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1 Title: Characterising hyperinsulinemia induced insulin resistance in human skeletal
2 muscle cells.

3 Authors: Mark C Turner[1,2*], Neil R.W Martin[1], Darren J Player[3], Richard A Ferguson[1],
4 Patrick Wheeler[1,2], Charlotte J Green[4], Elizabeth C Akam[1], Mark P Lewis[1,2]

5 [1]School of Sport, Exercise and Health Sciences, National Centre for Sport and
6 Exercise Medicine, Loughborough University, Loughborough, LE11 3TU

7 [2]University Hospitals of Leicester NHS Trust, Infirmary Square, Leicester LE1 5WW

8 [3]Division of Surgery and Interventional Science, Faculty of Medical Sciences,
9 University College London, United Kingdom

10 [4]Drug Discovery Unit, School of Life Sciences, University of Dundee, Dundee, DD1
11 5EH

12 *Corresponding Author and present address:

13 Dr Mark C Turner

14 Centre for Sport, Exercise and Life Sciences,

15 Faculty of Health and Life Sciences,

16 Coventry University,

17 Priory Street, Coventry, CV1 5FB

18 mark.turner@coventry.ac.uk

19

20 Running Head: Human skeletal muscle exposure to insulin *in vitro*.

21 Word Count:

22 Abstract:

23 Hyperinsulinemia potentially contributes to insulin resistance in metabolic tissues,
24 such as skeletal muscle. The purpose of these experiments was to characterise
25 glucose uptake, insulin signalling and relevant gene expression in primary human
26 skeletal muscle derived cells (HMDCs), in response to prolonged insulin exposure
27 (PIE) as a model of hyperinsulinemia induced insulin resistance. Differentiated
28 HMDCs from healthy human donors, were cultured with or without insulin (100nM)
29 for three days followed by an acute insulin stimulation. HMDC's exposed to PIE were
30 characterised by impaired insulin stimulated glucose uptake, blunted IRS-1
31 phosphorylation (Tyr^{612}) and Akt (Ser^{473}) phosphorylation in response to an acute
32 insulin stimulation. Glucose transporter 1 (GLUT1), but not GLUT4, mRNA and
33 protein increased following PIE. The mRNA expression of metabolic (PDK4) and
34 inflammatory markers (TNF-α) was reduced by PIE but did not change lipid
35 (SREBP1 and CD36) or mitochondrial (UCP3) markers. These experiments provide
36 further characterisation of the effects of PIE as a model of hyperinsulinemia induced
37 insulin resistance in HMDCs.

38 Keywords: Hyperinsulinemia, Insulin Resistance, Diabetes Mellitus, Primary Skeletal
39 Muscle Cells,

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Introduction:

The inability to maintain glucose homeostasis in response to physiological insulin concentrations, leads to an increase in blood glucose (hyperglycaemia) and consequently prolonged raised insulin concentrations (hyperinsulinemia). Hyperinsulinemia has been causally linked to the onset of diabetes in the early stages of the insulin resistance and in type 2 diabetes mellitus (Corkey 2012; Templeman *et al.* 2017), negatively affecting insulin sensitive tissues such as liver, adipose and skeletal muscle (Page & Johnson 2018). In humans, prolonged administration of insulin can attenuate insulin responsiveness, independent of hyperglycaemia. This would therefore suggest a potential role of hyperinsulinemia as a cause of insulin resistance (Marangou *et al.* 1986; Del Prato *et al.* 1994).

In vitro research using human skeletal muscle derived cells (HMDCs) has routinely been used to investigate various aspects of metabolic physiology (Aas *et al.* 2013). Consequently, it has been possible to investigate some of the cellular and molecular characteristics of skeletal muscle insulin resistance (Ciaraldi *et al.* 1995; Henry *et al.* 1995), as well as the potential causes of insulin resistance in skeletal muscle cells in response to other cells types, fatty acids and inflammatory cytokines (Dietze *et al.* 2002; Mäkinen *et al.* 2017).

Despite the development of relevant *in vitro* models to study metabolic disease, there is limited information regarding the effects of chronic insulin exposure on glucose metabolism in human skeletal muscle cells. Models of hyperinsulinemia induced insulin resistance through prolonged insulin exposure (PIE), using murine C2C12 skeletal muscle cells, have been shown to impair downstream insulin signalling and glucose uptake (Kumar & Dey 2003; Turner *et al.* 2018; Cen *et al.*

2019). In addition, work in HMDCs has shown that exposure to a chronic insulin exposure can ablate the fractional velocity of glycogen synthase activity (Henry *et al.* 1996; Gaster *et al.* 2001) and therefore, could contribute to the development of insulin resistance in skeletal muscle (Nikoulina *et al.* 1997).

Whilst previous literature has alluded to the physiological effects of hyperinsulinemia induced insulin resistance in human skeletal muscle tissue and primary cells (Del Prato *et al.* 1994; Gaster *et al.* 2001), currently the molecular characteristics which potentially underpin previously observed changes to PIE induced insulin resistance, are yet to be investigated. The aim of the current investigation was to determine how PIE would affect glucose uptake, insulin signalling and gene expression in HMDCs from healthy donors. In these experiments, it was found that PIE resulted in attenuated insulin signalling and glucose uptake, however did not alter the mRNA expression of genes involved in metabolism, which are putatively indicative of insulin resistant skeletal muscle.

Methods:

Participants

Healthy male volunteers (age 24.4 ± 1.1 years, height 1.78 ± 0.04 cm, weight 70.6 ± 2.9 kg, body mass index (BMI) 22.3 ± 1.5 kg·m^2) who did not report any family history of metabolic disease were recruited for this study. All procedures were conducted at Loughborough University, UK under ethical approval and in accordance with the Declaration of Helsinki, 2008.

Isolation and culture of human muscle derived cells (HMDCs)

Percutaneous skeletal muscle biopsies were obtained from the vastus lateralis by micro-biopsy technique (Acecut 11-gauge Biopsy Needle; TSK, Tochigi-Ken, Japan),

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as previously described (Ferguson *et al.* 2018). Skeletal muscle biopsies were scissor minced into small pieces, placed in tissue culture flasks coated in 0.2% Gelatin/PBS and maintained at 37°C and 5% CO_2 in growth media (GM, consisting of high glucose DMEM (Sigma, Dorset, UK) supplemented with 20% Foetal bovine serum (Pan Biotech UK Ltd, Dorset, UK) and 1% penicillin/streptomycin, Fisher Scientific, Loughborough, UK). The HMDCs which migrated out of the muscle tissue and adhered to the tissue culture plastic were harvested and expanded through serial passages to increase cell numbers prior to experimentation. For experimentation, HMDCs were used between passages 3-7 (7-10 population doublings).

Experimental Protocol

2,500 cells·cm^2 of HMDCs were seeded into 12 well plates and cultured in GM until 80% confluent. Media was subsequently changed to low serum differentiation media (DM) which consisted of high glucose DMEM (Sigma, Dorset, UK) supplemented with 2% horse serum (Fisher Scientific, Loughborough, UK), and 1% penicillin/streptomycin (Fisher Scientific, Loughborough, UK). Following differentiation, (5-7 days) into multinucleate myotubes (as evidenced by light microscopy), cultures were subjected to prolonged insulin exposure (PIE), consisting of DM supplemented with the addition of 100nM human recombinant insulin (Sigma, Dorset, UK). Cultures were then serum starved for 4 hours, before being acutely stimulated with or without insulin (100nM) for 30 minutes.

Protein quantification and immunoblotting

HMDCs were washed with PBS before being lysed in RIPA buffer (Sigma, Dorset, UK) containing a protease and phosphatase inhibitor cocktail mix (Fisher Scientific, Loughborough, UK). Protein concentrations were determined using the Pierce

660nm protein assay (Fisher Scientific, Loughborough, UK) and thereafter samples were mixed with 4X Laemmli buffer (Bio-Rad, Herts, UK), boiled for 5 minutes at 95ºC and separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Whatman Proton, Sigma-Aldrich, Dorset, UK) and blocked for 1 hour at room temperature in 5% bovine serum albumin (BSA) in TBST (Sigma, Dorset, UK), before being incubated with primary antibody overnight at 4ºC in BSA or Milk. Primary antibodies used for analysis were, Akt (#9272), phosphor Akt (Ser^{473}) (#4060), GSK-3β (#9315), GSK-3β (Ser^{9}) (#9336), AS160 (Ser^{588}) (#8730), Glucose Transporter 4 (GLUT4) (#2213) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#2118) purchased from Cell signalling (NEB, Herts, UK). Glucose transporter 1 (GLUT1) (#07-1401), Insulin Receptor Substrate-1 (IRS-1) (#05-784R) and phospho IRS-1 (Tyr^{612}) (#09-432) were purchased from Merck Millipore (Dorset, UK). Following overnight incubation, membranes were washed in TBST and subsequently incubated with anti-rabbit (#7074) or anti-mouse (#7076) horseradish peroxidase-conjugated secondary antibody (NEB, Herts, UK) at concentration of 1:2000 in milk. Proteins were visualised using chemiluminescence substrate (Bio-Rad, Herts, UK) and band densities were quantified using Quantity One image analysis software (Quality One 1-D analysis software version 4.6.8). Where appropriate, following visualisation of phosphorylated proteins, membranes were washed in TBST and incubated in stripping buffer (Fisher Scientific, Loughborough, UK) before being blocked and probed as outlined above for their corresponding total proteins. Phosphorylation was normalised to its corresponding total protein, with the exceptions of GLUT1, GLUT4 and AS160 (Ser^{588}) which were normalised to GAPDH.

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RNA extraction and qPCR analysis

RNA extraction was performed using TRI Reagent (Sigma, Dorset, UK) according to the manufacturer's instructions and quantified using UV spectroscopy (NanoDrop, Fisher Scientific, Loughborough, UK). Gene expression was analysed by one-step reverse transcription-qPCR (Quantifast SYBR Green Mix (Qiagen, Crawley, UK)) using a Viia 7 thermocycler (Applied Biosystems, Loughborough, UK). Each reaction consisted of 20ng of RNA in a final 10 µL reaction volume (Qiagen, Crawley, UK). Master mixes were made according to the manufacturer's instructions using primers outlined in Table 1. Fluorescence was detected after every cycle (40 cycles) and data was analysed using the $\Delta\Delta C_t$ method, using RNA polymerase II beta (POLR2B) as an endogenous control gene. Samples were normalised to each individual donor control sample, with each donor performed in duplicate for each condition and each sample was ran in triplicate.

[INSERT TABLE 1]

Cell Based Glucose uptake assay

HMDCs were plated into black, clear bottom 96 well plates (Fisher Scientific, Loughborough, UK) and cultured as described in the experimental protocol. The measurement of 2-deoxyglucose (2DG) uptake was performed using a commercially available Glucose Uptake-Glo™ Assay kit (Promega, Southampton, UK). Firstly, cultures were washed with PBS and incubated overnight in serum free media with/without 100nM insulin. Briefly, HMDC's were washed with PBS before being stimulated with/without 1mM insulin in PBS. 0.1M 2DG was added to all of the wells for 30 minutes at 25°C. The reaction was arrested with the addition of stop and neutralization buffer, before the addition of 2DG6P detection reagent. Values were normalised to total protein concentration analysed on plates following glucose uptake

measurement, using the Pierce 660nm protein assay (Fisher Scientific, Loughborough, UK).

Statistical Analysis

Statistical analysis was performed using SPSS (Version 23). Insulin signalling proteins was analysed by one-way ANOVA with Bonferroni *post-hoc* correction. Differences in glucose uptake and gene expression between control and PIE conditions, were analysed by independent samples t-test. The number of donors used for each analysis is outlined in the figure legends. Data is presented and mean ± standard error the mean (S.E.M) and statistical significance was set at $p < 0.05$.

Results:

Glucose Uptake in human skeletal muscle cells following insulin exposure

To investigate the physiological effects of exposure to PIE, we measured glucose uptake using a commercially available assay. Acute insulin-stimulation increased glucose uptake by approximately 1.5-fold in control HMDCs ($p <0.05$, figure 1a). However, HMDCs cultured with PIE exhibited no significant increase in glucose uptake following acute insulin stimulation ($p >0.05$, figure 1b).

[INSERT FIGURE 1]

Prolonged exposure to insulin alters phosphorylation of insulin signalling proteins IRS-1 (Tyr^{612})

Following evidence of altered glucose uptake following PIE, we analysed the phosphorylation of both Insulin receptor substrate 1 (IRS-1) and Akt as critical nodes of insulin signalling. Acute insulin stimulation significantly increased tyrosine phosphorylation of IRS-1 (Tyr^{612}) above basal levels in control HMDCs ($p <0.01$,

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figure 2A). In contrast, we observed elevated basal IRS-1 (Tyr^{612}) phosphorylation in PIE condition which was not increased further upon acute insulin stimulation ($p > 0.05$, Figure 2). In addition, IRS-1 mRNA expression was analysed however was not different between control and PIE conditions ($p > 0.05$, Figure 2B).

[INSERT FIGURE 2]

Prolonged exposure to insulin alters phosphorylation of Akt (Ser^{473}).

Akt (Ser^{473}) was responsive to acute insulin stimulation when HMDC's were cultured in control conditions ($p < 0.05$ figure 3a), however this response was blunted following PIE ($p > 0.05$; Figure 3a). GSK-3β (Ser^{9}) or AS160 (Ser^{588}) phosphorylation was not different following acute stimulation or different between conditions (both $p > 0.05$; Figure 3b and 3c respectively).

[INSERT FIGURE 3]

Exposure to insulin results in changes to GLUT1 but not GLUT4 mRNA and protein in HMDCs.

PIE increased the mRNA expression of GLUT1 by approximately 1.6-fold above HMDCs cultured in control conditions ($p < 0.01$; figure 4a), but did not alter the mRNA expression of GLUT4 ($p > 0.05$; figure 4b). Protein expression analysis of GLUT1 was also increased in the PIE condition compared to CON ($p < 0.05$ figure 4c), but there was no difference in GLUT4 protein expression between conditions ($p > 0.05$ figure 4d).

[INSERT FIGURE 4]

The effects of insulin exposure on the mRNA expression of metabolism markers.

Hexokinase II and pyruvate dehydrogenase kinase isoform 4 (PDK4), two enzymes which regulate glucose oxidation, have previously been shown to be regulated by insulin. Here, HKII mRNA expression did not change in HMDC's exposed to PIE ($p > 0.05$), however PDK4 mRNA expression was significantly reduced following PIE ($p < 0.01$), Glycogen synthase kinase-3β (GSK-3β) mRNA expression was increased following PIE, but did not reach statistical significance ($p = 0.054$). The mRNA expression of the lipid metabolism markers, sterol regulatory element binding protein 1 (SREBP-1) and fatty acid translocase (cluster of differentiation 36 (CD36)), was not different between conditions ($p > 0.05$). Similarly, the mRNA expression of mitochondrial uncoupling protein -3 (UCP-3), was not different between conditions ($p > 0.05$). However, the mRNA expression of the pro-inflammatory marker TNF-α was significantly lower in PIE compared to control ($p < 0.05$, figure 5b).

[INSERT FIGURE 5]

Discussion:

Hyperinsulinemia is a symptom in the early stages of insulin resistance and type 2 diabetes *mellitus* (Shanik *et al.* 2008; Page & Johnson 2018). To decipher how prolonged exposure to high concentrations of insulin could contribute to skeletal muscle insulin resistance, *in vitro* experiments have been used to investigate some of the physiological effects of hyperinsulinemia in cell lines (Kumar & Dey 2003; Turner *et al.* 2018; Cen *et al.* 2019), and primary human skeletal muscle cells (Henry *et al.* 1996; Gaster *et al.* 2001). Our initial experiments used prolonged insulin exposure (PIE) as a model of hyperinsulinemia and demonstrated that this was able to induce impaired glucose uptake in response to an acute insulin stimulation, thus

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confirming our own previous findings in C2C12 skeletal muscle cells (Turner *et al.* 2018) and those of Henry and colleagues who have shown hyperinsulinemia to impair glucose synthase activity and insulin stimulated glucose uptake in primary human skeletal muscle cells when chronically exposed to insulin (Ciaraldi *et al.* 1995) and is a phenomenon which occurs in both healthy and insulin resistant skeletal muscle.

To examine the molecular responses to this impaired insulin-stimulated glucose uptake we initially examined critical nodes of the insulin signalling cascade. Indeed, impaired insulin signalling contributes to skeletal muscle insulin resistance (Taniguchi *et al.* 2006). While the physiological changes in human skeletal muscle cells have been previously described (Henry *et al.* 1996; Gaster *et al.* 2001), we observed an attenuated IRS-1 phosphorylation (Tyr^{612}) to acute insulin stimulation. This was due to an increase in basal phosphorylation which has previously be reported in rat skeletal muscle (Kanety *et al.* 1994). This response has been attributed to multisite phosphorylation of the insulin receptor in a cell lines, including skeletal muscle (Kumar & Dey 2003; Cen *et al.* 2019), although further experiments would be required to determine the phosphorylation sites in human skeletal muscle cells. The attenuation in the phosphorylation of Akt is indicative of insulin resistance (Krook *et al.* 1998a; Karlsson *et al.* 2005), which has been reported by our group in C2C12 skeletal muscle cells following sustained exposure to insulin (Turner *et al.* 2018). In addition, while our findings did not show any changes in phosphorylation of downstream target AS160, it's phosphorylation of multiple serine sites in response to insulin (Kramer *et al.* 2006) mean that further investigation is required to elucidate what sites are specifically inhibited following prolonged insulin.

Exposure to insulin can increase glucose transporter (GLUT) mRNA and protein expression (Walker *et al.* 1989, 1990), a finding which has also been shown in primary human skeletal muscle cells (Ciaraldi *et al.* 1995). The increase in expression is mostly likely due to an increase in GLUT1 mRNA and protein expression, which was increased following exposure to PIE in the present set of experiments. Basal glucose uptake has previously been observed in human (Ciaraldi *et al.* 1995), and murine skeletal muscle cells (Turner *et al.* 2018), and is linked to the increase in GLUT1 mRNA and protein expression. In contrast, GLUT4 mRNA and protein expression was not altered in response to PIE, which has previously been reported in human skeletal muscle following hyperinsulinemia-euglycemia clamp (Postic *et al.* 1993). Despite contradictor findings in rodent skeletal muscle (Cusin *et al.* 1990), our findings provide further evidence that that hyperinsulinemia is not a mediator of GLUT4 protein or mRNA expression in human skeletal muscle. Insulin regulates the expression of genes involved in skeletal muscle metabolism (Rome *et al.* 2003), of which the expression is altered in metabolic disease (Ducluzeau *et al.* 2001). HKII mRNA expression has previously been shown to be sensitive to insulin stimulation (Osawa *et al.* 1996), and it's response is attenuated in insulin resistant skeletal muscle (Ducluzeau *et al.* 2001). However, this is in contrast to our experiments, which may reflect the differences in metabolic demands of cells and tissues. The reduction in PDK4 mRNA expression in these experiments, could be considered indicative of a physiological and not pathophysiological response to insulin in skeletal muscle (Kim *et al.* 2006; McAinch *et al.* 2015). In addition, a number of genes which are associated with skeletal muscle insulin resistance, such as SREBP1, CD36 and UCP3 (Krook *et al.* 1998b; Ducluzeau *et al.* 2001; Wallberg-Henriksson *et al.* 2007), were not altered in these experiments. This indicates while

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hyperinsulinemia can induce insulin resistance in healthy HMDC's, it might not contribute to the transcriptional changes which have previously been observed in disease states (Ducluzeau et al. 2001). These changes could be mediated by other factors, such as low grade chronic inflammation and the expression of pro-inflammatory cytokines (Ruge et al. 2009).

With the previously documented role of hyperinsulinemia in skeletal muscle insulin resistance, for the first time these experiments were able to confirm previous findings that PIE can attenuate insulin stimulated glucose uptake, attenuate insulin signalling and the induce compensatory changes in glucose transporter expression in HMDC's from healthy donors. While we appreciate that the concentrations of insulin used in these experiments are in excess of physiological hyperinsulinemia in humans and therefore further experiments would elude to impact of lower insulin concentrations upon skeletal muscle insulin sensitivity *in vitro*, these findings provide insight into the specific impact of hyperinsulinemia induced insulin resistance in primary human skeletal muscle cells.

[INSERT FIGURE 6]

Conflict of Interest:

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author Present Address: M C Turner, Centre for Sport, Exercise and Life Sciences, Faculty of Health and Life Sciences, Coventry University, Priory Street, Coventry, CV1 5FB

Author Contributions:

MCT performed the experiments. MCT and MPL developed the experiments. MCT, EA, DJP, CJH and NRWM analysed the data. RAF and PW took the skeletal muscle biopsies from which MCT, DJP, NRWM and extracted and cultured the cells. All authors read and approved the final manuscript for submission.

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Figure Legends

Figure 1: 2-deoxyglucose (2DG) uptake and phase contrast images of HMDCs cultured in control (CON) or prolonged insulin exposure (PIE) for three days. Cells were stimulated without (open bar) or with (doted bar) insulin. Data is mean ± s.e.m from 3 donors. *Significant difference between basal and stimulation ($p < 0.05$).

Figure 2: **A**; IRS-1 phosphorylation (Tyr^{612}) of HMDC cultured in CON or PIE media. Cells were stimulated without (open bar) or with (doted bar) insulin (100nM) for 30 minutes. **B;** IRS-1 mRNA expression ($\Delta\Delta Ct$) of basal samples cultured in CON or PIE media. Data is mean ± s.e.m from 2-4 donors. *Significant difference between basal and acute insulin stimulation ($p < 0.05$).

Figure 3: A; Akt phosphorylation (Ser^{473}), **B**; GSK-3β phosphorylation (Ser^{9}) of HMDC cultured in CON or PIE media, **C**; AS160 phosphorylation (Ser^{588}) of HDMC's cultured in CON or PIE media before being stimulated without (open bar) or with (doted bar) insulin (100nM) for 30 minutes. Data is mean ± s.e.m from 2-5 donors as represented by in the graph. *Significant difference between basal and acute insulin stimulation ($p < 0.05$).

Figure 4: **A;** glucose transporter 1 (GLUT1) and **B;** glucose transporter4 (GLUT4) mRNA expression ($\Delta\Delta Ct$). **C;** GLUT1 protein expression and **D**; GLUT4 protein expression normalised to GAPDH in HMDC cultured CON or PIE media. Data is mean ± s.e.m from 3 donors. Significant different between conditions ** ($p < 0.01$).

Figure 5: mRNA expression ($\Delta\Delta Ct$) of genes in HMDCs cultured CON or PIE media. Data is mean ± s.e.m from 3-4 donors. Significantly different between conditions * ($p < 0.05$), ** ($p < 0.01$).

465 **Figure 6**: Summary of human muscle derived cell (HMDC) responses to prolonged
466 insulin exposure (PIE) as a model of hyperinsulinemia induced insulin resistance.

467 **Table 1**: Primer sequences of genes analysed by qPCR.

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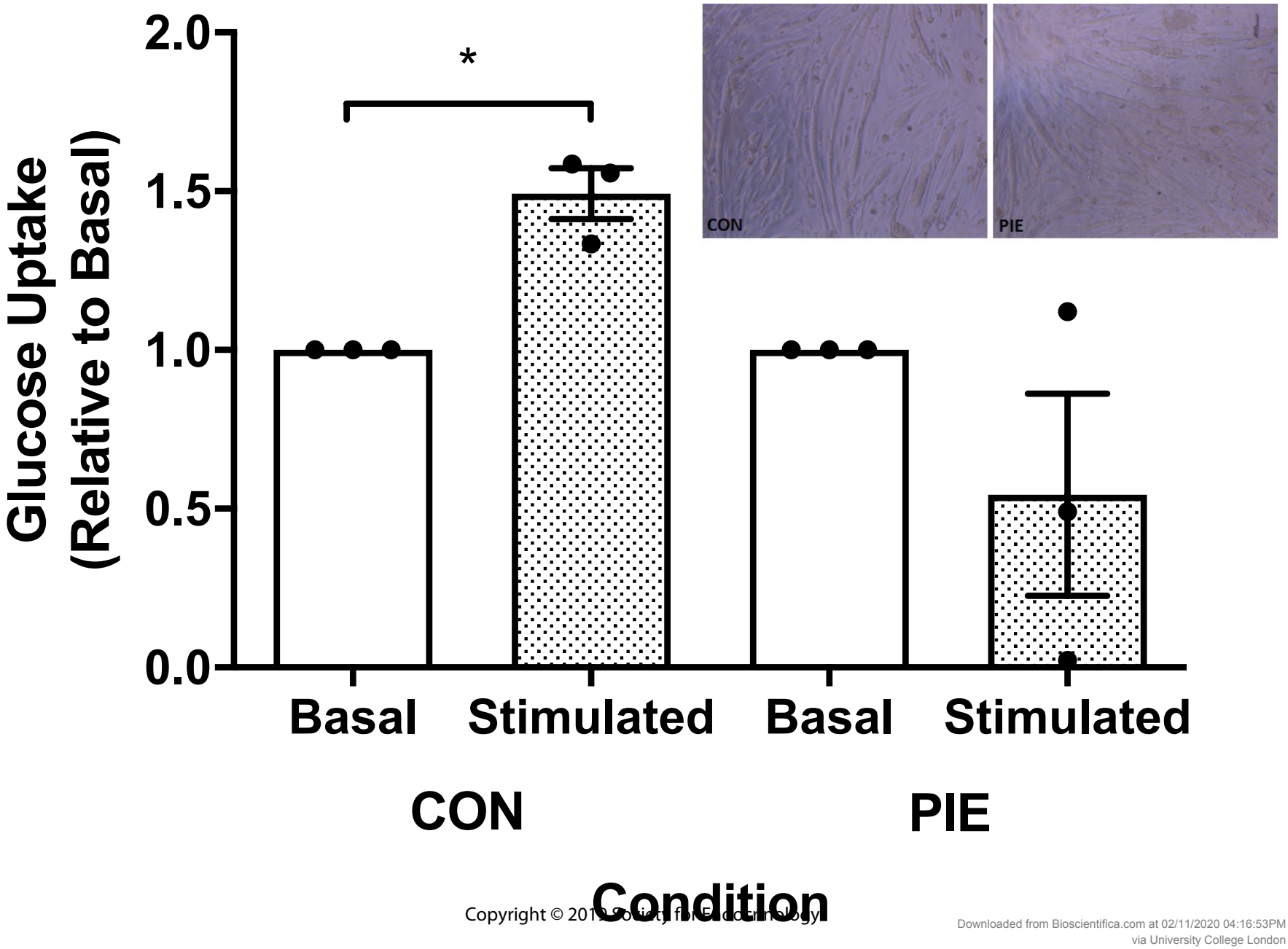

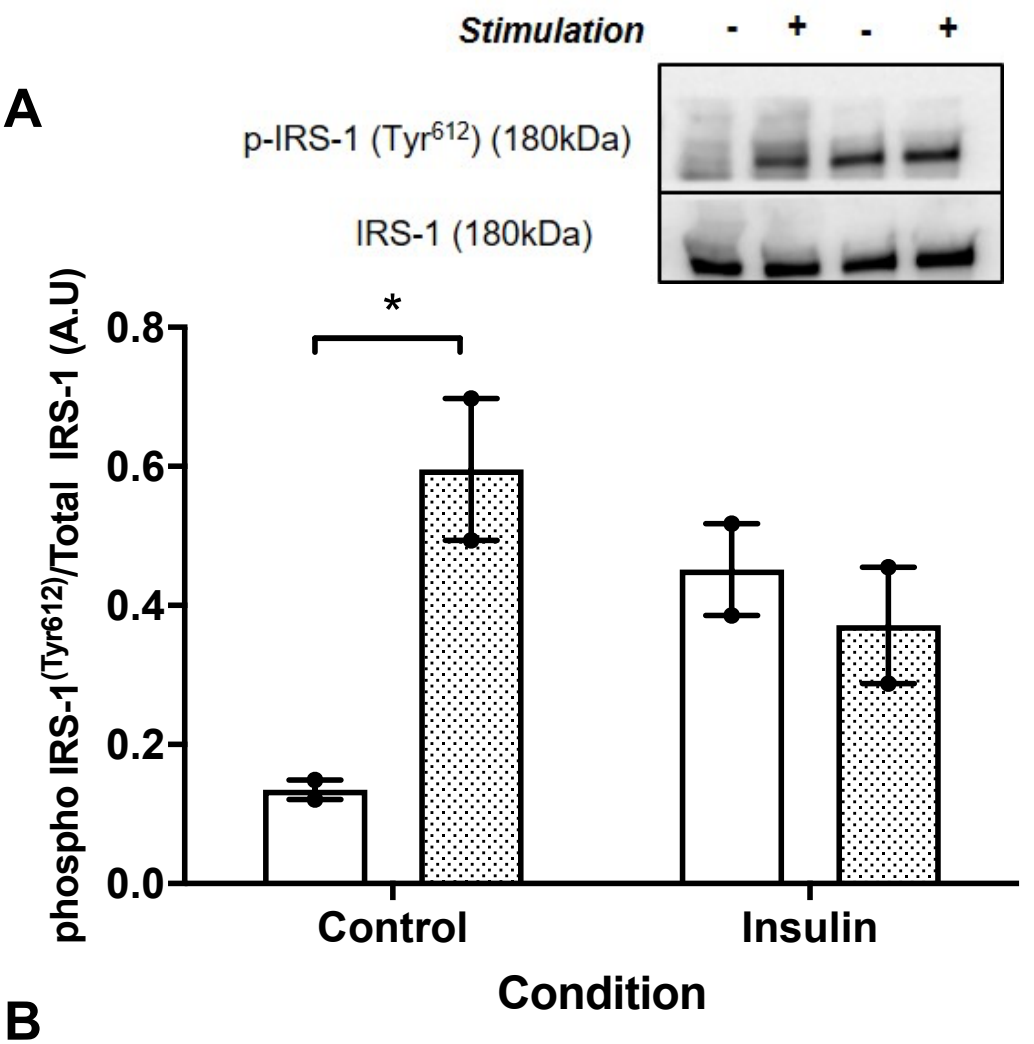

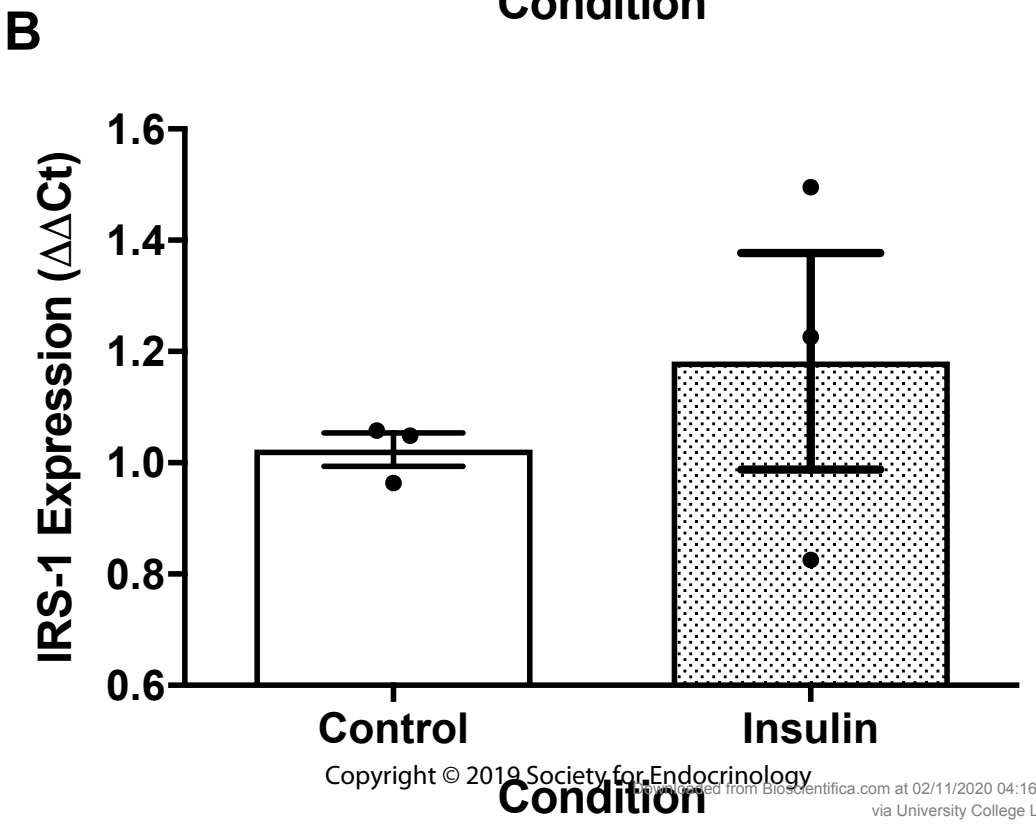

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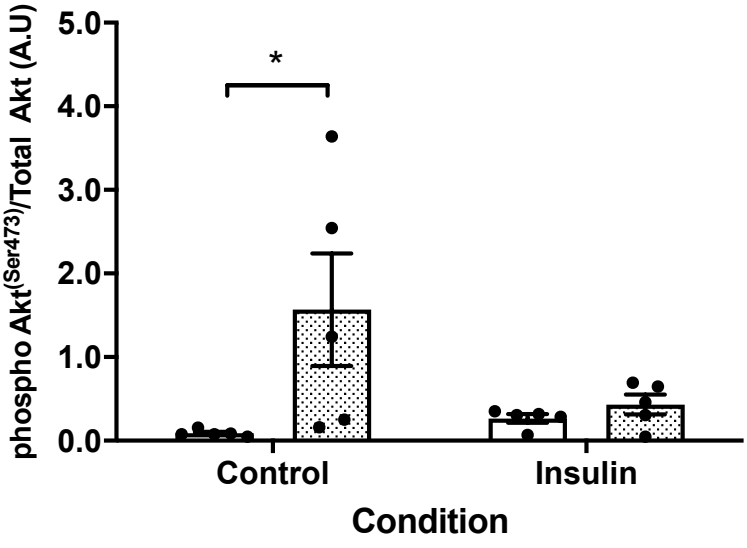

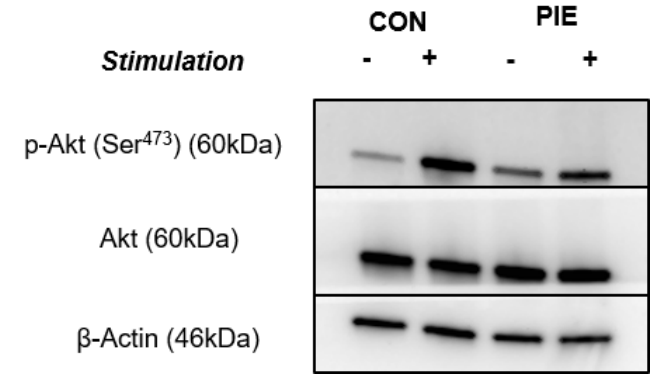

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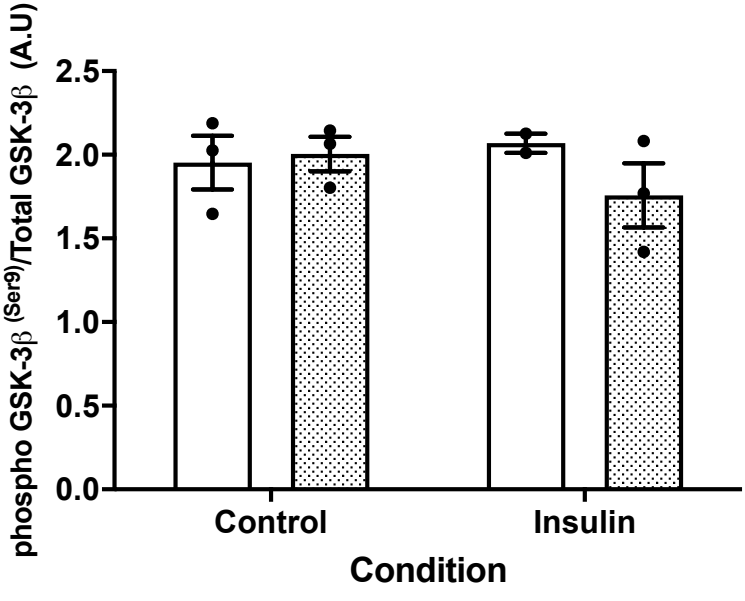

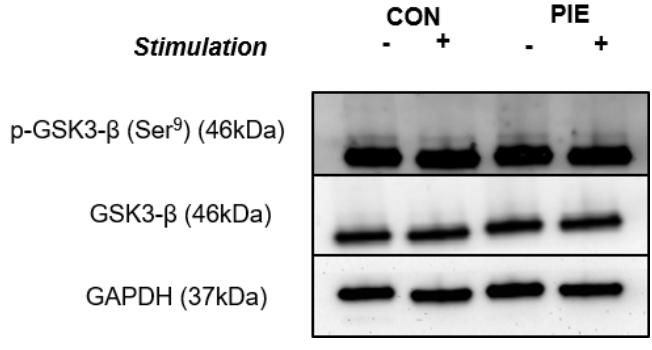

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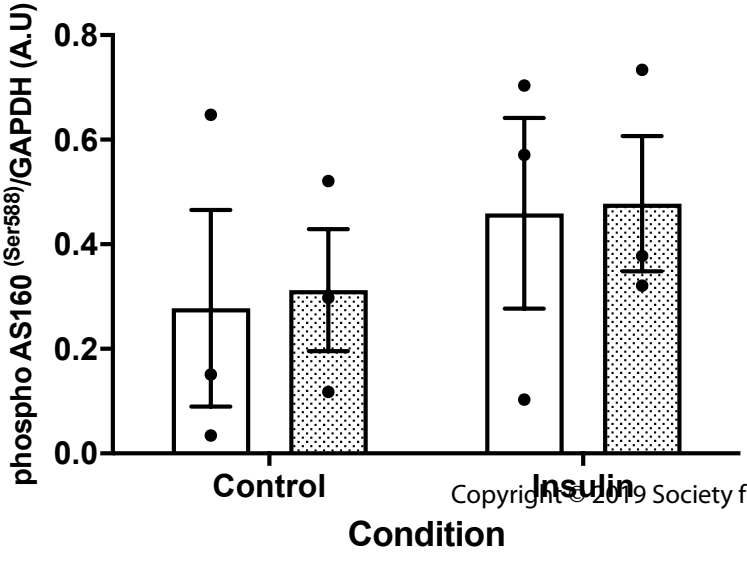

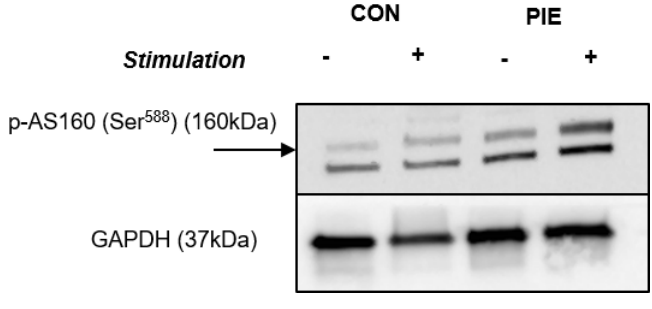

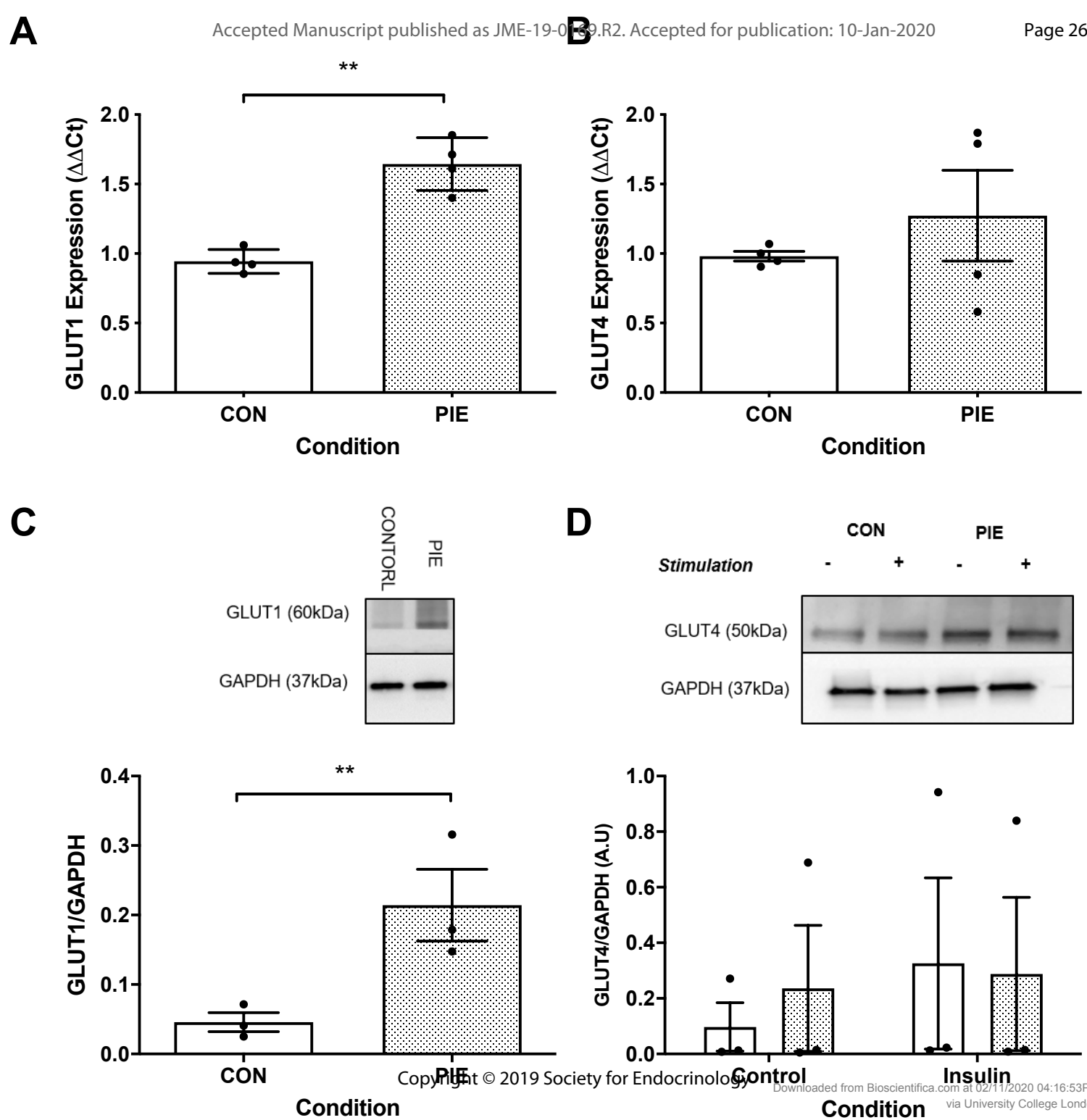

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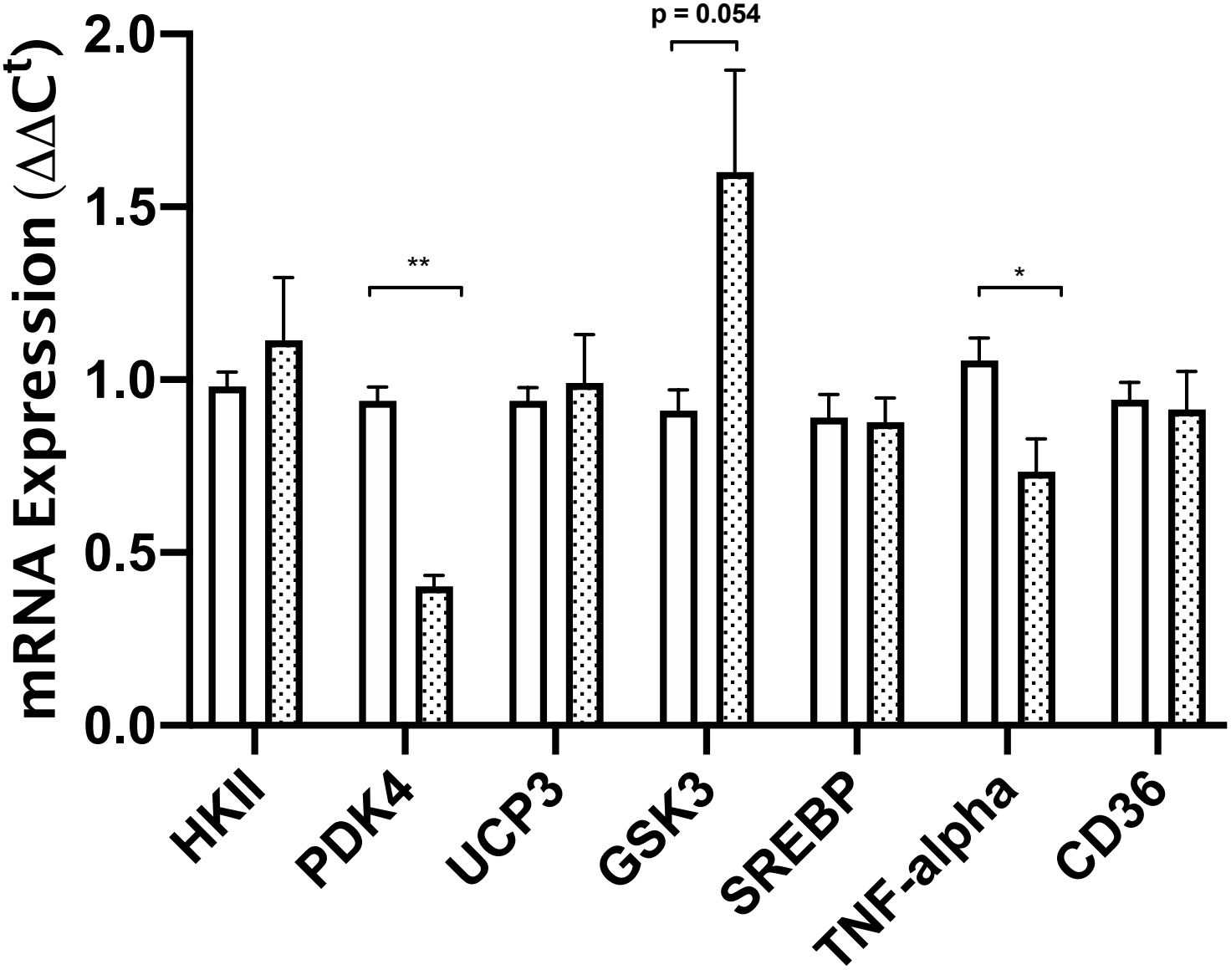

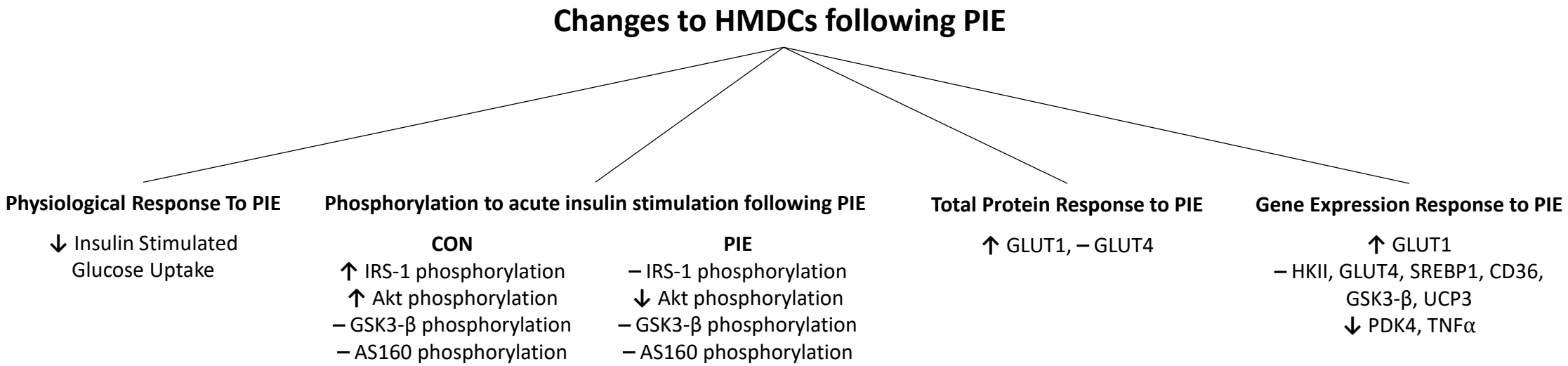

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Gene of Interest	Symbol	Manufacturer	Assertion No.
RNA polymerase II	POLRB	Sigma	*NM_000938*
Glucose Transporter 4	GLUT4 (SLC2A4)	Sigma	*NM_001042*
Hexokinase II	HKII	Qiagen	*NM_000189*
Pyruvate dehydrogenase lipoamide kinase isozyme 4	PDK4	Sigma	*NM_002612*
Glucose Transporter 1	GLUT1 (SLC2A1)	Qiagen	*NM_006516*
Uncoupling protein 3	UCP-3	Qiagen	*NM_003356*
Glycogen Synthase Kinase 3 beta	GSK3-β	Qiagen	*NM_001146156*
Tumour necrosis factor alpha	TNF-α	Sigma	*NM_000594*
Insulin receptor substrate 1	IRS-1	Qiagen	*NM_005544*
Cluster of differentiation 36 (Fatty acid translocase)	CD36	Sigma	*NM_000072*
Sterol regulatory element binding protein factor 1	SREBPF1	Qiagen	*NM_004176*