Effects of Visfatin on Brown Adipose Tissue Energy Regulation using T37i cells

*Georgios K Dimitriadis[1,2,3], *Raghu Adya[1], Bee K Tan[1,4,5,6], Terence A Jones[1,7], Vinod S Menon[8], Manjunath Ramanjaneya[1], Gregory Kaltsas[1], Alexander D Miras[2], Harpal S Randeva[1,3,9]

[1]Division of Translational and Experimental Medicine, Warwick Medical School, University of Warwick, Coventry, CV4 7AL, UK;

[2]Division of Endocrinology and Experimental Medicine, Imperial College London, Hammersmith Campus, London, W12 0NN, UK;

[3]WISDEM Centre, Human Metabolism Research Unit, University Hospitals Coventry and Warwickshire NHS Trust, Coventry CV2 2DX, UK;

[4]Departments of Cardiovascular Sciences, Health Sciences and Leicester Diabetes Centre, College of Life Sciences, University of Leicester, University Road, Leicester, LE1 7RH, UK;

[5]Department of Obstetrics and Gynaecology, University Hospitals of Leicester NHS Trust, Leicester Royal Infirmary, Infirmary Square, Leicester, Leicestershire, LE1 5WW, UK;

[6]Department of Obstetrics and Gynaecology, University Hospitals Birmingham NHS Foundation Trust, Bordesley Green East, Birmingham B9 5SS.

[7]Department of Radiology, University Hospitals Coventry and Warwickshire, Coventry, CV2 2DX, United Kingdom.

[8]Department of General Surgery, University Hospitals Coventry and Warwickshire, Coventry, CV2 2DX, United Kingdom.

[9]Division of Life and Health Sciences, Aston University, Birmingham, B4 7ET, UK;

G.K.D. and R.A. contributed equally to this work

Running Title: Visfatin and effects on Brown Adipocyte Biology.

Word count: Abstract: 247 words, Main Text: 3353 words, Tables: 1, Figures: 5

CORRESPONDENCE:

Professor Harpal S Randeva MBCHB, FRCP, FAcadTM, PhD
Division of Translational and Experimental Medicine - Metabolic and Vascular Health

Warwick Medical School, University of Warwick, Coventry CV4 7AL, UK

Tel: +44-24-76968662

Fax: +44-24-76968653

E-mail: Harpal.Randeva@warwick.ac.uk

Abstract

The role of brown adipose tissue (BAT) in pathological states of energy homeostasis and impaired adipocyte function, such as obesity has been a major area of research interest in recent years. Herein, we sought to determine the direct effects of adipokines, visfatin and leptin on BAT thermogenesis.

The effects of mouse recombinant visfatin, nicotinamide mononucleotide (NMN) and leptin with or without FK866 were studied on differentiated T37i cells. Treated cells were analyzed for key genes and proteins regulating BAT [UCP-1, PRD1-BF1-RIZ1 homologous domain-containing 16 (PRDM-16), PPARgamma-coactivator-1alpha (PGC-1α) and receptor-interacting protein 140 (RIP-140)] using quantitative PCR and western blot analysis. Data is presented as mean *P*-values.

Both visfatin and leptin had significant concentration dependent effects on thermogenesis in brown pre-adipocytes and at physiological levels, increased uncoupling protein-1 (UCP-1) levels in brown adipocytes. These effects of visfatin were similar to that of nicotinamide mononucleotide (NMN), further strengthening the enzymatic role of visfatin. We also showed that leptin induced UCP-1 mRNA expression and protein production appears to be mediated by visfatin. High concentrations of both visfatin and leptin led to a dramatic decrease in UCP-1 protein levels, supporting the notion that visfatin levels are raised in obesity and that obese people have reduced BAT activity, plausibly through a reduction in UCP-1 levels. Additionally, we found differential regulation of key brown adipogenic genes, specifically, PRD1-BF1-RIZ1 homologous domain-containing 16 (PRDM-16), PPARgamma-coactivator-1alpha (PGC-1α) and receptor-interacting protein 140 (RIP-140) by visfatin. Our observations provide novel insights in the potential actions of visfatin in BAT.

Keywords: brown adipose tissue, obesity, thermogenesis, UCP-1, visfatin.

Highlights

- Both visfatin and leptin have significant concentration dependent effects on thermogenesis in brown pre-adipocytes.
- At physiological levels, both visfatin and leptin increased uncoupling protein-1 (UCP-1) levels in brown adipocytes.
- High concentrations of both visfatin and leptin decreased UCP-1 protein levels.
- Our research suggests that visfatin levels are raised in obesity and that obese people have reduced BAT activity, plausibly through a reduction in UCP-1 levels.

1.1 Introduction

Adipose tissue (AT) functions as a key energy metabolic regulator [1]. White adipose tissue (WAT) serves as an energy reservoir, whilst brown adipose tissue (BAT) is involved in ß-adrenergically mediated thermogenesis [2]. Recent studies have implicated the importance of this metabolic activity in BAT in states of insulin resistance and obesity (inversely correlated with body mass index and age) [3]. BAT has been regarded as a key target in developing anti-obesity treatment. Although stimulants of the adrenergic system are excellent agents for enhancing BAT activity, their use has been limited by harmful cardiovascular side-effects. A safer approach employing endogenous molecules, specifically, AT derived molecules (adipokines), as potential thermogenic agents stimulating BAT is currently under focus, being investigated extensively [4].

Studies have elucidated the link between adipokines and AT function. Dysmetabolic states are characterized by altered circulating adipokine levels e.g. leptin and visfatin, levels of which are raised in obesity and metabolic syndrome, [5,6] influencing BAT function. Enriori *et al*. had demonstrated that central and peripheral administration of leptin significantly increased BAT energy expenditure *via* increased expression of mitochondrial uncoupling protein-1 (UCP-1) [7]. Other researchers have also demonstrated that in brown adipocytes undergoing differentiation, visfatin (intracellular and extracellular) levels are increased [8]. However, no studies have been to date conducted to elucidate the direct effects of visfatin (pre-B cell colony-enhancing factor or Nicotinamide phosphoribosyltransferase) a crucial energy regulator, on brown adipocytes. Moreover, we have previously demonstrated both *in vivo* and *ex vivo* regulation of visfatin production by leptin in WAT [9]. With this in mind, we sought to investigate the effects of leptin and visfatin in differentiated T37i cells , mitochondrial respiration and key genes involved in brown adipocyte energy regulation.

2.1 Materials and Methods

We studied the effect of mouse recombinant visfatin (Axxora, Nottingham, UK), nicotinamide mononucleotide (NMN) (Sigma-Aldrich, Gillingham, UK) and leptin (PeproTech, Rocky Hill, NJ, USA) with or without FK866 [a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase (AxonChem, Groningen, Netherlands)] on differentiated T37i cells (a kind gift provided by Dr Marc Lombes, INSERM, Paris, France) cultured in standard DMEM:HAM's F12 medium (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum, 2mM glutamine, 100IU/ml penicillin, 100µg/ml streptomycin, 20mM HEPES and grown at 37°C in a humidified atmosphere with 5% CO_2. Cells were differentiated with 2nM triiodothyronine (Sigma-Aldrich, Gillingham, UK) and 20nM insulin (Invitrogen, Paisley, UK) for 8 days [10,11]. The treated cells were analysed for key genes and proteins regulating BAT conversion [UCP-1, PRD1-BF1-RIZ1 homologous domain-containing 16 (PRDM-16), PPARgamma-coactivator-1alpha (PGC-1α) and receptor-interacting protein 140 (RIP-140)] using quantitative PCR and Western blot analysis [12,13]. Isoproterenol (Sigma-Aldrich, Gillingham, UK) was used as a positive control in some experiments. Lipid accumulation was performed in these treated cells by using Oil Red O (Sigma-Aldrich, Gillingham, UK) staining (see Supplementary Information).

2.1.1 Bioenergetic analysis of brown adipocytes

The XF24 Extracellular/Flux Analyser (Seahorse Biosciences, North Billerica, MA, USA) was employed for bioenergetic analysis of T37i differentiated cells. All the chemicals required for these experiments were supplied by Seahorse Biosciences, North Billerica, MA, USA. The XF24 Extracellular/Flux Analyser measures oxygen consumption rate (OCR) in a 24-well format by sensing changes in oxygen content (in a 7µl volume) above the plated cells with a fluorescence biosensor. T37i cells were seeded at a density of $3x10^4$ cells/well/500µl of Agilent Seahorse XF Assay Medium (Agilent Technologies) in an extracellular flux 24-well cell culture plate and incubated at 37ºC/5% Co_2 for 8 days. At

the end of day 7, cells were stimulated with either visfatin (100ng/mL) or leptin (10^{-9}M) or isoproterenol and cultured for a further 24 hours. Assays were initiated by removing the growth medium and replacing it with pre-warmed assay buffer medium. The microplates were incubated at 37ºC for 30 minutes to equilibrate temperature and pH prior to OCR measurements. The XF24 microplate was then transferred to a temperature-controlled (37°C) Seahorse analyzer where it was subjected to a further 10-minute equilibration period and 4 assay cycles, each comprising a 1-minute mix, 2-minute wait and 3-minute measure period cycle. Following this, Oligomycin A (1μM) was added by automatic pneumatic injection to inhibit ATP synthase activity and thus approximate the proportion of respiration used to drive ATP synthesis (coupling efficiency). After 4 assay cycles, 7.5μM carbonyl cyanide 4 trifluoromethoxy-phenylhydrazone (FCCP) was added. Each experimental trace was ended following addition of oligomycin A (5μM) and rotenone (5μM). At the end of the incubation period, the plates were used to assess protein concentration in each well by BCA protein assay. OCR (pmol/min) was normalised with protein content.

2.1.2 T37i cell culture and differentiation

T37i cells (a kind gift provided by Dr Marc Lombes, INSERM, Paris, France) were cultured in standard DMEM:HAM's F12 medium (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum, 2mM glutamine, 100IU/ml penicillin, 100μg/ml streptomycin, 20mM HEPES and grown at 37°C in a humidified atmosphere with 5% CO_2. Differentiation into mature brown adipocytes was achieved under standard conditions by incubating sub-confluent undifferentiated T37i cells with 2nM triiodothyronine [T3] (Sigma-Aldrich, Gillingham, UK)] and 20nM insulin (Invitrogen, Paisley, UK) for 8 days. At day 8 of differentiation, the cells were harvested for RNA (RT-PCR) and protein (Western blot). T37i cells were cultured in the presence of only differentiation stimuli (insulin + T3) for the control group and differentiation stimuli with or without visfatin (Axxora, Nottingham, UK), nicotinamide

mononucleotide (NMN) (Sigma-Aldrich, Gillingham, UK) or FK866 (AxonChem, Groningen, Netherlands) for the treatment group with two incubation time points as mentioned in the figure legend (Figure 1). Before stimulation, the differentiated cells were cultured overnight in the same media in the absence of serum. For UCP-1 mRNA expression and protein production studies, cells were incubated with media supplemented with 1μM isoproterenol for 6 hours prior to stimulation with peptides. The treated cells were analysed for key genes and proteins regulating brown adipose tissue conversion [UCP-1, PRD1-BF1-RIZ1 homologous domain-containing 16 (PRDM-16), PPARgamma-coactivator-1alpha (PGC-1α) and receptor-interacting protein 140 (RIP-140)] using quantitative RT-PCR and Western blot analysis.

2.1.3 RNA Isolation and Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted using the QIAGEN RNeasy Mini Kit (Qiagen Ltd, West Sussex, UK), DNAse treated and reverse-transcribed into cDNA using a reverse transcription kit according to the manufacturer's instructions (Fermentas Life Sciences, York, UK). The sequences for the sense and antisense primers (respectively) are listed in table 1. SYBR Green® real-time PCR was performed on a Biorad IQ5 realtime PCR (Bio-Rad, Hertfordshire, UK) using the primers listed above. PCR was performed using 2.5μl cDNA in 5.5μl PCR SYBR Green-1 Light Cycler Master Mix (Biogene, Cambridgeshire, UK), and 1μl each of sense and antisense primers (primer sequences as mentioned in table 1 below). A series of three dilutions for each cDNA was used to ensure linear amplification and to measure primer efficiency. Protocol conditions consisted of denaturation of 95ºC for 60 secs, followed by 40 cycles of 94ºC for 1 sec, 60ºC for 8 sec, and 72ºC for 15 sec, followed by melting-curve analysis. For analysis, expression of genes of interest were normalised against the expression of the housekeeping gene GAPDH. Negative controls for all the reactions

included preparations lacking cDNA or RNA-lacking reverse transcriptase in place of the cDNA. The relative mRNA levels were expressed as a ratio using the "2-Δct method" for comparing relative expression results between treatments in real-time PCR[1]. The PCR products from all samples were purified from the 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen Ltd, West Sussex, UK). PCR products were then sequenced in an automated DNA sequencer, and the sequence data were analyzed using Blast Nucleic Acid Database Searches from the National Centre for Biotechnology Information, confirming the identity of our products.

Semi-quantitative PCR analysis was employed for the identification of long form of leptin receptor (Ob-Rb) in T37i cells. Following RNA extraction and cDNA conversion, using specific primers for Ob-Rb gene (mentioned in the table 1 below) PCR was carried out for 40 cycles comprising of denaturation at 95° C for 1 min, annealing at 60° C for 30 sec, and extension at 72° C for 1 min. Amplification was terminated following a final extension step at 72 C for 10 min. PCR products were separated by agarose electrophoresis (1.5 % agarose gel), stained with ethidium bromide, and visualized under UV light.

***Table 1*. List of genes and sequences for the sense and antisense primers**

Gene/product size (bp)	Sense primer	Antisense primer
UCP-1 (83)	5′-ggcctctacgactcagtcca-3′	5′- taagccggctgagatcttgt-3′
PRDM-16 (180)	5′- atgcgaggtctgccacaagt-3′	5′- ctgccaggcgtgtaatggtt-3′
RIP-140 (173)	5′-tgcggatacttccacaggtc-3′	5′-gcattcctcacagccaacag-3′
PGC-1 alpha (163)	5′- tgcagccaagactctgtatg-3′	5′- attggtcgctacaccacttc-3′
GAPDH (185)	5′-gagtcaacggatttggtcgt-3′	5′-gacaagcttcccgttctcag-3′

Gene/product size (bp)	Sense primer	Antisense primer
Ob-Rb (182)	5′-aggcgcagcctgtattgtcc-3′	5′-cacgttggtggcgagtcaag-3′
CtBP1 (171)	5′-ttgggcatcattggactaggt-3′	5′- taacgcagtcactgtggaaga-3′
CtBP2 (162)	5′- atagaacgatctctgggcctg-3′	5′- aatgcaccttgctctatctgc-3′
BMP-7 (99)	5′- cttggctggcaggactggat-3′	5′- gtctggacgatggcgtggtt-3′

221

222 **2.1.4 Western Blot Analysis**

223 For UCP-1, PRDM-16 and BMP-7 protein analyses, following differentiation, T37i cells were
224 incubated with media supplemented with 1μM isoproterenol for 6 hours prior to
225 stimulation with only differentiation stimuli (insulin + T3) for the control group and
226 differentiation stimuli [with or without visfatin, leptin, NMN or FK866] for the treatment
227 group with various incubation time points as mentioned previously. Cells were then lysed
228 with Laemmli buffer [5M urea, 0.17M SDS, 0.4M dithiothreitol and 50mM Tris-HCl (pH
229 8.0)], mixed, sonicated, boiled, centrifuged (5,000rpm for 2 minutes), and stored at -80° C
230 until use. Twenty micrograms of each sample were separated on a 10% Sodium Dodecyl
231 Sulfate (SDS)-polyacrylamide gel, and electro-blotted onto a polyvinylidene fluoride
232 (PVDF) membrane (Millipore, Bedford, MA, USA). The PVDF membrane was then incubated
233 with 5% Bovine Serum Albumin (BSA) (Sigma-Aldrich, Gillingham, UK) in 1M Trizma/base,
234 1.54M NaCl, 0.05% Tween 20 (Tris buffered solution plus Tween 20, TBST, pH 7.4) for one
235 hour at room temperature, and then exposed overnight at 4°C to TBST containing UCP-1
236 primary antibody [Abcam, Cambridge, UK (AB23841, dilution 1:1000)]. The membranes
237 were then washed thoroughly for 60 min with TBS/0.1% Tween before incubation with
238 anti-mouse secondary antibody, horseradish-peroxidase-conjugated Ig (1:2000) (Dako Ltd,

Cambridge, UK) for 1 hour at room temperature. Antibody complexes were visualized using chemiluminescence (ECL; GE Healthcare, Little Chalfont, UK). The densities were measured using a scanning densitometer coupled to scanning software ScionImage™ (Scion Corporation, Frederick, Maryland, USA). Standard curves were generated to ensure linearity of signal intensity over the range of protein amounts loaded into gel lanes. Comparisons of densitometric signal intensities for proteins of interest were made only within this linear range.

2.1.5 Oil Red O stain

As mentioned previously, T37i cells were differentiated with insulin and T3 for the control group and differentiation stimuli with or without visfatin, leptin, NMN or FK866 for the treatment group for 8 days. Following differentiation, the cells were washed with PBS and fixed with 10% formalin (Sigma-Aldrich, Gillingham, UK). This was followed by washes with distilled water and 60 % isopropanol (Sigma-Aldrich, Gillingham, UK). The cells were allowed to air dry, Oil Red O (Sigma-Aldrich, Gillingham, UK) working solution was added and incubated at room temperature for 10 minutes. The cells were washed 4 times with distilled water and images were acquired.

2.1.6 Statistics

Data were analyzed by Mann-Whitney *U* test or Friedman's ANOVA (*post hoc* analysis, Dunn's test) according to the number of groups compared. All statistical analyses were performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered significant.

265 **3.1 Results**

266 Visfatin and leptin significantly increased UCP-1 mRNA and protein expression in
267 differentiated T37i cells (Figures 1A-D). Visfatin (100ng/mL) showed the maximal response
268 in contrary to visfatin (400ng/mL), which decreased UCP-1 levels. NMN (100μM) showed a
269 comparable increase of UCP-1 levels to visfatin (100ng/mL). Pre-incubation of FK866
270 (10μM) abolished visfatin (100ng/mL) induction of UCP-1 (Figures 1A-B). Leptin increased
271 UCP-1 protein levels (maximal response at 10^{-9}M), which was significantly negated by pre-
272 incubating with FK866 (10μM) (Figures 1C-D). Following differentiation protocol, T37i cells
273 were serum starved for 12 hours and treated with or without visfatin (100, 200 and
274 400ng/mL,), NMN (100μM) and FK866 (10μM) for two time points (4 and 24 hours). Visfatin
275 (100ng/mL) induced maximal expression of PRDM-16 mRNA at 4 hours. This effect of
276 visfatin was not negated when pre-incubated with FK866 (10μM). NMN (100μM) induced a
277 significant increase with comparable potency to visfatin (100ng/mL) (Figure 2A).
278 Furthermore, PGC-1α mRNA expression was significantly increased in a concentration
279 dependent manner by visfatin (maximal response at 400ng/mL). This effect was negated
280 by pre-incubating FK866 (10μM). Once again, NMN (100μM) induced a significant increase
281 with comparable potency to visfatin (400ng/mL) (Figure 2B). Also, visfatin (100ng/mL)
282 significantly decreased RIP-140 mRNA expressions (Figure 2C). Interestingly, visfatin
283 (400ng/mL), at 4 hours, resulted in a significant increase in RIP-140 mRNA expression.
284 However, no effect was observed with NMN (100μM) treatment. Pre-incubation with FK866
285 (10μM) resulted in a non-significant decrease of visfatin (400ng/mL) induced RIP-140 mRNA
286 expression (Figure 2C). Leptin increased mRNA expression levels of PDRM-16 and PGC-1α;
287 however, similar to visfatin, the maximal response dose of leptin i.e. 10^{-9}M, decreased
288 RIP-140 mRNA expression levels (data not shown). Following differentiation protocol, T37i
289 cells were serum starved overnight, and treated with or without leptin (10^{-7}, 10^{-9} and 10^{-11}M) for various time points (4-24 hours - data not shown). Leptin significantly increased
291 mRNA expression and protein production of visfatin (Maximal response was at 4 hours and

leptin 10^{-7}M) (Figures 4A-B). Finally, functional assays showed that visfatin (100ng/mL) caused a significant increase in lipid accumulation (Oil Red O stain) in pre-adipocytes and oxygen consumption (mitochondrial respiration) in mature brown adipocytes (Figures 1E-F).

3.1.2 Visfatin induces visfatin mRNA expression levels of brown adipogenic genes- CtBP-1, CtBP-2 and BMP-7.

In order to evaluate overall visfatin induced effects in brown adipogenesis, we sought to determine the effects of visfatin on key genes including CtBP-1 (C-terminal-binding protein 1), CtBP-2 and BMP-7 (Bone morphogenetic protein 7). Visfatin (400ng/mL) showed the maximal response in up-regulating all three genes, comparable with NMN (100μM). However, it is interesting to note that pre-incubation of FK866 (10μM) abolished visfatin (400ng/mL) induced CtBP-1 and BMP-7 mRNA expression levels (Fig-2D and Fig-2F), failed to do so with CtBP-2 mRNA expression (Fig-2E)

3.1.3 Visfatin increases protein expression levels of BMP-7 and PRDM-16 in differentiated T37i cells.

Visfatin concentration dependently increased protein expression levels of BMP-7 at 24 hours, promptly decreased by pre-incubation with FK-866. This increase induced by visfatin (400ng/mL) was comparable to the effects of NMN (100uM) (Fig-3A). However, in contrary, visfatin (100ng/mL) showed a significant increase in PRDM-16 protein levels and pre-incubation with FK-866 did not cause any significant effect (Fig-3C). To further study the concentration dependent effects of visfatin on protein expression levels of PRDM-16, we employed additional doses of 10, 25 and 50ng/mL of visfatin. The maximal response was observed at 100ng/mL of visfatin (Fig-3B).

3.1.4 Leptin induces visfatin mRNA and protein expression levels in a concentration dependent manner.

We used leptin in concentration ranges of 10^{-7} – 10^{-11} M in accordance with studies by others [14,15], to include physiological and pathological concentrations [2,3]. Leptin increased visfatin mRNA expression and protein expression levels in T37i cells concentration dependently with a maximum response at leptin 10^{-7} M (Fig.4A and 4B).

3.1.5 Identification of Ob-Rb receptor in T37i cells and mouse BAT

We employed RT-PCR analysis and gene specific primers to validate the presence of Ob-Rb gene in T37i cells and BAT (Fig.5) in line with previous reports [4].

4.1 Discussion

We present novel data indicating that visfatin significantly increased oxygen consumption (mitochondrial respiration) and lipid accumulation in differentiated T37i cells . Furthermore, visfatin, at physiological levels, significantly increased UCP-1 and PDRM-16 and decreased RIP-140 levels in T37i cells. Interestingly, FK-866 had no effect on visfatin induced PDRM-16 up-regulation. This may be due to the involvement of an undefined visfatin receptor. Also, this may be explained by the observation that eNampt (visfatin) blocks macrophage apoptosis through activation of IL-6/STAT3 pathway. This effect was not blocked by FK866, suggesting a non-enzymatic mechanism of action [16]. Moreover, like others, [7] we found that leptin significantly increased oxygen consumption and significantly increased UCP-1 levels in T37i cells . Interestingly, higher concentrations of leptin resulted in a significant decrease in UCP-1 levels in T37i cells. Importantly, we showed that leptin induced UCP-1 mRNA expression and protein production appears to be

mediated by visfatin. UCP-1 drives BAT thermogenesis, PDRM-16 promotes brown adipogenesis and BAT thermogenesis *via* increased stimulation of UCP-1 [17] whereas RIP-140 is an important co-repressor and inhibitor of brown adipogenesis and BAT thermogenesis [18]. However, higher concentrations of visfatin had opposite effects. This supports the observations that visfatin levels are raised in obesity [5] and that obese people have reduced BAT activity, [19] plausibly through a reduction in UCP-1 levels. Others were able to demonstrate that visfatin was preferentially expressed in mature adipocytes and that this expression was higher in brown adipose tissue of rodents compared to other fat depots following biopsies [20]. The situation was different in obese humans where visfatin expression was found to be equivalent between white and brown or brite adipocytes *in vivo* and *in vitro* supporting that BAT thermogenic properties are blunted during obesity [20]. Interestingly, we also found that visfatin concentration dependently increased PGC-1α (a strong inducer of UCP-1) mRNA expression in T37i cells. PGC-1α promotes BAT thermogenesis but not brown adipogenesis [21]; additionally, we elucidated PGC-1α independent UCP-1 gene induction. Studies have implicated a hyperglycemic state of impaired glucose metabolism induced by PGC-1α inhibiting insulin signaling and glucose utilization [22]. Taken together; our novel findings potentially suggest that visfatin could induce insulin resistance in dysmetabolic states such as obesity and diabetes *via* PGC-1α without a marked change in UCP-1 levels. However, further studies are required in brown adipocytes and *in vivo*.

4.2 Conclusion

In conclusion, our significant observations provide novel insights with respect to the potential actions of visfatin in brown adipocytes using T37i cell line. Future *in vivo* research should also seek to clarify whether visfatin could moderate thermogenesis and

adipogenesis, and whether it would be beneficial in the management of obesity and its dysmetabolic sequelae.

5.1 Declaration of Interest

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

6.1 Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

7.1 Acknowledgements

Dr Marc Lombes, INSERM, Paris, France kindly provided the T37i cells. HSR would like to acknowledge S. Waheguru, University of Warwick for his continual support.

8.1 References

[1] Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* 2004; **89:** 2548-2556.

[2] Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* 2004; **84:** 277-359.

[3] Cypess AM, *et al*. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* 2009; **360:** 1509-1517.

[4] Whittle AJ, López M, Vidal-Puig A. Using brown adipose tissue to treat obesity - the central issue. *Trends Mol Med* 2011; **17:** 405-411.

[5] Berndt J, *et al*. Plasma visfatin concentrations and fat depot-specific mRNA expression in humans. *Diabetes* 2005; **54:** 2911-2916.

[6] van Dielen FM, van't Veer C, Buurman WA, Greve JW. Leptin and soluble leptin receptor levels in obese and weight-losing individuals. *J Clin Endocrinol Metab* 2002; **87:** 1708-1716.

[7] Enriori PJ, Sinnayah P, Rudaz CG, Cowley MA. Leptin Action in the Dorsomedial Hypothalamus Increases Sympathetic Tone to Brown Adipose Tissue in Spite of Systemic Leptin Resistance. *J Neurosci* 2011; **31:** 12189-12197.

[8] Revollo JR, *et al*. Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab* 2007; **6:** 363-375.

[9] Tan BK, *et al*. In vivo and ex vivo regulation of visfatin production by leptin in human and murine adipose tissue: role of mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways. *Endocrinology* 2009; **150:** 3530-3539.

[10] Viengchareun S, Penfornis P, Zennaro MC, Lombès M. Mineralocorticoid and glucocorticoid receptors inhibit UCP expression and function in brown adipocytes. Am J Physiol Endocrinol Metab. 2001 Apr;280(4):E640-9.

[11] Kuhn E, Lombès M. The mineralocorticoid receptor: a new player controlling energy homeostasis. Horm Mol Biol Clin Investig. 2013 Sep;15(2):59-69. doi: 10.1515/hmbci-2013-0033. Review.

[12] Richard D, Picard F. Brown fat biology and thermogenesis. Front Biosci (Landmark Ed). 2011 Jan 1;16:1233-60.

[13] Whittle AJ, *et al*. BMP8B increases brown adipose tissue thermogenesis through both central and peripheral actions. Cell. 2012 May 11;149(4):871-85. doi: 10.1016/j.cell.2012.02.066.

[14] Mahabir S, *et al*. Body Mass Index, percent body fat, and regional body fat distribution in relation to leptin concentrations in healthy, non-smoking postmenopausal women in a feeding study. Nutr J. 2007 Jan 17;6:3.

[15] Hwa JJ, *et al*. Leptin increases energy expenditure and selectively promotes fat metabolism in ob/ob mice. Am J Physiol. 1997 Apr;272(4 Pt 2):R1204-9.

[16] Li Y, *et al*. Extracellular Nampt promotes macrophage survival via a nonenzymatic interleukin-6/STAT3 signaling mechanism. *J Biol Chem*. 2008; 283(50):34833-43.

[17] Seale P, *et al*. Transcriptional control of brown fat determination by PRDM16. *Cell Metab* 2007; **6:** 38-54.

[18] Christian M, Kiskinis E, Debevec D, Leonardsson G, White R, Parker M.G. RIP140-targeted repression of gene expression in adipocytes. *Mol Cell Biol* 2005; **25:** 9383-9391.

[19] Vijgen GH, Bouvy ND, Teule GJ, Brans B, Schrauwen P, van Marken Lichtenbelt WD. Brown Adipose Tissue in Morbidly Obese Subjects. *PLoS One* 2011; **6:** e17247.

[20] Pisani DF *et al*. Visfatin expression analysis in association with recruitment and activation of human and rodent brown and brite adipocytes. *Adipocyte*. 2015 Dec 9;5(2):186-95.

[21] Uldry M, Yang W, St-Pierre J, Lin J, Seale P, Spiegelman BM. Complementary action of the PGC-1 coactivators in mitochondrial biogenesis and brown fat differentiation. *Cell Metab* 2006; **3:** 333-341.

[22] Wende AR, Huss JM, Schaeffer PJ, Giguère V, Kelly DP. PGC-1alpha coactivates PDK4 gene expression via the orphan nuclear receptor ERRalpha: a mechanism for transcriptional control of muscle glucose metabolism. *Mol Cell Biol* 2005; **25:** 10684-10694.

Figure Legends

Figure 1. Serum-starved differentiated T37i cells were pre-incubated with or without FK866 (10uM). On treatment with or without visfatin (100-400ng/mL) or NMN (100uM) or isoproterenol (1µM) for 4 hours, mRNA levels of (**A**) UCP-1 was analyzed by real-time PCR and normalized with the housekeeping gene GAPDH. Similarly, serum-starved differentiated T37i cells were treated for 24 hours, protein expression levels of (**B**) UCP-1 was analyzed by western blot analyses and densitometric analyses of UCP-1, normalized to

ß-actin and expressed as a fold increase over basal. Experiments were performed in triplicates. The values represented are relative to basal. **P < 0.01, *P < 0.05 vs. basal, $^{\#}$P < 0.01 vs. visfatin (100ng/mL) only treated, n = 6 per group. Likewise, serum-starved differentiated T37i cells were pre-incubated with or without FK866 (10uM). On treatment with or without leptin (10^{-11}, 10^{-9} and 10^{-7} M) or isoproterenol (1µM) for 4 hours, mRNA levels of (**C**) UCP-1 was analyzed by real-time PCR and normalized with the housekeeping gene GAPDH. Similarly, serum-starved differentiated T37i cells were treated for 24 hours, protein expression levels of (**D**) UCP-1 was analyzed by western blot analyses and densitometric analyses of UCP-1, normalized to ß-actin and expressed as a fold increase over basal. Experiments were performed in triplicates. The values represented are relative to basal. ***P < 0.001, **P < 0.01, *P < 0.05 vs. basal, $^{\#}$P < 0.01 vs. leptin (10^{-9} M) only treated n = 6 per group.

T37i cells were differentiated with insulin and T3 (6 days) for control group and differentiation stimuli with or without visfatin (100-400ng/mL) or NMN (100uM) or leptin (10^{-11}, 10^{-9} and 10^{-7} M) for treatment group for 2 days. One set of treatment group was pre-incubated with FK866 (10 uM). (**E**) Representative scanned images of Oil Red O stained brown adipocytes [1] and [2] Control (undifferentiated) [3] Control (differentiated) [4] Visfatin(100ng/mL) [5] Vis(100ng/mL)+FK866(10µM) [6] NMN(100µM).(**F**) Mitochondrial respiration [oxygen consumption rate (OCR)] in differentiated brown adipocytes (day 6) was assessed using the XF24 Extracellular/Flux Analyser. A representative graph of the OCRs of untreated, visfatin, leptin and isoproterenol treated cells in their basal states and on treatment with oligomycin, FCCP and rotenone used to demonstrate the specific components of the respiratory chain. Experiments were performed in triplicates. The values represented are relative to basal. **P < 0.01, *P < 0.05 vs. untreated cells (control of that particular group), n = 6 per group.

Figure 2. Serum-starved differentiated T37i cells were pre-incubated with or without FK866 (10uM). On treatment with or without visfatin (100-400ng/mL) or NMN (100uM) for 4 hours, mRNA levels of (**A**) PDRM-16, (**B**) PGC-1α, (**C**) RIP-140, (**E**) CtBP-1, (**F**) CtBP-2 and (**G**) BMP-7 were analyzed by real-time PCR and normalized with the housekeeping gene GAPDH. Experiments were performed in triplicates. The values represented are relative to basal. ***P < 0.001, **P < 0.01, *P < 0.05 vs. basal, $^{\#}$P < 0.01 vs. visfatin (100 or 400ng/mL) only treated, *NS*- Non-Significant vs. basal, n = 6 per group.

Figure 3. Serum-starved differentiated T37i cells were pre-incubated with or without FK866 (10uM). On treatment with or without visfatin (100-400ng/mL) or NMN (100uM) for 24 hours, protein levels of (**A**) BMP-7 and (**C**) PRDM-16 were analyzed by western blot analyses and normalized with the housekeeping protein GAPDH. Similar experiments were performed with concentration dependent visfatin (10, 25, 50 and 100 ng.mL) for 24 hours and protein levels of (**B**) PRDM-16 were analyzed by western blot analyses and normalized with the housekeeping protein GAPDH. Experiments were performed in triplicates. The values represented are relative to basal. ***P < 0.001, **P < 0.01, *P < 0.05 vs. basal, $^{\#}$P < 0.01 vs. visfatin (100 or 400ng/mL) only treated, *NS*- Non-Significant vs. basal, n = 6 per group.

Figure 4. Following time optimisation studies, serum-starved differentiated T37i cells were treated with or without leptin (10^{-11}, 10^{-9} and 10^{-7} M) for 4 hours; (**A**) mRNA levels of visfatin were analyzed by real-time PCR and normalized with the housekeeping gene GAPDH. Experiments were performed in triplicates. The values represented are relative to basal. ***P < 0.001, **P < 0.01, *P < 0.05 vs. basal, n = 6 per group. Similar treatments were done with leptin for 24 hours, (**B**) protein expression levels of visfatin were analyzed by western blot and densitometric analyses normalized to ß-actin and expressed as a fold increase over basal. Experiments were performed in triplicates. The values represented are relative to basal. ***P < 0.001, **P < 0.01, *P < 0.05 vs. basal, n = 6 per group.

Figure 5. RT-PCR amplification of Ob-Rb gene. Lane 1, DNA ladder marker; lane 2, cDNA from T37i cells; lane 3, cDNA from mouse BAT; lane 4, $-^{ve}$ control (RT $-^{ve}$ – cDNA preparation lacking reverse transcriptase).