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Endoplasmic Reticulum Stress Markers Are Associated With Obesity In Non-Diabetic Subjects †

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Abstract:

Objective: Adipocyte and hepatocyte endoplasmic reticulum (ER) stress response is activated in dietary and genetic models of obesity in mice. We hypothesized that ER stress was also activated and associated with reduced insulin sensitivity in human obesity.

Research Design and Methods: We recruited 78 healthy, nondiabetic individuals over a spectrum of body mass index (BMI) who underwent oral and intravenous glucose tolerance tests and fasting subcutaneous adipose and muscle biopsies. We tested expression of 18 genes and levels of total and phosphorylated eIF2α, c-jun and JNK1 in subcutaneous adipose tissue. We compared gene expression in stromal vascular and adipocyte fractions in paired samples from 22 individuals, and tested clustering on gene and protein markers.

Results: Adipocyte expression of most markers of ER stress, including chaperones downstream of *ATF6*, were significantly correlated with BMI and percent fat ($r>0.5$; $p<0.00001$). Phosphorylation of initiation factor EIF2α but not of JNK1 or c-jun was increased with obesity. ERSR was also increased with obesity in a second set of 86 individuals, and in the combined sample (n=161). The increase was only partially attributable to the stromal vascular fraction and macrophage infiltration. ER stress markers were only modestly correlated with insulin sensitivity. Clustering algorithms supported ER stress activation with high BMI but not low insulin sensitivity.

Conclusions: Multiple markers of ER stress are activated in human adipose with obesity, particularly for protective chaperones downstream of transcription factor ATF6α.

Introduction:

Despite the strong epidemiological linkage between obesity and type 2 diabetes, the pathophysiological mechanisms of that linkage remain uncertain. Considerable data suggest that obesity is a state of chronic inflammation, and that this inflammation in turn results in reduced insulin sensitivity (1), and recent genetic analyses point to inflammatory and immune pathways in insulin resistance, glucose intolerance, and metabolic syndrome (2;3). Whether inflammation is the proximal cause of diabetes or the bystander of another process such as oxidative stress (4) is unknown.

Recently, two studies suggested that increased endoplasmic reticulum stress may represent the proximal cause of the association between obesity, hepatic and adipocyte insulin resistance, and type 2 diabetes (5;6). The endoplasmic reticulum (ER) is contiguous with the nuclear membrane and is the location for synthesis and folding of membrane and secretory proteins. Conditions that impair protein folding, nutritional deficiency such as hypoglycemia, altered ER homeostasis such as induced by chemicals thapsigargin (altered calcium homeostasis) or tunicamycin (blocked glycosylation) all result in ER stress response and the elaborate unfolded protein response (UPR) (7). More recently fatty acids were also shown to induce ER stress in some cell lines (8;9).

The ER stress response is mediated by three proximal transmembrane proteins that in the absence of unfolded proteins or ER stress are held inactive by the chaperone heat shock protein A5 (HSPA5, also known as GRP78 or BiP). ER stress activates pancreatic eIF2α kinase (PERK, encoded by *EIF2AK3*), which by phosphorylating eukaryotic initiation factor EIF2α blocks most

translation but favors translation of pro-apoptotic factor ATF4 and transcription of pro-apoptotic factors CHOP and ATF3. Activation of inositol – requiring enzyme 1 (IRE1; encoded by human gene *ERN1*) acts as an endonuclease to splice transcription factor x-box binding protein 1 (XPB1), which in turn activates downstream chaperones. Finally, transcription factor ATF6α is transported to the golgi, where it is further processed by two site specific proteases (SP1 and SP2) to release the cytosolic fragment, which is a transcriptional coactivator that in turn increases transcription of downstream chaperones, including HSPA5 (10-12). Recent studies suggest that ATF6α activity is critical to upregulation of protective chaperone proteins including *HSPA5, HYOU1, CALR, DNAJC3 (*$p58^{IPK}$), and *HSP90B1* (GRP94) (13;14), as well as increased ER associated protein degradation (ERAD; encoded in part by gene *EDEM1*) (13). When up-regulation of protective chaperones in not successful, apoptotic pathways predominate (11;12).

Ozcan et al. (5) demonstrated increased markers of ER stress in both liver and adipose tissue from mice with either genetic (*ob/ob*) or dietary (high fat diet induced) models of obesity, including increased phosphorylation of PERK and eIF2α, increased transcript levels of HSPA5 (GRP78), and increased c-Jun N terminal kinase (JNK) activity (c-jun phosphorylation). XBP1heterozygous null mice were both insulin resistant and glucose intolerant (5). Nakatani and colleagues similarly showed increased ER stress markers in the livers of diabetic *db/db* mice (6). Conversely, over-expression of chaperone proteins such as HYOU1 (ORP150) (6) or use of small molecule chaperones (15) appear to reduce ER stress, improve insulin sensitivity and secretion, and improve diabetes control.

Despite convincing data supporting a role for ER stress response in the pathogenesis of diabetes in obese mice and in cell culture, the role of this pathway in human tissues unclear. Two other recently published studies support an increase in ER stress response in human obesity

(16;17). We tested the hypothesis that ER stress is activated in human subcutaneous adipose tissue with obesity independent of hyperglycemia, and in turn reduces whole body insulin action. We report on expression of both proximal sensors and downstream chaperones in subcutaneous adipose and muscle from individuals over a wide range of body mass index (BMI) and insulin sensitivity.

Materials and Methods:

Experimental Subjects:

The primary study was conducted in 78 individuals who were recruited by advertisement for good general health, age between 18 and 55 years, BMI between 19 kg/m^2 and 42.5 kg/m^2, and either self determined European American or African American ancestry. All participants had a screening visit, at which time they had measurement of height, weight, waist and hip measures, body fat determination by dual x-ray absortiometry (DXA) scan, fasting blood samples for lipid measures, and a standard 75 g oral glucose tolerance test with measurement of glucose and insulin at baseline and 30 min intervals for 2 hours. Participants with diabetic glucose tolerance tests were not enrolled, but 10 subjects had impaired glucose tolerance. Subjects returned for a second visit, generally within 2 months. Premenopausal women were studied in the follicular phase of the menstrual cycle. After initiating intravenous lines in both arms, adipose and muscle biopsies were performed using a Bergstrom needle under local (lidocaine) anesthesia. Biopsy samples were immediately rinsed in normal saline, cut, and quick frozen in liquid nitrogen. Following the biopsy an insulin-modified (0.04 U/kg), frequently sampled intravenous glucose tolerance test (FSIGT) was performed as described previously (18). The initial 62 participants had fasting insulin obtained before (2 baseline) and after (2 additional baseline samples) the biopsies to exclude altered insulin sensitivity induced by the biopsy. Pre- and post-biopsy insulin levels did not differ significantly (data not shown).

Adipose tissue cDNA was available for a second sample of 86 individuals who participated in several studies using a similar protocol, as described in detail elsewhere (19;20). FSIGT studies were available on 83 African-American or European American subjects. Table 1 shows the characteristics of the primary (Sample 1) and replication (Sample 2) groups. Of the 80

subjects included in the analysis, 34 had impaired glucose tolerance.

All study participants provided written, informed consent under protocols approved by the University of Arkansas for Medical Sciences or Central Arkansas Veterans Healthcare System Institutional Review Boards and the Central Arkansas Veterans Affairs Research and Development Committee.

Laboratory Measurements:

Insulin levels were measured using an immuno-chemiluminometric assay (MLT Assay, Wales, UK) and plasma glucose by a glucose oxidase assay. Standard clinical assays (lipids, glucose) were performed at LabCorp, Inc (LabCorp, Inc, Burlington, North Carolina).

Gene Expression:

Total RNA was isolated from adipose using the RNAeasy Lipid Tissue Mini kit (Qiagen Inc-USA, Valencia, CA), and from muscle using the Ultraspec RNA kit (Biotecx Laboratories, Inc, Houston, TX). The adipocyte fraction from 22 samples was separated from the stromal vascular fraction following collagenase digestion using the method of Rodbell (21). The 22 individuals had similar BMI and insulin sensitivity to the full sample (data not shown). Total RNA was isolated from the adipocyte fraction using the RNeasy lipid tissue minikit (Qiagen), and from the stromal-vascular fraction using the RNAqueous kit (Ambion, Inc, Austin, TX).

The quantity and quality of the isolated RNA were determined by ultraviolet spectrophotometry and electrophoresis using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), respectively, and 1 µg was reverse transcribed using random hexamer primers with TaqMan Reverse transcription reagents (Applied Biosystems, Foster City, CA). All RNA samples from a single study population and tissue were reverse transcribed with the same kit on the same day (see Supplemental Data, Table 1S for details). The standard curves were generated

using pooled RNA from the samples assayed. Primers were designed to capture most known splice variants and such that the amplicon spanned an intron, or one primer spanned two contiguous exons (Supplemental Data, Table 1S). The correlation between replicate runs (Sample 1) was >0.98 for *HSPA5*, *STC2*, and 18S RNA ($p<10^{-50}$).

Quantitative Western Blots:

Protein was isolated from 100-200 mg of subcutaneous adipose by lysis with a tissue homogenizer, sonication, and centrifugation (see Supplemental Data for detailed methods). Protein content was estimated by the Bradford method (22). Equal amounts (40-50 µg) of total protein were separated on 8% SDS-polyacrylamide gels, and transferred onto Trans-Blot nitrocellulose membrane (BioRad, Laboratories, Hercules, CA), and hybridized with the primary and secondary antibodies as described in Supplemental Data. Blots were scanned with the ChemiDoc XRS Image Analysis System (BioRad Laboratories Inc), and quantified by densitometry with Quantity One v 4.6.3 Image Analysis software (BioRad Laboratories). To facilitate comparison across multiple gels, approximately equal numbers of lean and obese individuals were loaded on each gel, including some samples in common between gels, and protein levels were normalized to β-actin.

Statistical Analysis:

S_I was estimated from the insulin and glucose data using the MinMod Millenium program (23), and acute insulin response to glucose (AIR_g) and disposition index (DI) were taken from the program output. Insulin sensitivity from the oral glucose tolerance test was calculated using the homeostatic index (24). Gene expression levels were normalized to 18-S RNA, and the ratio was used in all calculations. Data were assessed for normality separately within each study sample and within the combined data set, and non-normal variables natural

logarithmically transformed to normality. Partial correlation measures of obesity or insulin sensitivity with gene expression were calculated controlling for age, gender, and ethnicity. General linear regression models were examined with age, gender, ethnicity, and study identity in combined data sets as covariates. Obesity measures were evaluated as covariates in models with gene expression levels as the dependent variables. Insulin sensitivity (S_I) was assessed as the dependent variable with gene expression as a covariate to test the effects of ER stress on S_I, with BMI as an additional covariate to control for obesity effects on S_I. No interactions of study identifier with gene expression levels or measures of obesity were found. Protein levels were compared across gels after taking the protein or phospho-protein to β-actin ratio and using both the 2-tailed Student t-test, and to avoid problems with non-normal data, the Mann Whitney U test. Results were similar; we report only the Mann Whitney U results. Analyses were performed in SPSS for Windows v 12.0. Values are reported as means with standard deviations for normal data, or geometric means and 95% confidence intervals for data not normally distributed.

We performed the unsupervised hierarchical clustering for gene expression and phospho-protein levels using PermutMatrix v1.9.3 software (25). Euclidian distance matrix between each individual was generated using log normalized (LN) values and seriation under multiple fragment heuristics (MF). The hierarchical clustering based tree was generated using McQuitty's criteria (26).

Results:

Endoplasmic Reticulum Stress Markers Correlate with Total Adiposity

Characteristics of the study population are shown in Table 1. Among the primary study (Sample 1) of 78 individuals (28 men, 50 women; BMI 19 kg/m^2 - 42.5 kg/m^2) recruited specifically for this study, key proximal ER stress markers *HSPA5* (encoding GRP78)*, ATF6α,* and *EIF2AK3* (encoding PERK), and downstream chaperones and response genes *DNAJC3*, *HYOU1*, *HSP90B1*, *CALR*, *STC2*, *EDEM1*, and *NRF2* were strongly correlated with both BMI and DEXA-determined percent body fat (r>0.45; Table 2). Transcripts downstream of ATF4 (indirectly activated by PERK phosphorylation of EIF2α), including markers of apoptosis, were more variably associated with obesity measures. Thus, transcription factor *ATF3* (r>0.4) was strongly correlated with obesity measures, factors *ATF4, TRAF2*, and *GADD34* modestly correlated (r>0.3), and primary *CHOP* showed no activation with increasing obesity. Neither *ERN1* (encoding proximal sensor IRE1) nor total *XBP1* transcript levels were correlated with obesity measures. Selected regression lines predicting average expression for *HSPA5*, *HYOU1*, *DNAJC3*, and *TRAF2* given BMI by ethnicity are shown in Figure 1. Gender and measures of obesity (BMI, waist circumference, percent fat) were significant determinants of gene expression for most markers in general linear regression models. Ethnicity was not significant as a main effect, but ethnicity and measures of total obesity showed a significant interaction to determine gene expression levels (*HSPA5*, ethnicity vs BMI, p=0.01; Figure 1). The relationship of BMI and gene expression was reduced (lower slope) in African American compared with European American participants (Figure 1). Furthermore, measures of fat distribution (waist:hip ratio, trunk:leg fat from DXA) were less correlated (r<0.3) than obesity measures with transcriptional markers of ERSR (Table 2).

We stratified the population into lean (BMI<25 kg/m^2), overweight (BMI 25 kg/m^2 to 30 kg/m^2), and obese (BMI >30 kg/m^2). As shown in Figure 2, proximal sensors *ATF6*, *EIF2AK3* (PERK), and *HSPA5*, and factors downstream of *ATF6* (*EDEM1*; chaperone proteins *HYOU1*, *HSPA5*, *DNAJC3*, *HSP90B1, CALR, STC2*) showed increased gene expression by over 60% from lean to obese individuals.

Effects on insulin sensitivity are proposed to result from kinase action of IRE1 (encoded by *ERN1*) on JNK1. Similarly, PERK (encoded by *EIF2AK3*) is activated by phosphorylation, and in turn phosphorylates EIF2α to halt translation. We examined total and phosphorylated JNK1, c-jun, and EIF2α in the 15 leanest (lowest BMI) and 19 most obese (highest BMI) individuals (Figure 3). Initiation factor EIF2α (Figures 3a, 3c; p=3x10^{-7}) but not JNK1 or c-jun (Figure 3b) showed increased phosphorylated in the high BMI group. The increased EIF2α Phosphorylation was less significant (p<0.05) when normalized to total EIF2α rather than β-actin.

Activation of IRE1 in the acute state results in activation of transcription factor XBP1 by splicing of a 24 bp intron. No splicing was observed in lean or obese subjects using gel electrophoresis standard methods (Supplemental Data, Figure 2S), but spliced XBP1 transcript increased by 47% from the lean to obese subjects using a real time PCR assay (p=0.0002; Figure 2).

ER Stress and Obesity: Replication Study

We examined genes *HSPA5, EDEM1, DNAJC3, HYOU1,* and *TRAF2* in a second group of 86 individuals ascertained originally for other studies (Table 1), again categorizing individuals as lean (n=13, BMI<25 kg/m^2), overweight (n=16, BMI 25 – 30 kg/m^2), or obese (n=57, BMI>30 kg/m^2). As in Sample 1,*HSPA5* expression was significantly increased with obesity

(means and 95% confidence intervals 0.73 [0.63, 0.85], 0.84 [0.74, 0.95], and 1.07 [1.00, 1.16] for lean, overweight, and obese, respectively; $p<0.00001$). Chaperones *DNAJC3*, *HYOU1*, and *TRAF2* were also significantly increased in obese individuals ($p<0.05$), whereas *EDEM1* did not reach significance. Partial correlations with obesity measures are shown in Table 3S, and results were confirmed in linear regression analyses with BMI (data not shown). When Samples 1 and 2 were combined (n=161), the increase of ERSR transcripts with obesity class was highly significant, and increased from lean to obese classes by 31% for *TRAF2* ($p<0.0005$) to 53% for *HSPA5* ($p<1x10^{-11}$) (Table 2S).

Endoplasmic Reticulum Stress and Muscle Gene Expression

Muscle gene ERSR gene did not correlate with obesity measures (BMI, percent fat, or waist circumference; $p>0.35$) for genes *HSPA5, EDEM, DNAJC3,* and *TRAF2* examined in 77 individuals (Sample 1) for whom paired adipose and muscle biopsies were available. In both subcutaneous adipose and in muscle, expression of chaperone genes downstream of the ER stress response were mutually highly correlated ($r>0.60$ in muscle; $r>0.9$ for factors downstream of *ATF6α* in adipose), suggesting that all chaperones were similarly increased in adipose in response to obesity. In contrast, expression of ER stress response genes in adipose tissue correlated poorly with the same gene expression levels in muscle with the exception of *HSPA5*, for which the correlation in expression was nominally significance ($r=0.25$; $p=0.03$). Interestingly, muscle ER stress response gene expression tended to be higher in African Americans, reaching significance for *HSPA5* (HSPA5/18 S ratio 0.91, CI 0.83 – 1.0 in European Americans; 1.11, CI 0.97 – 1.28 in African Americans; $p=0.02$).

ER stress and Insulin Sensitivity

If the increased adipocyte ER stress in obese individuals contributes to insulin resistance,

we hypothesized that transcriptional markers would predict whole body insulin sensitivity (S_I). In Sample 1 (Table 2, Table 3), the strongest correlations with S_I were for *ATF6α* (r= -0.29; p=0.01), *GADD34* (r=-0.37, p=0.001), and *DNAJC3* (r=-0.23, p=0.05). Stronger correlations were observed in Sample 2 for HSPA5 (r=-0.43, p =0.00006), and in the combined sample the correlation was highly significant (p=0.00007; r=-0.31; Table 3). Partial regression or general linear models are standard approaches to determine which of several correlated variables is most probably driving an association. In a regression model which included *HSPA5* expression, BMI, age, gender, and protocol, BMI remained highly predictive ($p< 1x10^{-8}$), gender was nominally significant (p=0.02), but *HSPA5* expression failed to reach significance as a predictor of S_I. In a further attempt to control for the strong correlations of ER stress transcripts and obesity measures, and the known negative correlation of obesity and S_I, we calculated the residual for S_I controlling for BMI, age, gender, ethnicity, and protocol. Again, no individual marker of ER stress (*HSPA5, DNAJC3, EDEM1, GADD34,* or *TRAF2*) was significantly correlated with residual S_I ($r^2<0.01$, p>0.18). Surrogate measures of insulin sensitivity from the oral glucose tolerance visit were highly correlated with S_I, and like S_I were not significantly correlated with ER stress response (data not shown).

ERSR in Stromal Vascular vs Adipocyte Fraction

To exclude macrophage infiltration as the explanation for the findings of increased ERSR with obesity, we examined markers *ATF6α, HSPA5*, and macrophage marker *CD68* in paired adipocyte and stromal vascular fractions from 22 individuals (BMI 21.6 kg/m^2 to 39.9 kg/m^2). Mean BMI and S_I of these individuals was similar to the full study population (data not shown). In contrast to *CD68* expression, which showed 7.5 fold enrichment in the stromal vascular fraction (mean 0.16 vs 1.24, $p=3x10^{-13}$), *HSPA5* was only 1.4-fold enriched in the stromal

vascular fraction (1.30 vs 1.82, p=0.01) and *ATF6α* was not different between fractions (1.43 vs 1.27, p=0.3) (Figure 3S). We reexamined the correlation of ER stress transcripts with BMI and percent body fat, controlling for *CD68* gene expression by partial regression to further remove the macrophage contribution (Supplemental Data, Table 4S). Correlations with percent fat remained significant for *HSPA5, EDEM1, DNAJC3, HSP90B1, ATF6, PERK, HYOU1*, and *STC2*, ranging from r= 0.31 (*EDEM1*, p=0.009) to 0.54 (*STC2*, $p=1x10^{-6}$). In contrast, significant correlations were lost for pro-apoptotic makers *ATF4*, *ATF3*, *GADD34*, and *CHOP*.

Hierarchical Clustering Analysis of ER Stress Markers

The phospho-protein and transcript levels measured in this study represent the integrated downstream response of the three proximal sensors, and different pathways may be activated in response to different levels or substances inducing ER stress. To exclude the possibility that a single gene or phospho-protein analysis masked individual variation, we used the program PermutMatrix to examine clustering in the 34 individuals with low and high BMI tested for protein levels. As shown in Figure 4, ER stress markers clustered into two clear groups which clearly predicted BMI with only two exceptions. BMI between groups predicted by unbiased clustering was highly significantly different (p=0.00001), whereas S_I did not differ significantly between groups with low and high levels of ER stress markers (p=0.15).;

Discussion:

Induction of obesity activated the ER stress response in both liver and adipose tissue, but not in muscle from mice (5). Furthermore, in mice inactivation of XBP1(5) or reduced expression of HYOU1 (ORP150) (6) in the liver resulted in insulin resistance and glucose intolerance, whereas hepatic or systemic over-expression of HYOU1 (6;27), or XBP1 over-expression in mouse embryo fibroblast cells (5) improved insulin sensitivity, as did chemical chaperones (15). Hence, the implication of ER stress as a mediator of obesity-induced insulin resistance and glucose intolerance in humans would have clear therapeutic implications.

Marchetti et al (28) reported induction of ER stress in human β- cells from diabetic subjects with hyperglycemia. Two other studies have addressed the role of ER stress response in human adipose. We recently reported in a limited sample that marker HSPA5 was increased in obese individuals who correspond to the replication set in the current study (16). Boden and colleagues (17) examined subcutaneous adipose from the thigh in 6 lean and 6 obese subjects, and reported an increase in UPR proteins calreticulin, protein disulfide isomerase A3, and glutathione S transferase P with obesity in a proteomic analysis. Additional analyses supported these studies by showing obesity-related increases in spliced XBP1, calnexin, and phospho-JNK1. These published studies are considerably smaller than the present study, and present a more limited picture of ER stress. Furthermore, neither study attempted to distinguish the correlations with obesity and insulin sensitivity. The increased JNK1 phosphorylation in obese subjects observed by Boden et al (17) is in contrast to a study of Bashan et al (29), which found an increase in JNK1 and p38MAPK phosphorylation with obesity in omental but not subcutaneous adipose tissue. Our larger study (Figure 3) suggests considerable individual fluctuation. Hence, we propose that these apparent discrepancies represent sampling fluctuations

resulting from a small sample size.

We thoroughly evaluated the UPR pathways downstream of *EIF2AK3*, *ATF6α*, and *ERN1* across a spectrum of BMI from lean to obese in a large sample. We confirm the findings in mice by showing increased transcription of ER chaperone genes with increasing body fat, including *HSPA5* (GRP78), which is viewed as a key monitor of ER stress and the UPR (30). Particularly strongly induced were *ATF6α* and protective chaperones that are primarily under ATF6α control (13;14), including *HYOU1* (ORP150) and *DNAJC3* ($p58^{IPK}$) (10;31), and stanniocalcin 2 (STC2), the most strongly up-regulated transcript and also thought to be protective (32). We also find evidence that the EIF2AK3 (PERK) pathway may be activated, based on significantly increased phosphorylation of eukaryotic initiation factor eIF2α in obese individuals. However, other kinases are known to phosphorylate eIF2α, and as we could not demonstrate PERK phosphorylation, we cannot be certain that this increase is related to ER stress. We also observed modest increases in transcription factor *ATF4*, which is increased when eIF2α is phosphorylated. Like Ozcan et al (5), we found no increase in ER stress with obesity in muscle.

An unexpected finding was the much stronger correlation of UPR in adipose with obesity than with whole body insulin sensitivity. Separating S_I from obesity measures is challenging given the relatively strong and consistently observed negative correlation. Nonetheless, we used several standard statistical methods, including general linear and partial regression models, which consistently failed to find an association of UPR in adipose with S_I once we accounted for the known effects of obesity (BMI). If activation of JNK1 is indeed the mechanism for ER stress induced insulin resistance (5), this pathway appeared not to be activated in human subcutaneous adipose. Finally, results of our hierarchical clustering analysis strongly predicted BMI but not S_I, again suggesting a poor prediction of insulin sensitivity by subcutaneous adipose UPR genes.

The lack of correlation of UPR with S_I might reflect tissue specificity. ER stress genes in liver or visceral (omental) adipose might have been more predictive of S_I. This latter possibility is suggested by higher levels of phospho-JNK1 in omental than subcutaneous adipose (29). Alternatively, our results may reflect differences in the species. Sorting out these possibilities is essential in determining whether reduction of ER stress human liver or adipose will improve S_I or glucose tolerance.

Obesity is a state of mild inflammation with increasing macrophage infiltration into adipose with obesity (33). Indeed, two recent papers suggested that an inflammatory and immune response gene transcription network, particularly representing macrophages, was activated in obesity and metabolic syndrome in both mouse and human adipose (2;3). Hence, a prominent ER stress activation in infiltrating macrophages might explain the increased ER stress markers in obesity. We found that the stromal vascular fraction partially contributed to but could not totally explain the UPR transcripts measured in whole biopsy samples. Similarly, macrophage marker CD68 explained part but not all of the strong correlation of ER stress transcripts with obesity. Interestingly, the weaker association of apoptotic pathways with obesity (ATF4, GADD34, ATF3) was lost when corrected for CD68, suggesting that these markers may derive primarily from infiltrating macrophages. Whether ER stress is the cause of the immune activation or the result cannot be determined from the present study or from available published data.

Recent studies in cell culture have distinguished the acute ER stress response (cells treated with thapsigargin or tunicamycin) from the response to chronic, low grade exposure (10;34;35). Chronic, low grade stress up-regulated ER chaperones such as *HSPA5* and presumably other compensatory chaperones such as *DNAJC3* ($p58^{IPK}$), *HYOU1* (ORP150), and *HSP90B1* (GRP94), along with increased transcription of proteins involved in degradation of

unfolded proteins (*EDEM1*), but did not stimulate markers of apoptosis (*CHOP*) (10;34). Furthermore, during persistent ER stress as might be expected with obesity, ERN1 signaling was rapidly attenuated along with downstream *XBP1* splicing and JNK phosphorylation (35). Recent findings suggest that *ATF6α* is essential to these protective responses (14). These cell culture findings mirror our observations from obese humans, where the most striking correlations with obesity are in chaperones that are thought to protect against ER stress induced apoptosis. Indeed, in preliminary experiments, we observe a similar pattern of ERSR transcripts when HepG2 cells were conditioned with low doses of palmitate or oleate and then challenged with doses that typically induce an acute UPR. Such findings are markedly different from the acute UPR induced by thapsigargin, tunicamycin, or palmitate. One possible interpretation of our findings is that human obesity may be a state of chronic, compensated ER stress in adipose. Perhaps those individuals who compensate adequately with high levels of protective chaperones ameliorate the metabolic consequences observed in inbred mice.

Strengths of this study include the very broad assessment of each ER stress/UPR pathway by both protein phosphorylation and gene transcription and the replication of the key transcription findings in an independent sample. The association of ER stress factors with obesity measures is statistically convincing with p values well below 0.0001. The findings were robust to multiple methods of statistical analysis, and are confirmed by cluster analysis in a subset of samples with extremes of BMI. Insulin sensitivity in both the primary and replication populations was assessed directly using the insulin-modified FSIGT in addition to surrogate measures based on fasting insulin and glucose. Nonetheless, our conclusions have some necessary limitations. As noted above, work in mice focused on hepatic ER stress (5;6), whereas a comparable study would be difficult in humans. The UPR in tissues other than subcutaneous

adipose may be more predictive of S_I.

In conclusion, we show that ER stress markers, mostly representing the activation of the ATF6α pathway, are increased by 50% or more in subcutaneous adipose tissue from obese compared with lean humans. Furthermore, initiation factor eIF2α is phosphorylated, suggesting a possible decrease in subcutaneous adipose translation with obesity and possibly reflecting activation of the PERK pathway. JNK1 activation was not convincingly demonstrated in our study, nor could we demonstrate that the association of ER stress with S_I was independent of the well known inverse correlation of S_I and obesity. Future studies are needed to explore the implications of eIF2α phosphorylation and to determine whether individuals who develop type 2 diabetes show a different pattern of adipocyte ER stress response, perhaps with less compensation and increased activation of pro-apoptotic pathways.

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Figure Legends

Figure 1 Gene Expression vs BMI by Ethnicity. The figure shows regression plots for HSPA5 (A), TRAF2 (B), DNAJC3 (C), and HYOU1 (D). For all figures, both gene expression measures (ratio to 18S RNA) and BMI were ln-transformed, but BMI is shown as kg/m^2. Solid lines represent means for European American participants (n=54), and dotted lines represent means for African American participants (n=24). Bars represent 95% confidence intervals.

Figure 2. Gene Expression of Endoplasmic Reticulum Stress Response Genes by BMI Group. Gene expression, calculated as real time values normalized to 18 S RNA, are shown by body mass index divided into lean (BMI < 25 kg/m^2; n=21, black bars), overweight (25 kg/m^2 to 30 kg/m^2; n=29, white bars) or obese (BMI > 30 kg/m^2, n=28, hatched bars). Significant differences based on analysis of variance of ln-transformed values are marked by asterisks; no asterisks represent no significant difference. Other levels of significance are as follows: * <0.01; ** p<0.001; ***; p<0.0001; ****; p<0.00001 ***** p< 10^{-8}. The graph is shown on a linear scale.

Figure 3: Western Blot of Phospho-Proteins. Representative Western blots showing samples with high and low BMI. Antibodies are either total protein as noted, or phospho-proteins. **A**: Total and phosphorylated initiation factor eIF2α, with β-actin for normalization. Control is palmitate-treated HepG2 cells, which show acute endoplasmic reticulum stress response. Numbers below lane numbers 1-8 give BMI of individuals represented. **B**. Phosphorylated and unphosphorylated forms of JNK1 and JNK2, and phosphorylated c-jun. Control is from palmitate treated HepG2 cells. The gel is representative of Western blots on 34 individuals, as described in the Results, with each individual measured at least twice. **C.** Scatterplot for 34

samples for p-eIF2α and p-JNK1; LBMI, low BMI group (n=15); HBMI, high BMI group (n=19). Significance (p values) is based on Mann-Whitney-U test.

Figure 4: Hierarchical clustering of gene expression for ER stress transcripts phosphorylated EIF2a and JNK1. Figure 4 shows data on 15 individuals selected for lowest BMI and 19 individuals selected for highest BMI from the full sample of 76 individuals, as shown in Figure 3. Euclidian distance matrix between each individual was generated using log normalized values of all expression measures and was used for seriation under multiple fragment heuristics (MF).

Table 1: Study Population Characteristics

	N	Sample 1	N	Sample 2	p
Male/Female		28/50		14/72	
Ethnicity (White/Black/Hispanic)		54/24/0		72/13/1	
Age (years)	78	42.3 (8.8)	86	42.7 (10.0)	NS
BMI (kg/m^2)	78	28.7 (5.6)	86	31.0 (4.8)	0.005
PFAT (%)	77	32.9 (10.3)	86	38.4 (7.6)	0.0002
Waist Circ.(cm)	77	95.2 (13.5)	74	96.4 (14.4)	NS
Waist/Hip Ratio	77	0.879 (0.072)	74	0.886 (0.085)	NS
Systolic BP (mm Hg)	78	124.2 (13.2)	75	116.2 (21.4)	0.006
Diastolic BP (mm Hg)	78	77 (10)	75	75.7 (14.4)	NS
Total Cholesterol (mmol/l)	78	4.58 (0.93)	75	4.91 (1.05)	0.05
HDL cholesterol (mmol/l)	78	1.38 (0.83, 2.29)	75	1.31 (0.76, 2.28)	NS
LDL cholesterol (mmol/l)	78	2.49 (1.37,4.56)	69	2.69 (1.45,5.00)	0.11
Triglycerides	78	1.08 (0.39, 3.00)	75	1.32 (0.45, 3.87)	0.02
S_I ($x10^{-4}$ min^{-1} $[\mu U/ml]^{-1}$)	77	3.16 (1.08,9.26)	83	2.40 (0.69,8.39)	0.004
AIR_g (mg-min/dl)	77	370 (70.0, 1960)	83	328 (63,1697)	NS
Disposition Index	77	1169 (236,5805)	83	768 (160, 3690)	0.001

Table 1 shows the traits shared between the primary study population (Sample 1) and the replication population of previously examined individuals ascertained under other studies (Sample 2). Skewed variables are shown as mean (95% confidence intervals); normal variables are mean (standard deviation). P values are from 2-tailed t-test comparing the two populations. Note that numbers of subjects used in subsequent tables and figures removed some individuals who were not of Caucasian or African American ancestry, were studied under a different protocol, or could not be modeled for FSIGT data. BMI, body mass index; PFAT, percent fat from DXA; Waist circ, waist circumference. For conversion to mass units (mg/dl) divide cholesterol by 0.0259 and triglycerides by 0.0113.

Table 2A: Correlation of Metabolic Traits with Endoplasmic Reticulum Stress Response in Sample 1

Gene Type		Proximal ER Sensors				Protein Degrad.	
Trait	r/p	HSPA5	ATF6α	EIF2AK3	sp. XBP1	EDEM1	TRAF2
BMI	r	0.51	0.48	0.48	0.43	0.45	0.38
	p	$6x10^{-6}$	$2x10^{-5}$	$2x10^{-5}$	0.0001	0.00008	0.001
Waist Circ	r	0.46	0.43	0.44	0.39	0.38	0.31
	p	$5x10^{-5}$	0.0002	0.0001	0.0007	0.0009	0.009
Hip Circ	r	0.47	0.44	0.44	0.43	0.43	0.35
	p	$4x10^{-5}$	$9x10^{-5}$	0.0001	0.0001	0.00015	0.002
Waist/Hip	r	0.25	0.21	0.23	0.12	0.13	0.1
	p	0.037	0.07	0.05	0.32	0.26	0.4
PFAT	r	0.55	0.53	0.52	0.46	0.52	0.42
	p	$6x10^{-7}$	$2x10^{-6}$	$3x10^{-6}$	$5x10^{-5}$	$2x10^{-6}$	0.0002
Trunk:Leg	r	0.23	0.25	0.214	0.2	0.19	0.17
	p	0.06	0.037	0.07	0.1	0.12	0.16
S_I	r	-0.21	-0.29	-0.18	-0.26	-0.17	-0.16
	p	0.08	0.01	0.14	0.027	0.14	0.16
AIR_g	r	0.05	0.018	-0.01	0.03	0.05	0.059
	p	0.68	0.89	0.93	0.79	0.66	0.62
DI	r	-0.1	-0.19	-0.14	-0.16	-0.07	-0.05
	p	0.42	0.1	0.24	0.19	0.56	0.656
Fast. Gluc	r	0.2	0.25	0.19	0.19	0.18	0.1
	p	0.09	0.032	0.11	0.11	0.12	0.39
2 H Gluc	r	0.04	0.11	0.07	0.11	0.11	0.12
	p	0.75	0.33	0.54	0.36	0.36	0.32
Insulin AUC	r	0.11	0.19	0.1	0.15	0.15	0.09
	p	0.36	0.1	0.39	0.21	0.22	0.44
Ins/Glu AUC	r	0.08	0.15	0.07	0.16	0.12	0.04
	p	0.5	0.2	0.55	0.18	0.33	0.73

Table 2B: Correlations of ERSR genes with metabolic traits, Chaperones and Apoptosis Pathway Genes

Gene Type		Chaperones				Apoptosis Pathway		
Trait	r/p	HYOU1	DNAJC3	CALR	STC2	ATF4	GADD34	ATF3
BMI	r	0.51	0.53	0.53	0.62	0.3	0.36	0.48
	p	$6x10^{-6}$	$2x\ 10^{-6}$	$2x10^{-6}$	$6x10^{-9}$	0.01	0.002	$2x10^{-5}$
Waist Circ	r	0.51	0.47	0.5	0.62	0.24	0.35	0.5
	p	$6x10^{-6}$	$3x\ 10^{-5}$	$1x10^{-5}$	$8x10^{-9}$	0.045	0.003	$1x10^{-5}$
Hip Circ	r	0.52	0.5	0.49	0.59	0.26	0.4	0.44
	p	$3x\ 10^{-6}$	$9\ x\ 10^{-6}$	$1x10^{-5}$	$3.8x10^{-8}$	0.03	0.0006	$9x10^{-5}$
Waist/Hip	r	0.25	0.21	0.27	0.37	0.1	0.13	0.34
	p	0.03	0.07	0.02	0.001	0.42	0.29	0.003
PFAT	r	0.54	0.57	0.52	0.65	0.31	0.38	0.43
	p	$1x\ 10^{-6}$	$1x\ 10^{-7}$	$2x10^{-6}$	6.10^{-10}	0.008	0.0009	0.0001
Trunk:Leg	r	0.23	0.23	0.27	0.37	0.19	0.4	0.37
	p	0.06	0.05	0.02	0.001	0.11	0.0004	.0001
S_I	r	-0.2	-0.23	-0.25	-0.35	-0.21	-0.37	-0.26
	p	0.084	0.05	0.04	0.002	0.08	0.001	0.025
AIR_g	r	0.2	0.06	0.11	0.21	-0.006	0.052	-0.07
	p	0.083	0.64	0.36	0.07	0.96	0.66	0.54
DI	r	0.075	-0.1	-0.06	-0.02	-0.16	-0.21	-0.27
	p	0.53	0.38	0.6	0.83	0.18	0.08	0.02
Fast. Gluc	r	0.17	0.22	0.26	0.13	0.001	0.2	0.19
	p	0.16	0.06	0.03	0.28	0.99	0.1	0.11
2 H Gluc	r	0.07	0.08	0.11	0.16	0.13	0.24	0.15
	p	0.55	0.51	0.35	0.18	0.27	0.038	0.21
Insulin AUC	r	0.19	0.17	0.15	0.28	0.12	0.3	0.23
	p	0.11	0.15	0.22	0.02	0.3	0.009	0.05
Ins/Glu AUC	r	0.19	0.14	0.1	0.25	0.1	0.26	0.21
	p	0.1	0.23	0.42	0.04	0.38	0.03	0.08

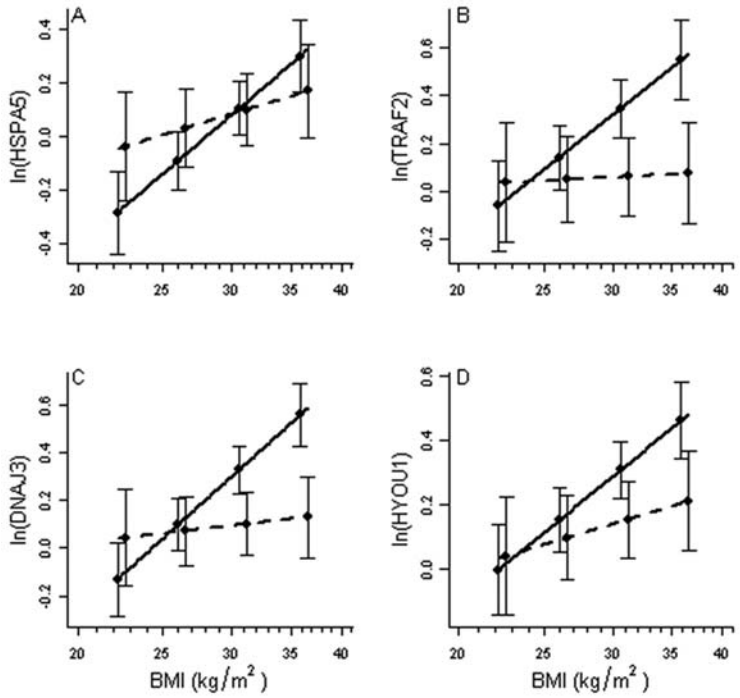
Table 2 Legend: Correlations of traits with expression of ER stress genes in subcutaneous adipose. Shaded cells are statistically significance. The second column (r/p) gives either the partial correlation coefficient (r) or the attached significance (p). For space considerations, genes with no significant correlations are not shown: ERN1, total XBP1, CHOP. BMI, body mass index; Waist circ, waist circumference; Hip Circ, hip circumference; Waist/Hip, waist to hip ratio; PFAT, percent fat from DEXA; Trunk:Leg ratio of mean percent fat in both legs to trunk from DEXA; S_I insulin sensitivity from

MinMod; AIRg acute insulin response to glucose (MinMod); DI, disposition index; Fast. Gluc, fasting glucose; 2H gluc., 2 hour glucose; insulin AUC, insulin area under curve from OGTT study; Ins/Glu AUC, ratio of insulin to glucose area under curve from OGTT visit; sp XBP1, spliced XBP1 by real time assay. Note that approved human gene codes are used throughout. Commonly used aliases are GRP78/BIP (HSPA5), PERK (EIF2AK3), $p58^{IPK}$ (DNAJC3), and ORP150 (HYOU1).

Table 3: Correlation of Endoplasmic Reticulum Stress Factors Gene Expression in Adipose with Insulin Sensitivity (S_I)

	Sample 1 (n=78)		Sample 2 (n=83)		Combined Sample (n=161)	
Gene	**r**	**p**	**r**	**p**	**r**	**p**
HSPA5	-0.21	0.08	-0.43	5.9E-05	-0.31	7.3E-05
EDEM1	-0.18	0.14	-0.22	0.042	-0.18	0.03
TRAF2	-0.16	0.16	-0.11	0.33	-0.08	0.29
DNAJC3	-0.23	0.052	-0.16	0.15	-0.15	0.06
HYOU1	-0.20	0.08	-0.29	0.009	-0.22	0.006

Correlation of insulin sensitivity with subcutaneous adipose in the primary study (Sample 1) and replication study (Sample 2) and combined sample. Note that compared with Tables 1 and 3, only individuals who had successfully completed the insulin modified frequently sampled glucose tolerance test and were either African American or Caucasian were included. Columns "r" are the partial correlation coefficient with S_I; columns "p" are the attached significance level.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B
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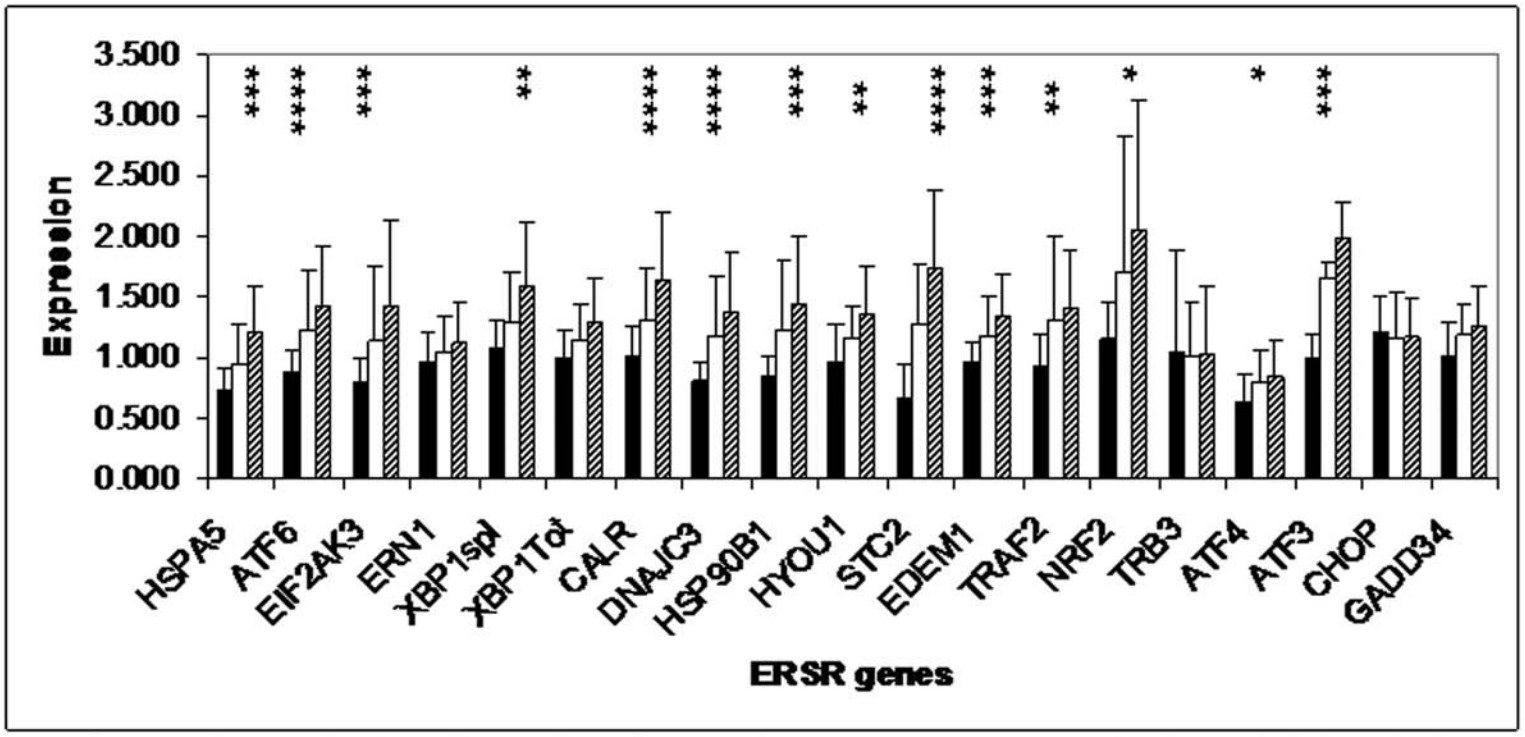
ln(DNAJ3)
D
ln(HYOU1)
BMI (kg/m^2)
BMI (kg/m^2)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HSPA5
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ERSR genes

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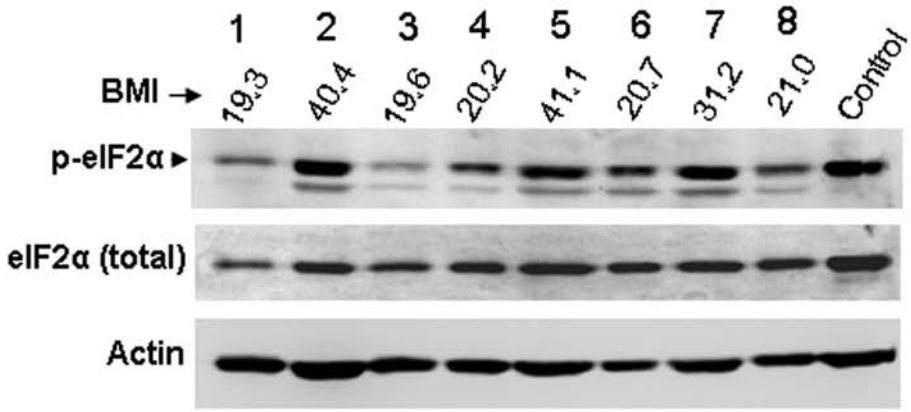
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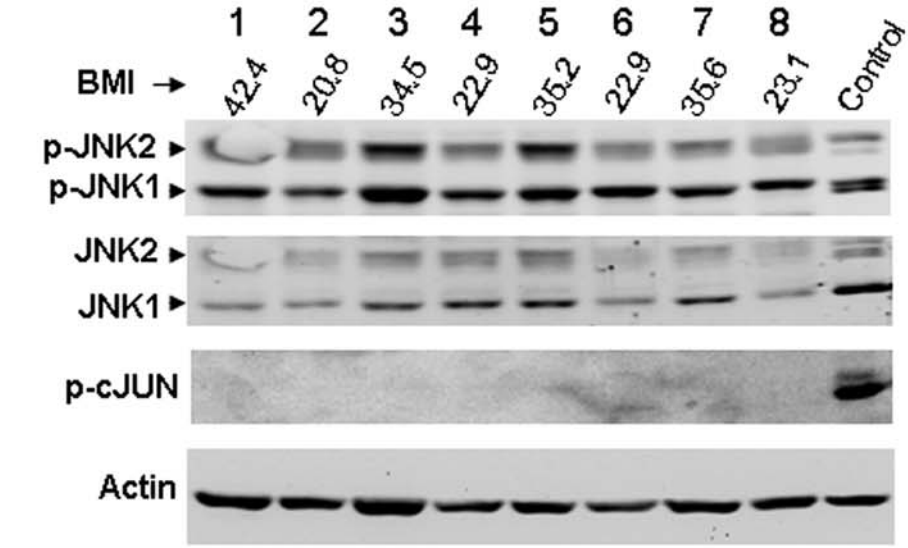
p-eIF2α ▸
eIF2α (total)
Actin

B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BMI →
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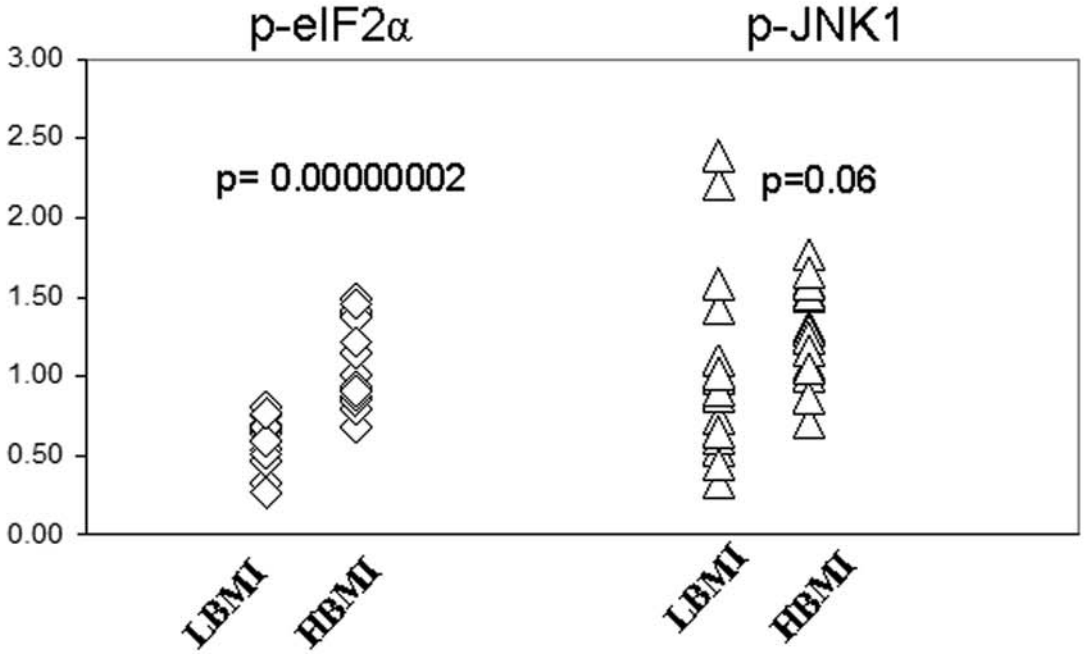
JNK1 ▸
p-cJUN
Actin

C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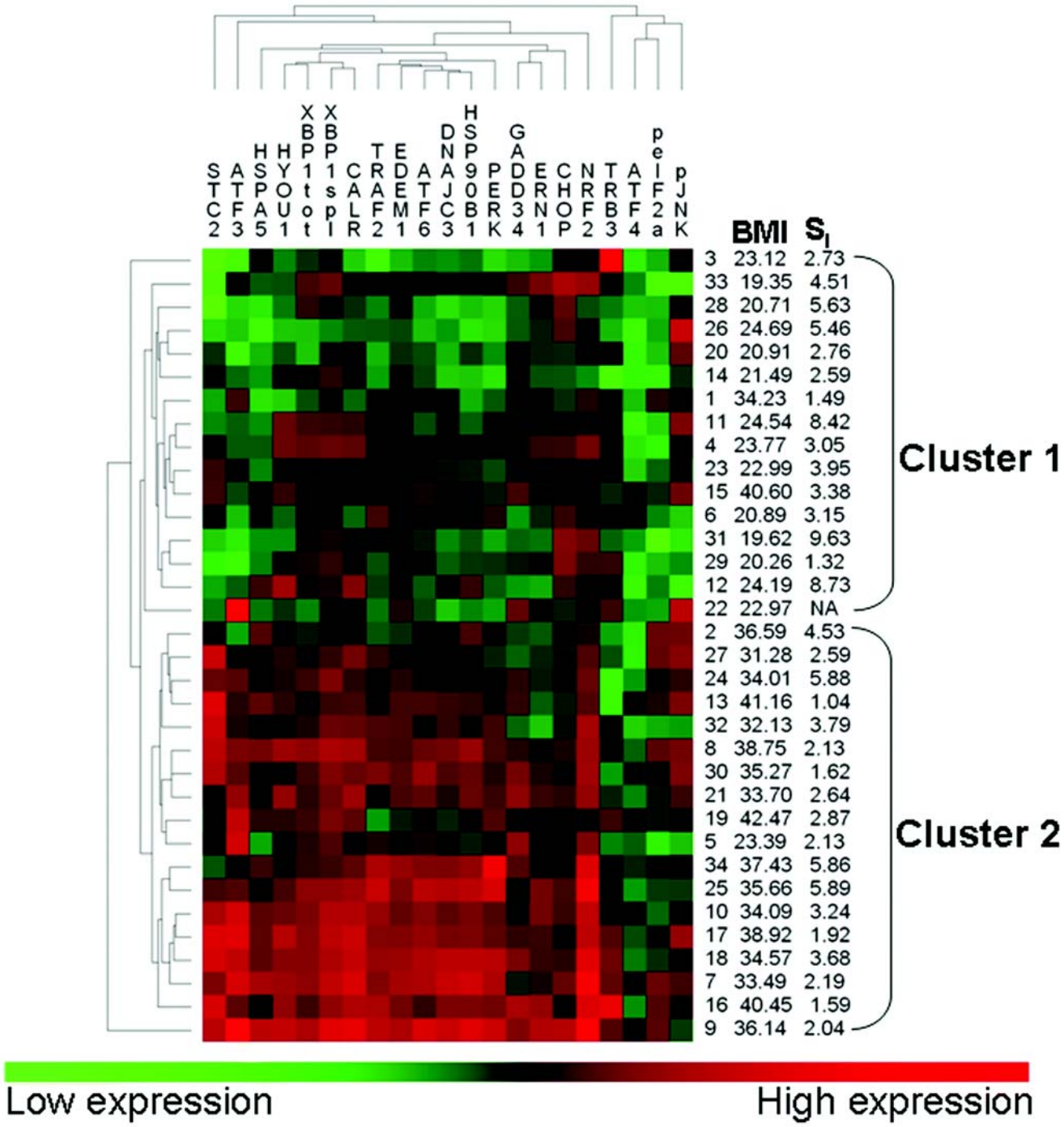
HBMI
LBMI
HBMI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pJNK
BMI
S_I
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Cluster 1
Cluster 2
Low expression
High expression