Differential Unfolded Protein Response in skeletal muscle from non-diabetic glucose tolerant or intolerant patients with obesity before and after bariatric surgery

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

Abstract

Aims: Not all people with obesity become glucose intolerant, suggesting differential activation of cellular pathways. The Unfolded Protein Response (UPR) may contribute to the development of insulin resistance in several organs but its role in skeletal muscle remains debated. Therefore, we explored the UPR activation in muscle from non-diabetic glucose tolerant or intolerant patients with obesity, and the impact of bariatric procedures. *Methods:* Muscle biopsies from 22 normoglycemic (NG, blood glucose measured 120min after an oral glucose tolerance test, G120 < 7.8mM) and 22 glucose intolerant (GI, G120 between 7.8 and 11.1mM) were used to measure UPR activation by RTqPCR and western-blot. Then, UPR was studied in biopsies from 7 NG and 7 GI patients before and one year after bariatric surgery. *Results:* Binding Immunoglobulin Protein (BIP) protein was ~40% higher in the GI compared to NG subjects. Contrastingly, expression of the UPR-related genes *BIP*, *Activating Transcription Factor 6* (*ATF6)* and unspliced *X-box Binding Protein 1* (*XBP1u*) were significantly lower and *C/EBP HomolOgous Protein* (*CHOP*) tended to decrease (p=0.08) in GI individuals. While BIP protein positively correlated with fasting blood glucose (r=0.38, p=0.01), *ATF6* and *CHOP* were associated with G120 (r=-0.38 and r=-0.41, p<0.05) and the Matsuda index (r=0.37 and r=0.38, p<0.05). Bariatric surgery improved metabolic parameters, associated with higher *CHOP* expression in GI patients, while *ATF6* tended to increase (p=0.08). *Conclusions: CHOP* and *ATF6* expression decreased in non-diabetic GI patients with obesity and was modified by bariatric surgery. These genes may contribute to glucose homeostasis in human skeletal muscle.

Keywords: unfolded protein response, skeletal muscle, obesity, glucose intolerance, bariatric surgery

Introduction

The World Health organization estimates that almost 2 billion adults were overweight, of which one third is obese, in 2016. Obesity predisposes to several complications including cardiovascular diseases, cancers, musculoskeletal disorders and diabetes [1]. However, not all obese people develop type 2 diabetes mellitus (T2D) [2], which is characterized by insulin secretion failure and peripheral resistance. Therefore, certain cellular pathways may be differently expressed between obese patients with normal glucose homeostasis *vs.* those in a prediabetic state [3], characterized by an impaired glucose tolerance, *i.e.* having a 2-hr glucose level after a 75g oral glucose tolerance test (G120) comprised between 7.8 and 11.1mmol/L. Most studies focused on the pancreas, liver and adipose tissue to explain alterations in whole-body glucose handling. Skeletal muscle has surprisingly been neglected while it accounts for 30% of body weight and for up to 80% of postprandial glucose uptake [4].

Recent studies indicate that the Endoplasmic Reticulum (ER) stress-induced Unfolded Protein Response (UPR) may contribute to the development of insulin resistance in adipose tissue, liver or pancreas [5-7]. This cellular response involves three major pathways [8] implicating Protein kinase RNA-like ER Kinase (PERK), Inositol-REquiring protein-1α (IRE-1α) and Activating Transcription Factor 6 (ATF6). Under basal conditions, these three proteins are sequestrated by the Glucose-Regulated Protein of 78kDa (GRP78), also known as Binding Immunoglobulin Protein (BIP). Consecutive to ER stress, BIP goes to the lumen to participate in protein folding, thereby releasing PERK, IRE-1α and ATF6, and activating their respective signaling pathways [9,10] promoting the expression of protein chaperones and foldases or anti-oxidant defenses. Sustained activation of the UPR drives apoptosis signaling mediated by the CCAAT/enhancer-binding protein HOmologous Protein (CHOP) and caspase-12. In the liver, transcription factors activated in response to ER stress (*e.g.* SREBP1 and CREBH) modulate lipogenic and glycogenic pathways [11,12]. In addition, phosphorylation of IRE-1α may, in turn, activate c-Jun N-terminal Kinase that will interfere with insulin signaling by phosphorylating Insulin Receptor Substrate 1 IRS1 [13]. However, whether it plays a role in skeletal muscle insulin resistance and glucose intolerance is still debated. While most of the studies detect UPR activation in muscle from obese animals or fatty acids-treated myocytes [14,10,15-17], inhibition of ER stress by the use of chemical chaperones does not block palmitate-induced insulin resistance in cultured muscle cells [15,16]. In humans, although skeletal muscle UPR is increased in obesity and T2D [17], it remains unknown whether differential activation of this cellular pathway occurs between glucose tolerant and intolerant non-diabetic patients. Moreover, weight loss obtained by nutritional or surgery strategies in patients with obesity changes UPR signaling in liver and adipose tissue [18,19], but its consequences on muscle UPR have not been evaluated.

Here, we aim (i) to evaluate whether UPR-related genes are differentially expressed in human muscle from normoglycemic *vs.* non-diabetic glucose intolerant patients with obesity, (ii) to determine whether UPR-related gene expression correlates with glucose homeostasis parameters (iii) to find out whether UPR expression is modified by bariatric surgery, which induces weight loss and ameliorates insulin sensitivity.

Methods

Experimental subjects

Biological data and biopsies from patients obesity (Body Mass Index BMI>35) were obtained from the ABOS (Atlas Biologique de l'Obésité Sévère) cohort (NCT01129297). 22 obese normoglycemic and 22 obese glucose intolerant but non-diabetic, defined by a normal or mildly raised Fasting Blood Glucose (FBG) and a 2-hr glucose level after a 75g oral glucose tolerance test (G120) comprised between 140 and 199 mg/dL (7.8 and

11.1mmol/L), subjects were randomly selected, biological parameters were extracted from the database and transverse abdominal muscle biopsies were obtained. Patients were subjected to an oral glucose tolerance test (75g); blood glucose and insulin levels were measured before and 30 and 120 min after glucose ingestion. In a second set of experiments, gastrocnemius needle biopsies from 7 normoglycemic and 7 glucose intolerant patients with obesity were obtained before and after bariatric surgery (Roux-en-Y Gastric Bypass).

Matsuda index

Mastuda index [20], which estimates hepatic and muscle insulin sensitivity, was calculated as follows: $\frac{10{,}000}{\sqrt{G0*I0*Gmean*Imean}}$ where G0 and I0 indicate FBG and insulin levels (mg/dL and mIU/L), respectively, and Gmean and Imean designate mean blood glucose and insulin concentrations from 0 to 120 min during the Oral Glucose Tolerance Test, respectively.

Laboratory measurements

Blood glucose and insulin levels were measured as reported in the literature [21].

Gene expression

RNA was extracted from abdominal and gastrocnemius muscles using the Trizol (Invitrogen)/Chloroform/Isopropanol protocol. After DNase treatment, the High-Capacity cDNA Reverse Transcription Kit (Thermofischer Scientific, Montigny Le Bretonneux, France) was used to obtain cDNA. Quantitative PCRs were performed with the SYBR® Green Real-Time PCR Master Mix kit (Agilent Technologies, Les Ulis, France) on a MX3005 apparatus (Agilent Technologies). Human specific primers are shown in supplementary table 1. Gene expression was normalized to cyclophilin A (*PPIA*).

Protein expression

Using a Polytron tissue homogenizer, 40-50 mg of abdominal muscle were lyzed in RIPA buffer (Merck-Sigma Aldrich, St. Quentin Fallavier, France) containing anti-protease and anti-phosphatase cocktails (Merck-Sigma Aldrich). Once centrifuged at 15 000*g* at 4°C for 15 min, proteins in the supernatant were quantified according to the Bradford method. Total proteins (30µg) were separated on a 10% SDS-polyacrylamide gel and then transferred onto a PVDF membrane. After blocking with a 5% BSA-TBS solution, primary antibodies - purified mouse anti-BIP/GRP78 #610979 (BD Biosciences, Le Pont de Claix, France) and rabbit monoclonal anti-GAPDH #5174 (Cell Signaling Technology, Leiden, The Netherlands)- were hybridized overnight at 4°C. After incubation with specific secondary antibodies coupled with IRDyes (LI-COR Biosciences GmbH, Bad Homburg, Germany), membranes were scanned and analyzed using the Odyssey CLX and Image Studio software (LI-COR Biosciences GmbH). Results were normalized to GAPDH.

Statistical analysis

According to the Shapiro-Wilk normality test results, comparisons between NG and GI patients were performed using a two-tailed Student t-test for Gaussian distribution or a Mann-Whitney U-test otherwise. Frequency distribution was tested by Chi-squared test. Pearson correlations were generated to determine a linear link between two variables. Parametric paired t-test or non-parametric Wilcoxon signed-rank were used to analyze data obtained before and after bariatric surgery. Statistics were performed by the use of GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Results

ER stress markers are differentially expressed according to the glucose tolerance status in skeletal muscle from non-diabetic patients with obesity

The study was designed to obtain two subgroups of non-diabetic patients with a BMI>30: one with normal glucose tolerance (NG), the other with glucose intolerance (GI). As depicted in supplementary table 2, while there was no significant differences in sex ratio, age, BMI and Hb1Ac, FBG and G120 were significantly higher in GI compared to NG patients. Consistently, the Matsuda index was markedly lower in GI patients (supplementary table 2), indicating alterations in peripheral insulin sensitivity.

The UPR has been associated with metabolic alterations in liver or adipose tissue but its involvement in skeletal muscle glucose handling remains unclear. Therefore, we compared the expression of UPR-related genes *BIP*, *ATF4*, *ATF6*, unspliced *X-box Binding Protein 1 XBP1U*, spliced *XBP1S* and *CHOP* as well as BIP protein levels between NG and GI obese patients. While BIP protein level was significantly higher in the GI group (Fig. 1a, 1b), its transcript was less expressed in GI patients compared to NG individuals (Fig. 1c). *ATF4*, which depends on the PERK axis, was not different between NG and GI patients (Fig. 1d). *XBP1U*, but not spliced *XBP1 (XBP1S)*, was lower in the GI group (Fig. 1e, 1f). *ATF6* expression was significantly reduced in GI patients (Fig. 1g). Similarly, a decrease in *CHOP* expression was observed in GI patients (Fig. 1h), although the difference did not reach statistical significance.

Metabolic parameters correlate with UPR-related genes

Pearson's linear analysis was performed to determine whether differences in gene expression are associated with metabolic features (Table 1). Neither BIP, nor PERK-dependent ATF4, nor XBP1U mRNA correlated with BMI, weight, age, FBG, HbA1c, G120 and Matsuda Index. HbA1c level negatively correlated with XBP1S mRNA levels. FBG correlated positively with BIP protein expression. Interestingly, and consistent with the above data linking CHOP and ATF6 with the glucose tolerance status, HbA1c level was negatively correlated

with ATF6 mRNA levels, and CHOP and ATF6 were both significantly and negatively correlated with G120 and the Matsuda index.

Effects of bariatric surgery on skeletal muscle UPR

In addition to weight loss, bariatric surgery improves, almost immediately, glucose homeostasis [22,23]. Therefore, we tested the impact of bariatric surgery on skeletal muscle UPR in non-diabetic obese NG *vs.* GI patients. No differences were observed between the NG and GI individuals for weight, BMI and HbA1c before surgical intervention, whereas FBG, G120 and the Matsuda index were significantly different between the NG and GI (Table 2). One year after bariatric surgery, weight, BMI and HbA1c were reduced in both the NG and GI groups (Table 2). The surgical procedure improved FBG, G120 and the Matsuda index and abolished the differences in glucose homeostasis between NG and GI patients (Table 2).

We then explored whether bariatric surgery modified skeletal muscle UPR. BIP protein significantly increased after bariatric surgery in both groups (Fig. 2a, 2b). While no differences in *BIP*, *ATF4*, *XBP1U* and *XBP1S* were detected (Fig. 2c-2f), bariatric surgery in GI patients tended to raise *ATF6* (Fig. 2g) and induced a significant increase in *CHOP* mRNA expression (Fig. 2h).

Discussion

We first aimed to determine whether UPR signaling is differentially expressed in skeletal muscle from non-diabetic glucose tolerant *vs.* intolerant obese patients and whether it correlates with metabolic parameters. The most striking result was that *CHOP* and *ATF6* expression, which is reduced in GI patients, was correlated with G120 as well as the Matsuda index. Bariatric surgery, which greatly improved glucose homeostasis and peripheral insulin sensitivity in GI patients, increased *CHOP* and *ATF6* expression.

We specifically studied the UPR in obese patients without T2D to avoid interferences with medical treatment or duration, evolution and severity of T2D. To prevent any confounding effects of weight on the measured parameters, we compared two obese populations with normal and abnormal glucose tolerance rather than matching our data with lean NG individuals. Therefore, the major criteria differentiating our two populations is the glucose tolerance status evaluated by G120, hence the Matsuda index. This setting was also chosen to determine whether changes in UPR could occur before the occurrence of diabetes.

Data from the literature indicate that skeletal muscle of obese and diabetic patients expressed more BIP and CHOP proteins, while the IRE-1 pathway is not activated [17]. Our findings also show that BIP protein is more expressed in GI patients and that IRE1 activation, observed through XBP1 splicing, is unlikely to contribute to insulin resistance as spliced *XBP1* is not different between NG and GI subjects. Nevertheless, conflicting data

have been reported in obese pregnant women [24]. We also found that UPR-related genes, namely *BIP*, *ATF6* and, albeit to a lower extent, *CHOP* are less expressed in GI compared to NG obese patients. This may be related to the higher amount of BIP protein since BIP buffering capacity [25] on IRE1, PERK and ATF6 may reduce their activation, thus diminishing expression of their target genes such as *BIP*, *ATF6* and *CHOP*. Moreover, higher BIP protein level may also be related to the glucose intolerance status since BIP binds to Skeletal muscle- and Kidney-enriched Inositol polyphosphate 5-Phosphatase (SKIP) to favor insulin signaling termination [26]. Thus, the increase in BIP protein expression in GI patients may be either protective to avoid UPR overactivation although, the other way around, it may contribute to the glucose intolerance phenotype. In addition, *ATF6* and *CHOP* negatively correlated with G120 and positively with the Matsuda index. While ATF6 is beneficial for muscle adaptation to exercise [27,28], its participation in muscle glucose homeostasis remains elusive. Consistently, in liver, *Atf6* deletion promotes glucose intolerance in HFD-fed mice [29] and chronic induction of Atf6 translocation and activation improves glucose homeostasis in *db*/*db* male mice [30].

Bariatric surgical procedures represent effective strategies to lose weight and improve insulin sensitivity, notably in muscle [31]. As expected, we found a profound enhancement of insulin sensitivity and glucose tolerance, as observed with the normalization of the Matsuda index and G120, in GI compared to NG patients. In their skeletal muscle tissue, *CHOP* and *ATF6*, albeit close to significance, increased in GI patients one year after RYGB but the physiological consequences remain speculative. We can hypothesize that higher *ATF6* and *CHOP* levels may contribute to the amelioration of glucose handling obtained after bariatric surgery, as explained above. Moreover, higher CHOP expression could also participate to the loss of muscle mass observed after bariatric surgery [32-34]. Indeed, CHOP is a transcriptional factor which promotes cell apoptosis [35]. In muscle, CHOP also represses MyoD transcription [36], hence delaying myogenesis.

Conclusion

CHOP and *ATF6* expression decreased in non-diabetic GI patients and was modified by bariatric surgery. These genes may contribute to glucose homeostasis in human skeletal muscle.

Conflict of Interest Disclosure

The authors declare that they have no conflict of interest.

Ethical and consent statements

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its

later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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Figure legends

Fig. 1 UPR pathways are differentially expressed between normal glucose tolerant and glucose intolerant non-diabetic obese patients in skeletal muscle. (A) Representative western-blot of BIP and GAPDH proteins and (B) their quantification, n=22, unpaired t-test, *p<0.05. mRNA levels of (C) BIP, (D) ATF4, (E) XBP1U, (F) XBP1S, (G) ATF6 and (H) CHOP normalized to PPIA. n=22, *p<0.05, **p<0.01 vs. NG. Mean +/- SEM with unpaired t-test for BIP, XBP1U, ATF6, median and interquartile range with Mann-Whitney's U test for ATF4, XBP1S, CHOP

Fig. 2 Bariatric surgery changes skeletal muscle UPR in non-obese glucose intolerant patients. (A) Representative western-blot of BIP and GAPDH proteins and (B) their quantification in normal glucose tolerant (NG) or glucose intolerant (GI) patients before and after bariatric surgery, n=7, Wilcoxon signed-rank test for NG, paired t-test for GI, *p<0.05 *vs.* pre-surgery. NG and GI samples were run on different gels. mRNA levels of (C) *BIP*, (D) *ATF4*, (E) *XBP1U*, (F) *XBP1S*, (G) *ATF6* and (H) *CHOP* normalized by *PPIA*. n=7, *p<0.05, **p<0.01 *vs.* pre-surgery, Wilcoxon signed-rank test for XBP1S, paired t-test otherwise

Table 1 Pearson's correlations between metabolic features and the UPR

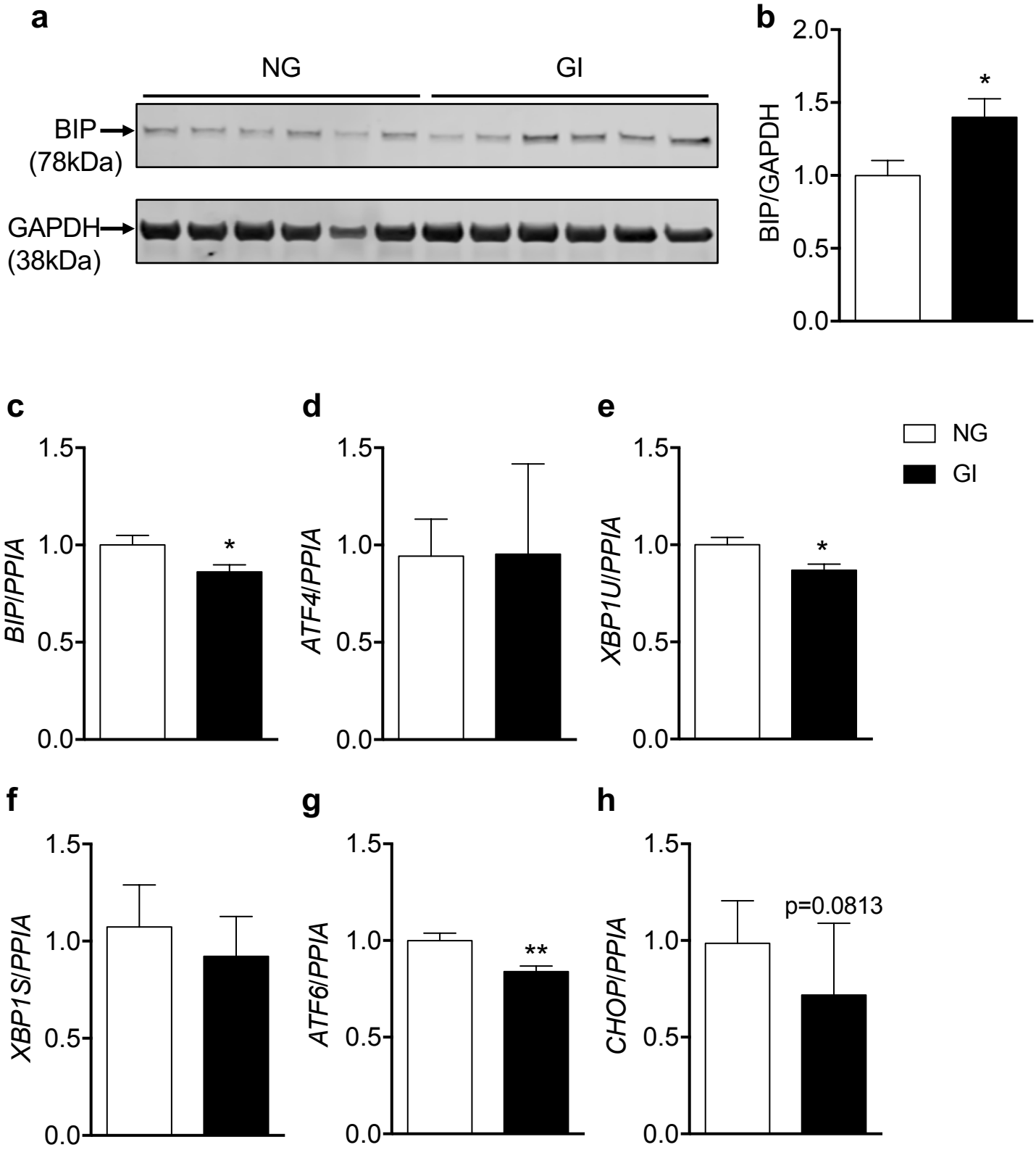
r	BMI	Weight	Age	FBG	HbA1c	G120	Matsuda	*ATF6*	*ATF4*	*CHOP*	*XBP1S*	*XBP1U*	*BIP*
BMI													
Weight	0.76***												
Age	-0.23	-0.16											
FBG	-0.17	0.02	0.28										
HbA1c	0.03	0.10	0.30*	0.34*									
G120	-0.23	-0.18	0.15	0.57***	0.31*								
Matsuda	0.03	-0.02	-0.08	-0.5***	-0.45**	-0.69***							
ATF6	0.18	0.24	-0.30*	-0.25	-0.34*	-0.38*	0.37*						
ATF4	-0.10	0.10	-0.23	0.19	-0.10	0.08	-0.16	0.41**					
CHOP	-0.17	-0.19	0.23	-0.06	-0.27	-0.41**	0.38*	0.15	0.05				
XBP1S	0.09	0.12	-0.07	0.07	-0.36*	-0.07	0.09	0.31*	0.33*	0.33*			
XBP1U	0.25	0.16	-0.07	-0.08	0.09	-0.20	-0.05	0.58***	0.41***	0.12	0.16		
BIP	0.04	0.11	-0.03	-0.03	0.15	-0.25	-0.13	0.42**	0.32*	0.04	0.05	0.66***	
BIP	-0.05	-0.04	0.36*	0.38**	0.21	0.27	-0.09	-0.19	-0.13	0.00	0.20	-0.13	-0.06

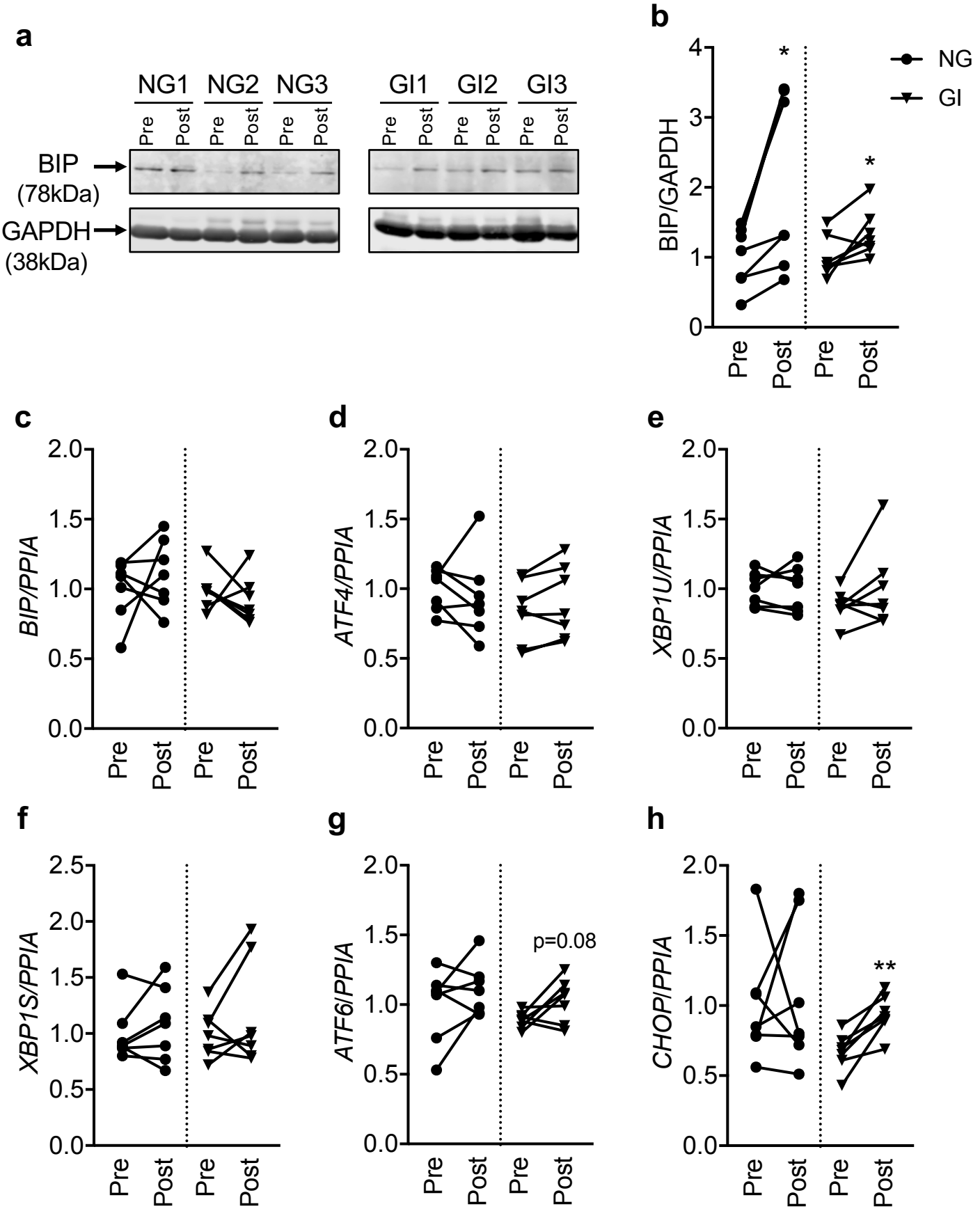
r: Pearson's correlation coefficient, BMI: Body Mass Index, FBG: Fasting Blood Glucose, G120: blood glucose concentration 120min after an oral glucose tolerance test, *BIP*: *Binding Immunoglobulin Protein*, *ATF6*: *Activating Transcription Factor 6*, *ATF4*: *Activating Transcription Factor 4*, *XBP1U*: *X-box Binding Protein 1 unspliced, XBP1S*: *X-box Binding Protein 1 spliced, CHOP*: *C/EBP HomolOgous Protein*, *p<0.05, *p<0.01, ***p<0.001

Table 2 Metabolic features of glucose tolerant and intolerant non-diabetic obese patients before and after bariatric surgery

	Before Surgery			After Surgery		
	NG	GI	P value	NG	GI	P value
Age (years)	37.1 (2.8)	45.0 (3.4)	0.098 (t test)	38.2 (2.9)	46 (3.4)	0.102 (t test)
Sex (male/female)	0/7	0/7	1 (χ^2 test)			
Weight (kg)	127.6 (5.5)	125.8 (6.2)	0.833 (t test)	94.4 (5.1)	81.6 (4.3)	0.0782 (t test)
BMI (kg/m^2)	43.1 [42.5-46.4]	45.3 [42.1-50.7]	0.646 (U test)	33.2 (1.7)	29.9 (1.8)	0.149 (t test)
HbA1c (%) (mmol/mol)	5.3 (0.1) 34 (0.9)	5.6 (0.2) 38 (1.8)	0.1116 (t test)	5.0 (0.1) 31 (0.9)	4.8 (0.1) 29 (0.9)	0.463 (t test)
FBG (mmol/L)	4.9 (0.04)	5.4 (0.1)	0.0011 (t test)	4.6 (0.1)	4.5 (0.2)	0.492 (t test)
G120 (mmol/L)	5.1 [5-6.4]	8.0 [7.8-8.1]	0.0006 (U test)	4.6 (0.7)	3.6 (0.4)	0.2217 (t test)
Matsuda index	7.3 (1.7)	2.1 (0.4)	0.0095 (t test)	15.0 (3.9)	31.5 (6.4)	0.0589 (t test)

BMI: Body Mass Index, FBG: Fasting Blood Glucose, G120: blood glucose concentration 120min after an oral glucose tolerance test, NG: Normal Glucose tolerance, GI: Glucose Intolerance. Data are Mean (SEM) or Median [25% percentile-75% percentile]. P values are provided along with the statistical test (t test: Student's t test, U test: Mann-Whitney's U test, χ^2 test: chi-squared test)


a
NG
GI
BIP
(78kDa)
GAPDH
(38kDa)
b
BIP/GAPDH
2.0
1.5
1.0
0.5
0.0
*
c
BIP/PPIA
d
ATF4/PPIA
e
XBP1U/PPIA
NG
GI
f
XBP1S/PPIA
g
ATF6/PPIA
**
h
CHOP/PPIA
p=0.0813


a
NG1
NG2
NG3
GI1
GI2
GI3
Pre
Post
BIP
(78kDa)
GAPDH
(38kDa)
b
NG
GI
BIP/GAPDH
*
*
c
BIP/PPIA
d
ATF4/PPIA
e
XBP1U/PPIA
f
XBP1S/PPIA
g
ATF6/PPIA
p=0.08
h
CHOP/PPIA
**

Supplementary Table 1 Human RTqPCR primers

target	Accession number	forward 5'-3'	reverse 3'-5'
BIP	NM_005347	TAGCGTATGGTGCTGCTGTC	TTTGTCAGGGGTCTTTCACC
ATF6	NM_007348	CAATTGGAAGCAGCAAATGA	ACCGAGGAGACGAGACTGAA
ATF4	NM_001675	TCAAACCTCATGGGTTCTCC	GTGTCATCCAACGTGGTCAG
XBP1U	NM_ 005080	GGAGTTAAGACAGCGCTTGGGGA	TGTTCTGGAGGGGTGACAACTGGG
XBP1S	NM_001079539	CTGAGTCCGCAGCAGGTG	GCTGATGACGTCCCCACTGA
CHOP	NM_001195053	GAACCAGGAAACGGAAACAGA	TCTCCTTCATGCGCTGCTT
PPIA	NM_021130	GCATACGGGTCCTGGCATCTTGTCC	ATGGTGATCTTCTTGCTGGTCTTGC

BIP: *Binding Immunoglobulin Protein*, *ATF6*: *Activating Transcription Factor 6*, *ATF4*: *Activating Transcription Factor 4*, *XBP1U*: *X-box Binding Protein 1 unspliced, XBP1S*: *X-box Binding Protein 1 spliced, CHOP*: *C/EBP HomolOgous Protein, PPIA*: *cyclophilin A*

Supplementary Table 2 Metabolic features in the first cohort of glucose tolerant and intolerant non-diabetic obese patients

	Glucose tolerant	Glucose intolerant	P value (test)
Age (years)	35.6 (2.4)	38.6 (2.5)	0.3883 (t test)
Sex (male/female)	3/19	4/18	1.0 (χ^2 test)
Weight (kg)	131.7 (5.9)	123.9 (3.2)	0.2531 (t test)
BMI (kg/m^2)	46.9 (1.4)	44.0 (0.9)	0.0776 (t test)
HbA1c (%) (mmol/mol)	5.6 (0.1) 38 (0.9)	5.7 (0.1) 39 (0.9)	0.236 (t test)
FBG (mmol/L)	4.8 (0.1)	5.3 (0.1)	<0.0001 (t test)
G120 (mmol/L)	5.3 [4.7-6.9]	8.2 [8.0-8.8]	<0.0001 (U test)
Matsuda	7.1 [3.1-10.4]	2.2 [1.4-3.3]	<0.0001 (U test)

BMI: Body Mass Index, FBG: Fasting Blood Glucose, G120: 2-hr glucose level after a 75g oral glucose tolerance test. Data are Mean (SEM) for normal distribution or Median [25% percentile-75% percentile] otherwise. P values are provided along with the test used (t test: Student's t test, U test: Mann-Whitney's U test, χ^2 test: chi-squared test). n=22 in each group