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Effects of short-term prednisolone treatment on indices of lipolysis and lipase signaling in abdominal adipose tissue in healthy humans☆

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ABSTRACT

Background: Glucocorticoid (GC) excess increases lipolysis, circulating free fatty acid concentrations and lipid oxidation rates in humans. In vitro and animal studies have shown that GCs increase adipocyte ATGL and HSL mRNA contents and HSL phosphorylations, but the effects of GC on in vivo lipase signaling in humans are uncertain. Our study was designed to test how GC administration affects ATGL and HSL related signals in human adipose tissue.
Material and methods: Nine healthy young men underwent 5 days administration of 37.5 mg prednisolone/d in a randomized, double-blinded, placebo-controlled crossover design. At the end of each 5 d period the subjects were studied after an overnight fast for 6.5 h including a basal period and a 2½ h hyperinsulinemic euglycemic clamp. Adipose tissue biopsies were sampled from the abdominal subcutaneous adipose tissue at the end of the basal period and the clamp.
Results: GC treatment increased serum FFA concentrations and comparative gene identification-58 (CGI-58) mRNA - an ATGL activator - and decreased G0/G1 switch 2 gene (G0S2) mRNA - an ATGL inhibitor - in adipose tissue biopsies. In addition, pro-lipolytic ser^{563} HSL phosphorylations and protein kinase A (PKA) phosphorylation of PLIN1 (Perilipin-1) increased. The transcripts of ANGPTL4 (Angiopoietin-like 4) mRNA - a regulator of circulating triglycerides - were elevated by GC; as were CIDE (Cell-death Inducing DNA fragmentation factor-α-like Effector)-A and CIDE-C mRNA transcripts indicative of concurrent stimulation of lipolysis and lipogenesis. Finally GCs reduced insulin receptor phosphorylation, and Akt protein levels.
Conclusions: High dose GC administration to humans leads to pro-lipolytic alterations of CGI-58, G0S2 and ANGPTL4 mRNA transcripts, increases PKA signaling to lipolysis and inhibits the insulin signal in adipose tissue. The increased CIDE-A and CIDE-C mRNA levels suggest concomitant stimulation of lipolysis and lipid storage.

1. Introduction

In humans a variety of hormones regulate triglyceride breakdown and storage and the overall switch between lipid storage and lipolysis is tightly regulated by insulin and catecholamines. Catecholamines stimulate lipolysis via activation of β-adrenergic receptors and via the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway. Activation of cAMP and PKA results in phosphorylation of the lipid droplet-associated protein PLIN1, which promotes activation of the lipolytic enzymes adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) [1]. ATGL is activated by association with its co-activator CGI-58, which is released from sequestration by PKA-mediated phosphorylation of PLIN1. The cAMP/PKA pathway is inhibited by insulin via the phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt) pathway, which activates phosphodiesterase 3B (PDE3B) [2]. PDE3B inhibits lipolysis by reducing cAMP levels and thereby downstream activation of the lipolytic enzymes.

Glucocorticoids (GCs) exert diverse effects in adipose tissue [3] including stimulation of lipolysis via β-adrenergic and insulin-antagonistic mechanisms [4,5]. GCs have been shown to upregulate

Abbreviations: ANGPTL4, Angiopoietin-like 4; Akt, protein kinase B; CGI-58, comparative gene identification-58; CIDE, Cell-death Inducing DNA fragmentation factor-α-like Effector; FOXO1, Forkhead box protein O1; G0S2, G0/G1 switch 2 gene; PDE3B, phosphodiesterase 3B; PLIN-1, Perilipin-1; PKA, protein kinase A; PTEN, Phosphatase and Tension homologue.

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intracellular cAMP levels and downregulate PDE3B expression in rat adipocytes [6]. Adipocyte stimulation with dexamethasone has also been shown to increase glycerol release [7] and therefore, GCs are believed to play a direct role on adipose tissue lipid breakdown (lipolysis) as well. Accordingly, GCs have been reported to increase adipocyte gene transcription of ATGL and HSL [7,8] as well as ANGPTL4 - a primary target gene of the GC receptor involved in triglyceride metabolism [9,10].

The majority of studies investigating the mechanisms by which GCs induce lipolysis have been experimental studies in cell systems or mice models. In order to define the in vivo effects of GC in humans, we conducted a randomized, placebo-controlled, cross-over study with 5 days of prednisolone (37.5 mg/day) in healthy subjects. Our specific aims were to test to which extent pro-lipolytic alterations or insulin-antagonistic alterations constitute the mechanisms by which GCs promote lipolysis.

2. Materials and methods

2.1. Subjects

Sample size was based on the basis of previous studies performed by our group dealing with glucocorticoids and lipolysis showing that lipolysis is clearly and robustly stimulated in $n = 7$ designs [11]. A total of nine healthy young men with an average age of 25 (23; 26) years, body mass index of 23 (23; 24) kg/m^2 and fasting plasma glucose of 5.1 (4.9; 5.6) mmol/l participated (Table 1). The subjects did not take prescription medicine. All participants gave written, informed consent in accordance with the Declaration of Helsinki II. The study was conducted after approval from The Regional Scientific Ethical Committee and Danish Health and Medicine Authority. This paper is part of a larger study reported at www.clinicaltrial.gov (identification number: NCT01762540, registration date: 03.01.2013.) and at the EU clinical trial register EUDRA-CT (identification number: 2012-003504-12). The study was performed at the Medical Research Laboratory, Aarhus University and Aarhus University Hospital, Denmark.

2.2. Study design and protocol

In a randomized, double-blinded, placebo-controlled, cross-over design, subjects underwent a 5-day-session of oral prednisolone treatment (37.5 mg/d) and a 5-day-session of placebo treatment. Each study session was separated by a wash-out period of 4–6 weeks. On day 5 of each treatment-arm, participants were examined in the morning after an overnight fast during a 4 h basal, and a 2.5 h hyperinsulinemic euglycemic clamp (HEC), throughout which the blood glucose levels were clamped at ~5 mmol/l with an insulin (*Insulin Actrapid; Novo-Nordisk, Copenhagen, Denmark*) infusion of 0.5 mU/kg/min. Adipose tissue biopsies were sampled from the abdominal subcutaneous adipose tissue using a lipid suction technique 1.5 h into the basal and HEC stimulated period. After sampling, biopsy tissues were immediately frozen in liquid nitrogen and stored at −80 °C until analyzed.

The study protocol is previously described [12]. In brief, screening and enrollment of study participants were conducted by the investigators and randomization by the hospital pharmacy. Tablets containing prednisolone or placebo (calcium supplement) were encapsulated to blind participants and investigators. The randomization list was kept at the pharmacy until trial end.

Table 1
Subject characteristics.

$N = 9$	
Age (years)	25 (23;26)
BMI (kg/m^2)	23 (23;24)
Fasting plasma glucose (mmol/l)	5.1 (4.9;5.6)
HbA1c (%)	5.2 (5.2;5.9)
Systolic BP (mmHg)	120 (114;126)
Diastolic BP (mmHg)	68 (66;75)

2.3. Prednisolone

The supraphysiological dosage of prednisolone 37.5 mg/d was chosen due to its clinical relevance and well-tolerance during short term treatment e.g. treatment of acute exacerbation of chronic obstructive pulmonary disease (COPD).

2.4. Blood analyses

Plasma glucose was immediately measured using the glucose oxidase method (YSI 2300 STAT Plus, YSI Life Sciences, Yellow Springs, OH, USA). FFAs were analyzed by a commercial kit (Wako Chemicals, Neuss, Germany). Triglyceride was analyzed with Advia Chemistry XPT from Siemens, Denmark. Insulin and C-peptide were measured by immunoassays and metanephrines were analyzed by ELIZA kit from Labor Diagnostika Nord GmbH & KG, Germany.

2.5. Western blot

50 mg frozen adipose tissues were homogenized in 200 μL lysis buffer (50 mM HEPES, 20 mM NaF, 5 mM EDTA, 5% SDS, HALT, 5 mM NAM, 10 μM TSA) using a Precellys homogenizer (Bertin Technologies). Following homogenization samples were incubated at 37 °C with continuous vortexing (1000 rpm) followed by centrifugation at 14,000*g* for 20 min at room temperature. The supernatant was carefully separated from the lipid layer and centrifuged again in clean tubes in order to further increase purification. This approach has been developed specifically for extraction of lipophilic proteins from adipose tissue and provides reliable and consistent extraction of cytosolic as well as lipid droplet-associated proteins [13]. Protein concentration of the supernatant was determined using a Bradford assay (BioRad). Samples were adjusted to equal concentrations with milli-Q water, denatured by mixing 1:1 with 2× Laemmli's buffer, and heating at 95 °C for 5 min.

Equal amounts of protein were separated by SDS-PAGE (TGX-Stain-Free 4–15% gels, CriterionXT-system, Bio-Rad) and transferred by electro-blotting to PVDF membranes (Trans-Blot, ®Turbo, Bio-Rad). Control for equal loading was performed using the Stain-Free technology [14]. The membranes were blocked for 2 h in TBS-T with 1% BSA before overnight incubation in primary antibody (Table 2) at 4 °C. After repeated washes the membranes were incubated in secondary antibody (Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit, Abcam, Cambridge UK) for 2 h at room temperature. Proteins were visualized by BioRad enhanced chemiluminescence and quantified using Image Lab (ver. 4.0.1 BioRad). Mean intensities were calculated and used for

Table 2
Primary antibodies used in Western blot analysis. ATGL adipose triglyceride lipase; HSL hormone-sensitive lipase; PLIN-1, Perilipin-1; CGI-58, comparative gene identification-58; G0S2; G0/G1 switch 2 gene; PTEN, Phosphatase and Tension homologue; PDE3B phosphodiesterase 3B; IR insulin receptor, PKA, protein kinase A.

Primary antibodies against:	Catalogue no	Manufacture
ATGL	GTX62840	Genetex, Bath, UK
HSL	4107s	Cell Signaling Technology, Beverly, CA, USA
pHSL Ser^{552}	4139s	Cell Signaling Technology, Beverly, CA, USA
pHSL Ser^{650}	4126L	Cell Signaling Technology, Beverly, CA, USA
PLIN1	9349s	Cell Signaling Technology, Beverly, CA, USA
CGI58	Ab183739	Abcam, Cambridge, UK
G0S2	Sc-133424	Santa Cruz Biotech, Santa Cruz, CA, USA
PTEN	9188	Cell Signaling Technology, Beverly, CA, USA
PDE3B	Ab99290	Abcam, Cambridge, UK
IR	3025	Cell Signaling Technology, Beverly, CA, USA
pIR Tyr^{361}	3023	Cell Signaling Technology, Beverly, CA, USA
PKA substrate	9624s	Cell Signaling Technology, Beverly, CA, USA

semi-quantitative analysis. Quantifications are expressed as the ratio between phosphorylated protein and the targeted protein measured on the same membrane. For determination of HSL activity antibodies recognizing the PKA regulatory sites Ser^{552} and Ser^{650} were used (Table 2). Human Ser^{552} and Ser^{650} are equivalent to Ser^{563} and Ser^{660} in rat, respectively [15,16]. For determination of PLIN1 phosphorylation, the same membrane was exposed to both Phospho-PKA Substrate antibody and PLIN1 antibody. When lipolysis is stimulated, the Phospho-PKA Substrate antibody detects a prominent band with a molecular mass of 62 kDa, of which the vast majority is PLIN1. This method for determination of PLIN1 phosphorylation has been validated by others using a PLIN1 knock-out model, showing that in the absence of PLIN1 no phospho-signal was detectable at 62 kDa [17]. Differences between interventions are expressed as the ratio change from the measurement made in the non-HEC stimulated period of the placebo day for each subject.

2.6. Quantitative PCR

RNA was extracted from the adipose tissue biopsies using Trizol reagent (Life technologies Inc.). RNA isolation was conducted according to the manufacture's protocol (Gibco BRL, Life Technologies). RNA was quantified by measuring absorbance at 260 and 280 nm using a NanoDrop 8000 (NanoDrop Products, Bancroft, DE, USA), and inclusion criteria were a ratio ≥1.8. Integrity of the RNA was checked by visual inspection of the two ribosomal RNAs, 18S and 28S, on an agarose gel. cDNA was synthesized with the TaqMan Gold RT-PCR Kit (PerkinElmer, Boston, MA, USA). Real-time PCR for PLIN1, CGI-58, ATGL, G0S2, HSL, CIDE-A, CIDE-C, PDE3B, and ANGPTL4 were assessed with mRNA levels of β-actin as an internal control. The following primers were used: *PLIN1*, 5′GGA GCG AGG ATG GCA GTC AAC 3′ and 5′TCT GGA AGC ATT CGC AGG T 3′. *CGI-58*, 5′TGT CAG CCG GCT TCG AGA TAA G 3′ and 5′ ACC AGT TAG CCA TCC TGA CCT CTC 3′. *ATGL*, 5′ACC TCA ATG AAC TTG GCA CC 3′ and 5′CAA CGC CAC GCA CAT CTA 3′. *G0S2*, 5′CGA GAG CCC AGA GCC GAG ATG 3′ and 5′AGC ACC ACG CCG AAG AG 3′. *HSL*, 5′GAA GGC GGC ACG GAC GCC 3′ and 5′GCT GGT GCG GCG GGA CAC 3′. *CIDE-A*, 5′CGG CTG CCT TAA CGT GAA3′ and 5′AGA TGA GAA ACT GTC CCG TCA 3′. *CIDE-C*, 5′CAT TGG CTG CCT GAA CGT GA 3′ and 5′GGA GGT GCC AAG CAG TAC GTG 3′. *ANGPTL4*, 5′ TAG TCC ACT CTG CCT CTC CC 3′ and 5′ GAG ATG GCC CAG CCA GTT 3′. *PDE3B*, 5′ TCT GAC AAC ACG GCC AGT TC 3′ and 5′ GAC AGG CAG CCA TAA CTC TCA 3′.

2.7. Statistics

All statistical analyses and figures were performed using Stata 13 (College Station, Texas, USA). Mixed-effect linear model was used to analyze effect of treatment (prednisolone vs. placebo) and HEC on dependent variables. Visit order, visit number, time, treatment, and the interaction between time and treatment were accounted for in the model. The interaction of treatment and subject was defined as a random factor (each subject gets both treatments so that treatment is nested in the subjects). Model validation was performed by comparing observed and expected within-subject standard deviations (SD) and correlations, and by inspecting QQ-plots of the residuals, and of scatterplots of the predicted versus the fitted values. If residuals were non-normally distributed, logarithmic transformation was performed. When variables showed interaction between prednisolone and HEC (*P*-value given as $P_{interaction}$), post-hoc analyses testing the effect of prednisolone and HEC were performed. When variables showed no interaction, the outcome was tested for main effect of prednisolone and HEC (*P*-value given as $P_{prednisolone}$ and P_{HEC}). Data are presented as means and confidence interval (CI) or as median and interquartile range (IQR 25%–75%) as appropriate. *P*-values <0.05 were considered significant.

3. Results

3.1. Hormone and metabolite concentrations

Concentrations of insulin, C-peptide, fasting glucose, FFA, triglyceride, metanephrine and normetanephrine are depicted in Table 3. Baseline levels of insulin and C-peptide were increased by prednisolone treatment as compared to placebo. During the HEC stimulation, insulin concentrations were indifferent between treatments. Prednisolone treatment increased baseline fasting glucose concentrations as compared to placebo. Circulating baseline FFA concentrations were increased by prednisolone as compared to placebo and suppressed by HEC stimulation ($P_{interaction}$ $P < 0.01$), however, this suppression was impaired by prednisolone as FFA levels during HEC remained higher as compared to placebo. Triglyceride concentrations were not affected by prednisolone administration but were significantly reduced by HEC. Five days of high dose prednisolone treatment decreased baseline concentrations of circulating normetanephrines compared to placebo, but not metanephrines.

3.2. GC, lipase related parameters and β-adrenergic pathway

Assessment of PKA mediated phosphorylation of PLIN1, a key step in β-adrenergic stimulation of lipolysis, revealed a prednisolone induced ~6 fold increase in PKA signaling that was reversed by HEC stimulation (Fig. 1A; $P_{interaction} < 0.05$; post hoc *basal period* $P < 0.05$).

Neither prednisolone nor HEC stimulation affected PLIN1 (Fig. 1B and C) or ATGL (Fig. 1D and E) at the mRNA or protein level.

Table 3
Baseline levels of insulin, C-peptide, FFA, triglyceride, metanephrine and normetanephrine as well as HEC levels of insulin, FFA and triglyceride. Data are mean(CI). Triglyceride is median (IQR). *#Triglyceride levels were significantly reduced by HEC as compared to baseline; $P < 0.01$.

N = 9	Baseline levels		*P*-value baseline	HEC levels		*P*-value HEC	*P*-value interaction
	Placebo	Prednisolone		Placebo	Prednisolone		
Insulin (pM)	30 (26; 34)	37 (33; 42)	*post hoc* <0.01	177 (158; 196)	174 (155; 192)	NS	<0.01
C-peptide (pM)	407 (338; 476)	504 (434; 574)	<0.05	–	–	–	–
Fasting glucose (mmol/l)	4.52 (4.24; 4.79)	4.91 (4.58; 5.25)	<0.05	–	–	–	–
Free fatty acid (mmol/l)	0.30 (0.22; 0.39)	0.39 (0.28; 0.49)	*post hoc* <0.01	0.02 (0.01; 0.03)	0.10 (0.07; 0.13)	*post hoc* <0.001	<0.01
Triglyceride (mmol/l)	*0.8 (0.5; 1.0)	#0.8 (0.6; 1.0)	NS	*0.6 (0.4; 0.7)	#0.5 (0.4; 0.6)	NS	
Metanephrine (ng/l)	28 (21; 34)	25 (20; 30)	NS	–	–	–	–
Normetanephrine (ng/l)	39 (33; 45)	29 (21; 36)	<0.05	–	–	–	–

Prednisolone, however, increased the levels of CGI-58 mRNA and levels were further enhanced by HEC stimulation (Fig. 2A; $P_{interaction}$ <0.05; post hoc *basal period* $P < 0.05$; post hoc *HEC stimulation* $P < 0.05$). CGI-58 protein concentrations were increased by prednisolone although it did not reach statistical significance (Fig. 2B; $P_{treatment} = 0.05$). HEC per se had no effect on CGI-58 concentrations. G0S2 mRNA levels declined during HEC stimulation in the prednisolone group (Fig. 2C; $P_{interaction} < 0.05$; post hoc *HEC stimulation* $P < 0.001$).

Phosphorylation of the regulatory sites Ser^{552} promotes translocation of HSL from the cytosol to lipid droplets, while phosphorylation of Ser^{650} activates the intrinsic enzyme activity [15,16,18,19]. Phosphorylation of Ser^{552} was increased by prednisolone and by HEC (Fig. 3A;

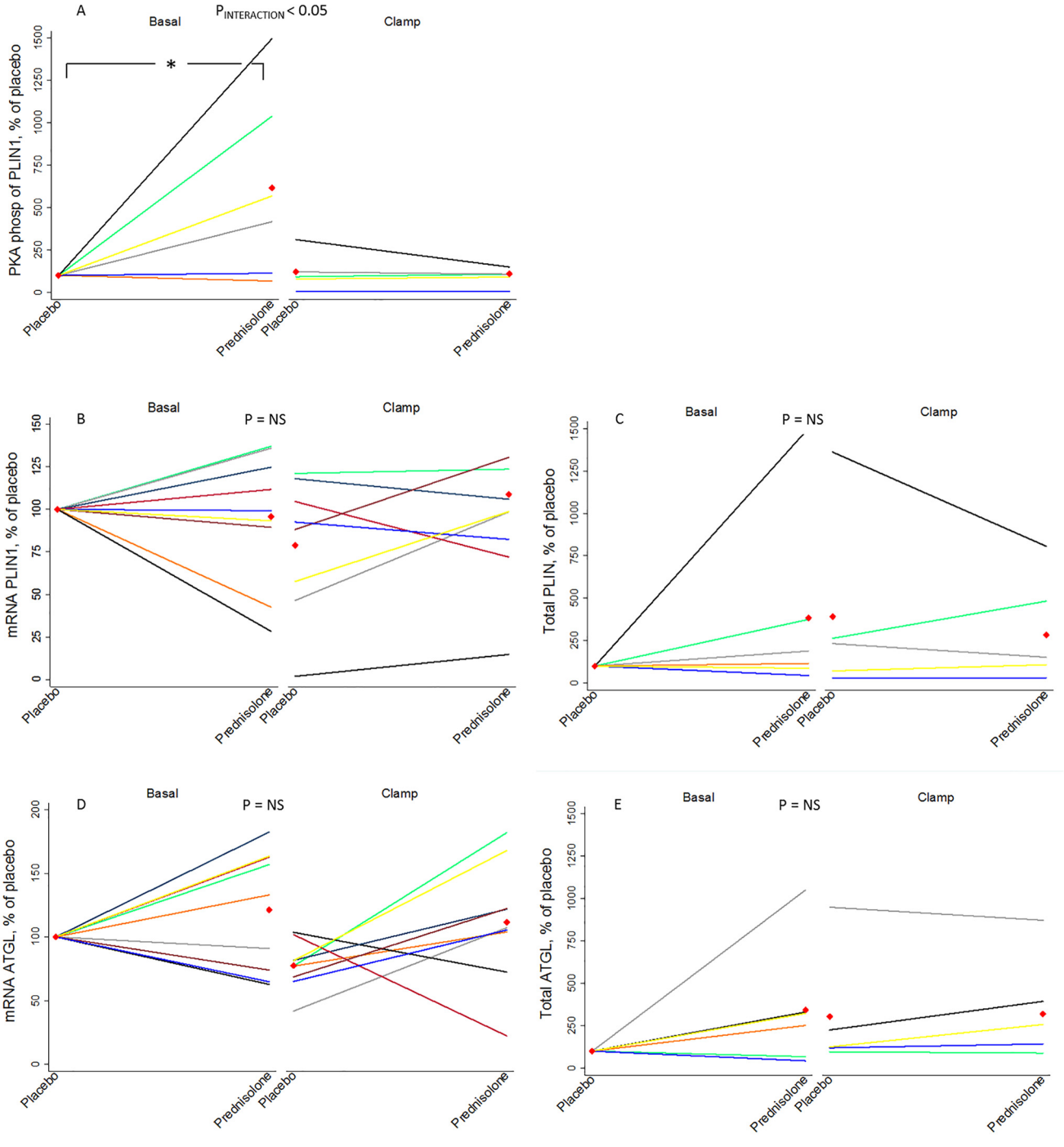

Fig. 1. Transcription and protein expression of ATGL parameters. A) PKA mediated phosphorylation of PLIN1, B) mRNA PLIN1, C) total PLIN1, D) mRNA ATGL, E) total ATGL. Data are presented as individual lineplots indicated by a unique color for each individual. The red dot indicates the mean value for each group. Complete dataset for Western blot is $n = 5$. Main effects of prednisolone ($P_{prednisolone}$) and/or HEC (P_{HEC}) or the interaction between prednisolone and HEC ($P_{interaction}$) are presented in figure. * post hoc basal period $P < 0.05$ prednisolone vs. placebo. NS = non-significant.

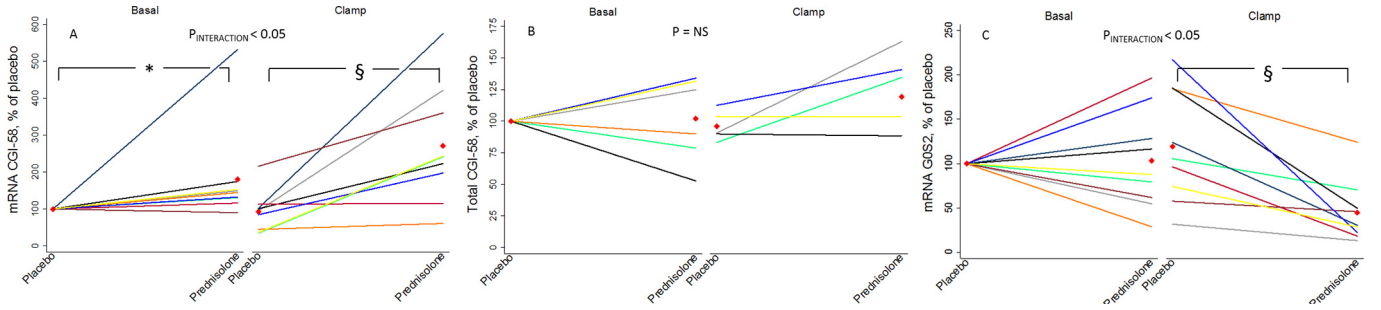

Fig. 2. Transcription and protein expression of ATGL regulators. A) mRNA CGI-58, B) total CGI-58, C) mRNA G0S2. Data are presented as individual lineplots indicated by a unique color for each individual. The red dot indicates the mean value for each group. Complete dataset for Western blot is $n = 5$. Main effects of prednisolone ($P_{prednisolone}$) and/or HEC (P_{HEC}) or the interaction between prednisolone and HEC ($P_{interaction}$) are presented in figure. * post hoc basal period $P < 0.05$ prednisolone vs. placebo. § post hoc HEC stimulation $P < 0.05$ for Panel A and $P < 0.001$ for Panel C prednisolone vs. placebo. NS = non-significant.

$P_{interaction}$ <0.05; post hoc *basal period* $P < 0.01$), but HEC did not amplify Ser^{552} phosphorylation during prednisolone stimulation. Phosphorylation of Ser^{650} was insignificantly increased by prednisolone treatment ($P_{prednisolone} = 0.07$). The phosphorylation level of Ser^{650} was significantly reduced during HEC stimulation (Fig. 3B; $P_{HEC} < 0.001$). No significant effects of prednisolone or HEC were observed on HSL mRNA and protein expression of total HSL (Fig. 3C and D).

ANGPTL4 mRNA was significantly increased during prednisolone treatment ($P_{prednisolone} < 0.01$) but was not affected by HEC (Fig. 4A).

The lipid droplet-associated CIDE proteins, CIDE-A ($P_{prednisolone} < 0.001$) and CIDE-C ($P_{prednisolone} < 0.01$) mRNA levels, were both significantly increased by prednisolone (Fig. 4B and C) and not affected by HEC stimulation.

3.3. GC and insulin signaling

To assess if prednisolone induced changes in insulin signaling to PDE3B in adipose tissue, protein expression of the insulin receptor

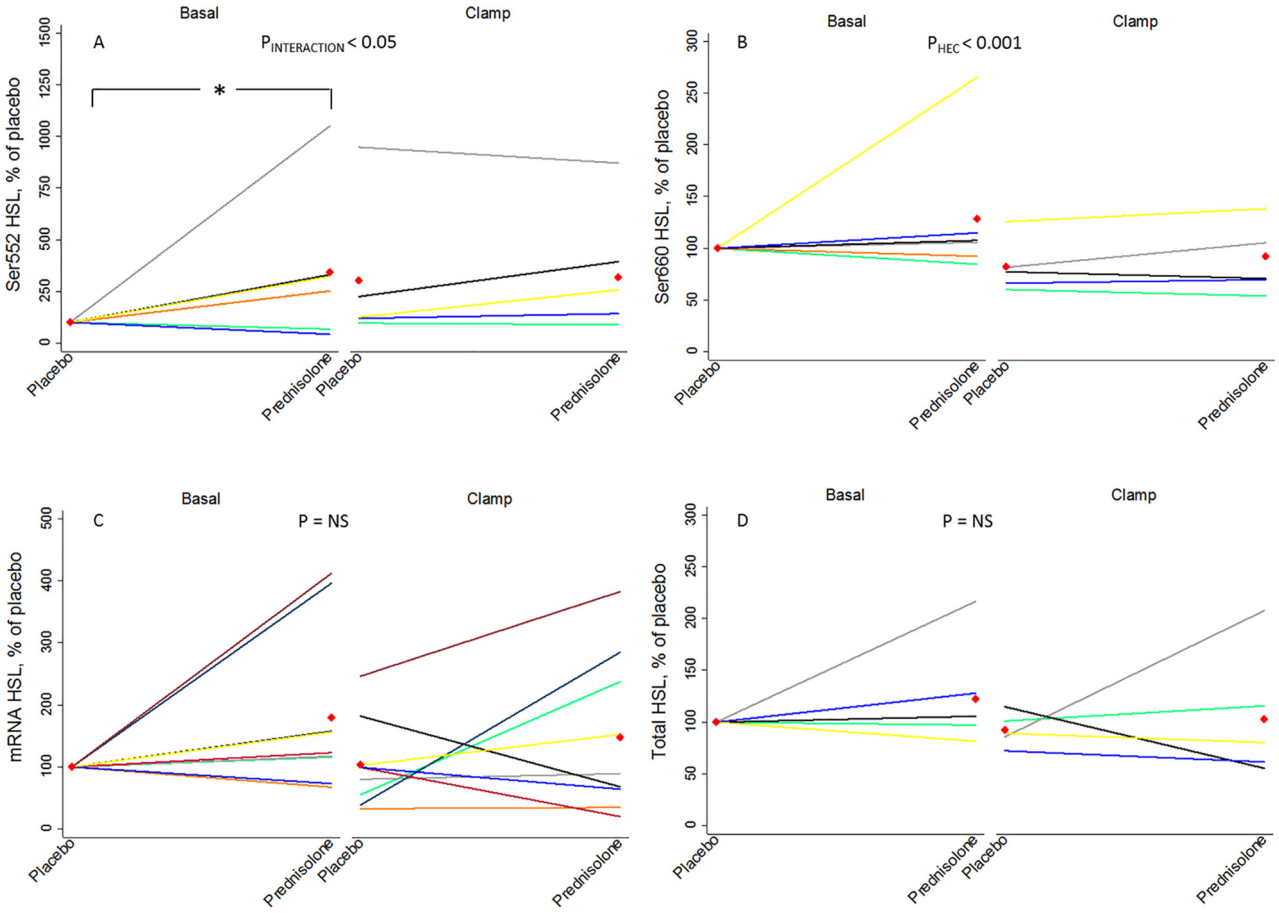

Fig. 3. Transcription and protein expression of HSL parameters. A) Phosphorylated Ser552 HSL, B) phosphorylated Ser650 HSL, C) mRNA HSL, D) total HSL Data are presented as individual lineplots indicated by a unique color for each individual. The red dot indicates the mean value for each group. Complete dataset for Western blot is n = 5. Main effects of prednisolone ($P_{prednisolone}$) and/or HEC (P_{HEC}) or the interaction between prednisolone and HEC ($P_{interaction}$) are presented in figure. Main effects of prednisolone ($P_{prednisolone}$) and/or HEC (P_{HEC}) or the interaction between prednisolone and HEC ($P_{interaction}$) are presented in figure. * post hoc basal period $P < 0.01$ prednisolone vs. placebo. NS = non-significant.

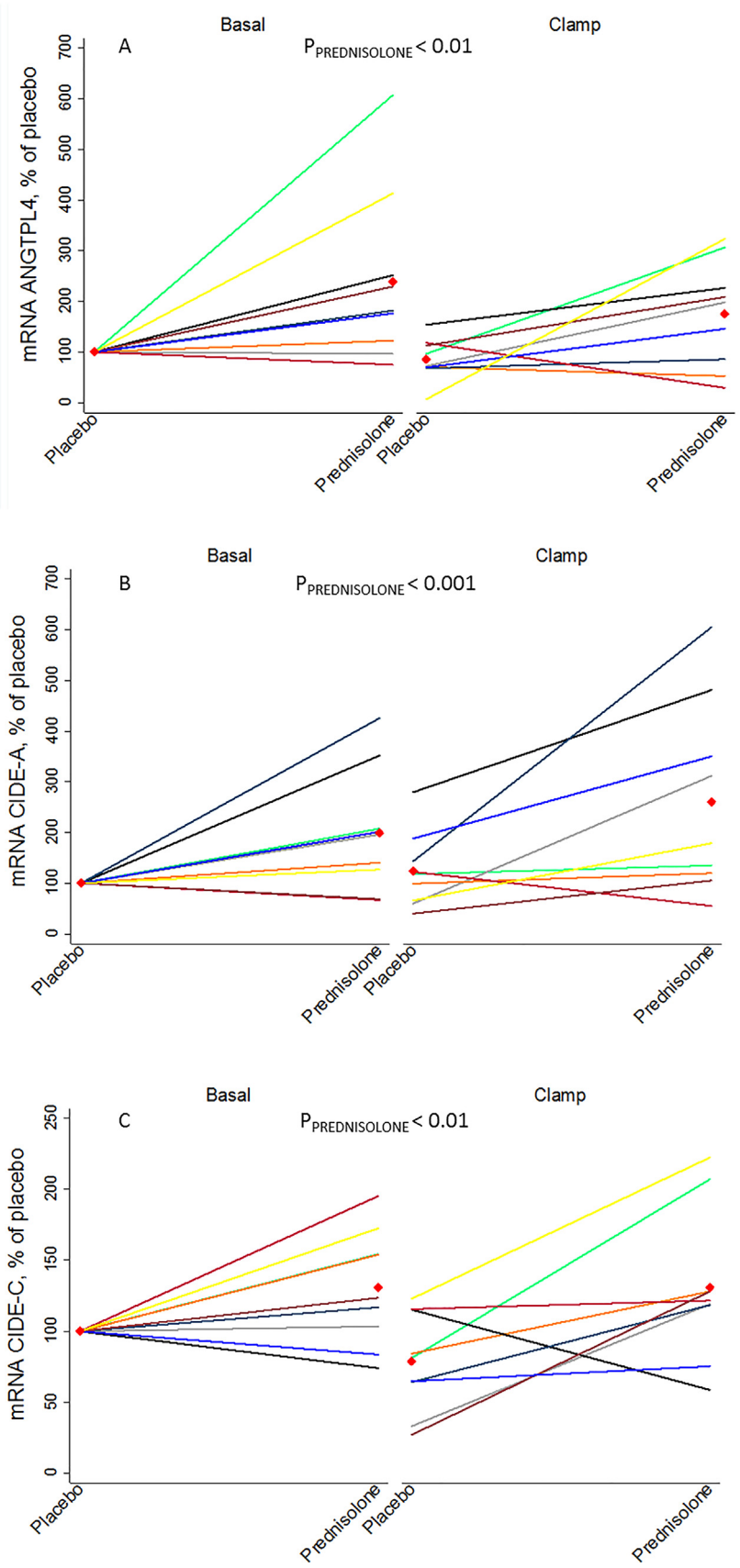

Fig. 4. Transcription of regulatory genes of lipid metabolism. A) mRNA ANGPTL, B) mRNA CIDE-A, C) mRNA CIDE-C. Data are presented as individual lineplots indicated by a unique color for each individual. The red dot indicates the mean value for each group. Main effects of prednisolone ($P_{prednisolone}$) and/or HEC (P_{HEC}) or the interaction between prednisolone and HEC ($P_{interaction}$) are presented in figure. NS = non-significant.

(IR) and the downstream signaling protein Akt was measured. Prednisolone did not affect the expression of IR (Fig. 5A), however, phosphorylation of the receptor both during the basal period and HEC stimulation was reduced by prednisolone despite compensatory hyperinsulinemia (Fig. 5B; $P_{prednisolone} < 0.05$). This was associated with decreased expression of total Akt (Fig. 5C; $P_{prednisolone} < 0.05$). As expected, Akt phosphorylation increased significantly during HEC stimulation (Fig. 5D; $P_{HEC} < 0.001$) however Akt phosphorylation did not transpire after prednisolone treatment.

Insulin regulated PDE3B mRNA increased during prednisolone treatment (Fig. 6A; $P_{prednisolone} < 0.05$), however we did not detect any changes in PDE3B protein expression (Fig. 6B). PTEN, a major negative regulator of insulin signaling, was increased by prednisolone treatment both at mRNA ($P_{prednisolone} = 0.0.7$) and protein ($P_{prednisolone} = 0.09$) levels; however neither reached statistical significance (Fig. 6C and D).

4. Discussion

Most previous studies investigating the lipolytic signaling effects of GC have been conducted in vitro. This study was performed to define the in vivo pharmacological effects of GC on lipolytic signaling in human subcutaneous adipose tissue with particular reference to the lipolytic enzymes ATGL and HSL, and the intracellular signaling of insulin and β-adrenergic pathway. Our main findings are that high dose GC induces pro-lipolytic effects in adipocytes through increased PKA signaling to PLIN1 and HSL phosphorylation together with alterations of CGI-58 and G0S2 mRNA transcripts. In addition, insulin signaling was impaired at the level of IR phosphorylation and Akt phosphorylation. Finally, we observed increased CIDE-A and CIDE-C mRNA accumulation intracellularly, which may promote lipogenesis and lipid storage.

It is a strength of our study that we included human subjects in vivo and performed extensive analyses of signaling events in adipose tissue. Conversely, it should be underlined that we used high dose prednisolone treatment (37.5 mg/d) for 5 days in healthy young subjects, which evidently restricts the general applicability of our data. Thus from a strict translational perspective our findings can only be extrapolated to the use of high dose GC treatment for shorter periods of time and it remains uncertain to which extent similar mechanisms may be active under more physiological conditions.

Studies have shown that the lipolytic effect of GC is more pronounced in peripheral adipose tissue than in central adipose depots, where lipogenic actions predominate [20]. In our study, adipose tissue was collected from the abdominal subcutaneous level since previous studies have shown that prednisolone in similar dosages stimulates lipolysis in this particular depot after treatment for 1 week [21]. Similar findings have been reported after acute exposure to hydrocortisone [11,22]. In support of this, we observed that five days of prednisolone

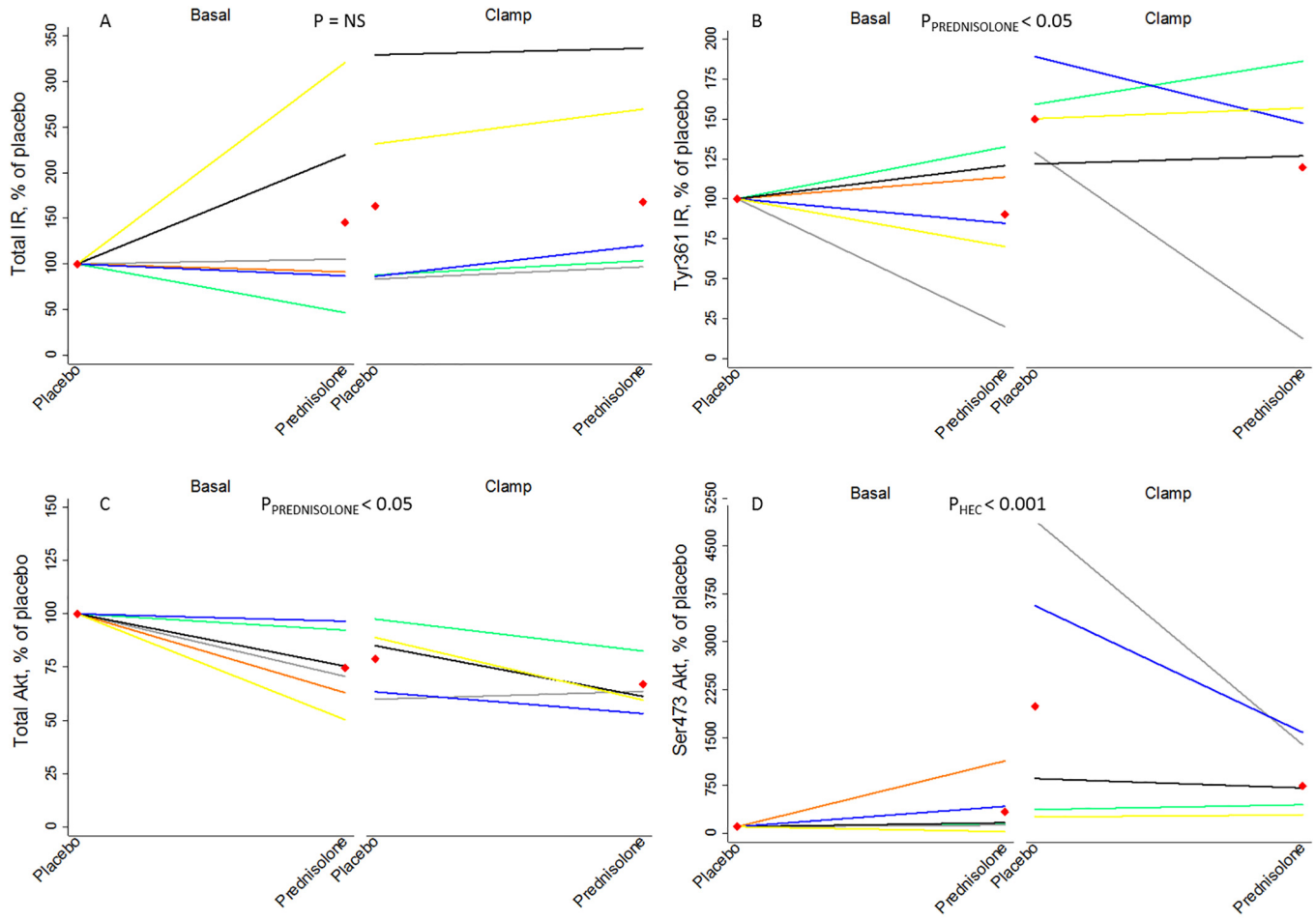

Fig. 5. Transcription and protein expression of insulin parameters. A) Total IR, B) phosphorylated Tyr361 IR, C) total Akt, D) phosphorylated Ser473 Akt. Data are presented as individual lineplots indicated by a unique color for each individual. The red dot indicates the mean value for each group. Complete dataset for Western blot is n = 5. Main effects of prednisolone ($P_{prednisolone}$) and/or HEC (P_{HEC}) or the interaction between prednisolone and HEC ($P_{interaction}$) are presented in figure. NS = non-significant.

treatment increased basal FFA significantly, and insulin-mediated suppression of FFA was impaired by prednisolone indicative of GC induced insulin resistance, as evidenced by elevated insulin and fasting glucose concentrations.

Although the current study did not include measurements of metabolic fluxes, the unchanged concentrations of triglycerides suggest that altered FFA conversion to triglycerides plays a minor role in this scenery.

GC induces insulin resistance in a dose- and time-dependent manner and in this study we used high doses of prednisolone. In vitro studies have shown that short-term dexamethasone exposure increased insulin signaling in human adipocytes whereas long-term, high-dose GC exposure led to insulin resistance [2,3]. In our study, 5 days of high-dose prednisolone treatment blunted adipocyte insulin receptor phosphorylation. It should be noted that lipolysis is highly sensitive to insulin [1] and prednisolone induced impairment of adipocyte insulin signaling occurred in the presence of discrete compensatory hyperinsulinemia. However, despite reduced phosphorylation of the IR and Akt as well as suppression of the PDE3B expression by prednisolone, we observed full suppression of PKA mediated PLIN1 phosphorylation during HEC conditions, indicating that GC-induced insulin resistance can be compensated by high levels of insulin.

It has been reported that GC primarily promotes lipolysis via a permissive effect on catecholamine dependent β-adrenergic signaling. In our study, we observed reduced levels of normetanephrine and increased PKA signaling through PLIN1 and HSL, both of which could indicate a direct stimulatory effect of GC. This is supported by the stimulation in basal lipolytic rates in cultured rat pre-adipocytes and adipocytes which increased ATGL protein and mRNA levels, HSL mRNA levels and HSL ser563 and 660 phosphorylations after 1–2 days of GC stimulation [6,8]. In our study we examined the equivalent human HSL activation sites Ser552 and Ser650, respectively, and found higher phosphorylation levels after prednisolone treatment indicative of increased HSL activity. This was supported by increased FFA concentrations. The observation of increased phosphorylation of Ser552 during insulin stimulation remains obscure.

Five days of high-dose prednisolone treatment increased ATGL cofactor CGI-58 mRNA and impaired the negative lipolytic regulator G0S2 mRNA but only during hyperinsulinemia. Similar observation is described in a clinical trial by Stimson et al. in which in vivo lipolytic changes in adipose mRNA levels of CGI-58 and G0S2 where induced by high cortisol concentration (1400 nM) only in the presence of high insulin concentration [23]. Although, prednisolone administration in our study induced pro-lipolytic changes in CGI-58 mRNA, we were not able to detect any significant changes at the protein level. Nor did we detect any alterations in ATGL expression. So far, the GC receptor binding regions for the ATGL gene have not been identified, but the ATGL gene is positively regulated by FOXO1 in 3T3-L1 adipocytes [24]. FOXO1 is inhibited by insulin through PI3K/Akt mediated phosphoryla-

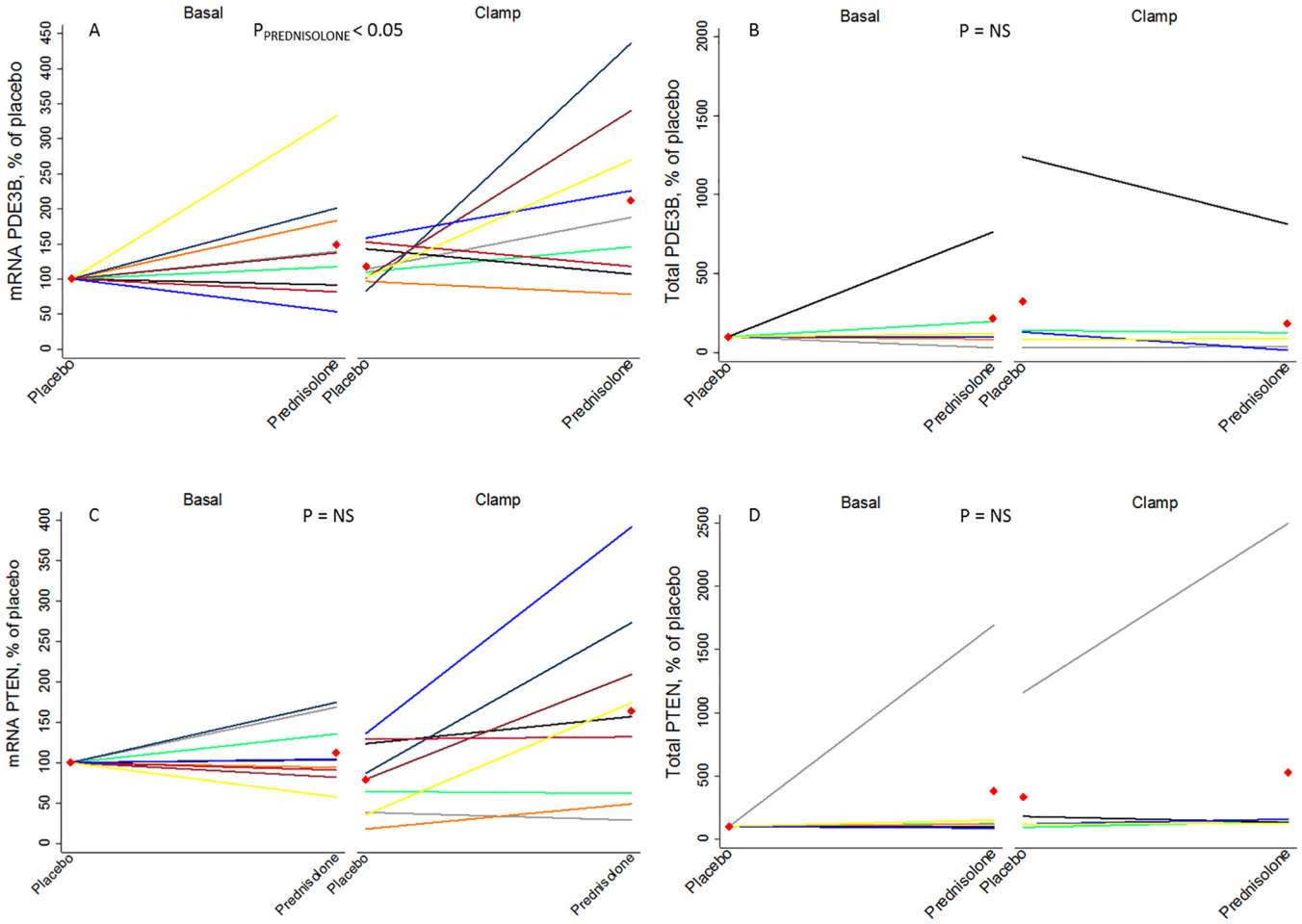

Fig. 6. Transcription and protein expression of regulatory genes of insulin signaling. A) mRNA PDE3B, B) total PDE3B, C) mRNA PTEN, D) total PTEN. Data are presented as individual lineplots indicated by a unique color for each individual. The red dot indicates the mean value for each group. Complete dataset for Western blot is n = 5. Main effects of prednisolone ($P_{prednisolone}$) and/or HEC (P_{HEC}) or the interaction between prednisolone and HEC ($P_{interaction}$) are presented in figure. NS = non-significant.

tion at three conserved Ser/Thr residues, which leads to retention of FOXO1 in the cytoplasm and thereby downregulation of RNA synthesis of target genes [25]. In our study, insulin signaling was impaired by prednisolone treatment suggesting that GC might activate ATGL transcription indirectly through FOXO1 by resistance to insulin mediated inhibition. In addition, GC is known to increase the transcription of FOXO1 in adipose tissue as well as other tissues [26–28]. Further studies are necessary in order to verify this hypothesis.

ANGPTL4 is a fasting induced adipose factor and a primary target gene of the GC receptor involved in lipid metabolism in liver and adipose tissue [10] ANGPTL4 suppresses serum triglyceride clearance via inhibition of extracellular lipoprotein lipase (LPL) causing elevated triglyceride concentration [29]. In our study, high-dose prednisolone administration increased expression of ANGPTL4 mRNA. However, a limitation to this finding is the lacking measurement of ANGPTL4 protein due to technicalities.

CIDE proteins play an important role in lipid metabolism as they are involved in regulation of lipid storage and lipolysis. The CIDE proteins promote lipid storage by formation and stabilization of lipid droplets [30] and by inhibition lipolysis. The mechanism by which CIDE proteins impair lipolysis is not fully outlined but the proteins have been shown to shield lipid droplets from lipase activity in a manner similar to PLIN1 [31] and to interact with PLIN1 to promote formation of large lipid droplets [32]. Moreover, CIDE proteins have been reported to impair ATGL-mediated lipolysis by downregulating ATGL transcription [33] and inhibit enzymatic activity of ATGL via a direct interaction with ATGL [34]. In our study high-dose prednisolone treatment significantly increased mRNA levels of CIDE-A and CIDE-C, which, to our knowledge, have not previously been reported. Of note, this result should be interpreted with caution as we were unable to measure protein levels. Although CIDE's are not consistent with a lipolytic effect this may reflect the dual nature of GC actions on lipid metabolism [35]. The net effect of GC on adipose tissue regulation depends on time and duration of exposure, the physiological condition as well as the depot-dependent attribute of GC [3]. E.g. numerous factors are known to contribute to this depot-dependent action of GC such as circulating level of GC, GC receptor expression and phosphorylation, depot differences in the inflammatory environment and levels of mineralocorticoid receptor expression [1–3,36].

In conclusion, our results indicate that 5 days of high dose GC administration exerts complex *in tandem* stimulation of lipogenesis and lipolysis in vivo. Pro-lipolytic actions were mediated by increased PKA signaling and HSL phosphorylations as well as alterations of CGI-58, G0S2 and ANGPTL4 mRNA levels. Reduced circulating levels of normetanephrine indicated a direct lipolytic effect of GC. GC increased CIDE-A and CIDE-C mRNA accumulation, which may promote lipogenesis and lipid storage, suggesting that insulin resistance in adipose tissue contributes to GC induced adipose tissue lipolysis.

Author contributions

N.R. and J.F. contributed to the study design and protocol. N.R. performed screening and enrollment of study participants, executed study day 5, performed statistics and interpretation of data and is the main writer of the manuscript. T.S.V. assisted in executing study day 5. N.J. assisted in Western Blot analyses and interpretation of data. S.B.P. assisted in quantitative real-time PCR analyses. N.M. was the assisting main writer of the manuscript and helped in interpretation of data. T.S.N. contributed in describing Western Blot procedure and to the discussion. J.O.J. contributed to the discussion. All authors revised and approved the manuscript prior to submission.

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Additional information

This paper is a part of a larger study reported at www.clinicaltrial.gov (identification number: NCT01762540, registration date: 03.01.2013.).

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.06.013.

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