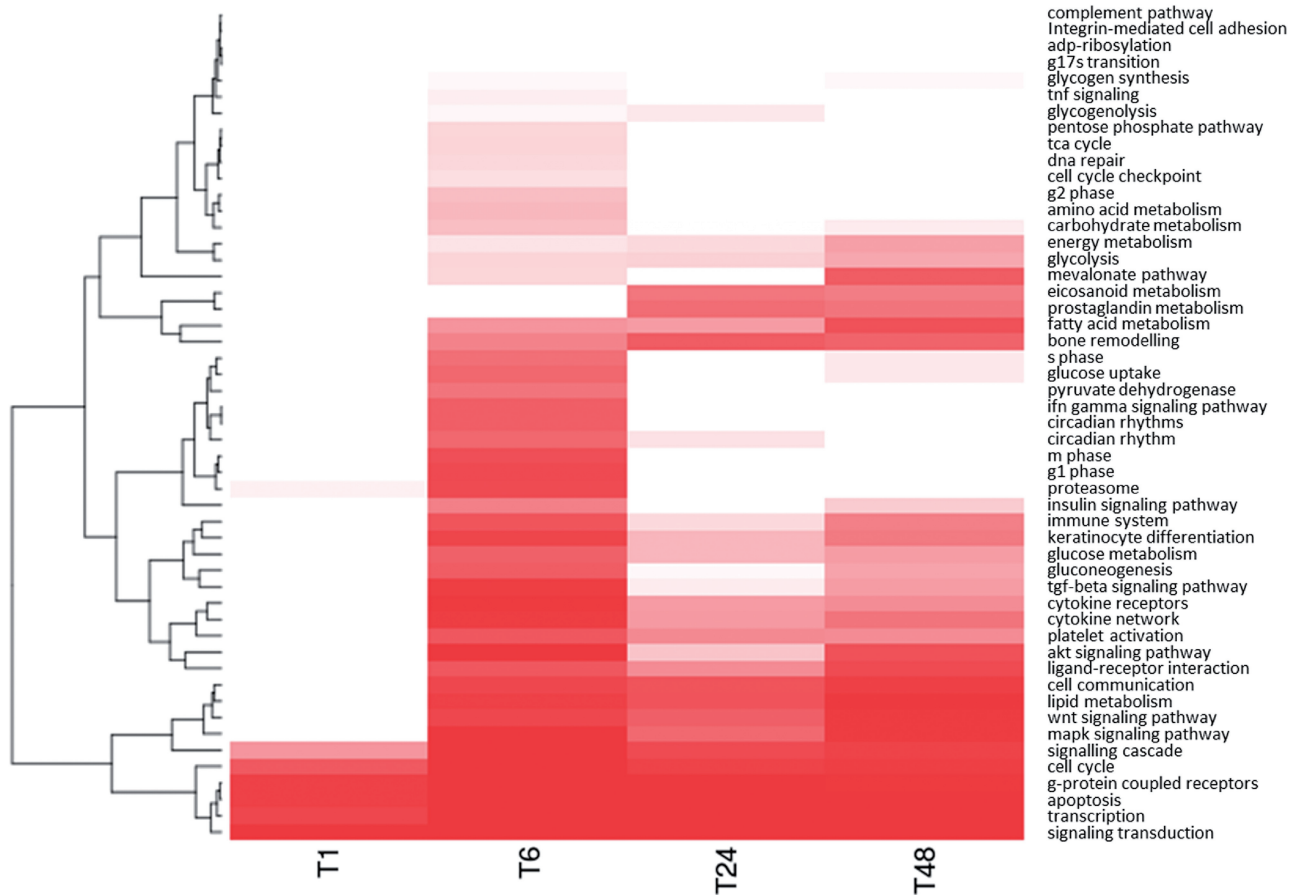


Figure 3. Heat map representation of biological pathways identified using CoPub analysis of regulated genes. The colours shading indicates the significance of the over-represented pathways (based on $p_{val_transformed}$), from the lowest value shown in red, to the highest value shown in white. Columns represent the time points at which the pathways have been found after incubation with prednisolone: 1 h (T1); 6 h (T6); 24 h (T24); 48 h (T48).

genes that had the most influence on this difference are shown. In this figure, the gene expression ratios (open dots) and protein ratios (black dots) as a result of prednisolone treatment are also shown. Genes that had an influence on the difference between DMSO and prednisolone treated samples and that showed a higher expression in DMSO treated samples (red bars) were down-regulated because of prednisolone treatment (open dots). The same effect on the Wnt signalling pathway was also seen after 48 h (p value = 0.064) of treatment (Supplementary Figure 3). This analysis indicates that in 3T3-L1 adipocytes, the entire Wnt signalling pathway is down-regulated by prednisolone.

Discussion and conclusions

The prolonged use of glucocorticoids is hampered by metabolic side-effects such as insulin resistance, which eventually leads to diabetes and obesity. Adipose tissue is thought to play an important role in GC-induced development of metabolic side effects. The mechanisms behind these side-effects are not completely understood. Key findings of our study indicate that prednisolone reduces insulin stimulated glucose uptake in 3T3-L1 cells, which is reflected by changes in expression of genes and proteins from the Akt/insulin, cytokine and Wnt signalling pathways. To our knowledge, this is the first work that uses a combination of gene expression and antibody arrays to study effects of prednisolone in adipocyte cells.

The set of 25 genes that showed regulation by prednisolone at all time points includes known target genes of the glucocorticoid receptor, such as *Ddit4*, *Dusp1* and *Cebpd* (Toonen et al., 2011; Yang et al., 2005), and genes such as *Fam107a* and *PDK4*, that were also found to be regulated by prednisolone in *in vivo* studies of mouse and human (Bastard et al., 2006; Frijters et al., 2010; Toonen et al., 2011). This indicates that prednisolone acts via its target genes and initiates a GC specific response in these 3T3-L1 cells.

Multiple cytokine molecules were regulated by prednisolone. Increasing evidence suggests that a chronic low-grade state of inflammation in adipose tissue contributes to the development of systemic insulin resistance and diabetes (Bastard et al., 2006; Harford et al., 2011; Heilbronn & Campbell, 2008). In the absence of IL1, mice are protected against high fat diet induced insulin resistance which is accompanied with a reduction in local adipose tissue inflammation (de Roos et al., 2009; McGillicuddy et al., 2011). Our results showed up-regulation of IL1 signalling by means of *IL1r1* and *IL1rap*. This suggests a role of IL-1 signalling in GC induced development of insulin resistance in 3T3-L1 cells.

Also other studies reported about the up-regulation of cytokines such as *Il6*, *Cxcl5*, *Ccl2* and *Cxcl10* in insulin resistance and obesity (Figure 6) (Bastard et al., 2006; Chavey et al., 2009; Lagathu et al., 2003; Murdolo et al., 2008; Rotter et al., 2003; Tateya et al., 2010). In contrast to

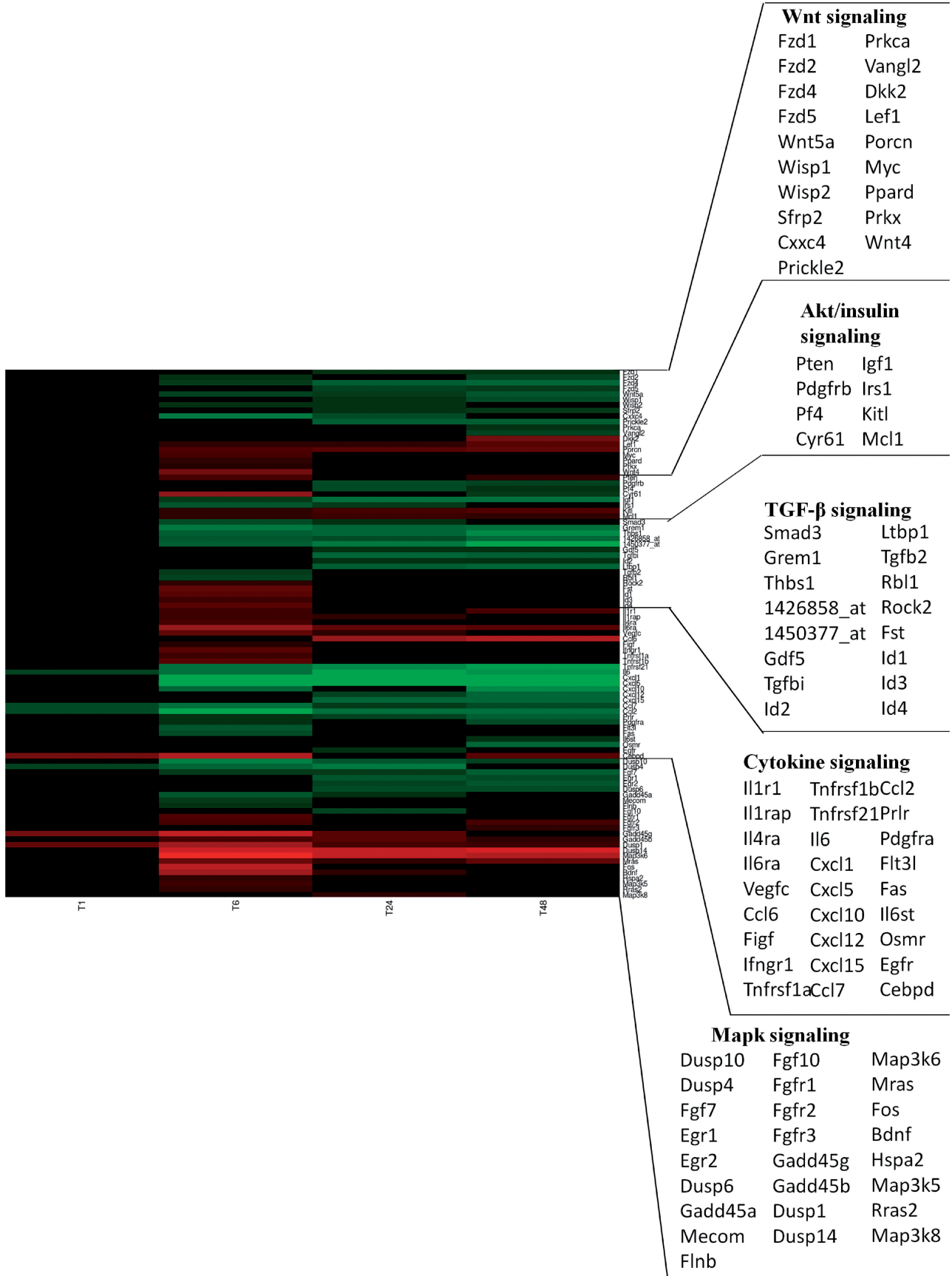


Figure 4. Prednisolone induced genes after 1 h (T1), 6 h (T6), 24 h (T24), 48 h (T48). Prednisolone-induced up-regulated genes are shown in red, prednisolone induced down-regulated genes are shown in green. For clarity genes have been assigned to major pathways. However, one should keep in mind that genes can belong to multiple pathways depending on the definition of a pathway.

Table 2. Regulated proteins after 48 hours of incubation with prednisolone. Inflammatory related proteins are indicated with a purple background, Wnt signaling related proteins with a blue background and insulin signaling related proteins with a green background. Up-regulated proteins are indicated with a red color. Down-regulated proteins are indicated with a green color.

Name	Protein Symbol	Mean log 2 ratios	P-value	Function description derived from Uniprot
BCMA/TNFRSF17	TNFRSF17	0.99	0.045	Receptor for TNFSF13B/BLyS/BAFF and TNFSF13/APRIL. Promotes B-cell survival and plays a role in the regulation of humoral immunity.
CCR6	CCR6	0.87	0.035	Receptor for a C-C type chemokine. Binds to MIP-3-alpha/LARC and subsequently transduces a signal by increasing the intracellular calcium ions level.
Artemin	ARTN	0.85	0.015	Ligand for the GFR-alpha-3-RET receptor complex but can also activate the GFR-alpha-1-RET receptor complex.
Decorin	DCN	0.84	0.003	May affect the rate of fibrils formation.
TLR1	TLR1	0.78	0.024	Participates in the innate immune response to microbial agents. Cooperates with TLR2 to mediate the innate immune response to bacterial lipoproteins or lipopeptides. Acts via MYD88 and TRAF6, leading to NF-kappa-B activation, cytokine secretion and the inflammatory response
LIF	LIF	0.64	0.016	LIF has the capacity to induce terminal differentiation in leukemic cells. Its activities include the induction of hematopoietic differentiation in normal and myeloid leukemia cells, the induction of neuronal cell differentiation, and the stimulation of acute-phase protein synthesis in hepatocytes.
IL-12 p70	IL12B	0.64	0.024	Cytokine that can act as a growth factor for activated T and NK cells, enhance the lytic activity of NK/lymphokine-activated killer cells, and stimulate the production of IFN-gamma by resting PBMC
Dkk-4	DKK4	0.57	0.024	Antagonizes canonical Wnt signalling by inhibiting LRP5/6 interaction with Wnt and by forming a ternary complex with the transmembrane protein KREMEN that promotes internalization of LRP5/6.
CD27 / TNFRSF7	TNFRSF7	0.51	0.040	Receptor for CD70/CD27L. May play a role in survival of activated T-cells. May play a role in apoptosis through association with SIVA1.
CD30 L	TNFSF8	0.37	0.010	Cytokine that binds to TNFRSF8/CD30. Induces proliferation of T-cells.
Gremlin	GREM1	0.24	0.019	Cytokine that may play an important role during carcinogenesis and metanephric kidney organogenesis, as BMP a antagonist required for early limb outgrowth and patterning in maintaining the FGF-4-SHH feedback loop.
WISP-1 / CCN4	WISP1	-0.17	0.033	Downstream regulator in the Wnt/Frizzled-signalling pathway.
MIP-1 gamma	CCL9	-0.32	0.007	Monokine with inflammatory, pyrogenic and chemokinetic properties. Circulates at high concentrations in the blood of healthy animals.
IL-31	IL31	-0.35	0.035	Activates STAT3 and possibly STAT1 and STAT5 through the IL31 heterodimeric receptor composed of IL31RA and OSMR. IL31 may function in skin immunity.
VEGF	VEGFA	-0.35	0.040	Growth factor active in angiogenesis, vasculogenesis and endothelial cell growth. Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels.
MIP-2	CXCL2	-0.36	0.000	Chemotactic for human polymorphonuclear leukocytes but does not induce chemokinesis or an oxidative burst.
TCCR / WSX-1	IL27RA	-0.42	0.049	Receptor for IL27. Requires IL6ST/gp130 to mediate signal transduction in response to IL27. This signalling system acts through STAT3 and STAT1. Involved in the regulation of Th1-type immune responses. Also appears to be involved in innate defence mechanisms.
Resistin	RETN	-0.43	0.022	Hormone that seems to suppress insulin ability to stimulate glucose uptake into adipose cells. Potentially links obesity to diabetes.
VEGF R1	FLT1	-0.57	0.027	The VEGF-kinase ligand/receptor signalling system plays a key role in vascular development and regulation of vascular permeability.
LRP-6	LRP6	-0.60	0.008	Component of the Wnt-Fzd-LRP5-LRP6 complex that triggers beta-catenin signalling through inducing aggregation of receptor-ligand complexes into ribosome-sized signalosomes.
IGFBP-5	IGFBP5	-0.65	0.015	IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture.
MIP-1 alpha	CCL3	-0.65	0.016	Monokine with inflammatory, pyrogenic and chemokinetic properties. Has a potent chemotactic activity for eosinophils. Binding to a high-affinity receptor activates calcium release in neutrophils.
P-Selectin	SELP	-0.69	0.010	Ca ²⁺ -dependent receptor for myeloid cells that binds to carbohydrates on neutrophils and monocytes.
IGFBP-3	IGFBP3	-0.71	0.043	IGF-binding proteins prolong the half-life of the IGFs and have been shown to either inhibit or stimulate the growth promoting effects of the IGFs on cell culture.
Integrin beta 2 / CD18	ITGB2	-0.86	0.040	Integrin alpha-L/beta-2 is a receptor for ICAM1, ICAM2, ICAM3 and ICAM4. Integrins alpha-M/beta-2 and alpha-X/beta-2 are receptors for the iC3b fragment of the third complement component and for fibrinogen.

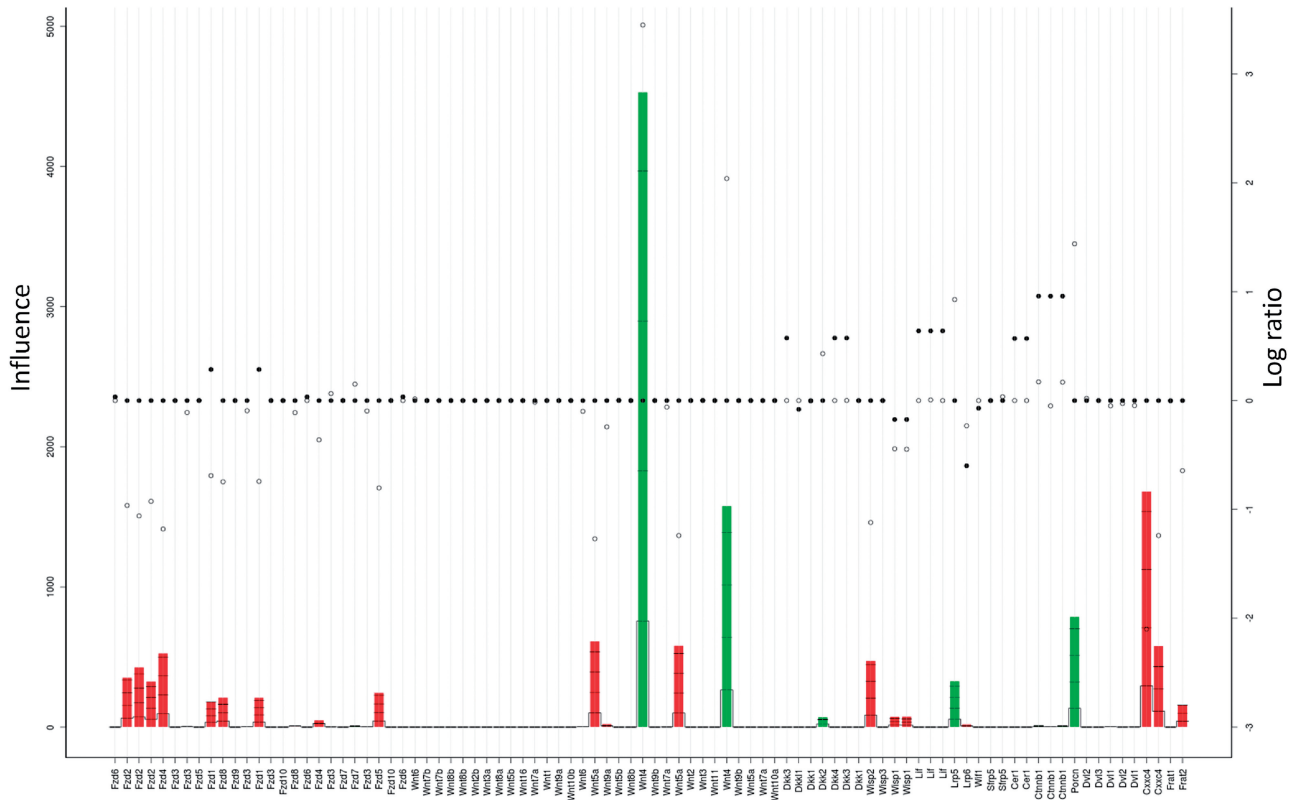


Figure 5. Wnt signalling genes that have an influence on the difference between prednisolone and DMSO, after 6 h of incubation. Genes that are up-regulated in DMSO treated samples are shown in red and genes that are up-regulated in prednisolone treated samples are shown in green. Ratios of prednisolone-induced genes are indicated with open dots. Prednisolone-induced ratios of the proteins are indicated with black dots. Ratios are indicated in log₂ fold change.

these studies, we observed a down regulation of expression of these genes by prednisolone. Based on these results it is difficult to deduce whether prednisolone-induced regulation of cytokines in adipocytes contribute to the development of insulin resistance or are the result of the immune suppressing properties of prednisolone or a combination of both.

Furthermore our results indicated that the reduction in insulin stimulated glucose uptake by prednisolone acts partly via the down regulation of insulin signalling. This regulation might be mediated by *Pten*, a known suppressor of insulin signalling that acts via the PI3K/Akt signalling pathway (Tang *et al.*, 2005). Additional studies confirm this observation and suggest an important role of *Pten* in the development of insulin resistance and diabetes (Ikubo *et al.*, 2009; Lazar & Saltiel, 2006; Nakashima *et al.*, 2000).

Again an apparent contrasting effect of prednisolone is the down regulation of proteins like *Igf3* and *resistin* that reduce glucose uptake in adipose tissue (Chan *et al.*, 2005; Kim *et al.*, 2007).

Inhibition of TGF- β /*Smad3* signalling results in diminished adiposity, improved glucose tolerance and insulin sensitivity and in the protection from insulin resistance, diabetes and obesity (Tan *et al.*, 2011; Tsurutani *et al.*, 2011; Yadav *et al.*, 2011). The observed down regulation of *Smad3* suggests that disturbance of glucose uptake and adiposity by prednisolone in 3T3-L1 cells acts via additional mechanisms.

Activation of the Wnt signalling pathway has been linked to inhibition of adipogenesis (Bennett *et al.*, 2002; Christodoulides *et al.*, 2006; Longo *et al.*, 2002; Ouchi *et al.*, 2010).

Recent studies toward the characterization of the adipocyte secretome have shown that multiple regulators of the activity the Wnt signalling are secreted by adipocytes, such as WISP2 and SFRP5 (Lehr *et al.*, 2012; Ouchi *et al.*, 2010). Interestingly, expression and circulating levels of these factors are altered in insulin resistance and obesity (Dahlman *et al.*, 2012; Hu *et al.*, 2012; Mori *et al.*, 2012; Ouchi *et al.*, 2010), underlying the suggestion that a dysregulated secretion of Wnt regulators could lead to metabolic disturbances and eventually into the development of obesity and diabetes (Ouchi *et al.*, 2010; Jin, 2008; Liu & Habener, 2010; Oh & Olefsky, 2010; Schinner, 2009). Yet, in the context of our study, it remains to be investigated to what extent the factors affected by prednisolone interfere with insulin action in adipocytes.

Prednisolone induced *Pten*, *Smad3* and *Lef1* which are able to mediate cross-talk between the signalling pathways (Guo & Wang, 2009). Via the alteration of the *Pten* function, TGF- β is able to influence the Akt activity, and via *Smad3* the Akt pathway is able to restrict the TGF- β pathway. Also Smad activity is involved in the cross-talk between Wnt signalling and TGF- β signalling, as part of the Smad/ β -catenin/Lef protein complex in the nucleus. These genes could play a central role in the development of prednisolone induced metabolic effects because of their ability to connect multiple pathways (Figure 6). The sometimes opposing effects of prednisolone on individual pathway members of *Smad3* and Akt in comparison with earlier work, requires additional *in vivo* studies in which the systemic effect of prednisolone on these pathways could be determined.

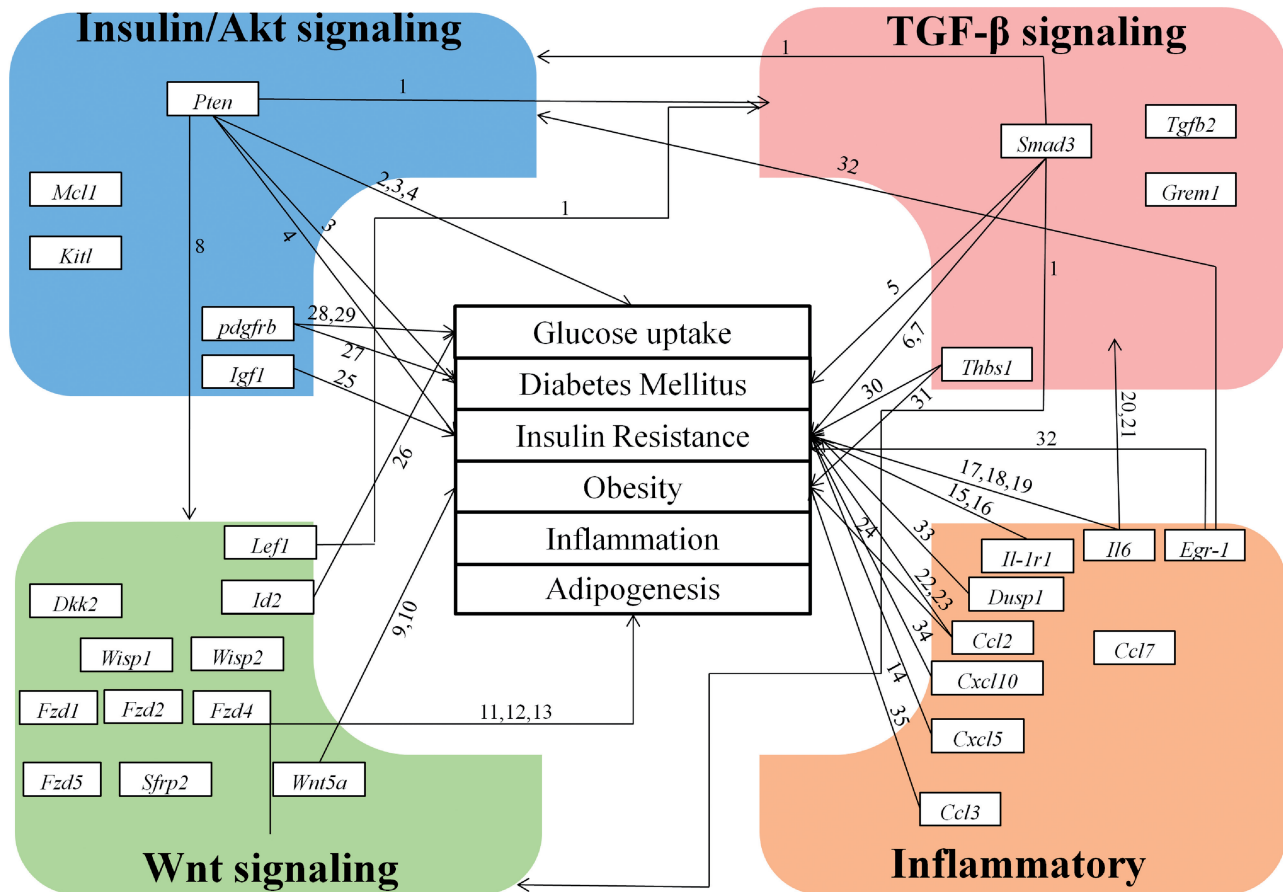


Figure 6. Overview of pathway members of insulin/Akt signalling, TGF- β , wnt signalling and inflammatory pathways that were regulated by prednisolone in 3T3-L1 cells. For these pathway members the literature links to metabolic disorders and other pathways are shown. The numbers at the arrows indicate the references in the literature, in which the association between pathway member and pathways/disease phenotype is mentioned. 1 (Guo & Wang, 2009); 2 (Nakashima *et al.*, 2000); 3 (Lazar & Satiel, 2006); 4 (Ikubo *et al.*, 2009); 5 (Lin *et al.*, 2009); 6 (Tan *et al.*, 2011); 7 (Tsurutani *et al.*, 2011); 8 (Zhang *et al.*, 2006); 9 (Oh & Olefsky, 2010); 10 (Ouchi *et al.*, 2010); 11 (Ross *et al.*, 2000); 12 (Longo *et al.*, 2002); 13 (Christodoulides *et al.*, 2006); 14 (Chavey *et al.*, 2009); 15 (de Roos *et al.*, 2009); 16 (McGillicuddy *et al.*, 2011); 17 (Lagathu *et al.*, 2003); 18 (Rotter *et al.*, 2003); 19 (Bastard *et al.*, 2006); 20 (Yamamoto *et al.*, 2001); 21 (Walia *et al.*, 2003); 22 (Tateya *et al.*, 2010); 23 (Kamei *et al.*, 2006); 24 (Sell and Eckel, 2007); 25 (Clemmons, 2004); 26 (Gronning *et al.*, 2006); 27 (Chen *et al.*, 2011); 28 (Yuasa *et al.*, 2004); 29 (Whiteman *et al.*, 2003); 30 (Alessi *et al.*, 2000); 31 (Garcia-Diaz *et al.*, 2011); 32 (Yu *et al.*, 2011); 33 (Fujimoto *et al.*, 2010); 34 (Mehta *et al.*, 2010); 35 (Bala *et al.*, 2011).

To our knowledge, for the first time it is shown that prednisolone affects the expression and secretion of Wnt regulators in adipocytes. The fact that Wnt signalling also participates in the development of metabolic disturbances in other tissues (Abiola *et al.*, 2009; Anagnostou & Shepherd, 2008) may direct future work towards a dedicated study of the Wnt signalling pathway in GC-induced metabolic effects. In an *in vivo* setting the Wnt signalling mediated GC effects in multiple glucose responsive tissues such as muscle tissue, liver tissue and adipose tissue could be determined. Hence new GC compounds with an improved efficacy/side effects ratio should have a reduced effect on components in the Wnt signalling pathway.

Declaration of interest

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