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Acyl-ghrelin mediated lipid retention and inflammation in obesity-related Type 2 diabetes

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Abstract

Acyl-ghrelin has various peripheral effects including the potential role in mediating cellular lipid removal and macrophage polarization. Previous reports are contradictory as to how glycaemia and acyl-ghrelin mediates lipid retention and inflammation within individuals with Type 2 diabetes (T2D). Our aim was to explore acyl-ghrelin levels and ghrelin expression in relation to lipid and inflammatory markers within an *ex vivo* human model, biopsied visceral adipose tissue.

Results indicated that acyl-ghrelin was associated with a decline in key lipid homeostasis genes *ABCG1* and *LXRβ* expression. Within T2D there was also a down regulation of these genes which was independent of acyl-ghrelin levels. Circulatory pro-inflammatory markers (IL-6 and TNFα) had no association with ghrelin expression nor circulating acyl-ghrelin levels. Anti-inflammatory marker (IL-10) and total antioxidant status (TAOS%) were positively associated with ghrelin expression across samples from all groups combined (total sample cohort) and specifically within the obesity sample cohorts.

Data supported the hypothesis that hyperglycaemia and acyl-ghrelin have a regulatory role in lipid retention. Furthermore, that both acyl- and desacyl-ghrelin is responsible for a protective inflammatory response; however this response is diminished in T2D.

Key words- Acyl-ghrelin; Type 2 Diabetes; Lipid retention; Inflammation

Introduction

The metabolic syndrome has a strong association with developing Type 2 diabetes (T2D). This syndrome is characterised by elevated blood pressure and glucose levels, abdominal adiposity and abnormal HDL-cholesterol or triglyceride levels [1]. Within the last decade, the orexigenic hormone ghrelin has gained scientific interest due to its association with the metabolic syndrome. Secreted from the X/A-like cells of the oxyntic glands of the gastric fundus, the 28 amino acid protein undergoes post-translational octanoylation to produce acyl-ghrelin (AG), which binds to growth hormone secretagogue receptors; 1α (GHSR1α) and 1β (GHSR1β) [2-4]. The unique post-translational modification of desacyl-ghrelin (DAG) into AG is dependent upon the bioavailability of a key octanoylation agents and de-octanoylating agent:- ghrelin O-acyltransferase (GOAT) and acyl-protein thioesterase 1 (APT1), respectively [5-7]. AG is often referred to as the active form, however recent studies suggest an independent role in homeostatic regulation for DAG [8-10]. Ghrelin has been reported to be present throughout the human body, indicative of a global homeostatic role, including an association with lipid and endocrine homeostasis [11, 12].

Acyl-ghrelin & lipogenesis

Peroxisome proliferator-activated receptor γ (PPARγ) induces the removal of cellular lipids by high density lipoproteins via the activation of liver X receptor isoform β (LXRβ) and in turn, ATP binding cassette G1 (ABCG1). The relationship between AG, lipid retention and lipid biosynthesis, is unclear within published studies discussed in detail within previous review [13]. This may be due to variations in dose and route of AG administration and evidence for a species-specific effect arising from murine data. Chronic intravenous

administration of centrally acting AG has been implicated in having a detrimental effect on the transcription of the PPAR-LXR-ABC pathway, resulting in increased white adipose tissue (WAT) depots in rodents [14]. Furthermore, AG administration is reported to activate the LXR-ABC pathway in a dose dependant manner in human THP-1 macrophages [15].

Acyl-ghrelin & inflammation

It is noted that AG and its target receptors (GHSR1α and GHSR1β) have been localized within various immune cells including macrophages, neutrophils and lymphocytes [16, 17]. Emerging evidence has linked GHSR1 presence to M1 and M2 macrophages, which might alter adipose tissue inflammation via macrophage polarization [18]. Alterations in macrophage polarization can result in changes in key pro-inflammatory cytokines such as TNFα and IL-6, and the anti-inflammatory cytokine IL-10 [19]. In addition to inflammatory markers, increased plasma levels of AG have been associated with a decrease in oxidative burden within obese subjects [20]. Furthermore, evidence linking AG to an increased adipose tissue mass may provide a plausible role for AG interaction within systemic oxidative stress, due to biomarkers of oxidative stress being correlated with fat accumulation [21, 22].

Acyl-ghrelin & endocrine homeostasis

Peripheral AG has been shown to have a direct metabolic action that influences endopancreatic function, altering glucose diabetogenic action [23, 24]. In obese individuals with T2D, a decreased plasma level of combined AG and DAG is associated with an increase

in abdominal adiposity and insulin resistance [25]. As previously mentioned, ghrelin's homeostatic action may play a role in T2D through lipid and glucose metabolism cross-talk. An increase in hepatic free fatty acid (FFA) oxidation as a result of adiposity, triggers insulin resistance and increased glucose output [26]. An AG infusion in healthy volunteers has been shown to increase circulating FFA levels [27], which promotes insulin resistance and a decline in insulin sensitivity via an increase of FFA, physical stress and reactive oxygen species associated with adipocyte hypertrophy. In line with this, ghrelin mediated-lipid retention, inflammation and glucose homeostasis may contribute to the pathophysiology of T2D. However, little data has been published to support the interaction of AG, lipid retention, inflammation and glucose homeostasis in humans.

Materials and Methods

Sample collection

30 human visceral adipose tissue (hVAT) samples categorised as; non-obese (BMI<30 kg/m^2 (NO [n=10])), obese (BMI > 30 kg/m^2 (O [n=10])), and obese with T2D (BMI > 30 kg/m^2, T2D diagnosis >6 months (OT2D [n=10])) were collected, with a corresponding fasting blood sample and additional clinical information (age, body weight, height, medical history and prescribed medication). All of which were collected within 24 hours prior of undergoing routine abdominal operations at Morriston and Singleton Hospitals, Swansea and after informed consent was retrieved. A hVAT biopsy was taken from the greater omentum during surgery and placed immediately into RNALater® (Ambion Inc, UK) to preserve tissue stability and RNA integrity. Analytical chemistries (glucose, total cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL) and triglycerides) were measured using

the Randox Daytona Plus and HbA1c measured using Tosoh GX HLC-723 (Tosoh Bioscience Ltd) from whole blood samples collected in Vacutainer™ EDTA-plasma tubes.

Real Time-PCR gene expression analysis

Expression of ghrelin axis and lipid profile genes were measured in hVAT samples. RNA was extracted using Qiagen™ Lipid extraction kits via standard manufacturers protocol. Reverse transcription was performed using 1000ng/µl of total RNA and reverse transcriptase kit (Ambion™) with oligo d(T) primers. Real Time-PCR was performed using SYBR Green chemistries on thermocycler (CFX connect; Biorad™). Primer sequences are reported in Table 1, for genes of interest analysed for exploration of ghrelin axis and lipid retention. The average CT value was taken from triplicate assays and normalised against the invariant expression of *β-actin* housekeeper gene. Result were analysed using the $2^{-\Delta\Delta CT}$ method to produce relative fold change values in comparison between groups, standard error of the mean (SEM) was calculated from the average CT value for each sample produced within the experiment cohort. Fold change range of -1.5 to 1.5 is indicative of no overall change in gene expression levels.

Measure of plasma acyl-ghrelin

AG was measured in plasma taken from whole blood sample treated with an irreversible serine protease inhibitor, 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AESBF) [0.02mg/ml], using a Human Ghrelin (active) ELISA (Merck Millipore™) following the manufacturer instructions. The complete assay was read at 450 nm and 590 nm absorbance on a SkanIt™ plate reader (ThermoScientific™).

Primer	Forward Primer	Reverse Primer
B-actin	GATGGCCACGGCTGCTTC	TGCCTCAGGGCAGCGGAA
GHRL	TGAGCCCTGAACACCAGAGAG	AAAGCCAGATGAGCGCTTCTA
PPARγ	ACAGCGACTTGGCAATATTTATTG	AGCTCCAGGGCTTGTAGCA
ABCG1	TCCTATGTCAGGTATGGGTTCG	GTCCAGGTACAGCTTGGCAT
LXRβ	CCTGCAGGTGGAGTTCATCA	CAGCTGGTCCTGCGGC
LYPLA1	GGTCCTATCGGTGGTGCTAA	ACATCCATCATTTCCTGTTGACAC
mBOAT4	TCTTTGTCTGAGCATGTGTGTAA	AAGCACTGGACCCTTGAACA

133 Table 1. Primer sequences generated through NCBI primer tools and PrimerDesign

134 *Measure of plasma cytokine levels*

135 IL-6, TNFα and IL-10 were measured in fasting plasma taken from whole blood sample using
136 ELISA (R&D systems™) following the manufacturer instructions. The complete assay was
137 read at 450 nm and 590 nm absorbance on a SkanIt™ plate reader (ThermoScientific™).

138 *Measure of total antioxidant status*

139 Relative total antioxidant status percentage (TAOS%) analysis was measured in plasma
140 taken from whole blood sample. Plasma TAOS, which is inversely related to oxidative stress,
141 was measured using the Sampson *et al* [28] protocol, a modification of Laight's photometric
142 microassay and as published by Prior *et al* [29, 30].

Statistical analysis

For gene expression data, statistical analysis (SPSS™; version 21) was run using one-way analysis of variance (ANOVA) on CT data normalised against *β-actin* housekeeper. All baseline characteristics were analysed using one-way ANOVA for parametric data and Kruskal Wallis for non-parametric data. Parametric data is given as mean and standard deviation shown in brackets. Non-paramteric data is presented as median and interquartile ranges [IQR] are shown in square brackets. Correlation analysis was performed using linear regression and Spearmans rank. P values less than 0.05 were deemed statistically significant.

Results

Total Sample Cohort

Baseline characteristics were compared across all three groups indicating significant differences in key metabolic markers (Table 2). Within the total sample cohort, circulating acyl-ghrelin levels were associated with ΔCt values of *GHRL* expression (r_s= -0.41, p<0.05), however, there was no association between circulating acyl-ghrelin levels and key octanoylation genes; *LYPLA1* (r_s= -0.01, p=0.62) or *mBOAT4* (r_s= -0.11, p=0.59). Baseline plasma acyl-ghrelin levels were significantly decreased in OT2D compared to both O (p<0.05) and NO groups (p<0.05) (Table 2). As shown in Figures 1A and 1B, acyl-ghrelin concentrations were inversely correlated with plasma glucose levels (r_s=-0.41, p<0.05) and body weight (r_s=-0.42; p<0.05), respectively. However, a positive correlation was seen when comparing plasma acyl-ghrelin levels with total cholesterol (r_s = 0.38, p<0.05) and LDL (r_s = 0.39, p<0.05) (Figures 1C and 1D respectively). In the total sample cohort, there was no

significant associations between inflammatory markers and plasma acyl-ghrelin (IL-6, r_s= -0.28, p=0.16; TNFα, r_s= 0.02, p=0.96; IL-10, r_s= 0.13, p=0.63; TAOS, r_s= 0.26, p=0.19). However, when inflammatory markers were analysed against *GHRL*, increased gene expression or a decline in ΔCt value as shown, was associated with increased plasma IL-10 (r_s= -0.48, p<0.05) and TAOS% (r_s= -0.40, p<0.05) and a non-significant reduction in TNFα (r_s = 0.44, p=0.06).

	Non Obese (NO) (n=10)	Obese (O) (n=10)	Obese Type 2 (OT2D) (n=10)	P-value
Age (Years) Mean (SD)	51.8 (15.5)	51.1 (12.0)	45.5 (6.8)	0.44
Weight (Kg)* Median [IQR]	72.2 [64-81]	90.7 [86-122]	131.4 [114-152]	**<0.001**
BMI (Kg/m^2)* Median [IQR]	26.2 [24-28]	34.9 [32-42]	47.3 [42-51]	**<0.001**
Glucose (mmol/L)* Median [IQR]	4.8 [4.5-6.5]	5.4 [4.9-6.5]	6.7 [6.0-12.6]	**<0.01**
HbA1c (%)* Median [IQR]	5.1 [4.6-5.7]	5.2 [4.9-5.5]	7.0 [5.4-8.4]	**<0.05**
HbA1c (mmol/mol)* Median [IQR]	30.6 [26-39]	36.1 [30-37]	52.5 [36-68]	**<0.05**
Statin prescription# % (n)	20 (2)	10 (1)	50 (5)	0.11
Acyl-ghrelin (pg/mL)* Median [IQR]	467.2 [326-508]	515.5 [309-701]	228.5 [98-439]	**<0.05**

Table 2. Baseline characteristics for total sample cohort. Mean and standard deviation (SD) shown for normally distributed data & p-value determined using one-way ANOVA (Age; F(1,28)=0.58, p=0.44). * Median and interquartile range [IQR] shown for data that is not normally distributed and non-parametric Kruskal Wallis used for p-value determination. #Categorical data tested using Pearson Chi-square analysis. Significant p-value are shown in bold.

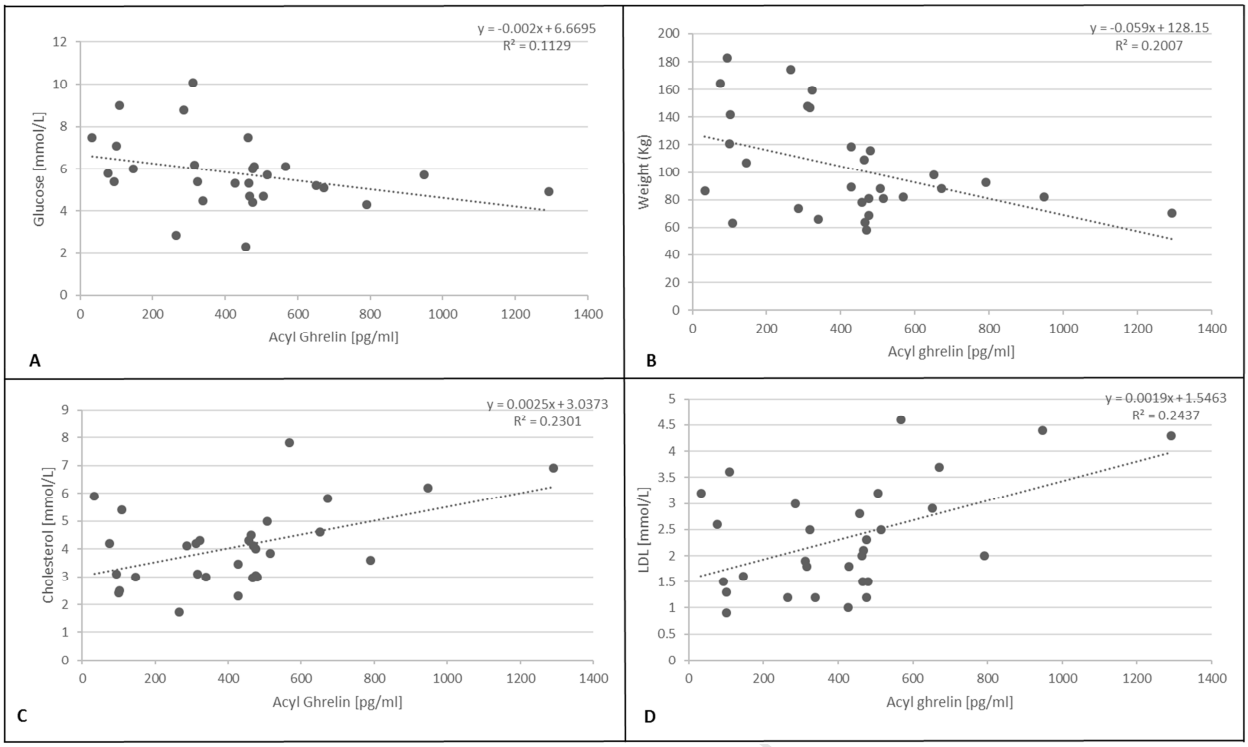

Figure 1. Scatter graphs showing correlations between AG levels and baseline characteristics. (A) Plasma glucose (R^2 = 0.113; $p<0.05$). (B) Body weight (R^2 = 0.2007; $p<0.05$). (C) Plasma total cholesterol (R^2 = 0.2301; $p<0.05$). (D) Plasma LDL (R^2 = 0.2437; $p<0.05$).

Since the baseline characteristics showed a significant difference in weight between O and OT2D ($p<0.05$), the total cohort was split into two further groups for data analysis to enable the investigation and exploration of adiposity versus glycaemic state (i) obesity effect ([OT2D+O] v NO) and (ii) diabetes effect ([O+NO] v OT2D).

Obesity effect

We observed that obese (OT2D+O) individuals had a significantly higher blood glucose concentration when compared to NO (6.1 [5.4-7.5] v 4.8 [4.5-5.7] mmol/L; $p<0.05$). Lipid profiles of total cholesterol ($F_{(1,28)}=0.6$, $p=0.45$), HDL ($F_{(1,28)}=0.6$, $p=0.43$), LDL (F

(1,28)=1.4, p=0.25) and triglycerides were unaltered (F (1,28)=2.3, p=0.14) (Figure 2A-D). *LXRβ*, *ABCG1, GHRL, mBOAT4, PPARγ* and *LYPLA1* gene expression showed no difference between obese (OT2D+O) and NO individuals (Figure 3A). Plasma AG showed no significant association with inflammatory cytokines within the obese group (IL-6, r_s=0.14, p=0.59; TNFα, r_s=0.26, p=0.47; IL-10, r_s=0.18, p=0.63). Furthermore, inflammatory cytokines, IL-6 (r_s=-0.03, p=0.92) and TNFα (r_s=0.42, p=0.20) had no association with *GHRL* expression, However, the pro-inflammatory marker IL-10, demonstrated a significant association with *GHRL* expression (r_s=-0.57, p<0.05). Additionally, TAOS% indicates oxidative stress was also associated with *GHRL* expression (r_s=-0.54, p<0.01), however, further down-stream when TAOS% is analysed versus circulating AG levels, no association was observed (r_s=0.24, p=0.32).

<u>*Diabetes effect*</u>

OT2D individuals, when compared with those with normoglycaemia (NO+O), were significantly different in weight (131.4 [116-148] v 82.0 [72-100] Kg: p<0.01), BMI (47.3 [43-50] v 29.9 [43-50] Kg/m^2; p<0.01), plasma glucose (6.7 [6.0-11.1] v 5.3 [4.6-5.9] mmol/L: p<0.01) and HbA1c (7.0 [5.5-7.6] v 5.2 [4.6-5.6] %: p<0.01). Lipid profiles of total cholesterol (F (1,28)=9.5, p<0.01), HDL (F (1,28)=7.1, p<0.05) and LDL (F (1,28)=12.4, p<0.01) were significantly decreased in the OT2D group, while triglycerides showed a non-significant increase (F (1,28)=3.0, p=0.10)(Figure 2A-D). However, gene expression data for diabetes effect (OT2D) indicated a marked decrease in *LXRβ, ABCG1* and *GHRL* expression levels (p<0.05) (Figure 3B). During analysis of inflammatory markers association with AG and *GHRL* gene expression, it was evident that within a diabetes effect all associations previously seen

had been diminished. AG showed no significant association with inflammatory cytokines (IL-6; r_s=-0.04, p=0.30, TNFα; r_s=-0.60, p=0.29 and IL-10; r_s=-0.70, p=0.19), nor with oxidative stress marker (TAOS%; r_s=-0.42, p=0.23). In addition, *GHRL* expression had also diminished all associations with inflammatory and oxidative stress markers, showing no significant correlation with IL-6 (r_s=-0.31, p=0.42), TNFα (r_s=0.67, p=0.22), IL-10 (r_s=0.82, p=0.09) or TAOS% (r_s=-0.17, p=0.65).

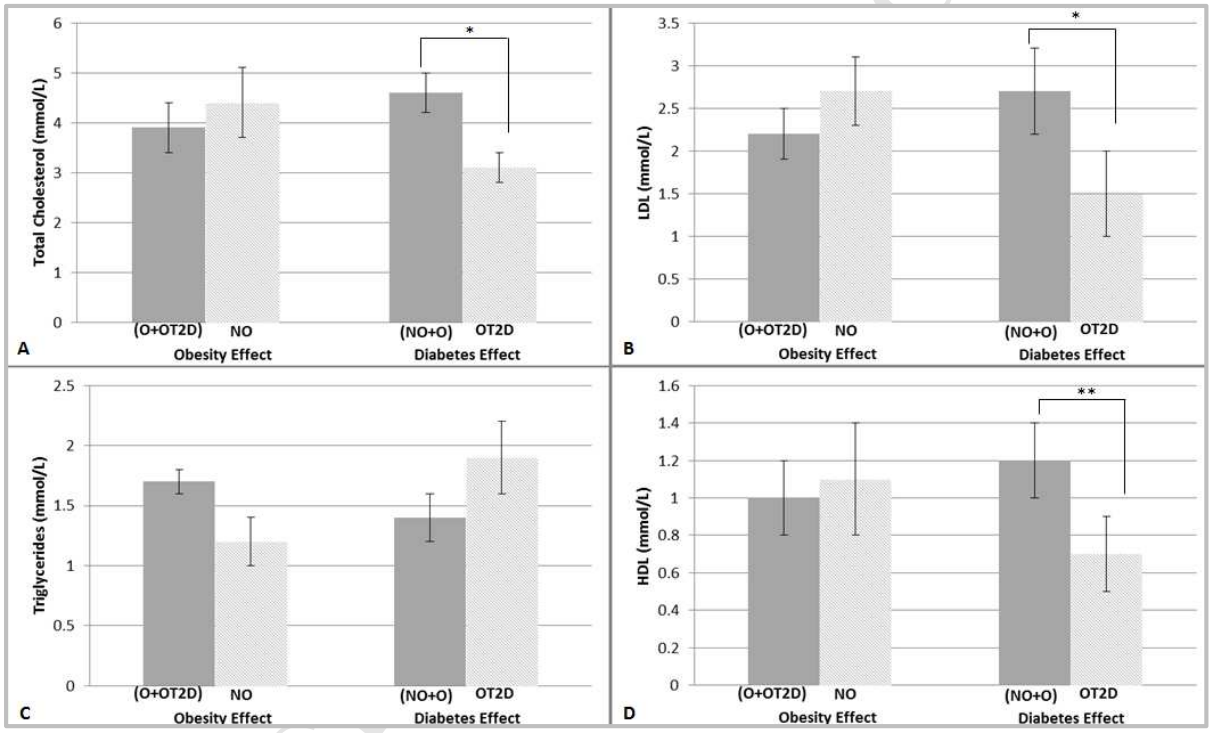

Figure 2. (A) Mean and standard error of total cholesterol levels (mmol/L) in comparison of control for the two-arm data analysis of obesity and diabetes effect. (B) Mean and standard error of LDL levels (mmol/L) in comparison of control for the two-arm data analysis of obesity and diabetes effect. (C) Mean and standard error of triglycerides levels (mmol/L) in comparison of control for the two-arm data analysis of obesity and diabetes effect. (D) Mean and standard error of HDL levels (mmol/L) in comparison of control for the two-arm data analysis of obesity and diabetes effect. P value determined using one-way ANOVA. * p <0.01. ** p <0.05.

	Non-Obese (NO)	Obese (O)	Obese Type 2 (OT2D)	P-value
Cholesterol (mmol/L)	4.4 (1.2)	4.8 (1.7)	3.1 (0.7)	**<0.05**
HDL (mmol/L)	1.1 (0.4)	1.3 (0.8)	0.7 (0.2)	**<0.05**
LDL (mmol/L)	2.7 (0.9)	2.8 (1.1)	1.5 (0.5)	**<0.01**
Triglycerides (mmol/L)	1.2 (1.0)	1.5 (0.6)	1.9 (0.8)	0.19

Table 3. Mean and standard deviation shown for lipid profiles for total cohort. Mean and standard deviation shown for normally distributed data & p-value determined using one way ANOVA. Significant values are shown in bold.

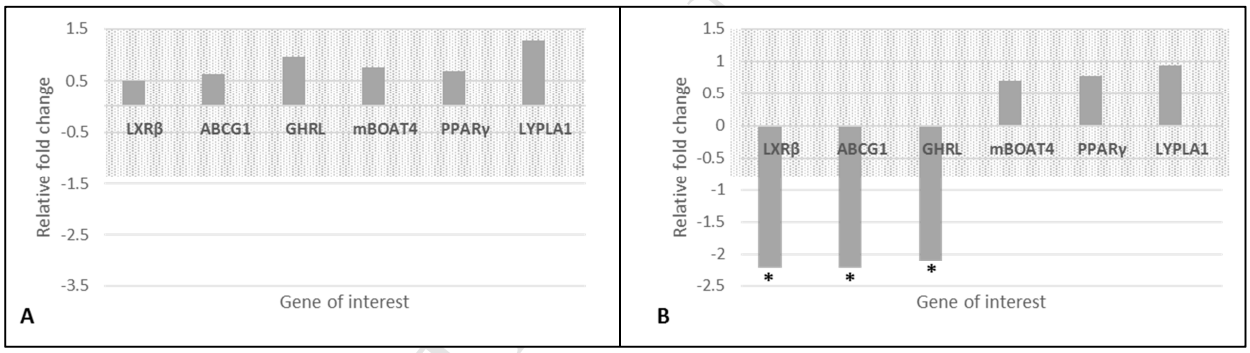

Figure 3. Relative fold change values for gene expression data; negative fold change indicates down-regulation, positive fold change indicates up-regulation and fold change between -1.5 and 1.5 is classed as no fold change, indicated by shaded area. (A) Obesity effect (NO v [O+OT2D]). (B) Diabetes effect ([NO+O] v OT2D). * indicates statistical significance ($p<0.05$) between ΔCt values between sample cohort for gene of interest.

Discussion

To date, pre-clinical studies into the regulation of lipid homeostasis via the ghrelin axis have yielded contradictory findings. The translation of these studies to humans suggests ghrelin is a mediator of lipid homeostasis, at least in hVAT. Correlation of AG with key lipid profile

markers advocates that in a high AG environment there is an increase in plasma lipid profiles. These findings are consistent with published data in humans, in the presence of low AG, suggesting there is a diminished LXR-ABC response [15], which could result in an increase in cellular lipid retention. However, it is important to acknowledge the significance of cell specific responses, and due to the complex make-up of hVAT it merely represents the profile of gene expression in adipose tissue. The down regulation of *LXRβ* within the diabetes cohort (OT2D) could indicate that AG promotes an altered immune function, as LXR isoforms have an anti-inflammatory response [31]. In accordance with published data, individuals with T2D have a significantly decreased level of circulating AG [25], which appears to be dependent on plasma glucose levels. With increased endogenous glucose levels present in those with T2D already shown to increase lipid concentrations within the cell, data suggests the cellular export mechanism that counterbalances the lipid increase is impaired due to the lack of AG present. These findings correspond to the observed low plasma lipid concentrations due to detainment, trapping lipids within the cell, and lowering the rate of release into the circulation, independently of statin usage.

Upon elucidation of a diabetes versus obesity effect, it is apparent that both obesity and T2D caused a marked decline in anti-inflammatory markers i.e. plasma IL-10 [32, 33], and an increase in surrogate oxidative stress markers i.e. TAOS levels [29, 34]. Furthermore, within both the total sample and obese effect cohorts, there was a significant association between *GHRL* expression and IL-10. However, when explored within the diabetes effect cohort it was apparent this association was diminished. Improvements in both IL-10 and TAOS% levels were associated with the up regulation of *GHRL* expression, corresponding with published studies that indicate a promotion of inflammatory health in the presence of ghrelin [20, 35]. It is not determined whether this inflammatory protection is due to an

increase in both AG and DAG via increased *GHRL* expression, or whether is the result of a shift in DAG or AG concentrations. Previous studies have linked AG with a plausible role in protecting human lens epithelial cells [36] and osteoblastic cells [37] against reactive oxygen species accumulation. In addition to AG, DAG treatment has also established a protective role from oxidative stress in microvascular endothelial cells via regulation of sirtuin 1 (SIRT1) catalytic activity [38] and within osteoblastic cells independent of GHSR1α [37].

Data demonstrates that circulatory AG concentration and action are dependent upon the mRNA expression of the *GHRL* gene. Furthermore, it is not altered due to the availability of APT1 or GOAT, with *LYPLA1* and *mBOAT4* gene expression remaining unchanged across the cohorts, respectively. However, an expansion of this analysis into the activity of the key des-octanoylation and octanoylation genes may elucidate a regulatory role. Further work is key to the exploration of whether endogenous glucose levels within T2D effects the ratio of AG to DAG and how this effects hypertrophy and consequent comorbidities.

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Highlights

- Hyperglycaemia and acyl-ghrelin have a role in the mediation of lipid retention
- Total ghrelin is responsible for a protective response to oxidative burden
- Pro-inflammatory markers had no association with ghrelin
- Anti-inflammatory markers are positively associated with ghrelin
- Low ghrelin levels in Type 2 diabetes extinguishes associations with inflammatory health