TRANSCRIPTOME PROFILING OF OMENTAL ADIPOSE TISSUES IN HUMAN OBESITY BY RNA-SEQ

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ABSTRACT

Recent scientific research has indicated that inflammation in adipose tissues plays a central role in the pathophysiology of obesity and related complications. However, despite extensive studies, the molecular mechanisms that trigger and aggravate the obesity-related inflammation remain unclear. In this study, transcriptome profiling of omental adipose tissues from three obese and four lean patients was performed using the RNA-Seq technology. Differential expression analysis identified 206 dysregulated genes (p-value < 0.05 and fold change ≥ 2) in obesity, that are known to be involved in a multitude of functions including stress response, inflammatory response and leukocyte adhesion. Furthermore, differential splicing analysis uncovered a possible role for *TLR4* RNA splicing in obesity, which is further validated with an independent cohort of samples (five obese and six lean patients) using RT-qPCR. These findings suggest that, as a person experiences weight gain leading to obesity, the adipose splicing pattern of *TLR4* transcripts changes in favor of activation of TLR4 signaling, which in turn, may contribute to the progression of obesity-related inflammation and complications. This study provides a glimpse at the transcriptome of disease-state adipose tissue in obesity, and demonstrates the potential importance of aberrant RNA splicing and expression in obesity-associated immune dysregulation.

ABSTRAK

Kajian saintifik baru-baru ini telah menjelaskan kepentingan keradangan tisu adipos dalam patofisiologi obesiti dan komplikasi yang berkaitan. Namun, mekanisme molekul yang mencetuskan dan menyulitkan keradangan metabolik masih tidak dapat difahami secara sepenuhnya. Kajian ini memprofilkan transkriptom tisu adipos (perut intra) daripada tiga orang pesakit obes dan empat orang pesakit kurus dengan penggunaan teknologi RNA-Seq. Analisis ekspresi mengenalpasti 206 gen dengan nilai $p < 0.05$ dan lipat perubahan ≥ 2; analisis pengayaan mendapati bahawa gen-gen tersebut terlibat dalam pelbagai fungsi, seperti tindak balas terhadap stres fisiologi/keradangan dan lekatan leukosit. Tambahan pula, analisis splicing alternatif menunjukkan bahawa splicing TLR4 memainkan peranan yang penting dalam obesiti; analisis RT-qPCR dengan 11 sampel tambahan (lima orang pesakit obes dan enam orang pesakit kurus) mengesahkan hasil kajian tersebut. Secara umumnya, analisis splicing alternatif ini menunjukkan perubahan trend splicing TLR4 apabila seseorang mengalami peningkatan berat badan/obesiti; perubahan ini mungkin mengaktifkan isyarat TLR4 dan mengakibatkan perkembangan keradangan metabolik dalam tisu adipos. Kesimpulannya, kajian ini mendapati bahawa splicing alternatif dan ekspresi gen memainkan peranan penting dalam keradangan yang berkaitan dengan obesiti.

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ACBP	Acyl-CoA binding protein
ADRA2A	Adrenoceptor alpha 2A
ADRB1	Adrenoceptor beta 1
ATF6	Activating transcription factor 6
BAT	Brown adipose tissue
BMI	Body mass index
C1QA	Complement component 1, q subcomponent, A chain
C1QB	Complement component 1, q subcomponent, B chain
C1QC	Complement component 1, q subcomponent, C chain
C3AR1	Complement component 3a receptor 1
C6	Complement component 6
CA3	Carbonic anhydrase III
cAMP	Cyclic adenosine monophosphate
CCL	Chemokine (C-C motif) ligand
CCN	Connective tissue growth factor, Cystein rich protein, and Nephroblastoma overexpressed gene
CD14	CD14 molecule
C_q	Quantification cycle
CTGF	Connective tissue growth factor
CYR61	Cysteine-rich, angiogenic inducer, 61
DAVID	Database for Annotation Visualization and Integrated Discovery
DBI	Diazepam binding inhibitor
DDRT-PCR	Differential display reverse transcriptase-PCR
DEG	Differentially expressed gene

DLK1	Delta-like 1 homolog
DO	Disease ontology
DSG	Differentially spliced gene
ER	Endoplasmic reticulum
FADD	Fas (TNFRSF6)-associated via death domain
FC	Fold change
FDR	False discovery rate
FFA	Free fatty acid
FPR3	Formyl peptide receptor 3
FunDO	Functional Disease Ontology
GEO	Gene Expression Omnibus
HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1
IGF	Insulin-like growth factor
IGFBP6	Insulin-like growth factor binding protein 6
IKKβ	IκB kinase β
IL	Interleukin
IRE1	Inositol-requiring enzyme 1
ISL1	ISL LIM homeobox 1
ITGAM	Integrin alpha M
ITGB2	Integrin beta 2
JNK	c-Jun N-terminal kinase
LEP	Leptin
LPS	Lipopolysaccharide
LY96	Lymphocyte Antigen 96
MAP2K1	Mitogen-activated protein kinase kinase 1
MAPK3	Mitogen-activated protein kinase 3

MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
NCAM2	Neural cell adhesion molecule 2
NCBI	National Center for Biotechnology information
NFAM1	NFAT activating protein with ITAM motif 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NPR3	Natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)
PCDHB7	Protocadherin beta 7
PERK	PKR-like ER kinase
PPARγ	Peroxisome proliferator-activated receptor *gamma*
PTAFR	Platelet-activating factor receptor
RNA-Seq	Whole-transcriptome shotgun sequencing
ROBO2	Roundabout, axon guidance receptor, homolog 2 (Drosophila)
RT-qPCR	Real-time quantitative reverse transcription PCR
SAGE	Serial analysis of gene expression
SI	Splicing index
smTLR4	Soluble mouse TLR4 protein
SNP	Single-nucleotide polymorphism
SPP1	Secreted phosphoprotein 1
SSH	Suppression subtractive hybridization
SSPO	SCO-spondin homolog (Bos taurus)
STEAP4	STEAP family member 4
SYK	Spleen tyrosine kinase
TCF7L2	Transcription factor 7-like 2
TLR	Toll-like receptor

TNFα	Tumor necrosis factor alpha
TNMD	Tenomodulin
TV	Transcript variant
UMMC	University of Malaya Medical Center
UPR	Unfolded protein response
WAT	White adipose tissue
WISP2	WNT1 inducible signaling pathway protein 2

CHAPTER 1: INTRODUCTION

Obesity is a medical condition characterized by accumulation of excess body fat to an extent that it may cause adverse effects to health. Approximately two billion adults worldwide were estimated to be either obese or overweight by 2008 (World Health Organization, 2014). Compared to the lean population, obese individuals are more prone to developing type 2 diabetes mellitus, hypertension, dyslipidemia, cardiovascular disease and other co-morbidities in the metabolic syndrome (Khaodhiar, McCowen, & Blackburn, 1999), leading to reduced life expectancy and increased economic burden for treating these disorders associated with obesity.

The pathophysiology of obesity and related complications implicates both intra-organ and generalized metabolic and immune dysfunctions that are the result of many different interrelated and interactive molecular mechanisms. The white adipose tissue that forms the large majority of fat depots in the human body has been recognized as a major endocrine organ that secretes adipokines (Frühbeck, Gómez-Ambrosi, Muruzábal, & Burrell, 2001) besides fatty acids, lipids and proteins. The adipokines participate in a wide range of functions including the regulation of appetite, energy balance, insulin sensitivity, angiogenesis, blood pressure, inflammation and immune response (Kwon & Pessin, 2013; Trayhurn, 2013). In obese individuals, the key pathological features are an aberrant adipogenesis and lipolysis, the occurrence of inflammation in adipose tissue, the dysregulated expression of pro- and anti-inflammatory adipokines, and the induction of serum resistance. These pathological processes are believed to contribute to the development of obesity-associated maladies (Redinger, 2007).

Inflammation in adipose tissue is characterized by the infiltration of various immune cells which include macrophages, T cells and mast cells (Altintas et al., 2011; Weisberg et al., 2003; Yang et al., 2010). Several contending hypotheses have been put forward to explain the development of inflammation. These include local hypoxia (Trayhurn, 2013), endoplasmic reticulum (ER) stress (Cnop, Foufelle, & Velloso, 2012) and oxidative stress (Bondia-Pons, Ryan, & Martinez, 2012). In all three conditions, dysregulated production of inflammatory adipokines has been observed (Bondia-Pons et al., 2012; Cnop et al., 2012; Trayhurn, 2013). More recently, it has also been shown that bacterial lipopolysaccharide (LPS) can trigger the low grade inflammation in human adipose tissue, leading to systemic insulin resistance (Mehta et al., 2010). LPS is the major structural component of the outer wall of all Gram-negative bacteria. It is recognized by toll-like receptor (TLR) 4 which interacts with extracellular proteins to induce a signaling cascade leading to the production of pro-inflammatory cytokines.

The complex gene-environment interplay in the development of obesity is being intensively researched. Transcriptome profiling permits the identification of dysregulated genes in adipose tissue. In the last decade, cDNA microarray has been the instrument of choice for comparing transcriptome profiles in adipose tissues of obese and non-obese groups (Gómez-Ambrosi et al., 2004; Grant, Vester Boler, Ridge, Graves, & Swanson, 2011; Morton et al., 2011). Nonetheless, microarray technology has some drawbacks, such as low sensitivity and restriction to the examination of only known genes (Wang, Gerstein, & Snyder, 2009). These limitations became irrelevant with the introduction of next-generation sequencing technology. Specifically, whole-transcriptome shotgun sequencing (RNA-Seq) allows millions of mRNA-derived cDNA molecules to be sequenced in parallel, without the need of bacterial clones; it also enables the detection of hitherto unknown transcripts and the generation of absolute

rather than relative gene-expression measurements (Wang et al., 2009). In addition, RNA-Seq can also detect changes in the splicing pattern of genes. Differential or alternative splicing generates multiple mRNA transcripts from a single gene to produce protein isoforms with different functions. Emerging evidence has suggested that the abnormal regulation of alternative splicing can result in the development of obesity and insulin resistance (Kaminska & Pihlajamäki, 2013).

In the present study, omental adipose tissues from obese and lean individuals were subjected to RNA-Seq for transcriptome profiling. Omental adipose tissue was selected because fat from this depot has been shown to be more closely related to the co-morbidities of obesity than fat from the subcutaneous depot (Montague & O'Rahilly, 2000).

The objectives of this study were: (i) to identify differentially spliced and expressed genes from omental adipose tissue of obese patients, in reference to the lean counterpart; and (ii) to derive new biological insights into the pathophysiology of obesity from the list of identified genes with alternative splicing and differential expression patterns.

CHAPTER 2: LITERATURE REVIEW

2.1 The Obesity Epidemic

Obesity is a disease characterized by accumulation of body fat to a sufficient magnitude to cause adverse consequences to health. A person with a body mass index (BMI) ≥ 30 is generally considered obese. According to the World Health Organization (2014), more than a third of the world's adult population (20 years old or above) was obese and overweight in 2008 (11 % was obese and the additional 24 % was overweight). Furthermore, obesity and overweight are also one of the leading risks for global deaths. Around 3.4 million adults are estimated to die each year due to the co-morbidities associated with obesity and overweight (World Health Organization, 2014).

It has been widely known that obesity is reaching epidemic proportions in Western countries. For example, a recently published report revealed that more than one-third of U.S. adults (34.9 %) were obese by 2012 (Ogden, Carroll, Kit, & Flegal, 2014). In many Asian countries, the prevalence of overweight and obesity has increased many-fold over previous decades (Ramachandran & Snehalatha, 2010). Malaysia was ranked sixth and first for obesity in the Asia-Pacific region and South-East Asia, respectively (Edwards, 2013). A national study in 2007 reported that the prevalence of obesity amongst adult Malaysians (18 years old or above) has markedly increased by 280 % since 1996 (Rampal et al., 2007). This alarming rate of obesity has been attributed to high energy and fat consumption together with a low level of physical activity, and is associated with a significant risk for insulin resistance and type 2 diabetes mellitus, dyslipidemia, hypertension, cardiovascular disease, stroke, sleep apnea and certain forms of cancer (Khaodhiar et al., 1999). National strategies are urgently needed to prevent the escalation of obesity-induced morbidity.

2.2 Obesity, Inflammation and Insulin Resistance

In recent years, much research efforts have been focused on delineating the association between obesity and insulin resistance. It has been suggested that inflammation contributes to the development of insulin resistance; the connection between the two conditions was established through a series of studies which observed the development of insulin resistance in diseases involving active inflammatory responses, such as sepsis, hepatitis C and human immunodeficiency virus infections, and rheumatoid arthritis (Bahtiyar, Shin, Aytaman, Sowers, & McFarlane, 2004; Clowes et al., 1978; Pao, Lee, & Grunfeld, 2008; Sidiropoulos, Karvounaris, & Boumpas, 2008). Interestingly, obese individuals are predisposed to developing insulin resistance, which is the primary defect leading to type 2 diabetes. Hotamisligil, Shargill, & Spiegelman (1993) discovered the first molecular link between obesity, inflammation and insulin resistance: increased level of tumor necrosis factor alpha (TNFα, a proinflammatory adipokine) mRNA and protein in adipose tissue of obese and/or diabetic mice compared to lean controls. Since then, accumulating literature have made it rapidly apparent that not only TNFα but an array of proinflammatory adipokines are elevated in obesity, leading to complications such as insulin resistance and diabetes mellitus (Berg & Scherer, 2005; Shoelson, Lee, & Goldfine, 2006).

2.3 Roles of Adipose Tissue in Obesity-induced Inflammation

Over the years, the biology of adipose tissue has been extensively studied in obesity-related research because it is the predominant site sustaining stresses and inflammatory insults under obese conditions (Gregor & Hotamisligil, 2011). There are two types of adipose tissues: white adipose tissue (WAT) and brown adipose tissue (BAT). BAT serves as a fat depot for heat generation in neonates and young children during cold exposure (Lean, 1989). As an individual ages, BAT regresses by transforming into

WAT (Heaton, 1972). WAT is the physiological site of energy storage as lipids and contributes to the inflammation associated with obesity. Previously considered to be an inert, loose connective tissue for the storage of fats, investigators now appreciate that WAT is an active endocrine and paracrine organ, consisting of adipocytes predominantly, and the stromal vascular fraction of cells (including preadipocytes, fibroblasts, vascular endothelial cells and a variety of immune cells). Among these cell types, adipocytes and macrophages are able to secrete a wide array of adipokines (cytokines secreted by adipose tissue), such as TNFα, interleukin (IL) 6, IL1β and chemokine (C-C motif) ligand (CCL) 2, which promote inflammation and impair insulin sensitivity (Berg & Scherer, 2005; Shoelson et al., 2006).

The anatomical distribution of WAT is strongly associated with co-morbidities of obesity. Many researchers have reported that intra-abdominal (visceral or omental) adipose tissue is more closely related to the co-morbidities of obesity than fat from the subcutaneous depot (Montague & O'Rahilly, 2000). The deleterious effect of omental fat is possibly due to its anatomical site with direct venous drainage to the liver, leading to full exposure of liver to undiluted repertoire of metabolites and secreted products by this fat depot (Montague & O'Rahilly, 2000). In addition, omental adipose tissue along with its resident macrophages is capable of producing more proinflamatory cytokines than the subcutaneous depot (Hamdy, Porramatikul, & Al-Ozairi, 2006).

2.4 Hallmarks of Obesity-related Inflammation

The inflammatory response in obesity is different from the classical inflammation, which is accompanied by the cardinal signs of redness, swelling, heat and pain (Hotamisligil, 2006). The classical response is often correlated with increased basal metabolic rate, resulting in a focused and rapid response by the immune system to the

site of infection or injury. Unlike the classical paradigm of inflammation, the obesity-related inflammatory state is evoked by nutrient overload and associated with reduced metabolic rate. Notably, the inflammatory signals (adipokines and other mediators) from obese tissues are significant but often "low-grade" when compared to that of an infection, trauma or acute immune response (Gregor & Hotamisligil, 2011). In the condition of obesity (nutrient surplus), inflammatory signals originated from metabolic cells (such as adipocytes) create a modified milieu, leading to the recruitment and infiltration of immune cells into the tissue. Changes in the population of various immune cells, including macrophages, T cells, natural killer T cells and mast cells, have been reported in obese adipose tissues (Altintas et al., 2011; Ohmura et al., 2010; Weisberg et al., 2003; Yang et al., 2010). Inflammatory signals from both metabolic cells and infiltrating immune cells ultimately lead to an unresolved and chronic inflammatory response within the obese tissue, which can only be reversed or improved by weight loss (Cancello et al., 2005; Capel et al., 2009).

2.5 Triggers to the Inflammatory Response Associated with Obesity

2.5.1 Stress

A key contributor to the metabolic inflammation of obesity is stress. In response to metabolic distress signals, the adipocyte is capable of engaging itself in inflammatory signaling events such as inflammasome and TLR activation, c-Jun N-terminal kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, and production of inflammatory cytokines/adipokines, all of which are normally viewed as the territory of specialized immune cells (Gregor & Hotamisligil, 2011).

During the development of obesity, adipocytes become hypertrophic, which subsequently leads to hypoxia because the oxygen availability does not match the demand of the surrounding tissue. The secretion and expression of a number of proinflammatory adipokines by adipocytes and local macrophages are upregulated in response to hypoxia, underpinning the occurrence of inflammation in adipose tissue during obesity (Wang, Wood, & Trayhurn, 2007; Ye, Gao, Yin, & He, 2007). Furthermore, hypoxia is known to inhibit macrophage migration from the affected region in tissues (Turner, Scotton, Negus, & Balkwill, 1999). An immunohistochemical study revealed the increased level of macrophage infiltration within hypoxic areas of obese adipose tissue (Rausch, Weisberg, Vardhana, & Tortoriello, 2008). Although the recruitment of macrophages may serve a beneficial role in removing dead and necrotic adipocytes (Cinti et al., 2005), macrophage activation inevitably causes the inflammation in obese adipose tissue (Weisberg et al., 2003). Remarkably, hypoxia is also able to induce ER stress in adipocytes (Hosogai et al., 2007).

Obese adipose tissue displays an increased level of ER stress in comparison to the lean counterpart (Ozcan et al., 2004). To alleviate ER stress, unfolded protein response (UPR) is activated through three main transmembrane sensors that reside on the ER, namely PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). By transmission of signals from the ER to the cytoplasm or nucleus through these factors, the UPR attempts to restore ER homeostasis by suppressing protein translation (Harding et al., 2000), increasing degradation of unfolded proteins (Travers et al., 2000), and increasing the level of chaperones to facilitate protein folding (Yoshida, Haze, Yanagi, Yura, & Mori, 1998). If these strategies fail, the UPR may also initiate apoptosis (Nakagawa et al., 2000). Prolonged activation of the UPR may trigger the production of reactive oxygen species (Santos,

Tanaka, Wosniak, & Laurindo, 2009), a process which is proinflammatory and apoptotic in nature. *In vitro* studies have demonstrated that free fatty acids (FFAs), which are elevated in obesity, are able to induce both ER and oxidative stresses in adipocytes (Furukawa et al., 2004; Jiao et al., 2011). FFA-induced ER and oxidative stresses have been shown to dysregulate the expression of IκB kinase β (IKKβ, a master kinase in regulating expression of inflammatory genes) and several proinflammatory adipokines, respectively.

2.5.2 Nutrient

There is evidence that inflammatory response may be induced, at least in part, by nutrients. For example, under normal conditions, the expression of *STEAP family member 4* (*STEAP4*) increases in adipose tissue during feeding (Wellen et al., 2007). Gene knockout of *STEAP4* in adipocytes and mice resulted in uncontrolled inflammatory response upon nutrient exposure, suggesting that STEAP4 may function as an immune suppressor in metabolic and immune cells in response to nutrient stimulation. Interestingly, the pattern of *STEAP4* upregulation upon feeding disappears under the obese condition, correlating with increased inflammatory conditions commonly seen in obesity. Also, it has been shown that nutrient overload may induce pathogen sensors, leading to inflammation and insulin resistance. In adipocytes and macrophages, infusion of saturated fatty acids leads to the activation of TLR4 (Shi et al., 2006), which is the sensor for LPS. Moreover, negative effects of systemic lipid infusion and high-fat diet is partially negated by mice lacking TLR4, resulting in improved insulin sensitivity, possibly through reduced inflammatory gene expression in liver and fat (Shi et al., 2006).

2.5.3 Gut Microbiota

Gut microbiota represents a new, promising area in the field of obesity research. The first important discovery in this area came from the observation of changes in the relative abundance of two dominant bacterial phyla, Bacteroidetes and Firmicutes, in the gut of obese animals (Ley et al., 2005). Interestingly, it has been shown that germ-free animals are resistant to diet-induced obesity (Bäckhed, Manchester, Semenkovich, & Gordon, 2007). However, features of metabolic syndrome manifest in non-obese, germ-free mice after being inoculated with the gut microbiota from obese mice (Turnbaugh et al., 2006). Conversely, this phenomenon was not observed when the germ-free mice were colonized with intestinal microbiota of lean donor mice. Several mechanisms for the role of gut microbiota in obesity have been proposed, including the provision of extra energy by the bacterial conversion of dietary fiber to short-chain fatty acids and effects on the production of gut hormones (Blaut & Klaus, 2012). In addition, the release of bacterial LPS through the leaky gut constitutes one of the triggers to the low-grade inflammation in obesity. Specifically, a study showed that obese animals have an elevated population of *Enterobacteriaceae* (important producers of inflammatory LPS) in the gut, when compared to both low-fat-fed and obesity-resistant high-fat-fed animals (de La Serre et al., 2010).

Insulin-resistant diet-induced and genetically obese mice as well as type 2 diabetic humans exhibit elevated circulatory LPS levels (Brun et al., 2007; Creely et al., 2007; Dasu, Devaraj, Park, & Jialal, 2010). It has been shown that circulatory LPS can trigger the adipose inflammation and systemic insulin resistance in humans, establishing the causal relationship between increased LPS level and impaired insulin sensitivity (Mehta et al., 2010). Interestingly, the increased level of serum LPS after feeding was reported in both mice and humans (Cani et al., 2007; Erridge, Attina, Spickett, & Webb, 2007).

This suggests that feeding increases the intestinal permeability to ensure maximum nutrient uptake, which in turn, paves the way for the release of inflammatory molecules from gut microbiota into the circulation. Incidentally, the intestine of obese animals is consistently more permeable than that of lean controls (Brun et al., 2007), corresponding to the increased level of circulatory LPS in obesity.

2.6 Transcriptome Profiling in Obesity-related Studies

The complex regulatory networks in adipose tissue are well-suited for an exploration with gene-expression (or transcriptome) profiling, which is an ideal tool for studying the molecular mechanisms of a polygenic disease like obesity. For instance, differential display reverse transcriptase-PCR (DDRT-PCR) had been employed to identify differentially expressed genes (DEGs) between subcutaneous and visceral adipose tissues from numerous animal models (Hishikawa et al., 2005). In 2007, Qiu et al. (2007) reported the use of another PCR-based method, suppression subtractive hybridization (SSH) to screen for dysregulated genes in omental adipose tissue of obese individuals. The most widely used instrument in gene-expression profiling over the last decade is the cDNA microarray. By comparing gene-expression profiles in adipose tissues from obese and non-obese subjects, many important insights into the pathogenesis of obesity and related diseases have been gained through microarray studies. For example, a microarray study on mouse adipose tissue revealed that the expression of adipogenic genes is decreased in obesity (Nadler et al., 2000). This provides one of the earliest indications that obese adipose tissue is associated with reduced adipogenesis and adipocyte hypertrophy, leading to complications such as ectopic lipid accumulation and insulin resistance. Nonetheless, the microarray technology has several inherent limitations, including dependence upon existing knowledge about genome sequences,

high background levels due to cross-hybridization and a limited dynamic range of detection owing to high background and saturation of signals (Wang et al., 2009).

On the other hand, the sequencing-based technique is another useful tool for gene-expression profiling. Bolduc et al. (2004) utilized the serial analysis of gene expression (SAGE) to examine the transcriptomic features of mouse adipose tissue. More recently, the advent of next-generation sequencing has dramatically changed the way that the functional complexity of transcriptomes can be studied. In particular, RNA-Seq can overcome the shortcomings of the microarray approach by being both sensitive and able to identify novel transcripts (Wang et al., 2009). Identification of candidate genes associated with obesity and nutrient stimulation has been reported in mice using the RNA-Seq approach (Zhang, Yao, Gao, & Abou-Samra, 2012; Zhang, 2012). To date, no RNA-Seq study that compares the mRNA profiles between obese and lean adipose tissues in humans has been reported (according to the PubMed database, using keywords: RNA-Seq, adipose tissue, obesity, and human, as of 23 November 2014).

2.7 Differential Splicing in Obesity

Alternative (or differential) splicing is a process which generates multiple transcripts from a single gene. This process increases the amount of protein isoforms, leading to changes in interactions of protein with other proteins, nucleic acids and membranes. The regulation of alternative splicing can be sequence-dependent. For instance, single-nucleotide polymorphisms (SNPs) associated with obesity at multiple loci were found to be located near to sequences regulating alternative splicing (Goren et al., 2008), suggesting that alternative splicing may be a missing link between the SNPs and risks related to obesity. Alternatively, environmental factors can also influence the regulation of alternative splicing, as exemplified by a recent study which demonstrated that

alternative splicing can be controlled by circadian clock and feeding/fasting in mice (McGlincy et al., 2012).

Several genes involved in adipogenic pathways have been found to be regulated by alternative splicing. Both peroxisome proliferator-activated receptor gamma (PPARγ) and delta-like 1 homolog (DLK1) are important regulators in the adipogenesis, with the former promoting and the latter inhibiting the process. Different protein isoforms of PPARγ and DLK1 were shown to have differential potential to activate and inhibit the adipogenic differentiation, respectively (Mei, Zhao, Chen, & Sul, 2002; Mueller et al., 2002). Diazepam binding inhibitor (DBI), also known as Acyl-CoA binding protein (ACBP), has been speculated to play a role in lipogenesis (Hansen, Andreasen, Mandrup, Kristiansen, & Knudsen, 1991) and adipogenesis (Mandrup et al., 1998). It has been shown that the splicing pattern of *DBI* changes during the differentiation of human preadipocyte cell strain (Ludewig, Klapper, Wabitsch, Döring, & Nitz, 2011), suggesting the potential importance of its isoforms in fat cell differentiation. Transcription factor 7-like 2 (TCF7L2) is a transcription factor playing an important role in canonical Wnt signaling, whereby the activation of this pathway inhibits adipogenesis. Protein isoforms encoded by spliced variants of *TCF7L2* exhibit differential ability to activate the Wnt signaling pathway (Weise et al., 2010). A recent study demonstrated that the short *TCF7L2* mRNA variant in subcutaneous adipose tissue is regulated by weight loss and is associated with hyperglycemia and impaired insulin sensitivity in human adipose tissue (Kaminska et al., 2012). Further analysis showed that the total *TCF7L2* expression remains unchanged, implying that the effect on RNA splicing is independent of regulation of transcription.

CHAPTER 3: METHODOLOGY

Figure 3.1 summarizes the workflow of the current study.

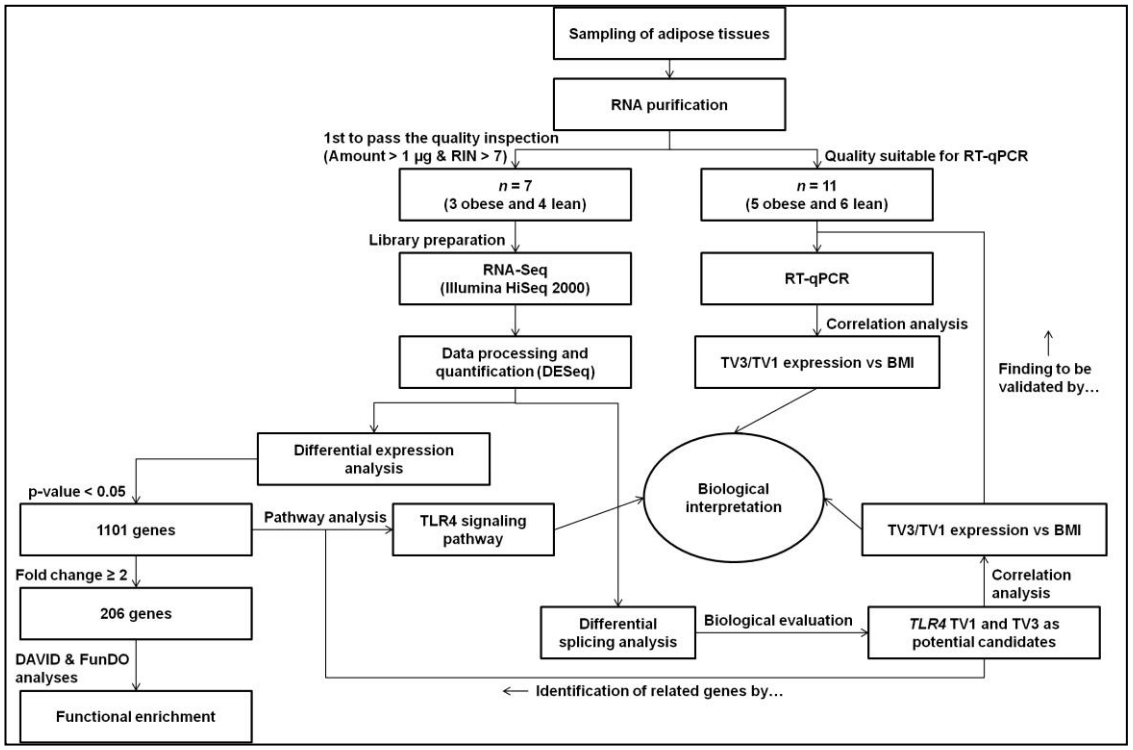


Figure 3.1: The workflow of this study. Abbreviation: DAVID for Database for Annotation Visualization and Integrated Discovery; FunDO for Functional Disease Ontology; TV for transcript variant

3.1 Sampling

Potential candidates were recruited from the patients who underwent laparoscopic abdominal surgery at the University of Malaya Medical Center (UMMC) between October 2012 and September 2013. Patients were excluded if they were suffering from diabetes mellitus, active infection or cancer and if they were either below 18 or above 60 years old. The BMI (weight [kg] divided by square of height [m]) was used to classify patients as obese or lean. Obesity was defined as BMI $\geq$ 30 kg/m^2, while leanness was BMI < 26 kg/m^2. A total of 18 patients (eight obese and 10 lean) were

recruited into the study. Their clinical characteristics are given in Table 3.1. The sampling protocol of this study was approved by the Medical Ethics Committee of the UMMC (reference number: 896.24). Written informed consent (Appendix A) was obtained from all patients, following a detailed explanation of the nature of the study.

Table 3.1: Clinical data of obese and lean patients

Analysis	Feature	Obese	Lean	p-value
RNA-Seq	*n* (male, female)	2, 1	1, 3	
	Age	39 ± 9	46 ± 4	NS
	Weight (kg)	105.0 ± 13.7	58.4 ± 2.7	*
	Height (m)	1.61 ± 0.03	1.59 ± 0.04	NS
	BMI (kg/m^2)	40.2 ± 4.8	23.0 ± 0.6	*
	Glucose (mmol/l)	5.5 ± 0.6	5.0 ± 0.4	NS
	Triglyceride (mmol/l)	1.3 ± 0.2	1.4 ± 0.3	NS
	Total cholesterol (mmol/l)	5.7 ± 1.2	5.3 ± 0.2	NS
	HDL (mmol/l)	1.3 ± 0.4	1.6 ± 0.2	NS
	LDL (mmol/l)	3.8 ± 0.9	3.1 ± 0.4	NS
RT-qPCR	*n* (male, female)	2, 3	4, 2	
	Age	46 ± 6	36 ± 7	NS
	Weight (kg)	107.0 ± 21.4	57.9 ± 4.1	*
	Height (m)	1.60 ± 0.06	1.63 ± 0.04	NS
	BMI (kg/m^2)	40.4 ± 5.6	21.8 ± 1.3	*
	Glucose (mmol/l)	5.4 ± 0.1	4.8 ± 0.3	NS
	Triglyceride (mmol/l)	1.8 ± 0.1	1.7 ± 0.4	NS
	Total cholesterol (mmol/l)	5.3 ± 0.5	4.9 ± 0.3	NS
	HDL (mmol/l)	1.1 ± 0.1	1.2 ± 0.1	NS
	LDL (mmol/l)	3.4 ± 0.4	2.9 ± 0.2	NS

Data are presented in mean ± standard error. Difference in mean between the obese and lean groups was analyzed using the two-sample t-test. Abbreviation: NS for not statistically significant; * for p-value < 0.05

3.2 RNA Preparation

During the laparoscopic surgery, the specimen of omental adipose tissue was excised under direct camera vision from the greater omentum by an experienced surgeon. The fat was then extracted through the sleeve of the 12-mm laparoscopic port without any

direct contact with the subcutaneous fat. Immediately, the tissue was cut and placed in Allprotect Tissue Reagent (Qiagen). With 0.1-0.15 g of stabilized adipose tissue as starting material, RNA purification was performed using RNeasy Lipid Tissue Mini Kit (Qiagen). To ensure the complete removal of contaminating DNA, on-column DNase digestion was carried out with RNase-free DNase (Qiagen). Quality inspection of each RNA sample was performed using the 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent) and Nanodrop 2000 (Thermo Scientific). Only the first seven RNA samples (three obese and four lean, Table 3.1) that passed the quality inspection (amount > 1 μg and RNA Integrity Number > 7) were further processed for RNA-Seq (Appendix B).

3.3 Library Preparation and RNA-Seq

The SureSelect Strand-specific RNA Library Prep Kit (Agilent) was used for the RNA-Seq library preparation. Briefly, 1 μg of total RNA was used for poly(A) RNA enrichment using oligo(dT) magnetic particles, followed by a chemical fragmentation of mRNA. The fragmented mRNA was then subjected to first-strand cDNA synthesis and second-strand cDNA synthesis with dUTP. This was followed by end repair, ligation of adaptors and finally, 14 cycles of PCR amplification with indexing primers and uracil-DNA glycosylase. The size distribution of the purified library was assessed with the 2100 Bioanalyzer DNA 1000 Assay (Agilent); a good library should have an expected size range of 200 to 600 bp and should not contain adaptor-dimers at the region of 100 to 150 bp. The libraries were sequenced by Ambry Genetics (USA). At a concentration of 6 pM each, seven indexed libraries were pooled and sequenced on three Illumina HiSeq 2000 lanes using 2 × 100 bp sequencing protocol.

With data generated from the sequencer, raw reads were mapped to a reference genome, National Center for Biotechnology information (NCBI) Build 37.2

(http://tophat.cbcb.umd.edu/igenomes.shtml) using Tophat2 version 2.0.4 (Kim et al., 2013). A GTF file containing the transcript location was downloaded from the Tohpat2 webpage and used to guide the Tophat2 mapper. The first run of Tophat2 was invoked with a `-G` option to build a transcriptome index using the GTF file. Transcriptome index from the first run was used to guide the Tophat2 mapper (with option `-transcriptome-index`) for the processing of subsequent samples. Tophat2 was invoked with two other option changes: `-b2-very-sensitive`, which maximizes the number of mappable reads and is equivalent to the `-very-sensitive` option for Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml); `-fr-firststrand`, which assumes that only the strand generated during first strand synthesis is sequenced, as assumed in the SureSelect Strand-specific RNA Library Prep Kit (Agilent). Other than that, all other Tophat2 options followed the default settings. The raw and processed data were deposited in Gene Expression Omnibus (GEO) under accession number: GSE55008.

3.4 Genespring Analysis

The BAM files generated from Tophat2 were imported into Genespring version 12.5 (Agilent, Appendix C) for further analysis. Prior to the differential expression and splicing analyses, the read data were filtered for duplicate reads (generated from PCR bias during the library preparation), average *Phred* score below 30 and nucleotide ambiguity above 0 %. Read count normalization was then carried out using DESeq (Anders & Huber, 2010).

Prior to performing differential expression analysis, genes with low-read coverage per condition (less than 50 raw reads in all samples in either one of the two phenotype classes) were filtered. The moderated t-test (Smyth, 2004) was performed for each gene

and only those genes with p-value < 0.05 were classified as DEGs. Because of the small sample size of the study, p-value adjustments that are usually done in multiple testing situations were not feasible, as very few or no genes at all could attain statistical significance at the nominal 5 % level. DEGs with fold change (FC) ≥ 2 were used for further functional analyses.

Differential splicing analysis was performed using the gene list generated after the removal of genes with low-read coverage per condition. To account for isoform read-mapping uncertainty, Genespring collects reads that cannot be mapped to any of the existing isoforms into a class called "unknown isoform", and estimation of the number of reads mapped to the isoform of interest under read-mapping uncertainty was done using the method of Li, Ruotti, Stewart, Thomson, and Dewey (2010). Isoform proportion was calculated by dividing the estimated reads mapped to each isoform with the total number of reads mapped to all isoforms of a gene. If the difference in proportion of the isoform (i.e. the splicing index, SI), was greater than 0.25 or less than -0.25 between the obese and lean groups, the gene was declared to be differentially spliced. The Mann-Whitney U test was used to determine whether the obese and lean groups differed significantly in their median proportions. The p-value of 0.1 was used as a cutoff for statistical significance because non-parametric methods are less powerful when the sample size is small (the smallest p-value attainable with $n = 7$ using the Mann-Whitney U test is 0.06).

3.5 Functional Enrichment Analyses

The Database for Annotation Visualization and Integrated Discovery (DAVID) version 6.7 (http://david.abcc.ncifcrf.gov/) was used to study biological functions enriched in the candidate DEGs of obesity (Dennis et al., 2003; Huang, Sherman, & Lempicki,

2009). The Functional Disease Ontology (FunDO) analysis (http://fundo.nubic.northwestern.edu/) was also performed on the candidate DEGs to investigate the association between disease and gene-expression changes, based on Disease Ontology and peer-reviewed evidence from GeneRIF (Osborne et al., 2009).

3.6 RT-qPCR

With 11 independent samples (five obese and six lean patients, Table 3.1), real-time quantitative reverse transcription PCR (RT-qPCR) was performed to validate the lead from differential splicing analysis of *TLR4*. Briefly, cDNA was synthesized from 300 ng of RNA using the High Capacity RNA-to-cDNA kit (Life Technologies). Each cDNA sample was diluted 1:10 for subsequent PCR analysis. The mRNA levels of *TLR4* transcript variants (TVs): TV1 and TV3, were measured in triplicate using the Rotor-Gene Q real-time PCR cycler (Qiagen) with inventoried Taqman Gene Expression assays (Life Technologies). For every patient, the expression of target TV was calculated using the formula: expression = $1/(\text{efficiency} + 1)^{Cq}$. Then, the ratio of TV3 to TV1 expression of each patient was computed. Reference genes were not included in this study because relative quantification was not performed. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) checklist (Bustin et al., 2009) for this study is given in Appendices D1 and D2.

3.7 Correlation Analysis

Using R version 2.15.2 (R Core Team, 2012) and the `ppcor` package (Kim & Yi, 2007), the association between TV3/TV1 ratio (obtained from RNA-Seq and RT-qPCR) and BMI was investigated. The partial Spearman correlation was used to control for the effects of gender and age. Data used in the partial correlation analysis are presented in Appendix E.

CHAPTER 4: RESULTS

4.1 Pre-analysis Data Processing

The total number of reads generated for each adipose tissue biopsy ranged from 24 to 41 million, with a median of 36.2 million and a mean of 35.4 ± 6 million (standard deviation). According to the key metrics extracted from Tophat2, 90.8 to 93.5 % of reads were aligned to the reference genome. For pre-analysis filtering using Genespring, between 18.2 to 29.6 % of duplicate reads and 1.8 to 1.9 % of reads with ambiguous nucleotide or low quality score (*Phred* score < 30) were removed.

4.2 Differential Expression Analysis

One thousand one hundred and one genes with significant difference (p-value < 0.05) in their expression between obese and lean adipose tissues were identified. Among these, 206 are differentially expressed by at least two-fold in the obese group. The overall gene-expression changes (expressed in FC) are between 23-fold downregulated and 15-fold upregulated (reference: lean group). Among the 206 DEGs with FC ≥ 2, the changes in their expression are skewed towards upregulation in the obese group (141 up and 65 down). The top 10 up- and down-regulated genes detected in obese adipose tissue are listed in Table 4.1.

Most of the microarray-based studies published to date used subcutaneous adipose tissue or cultured adipocytes or preadipocytes, which have important differences from omental adipose tissue of obese patients (Montague & O'Rahilly, 2000; Soukas, Socci, Saatkamp, Novelli, & Friedman, 2001). Since omental adipose tissues were used in this study, a direct comparison cannot be made between findings from this study and those from most previous microarray studies. Nonetheless, some concordances were observed

between gene-expression differences found with this RNA-Seq study and the few microarray studies published using omental adipose tissue. For example, in the present RNA-Seq and previous microarray studies, *leptin* (*LEP*), *secreted phosphoprotein 1* (*SPP1*), *tenomodulin* (*TNMD*), and *complement component 1, q subcomponent, A chain* (*C1QA*) are upregulated in obese omental adipose tissue while *complement component 6* (*C6*) and *cysteine-rich, angiogenic inducer, 61* (*CYR61*) are consistently downregulated (Baranova et al., 2005; del Pozo et al., 2011; Gómez-Ambrosi et al., 2004). In contrast, several genes identified in this study show a pattern of differential gene expression which is contradictory to previous microarray studies (Table 4.2).

A number of DEGs identified in this study have not been previously reported to be associated with obesity. For instance, *NFAM1* and *FPR3*, which encode for NFAT activating protein with ITAM motif 1 and formyl peptide receptor 3 respectively, might be functionally relevant to obesity because of their roles in leukocyte signaling and development.

Table 4.1: Top 10 up- and down-regulated genes in obese adipose tissue

Entrez ID	Gene Symbol	Description	p-value	FC	Lean group*	Obese group*
6696	*SPP1*	*Secreted phosphoprotein 1*	7×10^{-3}	14.9	96.3 ± 39.6	1436.2 ± 2656.2
64102	*TNMD*	*Tenomodulin*	1×10^{-2}	9.6	138.4 ± 124.0	1323.1 ± 1226.7
84966	*IGSF21*	*Immunoglobin superfamily, member 21*	3×10^{-3}	6.4	10.2 ± 5.9	65.1 ± 19.5
6362	*CCL18*	*Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)*	2×10^{-2}	6.1	36.3 ± 59.0	221.1 ± 49.5
3223	*HOXC6*	*Homeobox C6*	1×10^{-2}	5.5	41.2 ± 16.2	225.8 ± 165.2
9242	*MSC*	*Musculin*	5×10^{-2}	5.2	51.6 ± 34.7	268.2 ± 343.6
158800	*RHOXF1*	*Rhox homeobox family, member 1*	8×10^{-3}	5.1	14.2 ± 6.6	71.9 ± 49.9
377007	*KLHL30*	*Kelch-like family member 30*	4×10^{-2}	5.0	41.6 ± 36.9	209.4 ± 164.4
340443	*RPS5P5*	*Ribosomal protein S5 pseudogene 5*	4×10^{-2}	5.0	49.3 ± 50.1	247.3 ± 195.7
3952	*LEP*	*Leptin*	5×10^{-3}	4.9	4031.8 ± 1714.4	19755.5 ± 9888.5
761	*CA3*	*Carbonic anhydrase III, muscle specific*	3×10^{-3}	-23.0	2837.8 ± 2554.7	123.5 ± 80.8
579	*NKX3-2*	*NK3 homeobox 2*	9×10^{-3}	-14.7	111.6 ± 19.7	7.6 ± 13.8
80763	*C12orf39*	*Chromosome 12 open reading frame 39*	2×10^{-2}	-13.2	512.3 ± 620.8	38.9 ± 15.5
6096	*RORB*	*RAR-related orphan receptor B*	5×10^{-3}	-11.1	303.0 ± 179.3	27.3 ± 22.1
7138	*TNNT1*	*Troponin T type 1 (skeletal, slow)*	5×10^{-2}	-10.2	238.2 ± 238.5	23.3 ± 54.4
84675	*TRIM55*	*Tripartite motif containing 55*	1×10^{-2}	-6.5	138.1 ± 133.6	21.2 ± 4.1
3491	*CYR61*	*Cysteine-rich, angiogenic inducer, 61*	4×10^{-2}	-5.6	3726.5 ± 4551.1	660.5 ± 46.2
5744	*PTHLH*	*Parathyroid hormone-like hormone*	3×10^{-2}	-4.7	152.3 ± 36.7	32.7 ± 31.9
8913	*CACNA1G*	*Calcium channel, voltage-dependent, T type, alpha 1G subunit*	3×10^{-2}	-4.5	128.6 ± 67.3	28.8 ± 29.1
125336	*LOXHD1*	*Lipoxygenase homology domains 1*	2×10^{-2}	-4.2	328.7 ± 266.4	78.0 ± 8.3

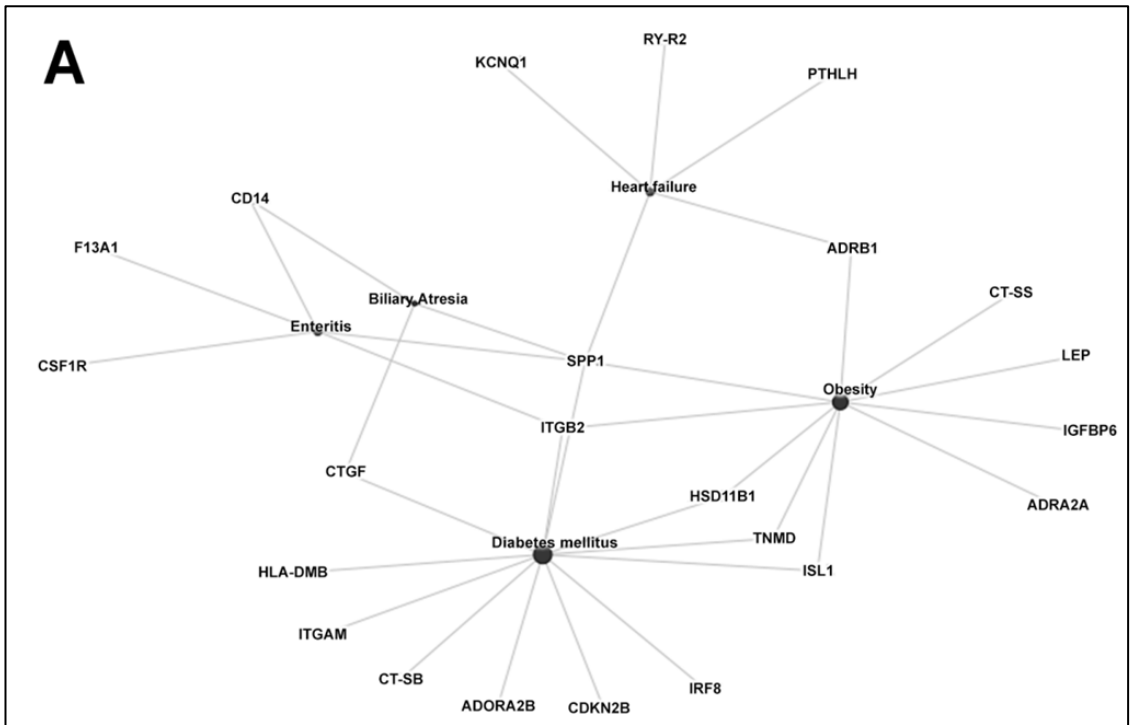
* Normalized read count data are presented in mean ± standard deviation

Table 4.2: Discordant findings of differential gene expression between studies using the RNA-Seq (current study) and the microarray approach

Gene symbol	Description	RNA-Seq	Micro-array	Reference
NPR3	*Natriuretic peptide receptor C/guanylate cyclase C*	Up	Down	Gómez-Ambrosi et al., 2004
CDKN2B	*Cyclin-dependent kinase inhibitor 2B*	Up	Down	Gómez-Ambrosi et al., 2004
DNER	*Delta/notch-like EGF repeat containing*	Down	Up	Baranova et al., 2005
ADRB1	*Adrenoceptor beta 1*	Up	Down	Baranova et al., 2005

4.3 FunDO Analysis

To check the veracity of the RNA-Seq analysis, candidate DEGs were categorized into different disease ontology (DO) terms using FunDO. Five disease nodes are evident (Figure 4.1A), with the size of each node proportional to the number of its constituent genes. As expected, obesity (corrected-p value = 10^{-6}) is the most over-represented DO term (Figure 4.1B), supporting the validity of the sample inclusion criteria and bioinformatic approach of this study in the biological context. Diabetes mellitus (corrected p-value = 2×10^{-5}), which has 12 hit genes and shares five of them (*SPP1*, *integrin beta 2* [*ITGB2*], *hydroxysteroid (11-beta) dehydrogenase 1* [*HSD11B1*], *TNMD*, and *ISL LIM homeobox* 1 [*ISL1*]) with obesity, is ranked second. This highlights the close link between the pathophysiology of the two diseases. Despite being statistically significant, the enrichment of biliary atresia (rank 3), enteritis (rank 4) and heart failure (rank 5) might not be biologically meaningful, as demonstrated by their relatively small disease nodes in comparison to those of obesity and diabetes mellitus.



B

Rank	DO Term	Hit Genes	Corrected p-value
1	Obesity	10	1×10^{-6}
2	Diabetes mellitus	12	2×10^{-5}
3	Biliary Atresia	3	4×10^{-4}
4	Enteritis	5	4×10^{-3}
5	Heart failure	5	5×10^{-3}

Figure 4.1: Correlations between candidate differentially expressed genes and the top five diseases identified by FunDO. (A) The map of the top five diseases over-represented with the genes showing dysregulated expression in diseased adipose tissue. (B) The number of hit genes and Bonferroni-corrected p-values of the top five enriched DO terms.

4.4 DAVID Analysis

The basis of enrichment analysis is that if a biological process is unusual in a given study, the co-functioning genes should be more likely (enriched) to be picked up as a relevant group by the high-throughput screening technologies. In this study, 13 clusters (Appendix F) were identified with enrichment scores passing the cutoff threshold of 1.3, which corresponds to a p-value of 0.05 (Huang et al., 2009). Only functions that are significantly enriched, defined as satisfying p-value < 0.05 and false discovery rate (FDR) at 10 %, are reported here. In general, the result suggests that the dysregulated genes in omental adipose tissue of obese patients are involved in multiple functions including cell adhesion, response to stress, inflammatory response, chemotaxis, leukocyte adhesion, insulin-like growth factor (IGF) binding, and regulation of adenylate cyclase activity.

Augmented leukocyte infiltration is well-documented in diseased adipose tissue of obesity (Altintas et al., 2011; Weisberg et al., 2003; Yang et al., 2010). In line with this observation, cell adhesion (cluster 1), chemotaxis (cluster 3) and leukocyte adhesion (cluster 10) were enriched in the candidate DEGs in obesity. A significant portion of genes mapped to these functions, including members of the integrin (transmembrane receptors) family (*ITGB2* and *integrin alpha M* [*ITGAM*]) are upregulated in obesity. Several genes enriched in the cell adhesion function, namely *neural cell adhesion molecule 2* (*NCAM2*), *roundabout, axon guidance receptor, homolog 2 (Drosophila)* (*ROBO2*), *protocadherin beta 7* (*PCDHB7*), and *SCO-spondin homolog (Bos taurus)* (*SSPO*), are also associated with neural development; some of the genes involved in neural development have previously been implicated in the adipogenic differentiation (Billon et al., 2010; Yang et al., 2011).

The most over- and under-expressed genes, *SPP1* and *carbonic anhydrase III* (*CA3*), are mapped to the response to stress cluster (cluster 2). The majority of genes involved in the inflammatory response (cluster 2), which includes those encoding members of complement systems (*C1QA*, *complement component 1, q subcomponent, B chain* [*C1QB*], complement component 1, q subcomponent, C chain [*C1QC*], and *complement component 3a receptor 1* [*C3AR1*]) and inflammatory cytokines (*CCL18* and *SPP1*), is overexpressed in obese adipose tissue. This signifies the positive regulation of immune response in obesity. Interestingly, both inflammatory response and response to stress are organized in the same cluster, whereby all the genes mapped to the former are also involved in the latter function. This reflects the frequent occurrence of stress-induced inflammation in obese adipose tissue.

Genes participating in IGF binding (cluster 7) are dysregulated in obese adipose tissue; these include those encoding members of IGF binding proteins (*insulin-like growth factor binding protein 6* [*IGFBP6*]) and Connective tissue growth factor, Cystein rich protein, and Nephroblastoma overexpressed gene (CCN) family proteins (*CYR61*/*CCN1*, *CTGF*/*CCN2* and *WISP2*/*CCN5*) that have been reported to be involved in the process of adipogenesis (Ng et al., 2008; Schutze, Noth, Schneidereit, Hendrich, & Jakob, 2005). While the downregulation of *CYR61* and *connective tissue growth factor* (*CTGF*) is in line with the promotion of adipogenesis, the upregulation of *WNT1 inducible signaling pathway protein 2* (*WISP2*) and downregulation of *IGFBP6* implies impairment of adipogenesis in obese adipose tissue; the impairment of adipogenesis has been shown to mediate adipocyte hypertrophy and ectopic lipid accumulation in obesity (Gustafson et al., 2009).

A number of candidate DEGs are enriched in the regulation of adenylate cyclase activity (cluster 13) that has been linked to the lipolytic defects in obese adipose tissue (Martin et al., 1990). The genes for both adrenoceptor alpha 2A (ADRA2A) and adrenoceptor beta 1 (ADRB1) are upregulated in obese adipose tissue. It has been reported that ADRB1 promotes while ADRA2A inhibits lipolysis in adipose tissue through the promotion and inhibition of cyclic adenosine monophosphate (cAMP) formation in catecholamine signaling (Alfredo Martínez et al., 2007; Stich et al., 2000). The paradoxical upregulation of *ADRB1* seems to contradict the idea of diminished lipolysis in obesity.

4.5 Differential Splicing Analysis

Forty-nine differentially spliced genes (DSGs) were found in obesity (p-value < 0.1, absolute SI > 0.25, Appendix G). DAVID functional annotation chart was used to identify enriched functions in these 49 DSGs; the top enriched function is immune response-activating signal transduction (p-value < 0.01, FDR = 13 %). One of the DSGs mapped to this function, *TLR4*, is of particular interest because of its widely reported relevance to obesity-associated inflammation (Ahmad et al., 2012; Kim, Gu, Lee, Joh, & Kim, 2012; Shi et al., 2006). In addition, *TLR4* was chosen because functions of its protein isoforms, generated through alternative splicing, have been described (Coats, Pham, Bainbridge, Reife, & Darveau, 2005); these information were used to facilitate the biological interpretation of the RNA-Seq data.

Using the Genome Browser in Genespring, a difference in expression level for the individual exons in *TLR4* was noticed (Figure 4.2A); the low level of reads that mapped to the intron regions are probably derived from pre-mRNA. According to the Refseq annotation, all three TVs, TV1 (NM_138554), TV3 (NM_003266), TV4 (NM_138557) share Exon I and IV but the additional exon II is present exclusively in TV3. The

proportion of TV3 in *TLR4* expression (TV3/sum of all TVs of *TLR4*) is decreased significantly in obesity (SI = -0.29, p-value = 0.06) (Figure 4.2B). Interestingly, the proportion of the canonical isoform TV1 is significantly higher in obese adipose tissue (p-value = 0.06), with SI marginally below the specified threshold (SI = 0.21). TV4 is generally expressed in very low abundance compared to the other two isoforms and the difference in its proportion between obese and lean adipose tissues is not statistically significant (p-value = 0.22). Hence, TV4 was excluded from further analysis. The percentage of the unknown isoform is 5.0 ± 2.8 % (1 standard deviation).

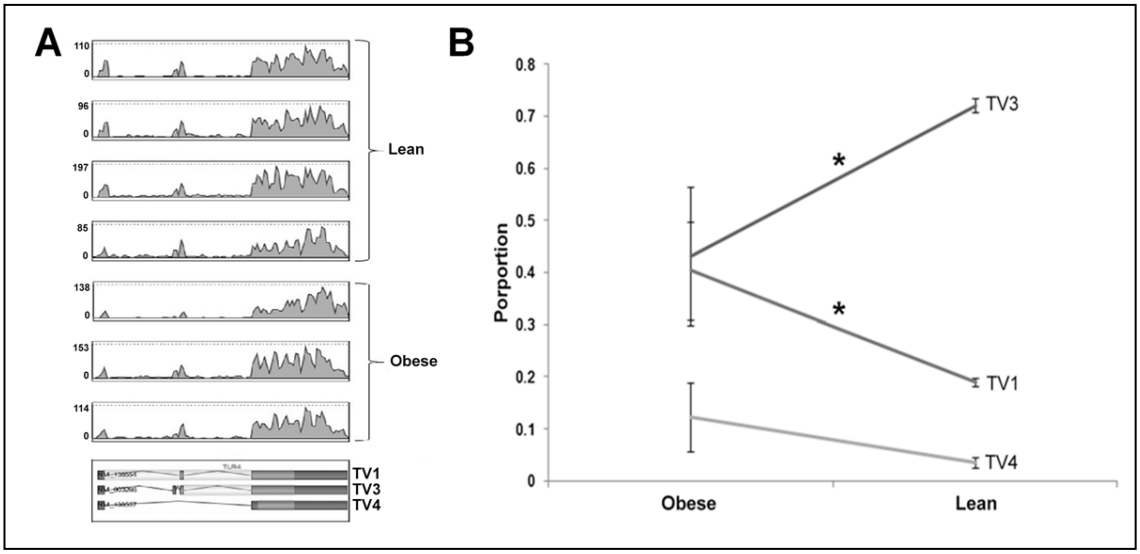


Figure 4.2: Differential splicing of *TLR4* transcript. (A) Reads mapped to the reference (Refseq annotation) for *TLR4*. (B) Proportion of isoforms in *TLR4* expression across obese and lean conditions. Data are expressed in mean ± standard error. Abbreviation: * for p-value = 0.06

Both isoforms under investigation have been reported to play antagonistic roles in TLR4 signaling, with TV3 attenuating while TV1 promoting the signaling pathway (Coats et al., 2005). To quantify the degree of inhibition/activation of TLR4 signaling, the ratio of TV3 to TV1 expression of each patient was computed. Using partial Spearman

correlation analysis, a strong negative association between TV3/TV1 ratio and BMI was detected (r = -0.81, p-value = 0.02; Figure 4.3A). RT-qPCR analysis with an independent, larger cohort of samples (five obese and six lean) further supports this finding (r = -0.82, p-value = 0.0002; Figure 4.3B).

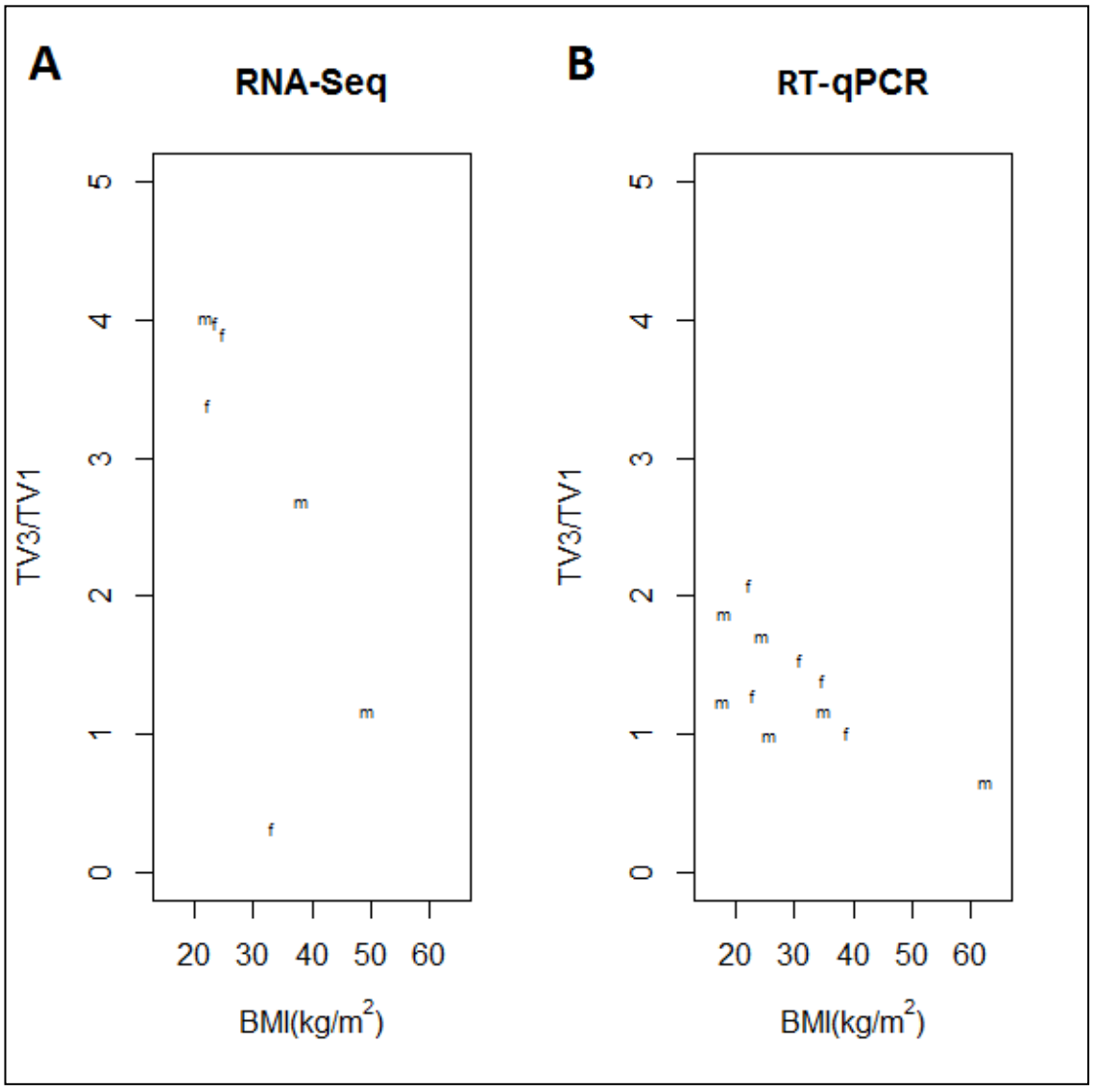


Figure 4.3: Association of TV3/TV1 ratio with BMI using data generated from (A) RNA-Seq (*n* = 7) and (B) RT-qPCR (*n* = 11). Abbreviation: m for male; f for female

CHAPTER 5: DISCUSSION

5.1 Discrepancy between Results Generated from RNA-Seq and Microarray Approaches

Many of the gene-expression changes observed between obese and lean omental adipose tissues in the current study are similar to those reported previously in microarray studies. Nonetheless, some discrepancies were also observed. For example, the upregulation of *NPR3* in this study is contradictory to the previous findings by Gómez-Ambrosi et al. (2004). A potential cause of the discrepancy between RNA-Seq data from this study and previously reported transcriptome profiles is the intrinsic limitation of microarray technology, such as high background noise of hybridization that limits the precision of microarray expression measurements (Wang et al., 2009). Interestingly, a transcriptomic analysis of mouse adipose tissues by microarray did demonstrate the overexpression of *NPR3* gene in obesity (Morton et al., 2011), as observed in the current study. In that study, the authors discussed the possible outcome from the upregulation of *NPR3* in obesity; *NPR3* encodes the signaling-deficient natriuretic peptide receptor C that functions mainly as a clearance receptor by sequestering atrial natriuretic peptide (a lipolytic ligand). Therefore, the upregulation of *NPR3* is likely to contribute to obesity by abrogating the lipolytic effect of atrial natriuretic peptide.

5.2 Enrichment Analysis Highlights the Likelihood of Stress-induced Inflammation in Obese Adipose Tissue

Obesity is associated with elevated stress level in adipose tissue (Bondia-Pons et al., 2012; Cnop et al., 2012; Trayhurn, 2013). In line with that finding, the DEGs identified in obesity are enriched in response to stress. In particular, the most up- and down-

regulated genes in obese adipose tissue, *SPP1* and *CA3* respectively, are among the DEGs mapped to this function. The product encoded by *SPP1* (secreted phosphoprotein 1 or more commonly known as osteopontin) is a multifunctional adipokine, known to regulate processes like adipogenesis and inflammation in obesity (Zeyda et al., 2011). A gene knockout study suggests that SPP1 is also a promoter of oxidative stress (Irita et al., 2011). In addition, increased secretion of this protein has been observed in dendritic cells cultured under hypoxic conditions (Yang et al., 2009). Thus, the upregulation of *SPP1* reflects the hypoxic, oxidative and pro-inflammatory microenvironment in adipose tissue of obese humans. On the other hand, *CA3* is one of the most abundant transcripts found in human (Gabrielsson, Carlsson, & Carlsson, 2000) and rodent (Bolduc et al., 2004) adipose tissues and has been speculated to play a role in the protection against reactive oxygen species generated in the metabolically active fat tissues (Räisänen et al., 1999). The overwhelming downregulation of *CA3* observed in this study is consistent with the finding of obesity-associated downregulation of *CA3* in adipose tissue of genetic/diet-induced models of obesity (Stanton, Ponte, Coleman, & Snyder, 1991). Underexpression of *CA3* implies the inability of obese adipose tissue to neutralize oxidative stress.

It has been suggested that stress is a trigger of immune dysregulation seen in obese adipose tissue (Bondia-Pons et al., 2012; Cnop et al., 2012; Trayhurn, 2013). In this study, the enrichment analysis supports this hypothesis as all 13 DEGs mapped to the inflammatory response are found to be involved in response to stress. Of these 13 DEGs, *C1QA*, *C1QB*, *C1QC* and *C6* are found to be enriched in complement activation, classical pathway (cluster 2). Interestingly, oxidative stress has been reported to initiate complement activation and result in immune dysregulation and organ injury (Collard, Lekowski, Jordan, Agah, & Stahl, 1999). Except for *C6*, all members of the classical

complement pathway are upregulated in obese patients. Although the underexpression of *C6* has been described in obese adipose tissue (del Pozo et al., 2011), its association with oxidative stress, complement activation and inflammatory response in obesity remains a subject for further investigation. Furthermore, *CD14 molecule* (*CD14*), an important member of TLR4 signaling, was detected to be upregulated in obesity. A recent study by Coope et al. (2012) has shown that *CD14* plays an important role in mediating unfolded protein response induced by LPS. The changes observed in *CD14* expression and *TLR4* splicing pattern in this study are relevant to inflammatory and ER stress responses observed in obesity (Coope et al., 2012; Pierre et al., 2013).

Genes involved in processes of chemotaxis and leukocyte adhesion are also significantly represented, which is consistent with the finding that obese adipose tissue is associated with augmented leukocyte infiltration. Upregulated genes included in these groups are those encoding for ITGB2, ITGAM, CCL18, FPR3, C3AR1, CD209, spleen tyrosine kinase (SYK), and platelet-activating factor receptor (PTAFR). Collectively, the upregulation of these genes in obese adipose tissue may enhance the chemotactic/adhesive activity of a wide array of immune cells, including but not limited to monocytes/macrophages, T cells, and neutrophils (information retrieved from Gene Ontology, Refseq, and Uniprot).

As for IGF binding and adenylate cyclase activity, the activity of these functions (increase or decrease) could not be predicted based on the differential expression patterns (up- or down-regulation) of enriched genes. Further studies will be needed to demonstrate conclusively that these functions are indeed associated with obesity in the Malaysian population.

5.3 Differential Splicing Analysis Uncovers the Potential Involvement of TLR4 Transcript Variants in Obesity-related Inflammation of Adipose Tissue

Evidence from animal studies suggests that translocation of bacterial LPS across the gut may initiate obesity-associated low-grade inflammation (Cani et al., 2007). This unusual phenomenon has also been reported in obese and diabetic humans (Hawkesworth et al., 2013). TLR4 plays an important role in innate immunity because it detects LPS from Gram-negative bacteria, which leads to the production of pro-inflammatory cytokines. Indeed, it has been shown that LPS can induce adipose inflammation and insulin resistance in humans (Mehta et al., 2010). Therefore, changes in TLR4 signaling are highly relevant in obesity and its co-morbidities.

TLR4 signaling is tightly regulated to prevent over-activation of immune response (Gray et al., 2010). One of the mechanisms to downregulate TLR4 signaling involves the synthesis of inhibitory isoforms through alternative splicing (Iwami et al., 2000; Jaresová et al., 2007). Alteration of the kinetics of *TLR4* splice variants expression has been reported to be associated with diseases like cystic fibrosis (Jaresová et al., 2007). Differential splicing of *TLR4* has not been reported previously in the disease-state adipose tissue in obesity.

TLR4 has three transcript isoforms. TV1 encodes protein isoform A, the canonical TLR4 bearing an N-terminal signal sequence which is absent in isoforms C and D, encoded by TV3 and TV4, respectively (Coats et al., 2005). In the present study, a significant decrease in TV3 proportion was observed in the obese group as compared to their lean counterparts. There are two hypotheses describing the function of this isoform of TLR4. According to Iwami et al. (2000), human *TLR4* exon II (which is specific to TV3) has an in-frame stop codon, which might give rise to a small protein isoform

without transmembrane and intracellular domains, analogous with the soluble mouse TLR4 protein (smTLR4); they have shown that smTLR4 is able to attenuate LPS-induced TLR4 signaling. On the other hand, Coats et al. (2005) found that the absence of the signal sequence at the N terminus renders the isoform C non-functional as it fails to associate with LY96, whereby this association confers responsiveness to LPS. In addition, through molecular cloning and transformation, they also demonstrated that only the protein encoded by TV1, but not by TV3 or TV4, illicits NF-κB activity in response to LPS in the HEK293 cell line. Both hypotheses support the inhibitory role of TV3 in TLR4 signaling. The altered kinetics in the alternative splicing of *TLR4* gene, as observed in the decrease in inhibitory TV3 and the increase in canonical TV1 proportion, signifies the up-regulation of TLR4 signaling in obesity.

To quantify these changes observed in obesity in a biologically meaningful way, the ratio of TV3 to TV1 expression was calculated and correlated with the BMI. There is a significant association between the TV3/TV1 ratio and BMI of the patients ($r = -0.81$, p-value = 0.02). Partial correlation was used to adjust for the effects of gender and age because TLR4 signaling can be affected by sex difference and aging (Renshaw et al., 2002; Roberts, Moussawi, & Huber, 2013). Collectively, these findings indicate that, as a person experiences weight gain or obesity, the kinetics of adipose splicing pattern for *TLR4* changes in favor of higher activation and lowering inhibition of TLR4 signaling. Further experimental validation using methods like protein immunoassay and RNA silencing is necessary for pursuing this line of thought.

To uncover more supporting evidence for the upregulation of TLR4 signaling in obesity, a pathway analysis in Genespring was performed (Appendix H). By mapping 1101 genes that are significantly different in expression level between obese and lean

groups (p-value < 0.05) to TLR signaling pathway (http://www.wikipathways.org/index.php/Pathway:WP75), five genes, namely *mitogen-activated protein kinase 3* (*MAPK3*), *Fas (TNFRSF6)-associated via death domain* (*FADD*), *lymphocyte Antigen 96* (*LY96*), *mitogen-activated protein kinase kinase 1* (*MAP2K1*), and *CD14* (in ascending order of their respective FC; as indicated by asterisks in Appendix H), were identified to be involved in the TLR4 signaling pathway. Notably, LY96 and CD14 are important members of this signaling pathway, which are known to be associated with TLR4 on the cell surface as co-receptors for bacterial LPS. On the other hand, FADD, MAPK3 and MAP2K1 are downstream regulators of the apoptosis pathway and the production of inflammatory cytokines in TLR4 signaling (Appendix H). Interestingly, gene-expression analysis revealed that all five of them are overexpressed in obesity (ranging from 1.2 to 2.5-fold). Along with the differential splicing of the *TLR4* gene, these findings support the upregulation of TLR4 signaling in obesity, which in turn might contribute to the progression of obesity-related inflammation and complications.

The result of the correlation analysis shows that the TV3/TV1 ratios obtained from RNA-Seq is much higher than the ratios from RT-qPCR, which is the gold standard in gene-expression quantification (Bustin et al., 2009). This is not entirely unexpected as taking ratio from isoform read counts (RNA-Seq) will generally be different compared to taking ratio from direct mRNA quantification (RT-qPCR), since the former are subjected to read-mapping uncertainty.

5.4 Limitations

For the interpretation of results, the subject matter constraints the study to be observational in nature. Although direct results from observational studies are known to

be problematic for causal inference (Glass, Goodman, Hernán, & Samet, 2013), they are useful for generating hypotheses to be tested. Experimental validation then provides the evidence for interpretation in the subject matter context.

Cost considerations and difficulty in obtaining sufficient omental adipose tissue biopsies mean that the study had to be cross-sectional and limited to a small sample size. The latter could result in low statistical power in differential expression analyses, particularly when intra-group variation of gene-expression level is large. In this study, optimization of statistical signals was endeavored using a set of carefully thought-out inclusion and exclusion criteria. The finding of significant enrichment of obesity-related DO terms with the set of mined candidate DEGs in the FunDO analysis suggests that the current results are unlikely to be an outcome of chance.

CHAPTER 6: CONCLUSION

This study has revealed an aspect of gene-disease association that has not been adequately investigated before in obesity. Apart from aberrant gene expression associated with perturbation in functions like stress and immune responses, obese adipose tissue was found to have a *TLR4* splicing pattern that is distinctive from the lean counterpart. TLR4 signaling is known to participate in the activation of immune response. In obesity and insulin resistance, most, if not all, of alternatively spliced genes reported are involved in lipid metabolism and preadipocyte proliferation/differentiation (Kaminska & Pihlajamäki, 2013). The finding from this study suggests that alternative splicing may be a key player in the development of obesity-associated inflammation in adipose tissue. Given the current paucity of available anti-obesity drugs, further investigation of the role of mRNA isoforms in adipose immune dysregulation may provide a new avenue for the discovery of new molecular therapeutic targets.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Two manuscripts drafted based on the findings of this study have been submitted to:

(i) International Journal of Genomics (formerly known as Comparative and Functional Genomics)

305732.v1 (Research Article)

Title	Adipose tissue TLR4 splicing pattern is associated with body mass index: A case study in the Malaysian population
Journal	International Journal of Genomics
Issue	Regular
Additional Files	Cover Letter
Manuscript Number	305732 (Research Article)
Submitted On	2015-02-26
Author(s)	Hien Fuh Ng, Tsung Fei Khang, Kin Fah Chin, Zhi Hui Li, Kok-Gan Chan, Siew Woh Choo, **Yun Fong Ngeow**
Editor	Jacques Camonis
Status	Under Review
Supplementary Materials	View Supplementary Materials

(ii) Genome

Manuscripts with Decisions

Manuscript ID	Manuscript Title	Date Submitted	Date Decisioned	Status	Actions
gen-2015-0028	The mRNA expression of soluble urokinase plasminogen activator surface receptor in human adipose tissue is positively correlated with body mass index [View Submission]	25-Feb-2015	23-Apr-2015	EA: Gordon, Brian • Major Revisions (23-Apr-2015) • Due on: 04-Jun-2015 view decision letter	create a revision Copyright Form submitted (27-Feb-2015) - view

top

Papers published during the candidature period:

Epidemiol. Infect. (2013), **141**, 1481 1487. © Cambridge University Press 2013
doi:10.1017/S0950268813000265

Tuberculosis in captive Asian elephants (*Elephas maximus*) in Peninsular Malaysia

B. L. ONG[1]*, Y. F. NGEOW[2]*, M. F. A. ABDUL RAZAK[3], Y. YAKUBU[1], Z. ZAKARIA[1], A. R. MUTALIB[1], L. HASSAN[1], H. F. NG[2] AND K. VERASAHIB[4]

genomeA
Journals.ASM.org

First Whole-Genome Sequence of *Mycobacterium iranicum*, a Newly Reported Mycobacterial Species

Joon Liang Tan,[a,c] Hien Fuh Ng,[a] Wei Yee Wee,[b,c] Mia Yang Ang,[b,c] Guat Jah Wong,[b,c] Yun Fong Ngeow,[a] Siew Woh Choo[b,c]

SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
COMPUTATIONAL BIOLOGY AND BIOINFORMATICS
BACTERIA

Received
13 August 2014

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4 November 2014

Comparative genomic analysis of *Mycobacterium iranicum* UM_TJL against representative mycobacterial species suggests its environmental origin

Joon Liang Tan[1,3], Yun Fong Ngeow[1], Wei Yee Wee[2,3], Guat Jah Wong[2,3], Hien Fuh Ng[1] & Siew Woh Choo[2,3]

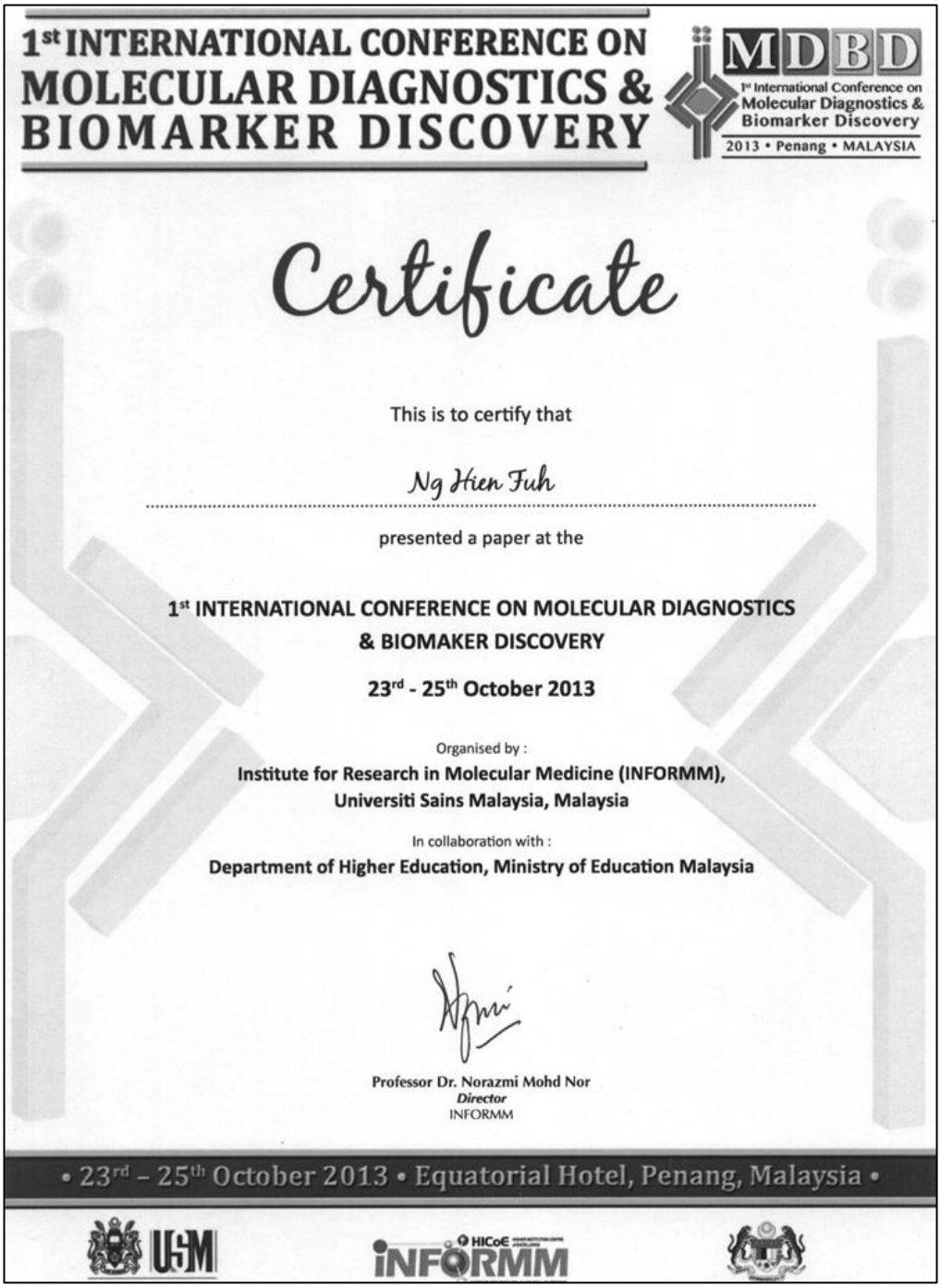
PLOS | ONE

RESEARCH ARTICLE

Identification of New Genomospecies in the *Mycobacterium terrae* Complex

Yun Fong Ngeow[1]*, Yan Ling Wong[1], Joon Liang Tan[1], Kar Wai Hong[2], Hien Fuh Ng[1¤a], Bee Lee Ong[3¤b], Kok Gan Chan[2]

The paper presented at the first International Conference on Molecular Diagnostics and Biomarker Discover on 23-25 October 2013:



1st INTERNATIONAL CONFERENCE ON MOLECULAR DIAGNOSTICS & BIOMARKER DISCOVERY

MDBD
1st International Conference on Molecular Diagnostics & Biomarker Discovery
2013 • Penang • MALAYSIA

Certificate

This is to certify that

Ng Hien Fuh

presented a paper at the

1st INTERNATIONAL CONFERENCE ON MOLECULAR DIAGNOSTICS & BIOMAKER DISCOVERY

23rd - 25th October 2013

Organised by :
Institute for Research in Molecular Medicine (INFORMM),
Universiti Sains Malaysia, Malaysia

In collaboration with :
Department of Higher Education, Ministry of Education Malaysia

Professor Dr. Norazmi Mohd Nor
Director
INFORMM

• 23rd – 25th October 2013 • Equatorial Hotel, Penang, Malaysia •

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APPENDIX A

Consent form (in English)

UNIVERSITY MALAYA MEDICAL CENTRE

CONSENT BY PATIENT FOR CLINICAL RESEARCH

I, ... *(Name of Patient)*

Identity Card No.....

of .....
(Address)

hereby agree to take part in the clinical research (clinical study/questionnaire study/drug trial) specified below:

Title of Study: Systems Analysis of Adiposity in Obesity

the nature and purpose of which has been explained to me by Dr. .....
(Name & Designation of Doctor)

and interpreted by .....
(Name & Designation of Interpreter)

to the best of his/her ability in ..... language/dialect.

I have been told about the nature of the clinical research in terms of methodology, possible adverse effects and complications (as per patient information sheet). After knowing and understanding all the possible advantages and disadvantages of this clinical research, I voluntarily consent of my own free will to participate in the clinical research specified above.

I understand that I can withdraw from this clinical research at any time without assigning any reason whatsoever and in such a situation shall not be denied the benefits of usual treatment by the attending doctors.

Date: ..... Signature or Thumbprint .....
(Patient)

I agree to have my left over tissues stored for use in future studies.....

I do not agree to have my left over tissues stored for use in future studies.....

IN THE PRESENCE OF

Name .....)
)
Identity Card No. .....) Signature

(Witness for Signature of Patient)

Designation .....)

I confirm that I have explained to the patient the nature and purpose of the above-mentioned clinical research.

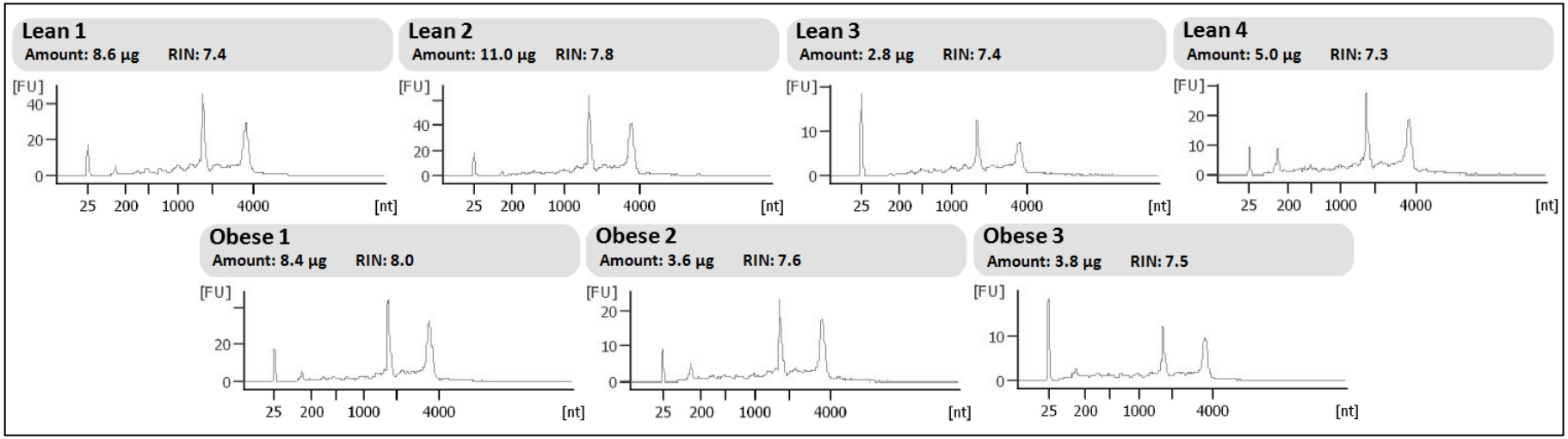
Date ..... Signature .....
(Attending Doctor)

CONSENT BY PATIENT FOR CLINICAL RESEARCH

R.N.
Name
Sex
Age
Unit

APPENDIX B

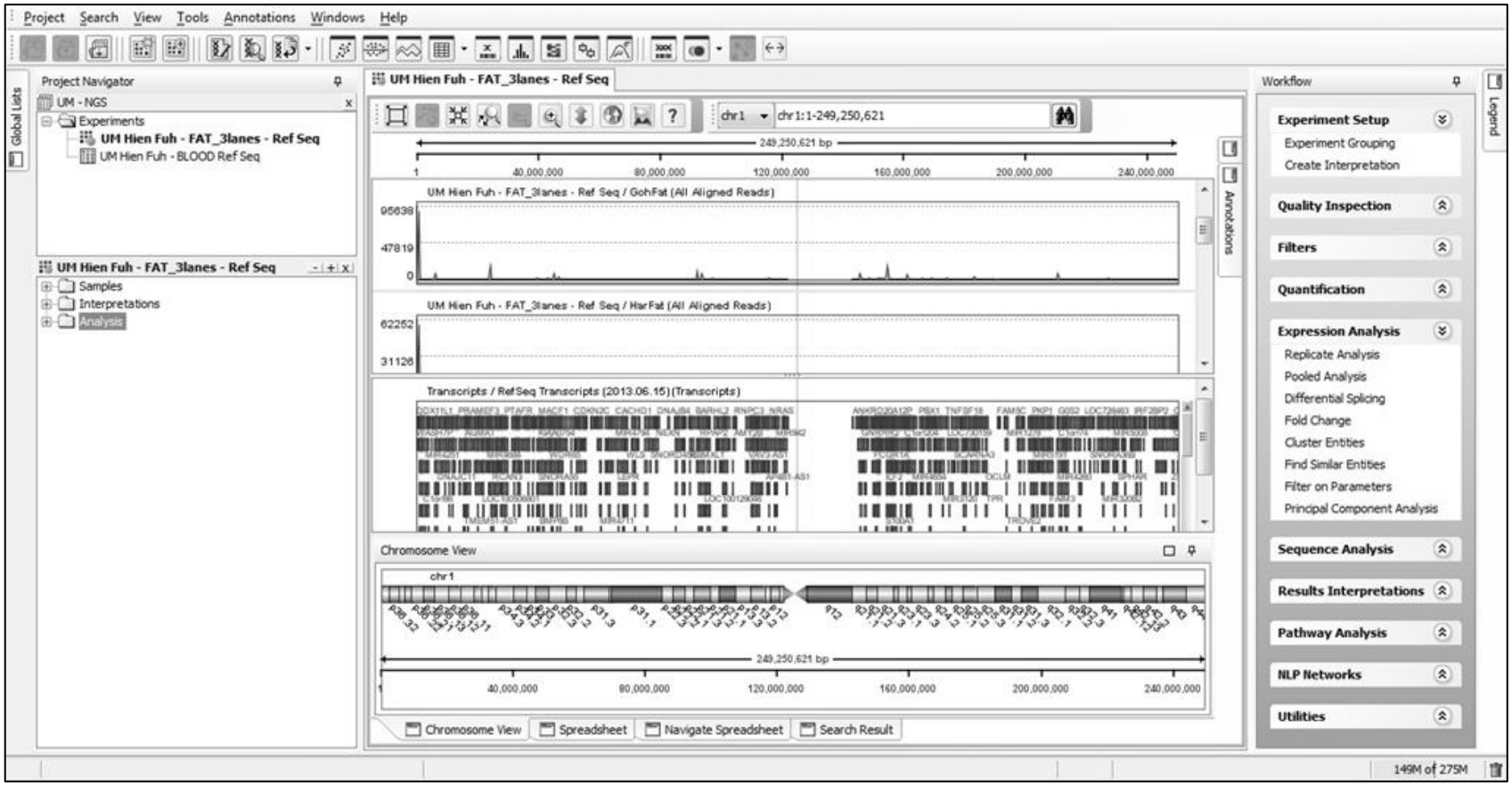
Quality and quantity of RNA samples used for RNA-Seq



Abbreviation: RIN for RNA integrity number

APPENDIX C

Graphic user interface of the Genespring 12.5



APPENDIX D1

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) checklist of this study

Category	Essential item to check	Description
Experimental design	Definition of experimental and control groups	Obesity was defined as BMI ≥ 30 kg/m^2, while leanness was BMI < 26 kg/m^2
	Number within each group	Five obese and six lean patients
Sample	Description	Omental adopose tissue biopsies extracted through laparoscopic abdominal surgeries
	Microdissection or macrodissection	Macrodissection
	Processing procedure	The tissue was immediately cut into small pieces and placed into the Allprotect Tissue Reagent (Qiagen)
	If frozen - how and how quickly?	Not applicable
	If fixed - with what, how quickly?	Not applicable
	Sample storage conditions and duration (especially for FFPE samples)	If RNA extraction was not performed on the same day, the stabilized tissue would be stored at -20 °C
Nucleic acid extraction	Procedure and/or instrumentation	Not applicable
	Name of kit and details of any modifications	RNA purification was performed using RNeasy Lipid Tissue Mini Kit (Qiagen)
	Details of DNase or RNAse treatment	During the extraction, on-column DNase digestion was carried out with RNase-free DNase set (Qiagen)
	Contamination assessment (DNA or RNA)	No amplification was observed in no-RT controls
	Nucleic acid quantification/instrument and method	Quantification done using Nanodrop 2000 (Thermo Scientific)
	RNA integrity method/instrument	RNA integrity inspection was performed using 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent)
	RIN/RQI or Cq of 3' and 5' transcripts	RNA samples with RIN of 6 or above were used for RT-qPCR
	Inhibition testing (Cq dilutions, spike or other)	Cq dilutions (five dilution series)

APPENDIX D1, continued

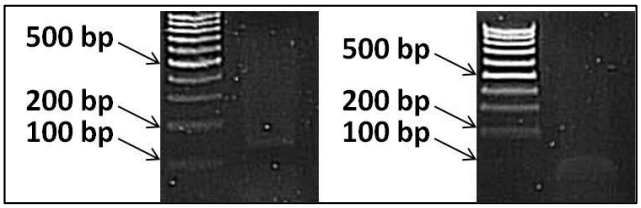
Category	Essential item to check	Description
Reverse transcription	Complete reaction conditions	High Capacity RNA-to-cDNA kit (Life Technologies) was used. Every reaction consists of 10 µl of 2 × RT Buffer, 1 µl of 20 × RT Enzyme Mix, and 9 µl of sample + nuclease-free water
	Priming oligonucleotide (if using GSP) and concentration	Not applicable
	Reverse transcriptase and concentration	20 × MultiScribe MuLV supplied with High Capacity RNA-to-cDNA kit (Life Technologies)
	Temperature and time	Step 1 (37 °C, 60 min), step 2 (95 °C, 5 min), and step 3 (4 °C, ∞)
	Cqs with and without RT	No amplification was observed in no-RT controls for both assays
qPCR target information	If multiplex, efficiency and LOD of each assay.	Not applicable
	Sequence accession number	TLR4 TV1 (NM_138554) and TV3 (NM_003266)
	Amplicon length	Hs01060206_m1: 125 bp; Hs00370853_m1: 68 bp
	In silico specificity screen (BLAST, etc)	According to the Taqman website, the assays are specific to its intended target
	Location of each primer by exon or intron (if applicable)	Hs01060206_m1 targets the boundary between exon 1 and 2 of TV1 (NM_138554); Hs00370853_m1 targets the boundary between exon 1 and 2 of TV3 (NM_003266)
	What splice variants are targeted?	Hs01060206_m1: TV1; Hs00370853_m1: TV3
qPCR oligonucleotides	Primer sequences	Not disclosed by the assay manufacturer
qPCR protocol	Complete reaction conditions	Each reaction consists of 10 µl of 2 × Taqman Fast Advance Mastermix, 1 µl of 20 × Taqman Gene Expression Assay, 2 µl of cDNA template (2 µl), and 7 µl of nuclease-free water
	Reaction volume and amount of cDNA/DNA	20 µl per reaction; 60 ng
	Primer, (probe), Mg++ and dNTP concentrations	20 × primer/probe; the concentrations of Mg^{2+} and dNTP are not disclosed by the product manufacturer
	Polymerase identity and concentration	AmpliTaq Fast DNA Polymerase, in 2 × Taqman Fast Advance Mastermix.
	Buffer/kit identity and manufacturer	Taqman Fast Advance Mastermix (Life Technologies)
	Additives (SYBR Green I, DMSO, etc.)	Not applicable
	Complete thermocycling parameters	UNG incubation (50 °C, 2 min), polymerase activation (95 °C, 20 s), 40 cycles of PCR: denature (95 °C, 10 s), and annealing and extension (60 °C, 30 s)
	Manufacturer of qPCR instrument	Qiagen

APPENDIX D1, continued

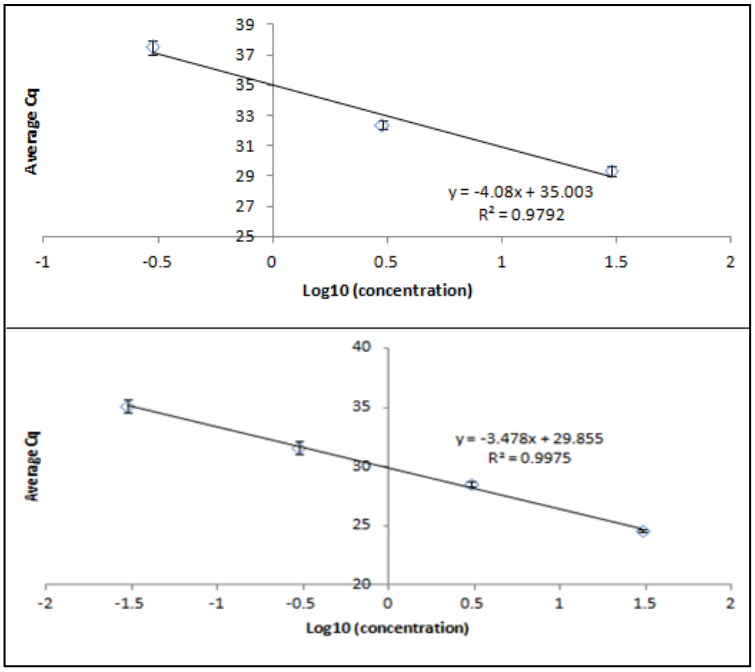
Category	Essential item to check	Description
qPCR validation	Specificity (gel, sequence, melt, or digest)	Refer to the gel picture in Appendix D2
	Standard curves with slope and y-intercept	Refer to the picture of standard curves in Appendix D2. Each standard curve was determined with 5 points, 10-fold dilution series run in triplicate
	PCR efficiency calculated from slope	Hs01060206_m1: 0.76; Hs00370853_m1: 0.94
	r^2 of standard curve	Refer to the picture of standard curves in Appendix D2
	Linear dynamic range	Hs01060206_m1: three orders of magnitude; Hs00370853_m1: four orders of magnitude
	Cq variation at lower limit	LOD measurements were not necessary for this study
	Evidence for limit of detection	LOD measurements were not necessary for this study
Data analysis	qPCR analysis program (source, version)	Rotor-Gene Q Series Software version 2.2.3 (Build 11) (Qiagen)
	Cq method determination	Quantitation Analysis Cycling A. Green
	Outlier identification and disposition	Not applicable
	Results of NTCs	No C_q observed in NTCs of both assays
	Justification of number and choice of reference genes	Not applicable
	Description of normalization method	Not applicable
	Number and stage (RT or qPCR) of technical replicates	In triplicates
	Repeatability (intra-assay variation)	For each sample, standard deviation (SD) was used to express the Cq variation between replicates. Refer to error bars in the picture of standard curves (Appendix D2)
	Statistical methods for result significance	For every patient, the expression of the target isoform was calculated using the formula: expression = $1/(\text{efficiency} + 1)^{Cq}$. Partial Spearman correlation was used to detect the significance of correlation between TV3/TV1 ratio and BMI of patients, controlling for the effects of gender and age
	Software (source, version)	R version 2.15.2

APPENDIX D2

RT-qPCR validation



Gel analysis of PCR amplicons. Ten-µl of PCR amplicons were separated by electrophoresis on 2 % w/v agarose gel. Left panel: TV1 amplicon generated by Hs01060206_m1 assay; right panel: TV3 amplicon generated by Hs00370853_m1 assay.



Standard curves. Top panel: standard curve for TV1 (Hs01060206_m1) assay; bottom panel: standard curve for TV3 (Hs00370853_m1) assay. The data are expressed in mean ± SD.

APPENDIX E

Data used for the partial correlation analysis

Method	Patient	TV3*	TV1*	TV3/TV1	BMI	Gender	Age
RNA-Seq	Obese 1	0.63	0.24	2.68	38.19	M	36
	Obese 2	0.49	0.42	1.16	49.38	M	26
	Obese 3	0.18	0.56	0.32	33.08	F	55
	Lean 1	0.69	0.17	4.01	21.97	M	46
	Lean 2	0.71	0.21	3.39	22.11	F	35
	Lean 3	0.75	0.19	3.99	23.37	F	56
	Lean 4	0.73	0.19	3.91	24.67	F	46
RT-qPCR	Obese 4	28.04	32.90	1.02	38.95	F	28
	Obese 5	27.00	31.91	1.16	35.08	M	57
	Obese 6	27.16	32.43	1.40	34.72	F	48
	Obese 7	28.05	32.12	0.65	62.36	M	39
	Obese 8	27.22	32.68	1.55	30.73	F	59
	Lean 5	28.41	33.68	1.24	17.80	M	18
	Lean 6	27.76	33.48	1.70	24.32	M	53
	Lean 7	26.83	32.75	2.08	22.09	F	52
	Lean 8	27.91	32.70	0.99	25.77	M	50
	Lean 9	27.59	32.78	1.28	22.77	F	19
	Lean 10	27.22	33.02	1.87	17.95	M	21

* For RNA-Seq analysis, proportion data of TV3 and TV1 are provided for every patient; for RT-qPCR analysis, C_q data are provided.

APPENDIX F

Top 13 functional clusters enriched by the differentially expressed genes (p-value < 0.05, fold change ≥ 2)

Cluster (Enrichment score)	Category	Term	p-value	FDR (%)
1 (3.94)	GOTERM_BP_ALL	GO:0007155~cell adhesion	4.67E-05	0.08
	GOTERM_BP_ALL	GO:0022610~biological adhesion	4.76E-05	0.08
	GOTERM_BP_ALL	GO:0016337~cell-cell adhesion	6.97E-04	1.15
2 (2.71)	GOTERM_BP_ALL	GO:0009605~response to external stimulus	7.77E-08	0.00
	GOTERM_BP_ALL	GO:0050896~response to stimulus	9.97E-06	0.02
	GOTERM_BP_ALL	GO:0006952~defense response	2.55E-05	0.04
	GOTERM_BP_ALL	GO:0006950~response to stress	1.31E-04	0.22
	GOTERM_BP_ALL	GO:0009611~response to wounding	1.51E-04	0.25
	GOTERM_BP_ALL	GO:0006954~inflammatory response	1.71E-04	0.28
	GOTERM_BP_ALL	GO:0002253~activation of immune response	4.75E-04	0.78
	GOTERM_BP_ALL	GO:0050778~positive regulation of immune response	8.70E-04	1.43
	GOTERM_BP_ALL	GO:0016064~immunoglobulin mediated immune response	2.50E-03	4.06
	GOTERM_BP_ALL	GO:0050776~regulation of immune response	2.75E-03	4.46
	GOTERM_BP_ALL	GO:0019724~B cell mediated immunity	2.86E-03	4.63
	GOTERM_BP_ALL	GO:0045087~innate immune response	3.43E-03	5.53
	GOTERM_BP_ALL	GO:0006958~complement activation, classical pathway	3.44E-03	5.55
	GOTERM_BP_ALL	GO:0048584~positive regulation of response to stimulus	3.58E-03	5.77
	GOTERM_BP_ALL	GO:0002684~positive regulation of immune system process	3.77E-03	6.06
	GOTERM_BP_ALL	GO:0002455~humoral immune response mediated by circulating immunoglobulin	4.17E-03	6.69
	GOTERM_BP_ALL	GO:0002449~lymphocyte mediated immunity	6.38E-03	10.05
	GOTERM_BP_ALL	GO:0016485~protein processing	6.66E-03	10.47
	GOTERM_BP_ALL	GO:0002682~regulation of immune system process	7.72E-03	12.04

APPENDIX F, continued

Cluster (Enrichment score)	Category	Term	p-value	FDR (%)
2 (2.71), continued	GOTERM_BP_ALL	GO:0002250~adaptive immune response	8.89E-03	13.75
	GOTERM_BP_ALL	GO:0002460~adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	8.89E-03	13.75
	GOTERM_BP_ALL	GO:0051604~protein maturation	9.46E-03	14.56
	GOTERM_BP_ALL	GO:0006959~humoral immune response	9.72E-03	14.92
	GOTERM_BP_ALL	GO:0006956~complement activation	9.79E-03	15.03
	GOTERM_BP_ALL	GO:0048583~regulation of response to stimulus	1.03E-02	15.80
	GOTERM_BP_ALL	GO:0002541~activation of plasma proteins involved in acute inflammatory response	1.04E-02	15.96
	GOTERM_BP_ALL	GO:0002443~leukocyte mediated immunity	1.30E-02	19.45
	GOTERM_BP_ALL	GO:0051605~protein maturation by peptide bond cleavage	1.30E-02	19.45
	GOTERM_BP_ALL	GO:0002252~immune effector process	5.35E-02	59.78
	GOTERM_BP_ALL	GO:0002526~acute inflammatory response	8.51E-02	77.07
3 (2.57)	GOTERM_BP_ALL	GO:0042330~taxis	4.97E-05	0.08
	GOTERM_BP_ALL	GO:0006935~chemotaxis	4.97E-05	0.08
	GOTERM_BP_ALL	GO:0040011~locomotion	1.89E-04	0.31
	GOTERM_BP_ALL	GO:0007626~locomotory behavior	6.59E-04	1.09
	GOTERM_BP_ALL	GO:0042221~response to chemical stimulus	3.99E-03	6.40
	GOTERM_BP_ALL	GO:0007610~behavior	1.10E-02	16.69
	GOTERM_BP_ALL	GO:0016477~cell migration	2.72E-02	36.65
	GOTERM_BP_ALL	GO:0006928~cell motion	2.91E-02	38.70
	GOTERM_BP_ALL	GO:0051674~localization of cell	4.43E-02	52.78
	GOTERM_BP_ALL	GO:0048870~cell motility	4.43E-02	52.78

APPENDIX F, continued

Cluster (Enrichment score)	Category	Term	p-value	FDR (%)
4 (2.48)	GOTERM_MF_ALL	GO:0004872~receptor activity	6.90E-05	0.10
	GOTERM_MF_ALL	GO:0060089~molecular transducer activity	1.03E-04	0.14
	GOTERM_MF_ALL	GO:0004871~signal transducer activity	1.03E-04	0.14
	GOTERM_MF_ALL	GO:0004888~transmembrane receptor activity	1.58E-02	19.76
	GOTERM_BP_ALL	GO:0007166~cell surface receptor linked signal transduction	1.69E-01	95.37
	GOTERM_MF_ALL	GO:0004930~G-protein coupled receptor activity	6.25E-01	100.00
5 (2.36)	GOTERM_BP_ALL	GO:0007165~signal transduction	1.17E-03	1.92
	GOTERM_BP_ALL	GO:0065007~biological regulation	3.04E-03	4.91
	GOTERM_BP_ALL	GO:0050789~regulation of biological process	5.22E-03	8.30
	GOTERM_BP_ALL	GO:0050794~regulation of cellular process	1.95E-02	27.79
6 (2.36)	GOTERM_CC_ALL	GO:0005886~plasma membrane	1.68E-04	0.21
	GOTERM_CC_ALL	GO:0016020~membrane	1.44E-03	1.81
	GOTERM_CC_ALL	GO:0044425~membrane part	1.01E-02	12.05
	GOTERM_CC_ALL	GO:0031224~intrinsic to membrane	1.87E-02	21.22
	GOTERM_CC_ALL	GO:0016021~integral to membrane	3.64E-02	37.42
7 (2.05)	GOTERM_MF_ALL	GO:0005520~insulin-like growth factor binding	1.80E-03	2.46
	GOTERM_BP_ALL	GO:0040008~regulation of growth	1.02E-02	15.67
	GOTERM_BP_ALL	GO:0001558~regulation of cell growth	1.70E-02	24.68
	GOTERM_MF_ALL	GO:0019838~growth factor binding	1.96E-02	23.94

APPENDIX F, continued

Cluster (Enrichment score)	Category	Term	p-value	FDR (%)
8 (1.77)	GOTERM_BP_ALL	GO:0002253~activation of immune response	4.75E-04	0.78
	GOTERM_BP_ALL	GO:0050778~positive regulation of immune response	8.70E-04	1.43
	GOTERM_BP_ALL	GO:0002429~immune response-activating cell surface receptor signaling pathway	6.36E-02	66.32
	GOTERM_BP_ALL	GO:0002768~immune response-regulating cell surface receptor signaling pathway	7.24E-02	71.20
	GOTERM_BP_ALL	GO:0002757~immune response-activating signal transduction	1.04E-01	83.86
	GOTERM_BP_ALL	GO:0002764~immune response-regulating signal transduction	1.18E-01	87.49
9 (1.74)	GOTERM_BP_ALL	GO:0051239~regulation of multicellular organismal process	3.31E-04	0.55
	GOTERM_BP_ALL	GO:0050793~regulation of developmental process	1.19E-02	18.02
	GOTERM_BP_ALL	GO:0045595~regulation of cell differentiation	1.52E-02	22.43
	GOTERM_BP_ALL	GO:0048518~positive regulation of biological process	2.06E-02	29.12
	GOTERM_BP_ALL	GO:0048522~positive regulation of cellular process	1.63E-01	94.79
	GOTERM_BP_ALL	GO:0051094~positive regulation of developmental process	1.71E-01	95.53
10 (1.71)	GOTERM_BP_ALL	GO:0007229~integrin-mediated signaling pathway	8.54E-04	1.40
	GOTERM_BP_ALL	GO:0007159~leukocyte adhesion	3.11E-03	5.03
	GOTERM_CC_ALL	GO:0043235~receptor complex	9.15E-03	10.97
	GOTERM_BP_ALL	GO:0030593~neutrophil chemotaxis	1.52E-02	22.34
	GOTERM_BP_ALL	GO:0016477~cell migration	2.72E-02	36.65
	GOTERM_BP_ALL	GO:0051674~localization of cell	4.43E-02	52.78
	GOTERM_BP_ALL	GO:0048870~cell motility	4.43E-02	52.78
	GOTERM_BP_ALL	GO:0030595~leukocyte chemotaxis	5.80E-02	62.78
	GOTERM_BP_ALL	GO:0060326~cell chemotaxis	6.36E-02	66.32
	GOTERM_BP_ALL	GO:0050900~leukocyte migration	1.21E-01	88.28

APPENDIX F, continued

Cluster (Enrichment score)	Category	Term	p-value	FDR (%)
11 (1.69)	GOTERM_CC_ALL	GO:0044459~plasma membrane part	8.90E-03	10.68
	GOTERM_CC_ALL	GO:0031226~intrinsic to plasma membrane	2.60E-02	28.35
	GOTERM_CC_ALL	GO:0005887~integral to plasma membrane	3.75E-02	38.26
12 (1.47)	GOTERM_BP_ALL	GO:0032501~multicellular organismal process	5.98E-03	9.45
	GOTERM_BP_ALL	GO:0032502~developmental process	9.26E-03	14.28
	GOTERM_BP_ALL	GO:0007275~multicellular organismal development	1.27E-02	19.14
	GOTERM_BP_ALL	GO:0048513~organ development	4.12E-02	50.17
	GOTERM_BP_ALL	GO:0009653~anatomical structure morphogenesis	4.75E-02	55.30
	GOTERM_BP_ALL	GO:0048856~anatomical structure development	4.99E-02	57.11
	GOTERM_BP_ALL	GO:0048869~cellular developmental process	8.63E-02	77.55
	GOTERM_BP_ALL	GO:0030154~cell differentiation	9.31E-02	80.17
	GOTERM_BP_ALL	GO:0048731~system development	9.76E-02	81.72
13 (1.44)	GOTERM_BP_ALL	GO:0045761~regulation of adenylate cyclase activity	3.47E-03	5.59
	GOTERM_BP_ALL	GO:0031279~regulation of cyclase activity	3.96E-03	6.35
	GOTERM_BP_ALL	GO:0030817~regulation of cAMP biosynthetic process	4.31E-03	6.90
	GOTERM_BP_ALL	GO:0051339~regulation of lyase activity	4.31E-03	6.90
	GOTERM_BP_ALL	GO:0030814~regulation of cAMP metabolic process	4.68E-03	7.48
	GOTERM_BP_ALL	GO:0030808~regulation of nucleotide biosynthetic process	6.18E-03	9.75
	GOTERM_BP_ALL	GO:0030802~regulation of cyclic nucleotide biosynthetic process	6.18E-03	9.75
	GOTERM_BP_ALL	GO:0030799~regulation of cyclic nucleotide metabolic process	6.91E-03	10.84
	GOTERM_BP_ALL	GO:0006140~regulation of nucleotide metabolic process	7.70E-03	12.01
	GOTERM_BP_ALL	GO:0007188~G-protein signaling, coupled to cAMP nucleotide second messenger	1.25E-02	18.76
	GOTERM_BP_ALL	GO:0019933~cAMP-mediated signaling	1.81E-02	26.10

APPENDIX F, continued

Cluster (Enrichment score)	Category	Term	p-value	FDR (%)
13 (1.44), continued	GOTERM_BP_ALL	GO:0007187~G-protein signaling, coupled to cyclic nucleotide second messenger	3.35E-02	43.12
	GOTERM_BP_ALL	GO:0050790~regulation of catalytic activity	3.37E-02	43.30
	GOTERM_BP_ALL	GO:0019932~second-messenger-mediated signaling	3.85E-02	47.78
	GOTERM_BP_ALL	GO:0044093~positive regulation of molecular function	4.61E-02	54.24
	GOTERM_BP_ALL	GO:0019935~cyclic-nucleotide-mediated signaling	4.89E-02	56.38
	GOTERM_BP_ALL	GO:0043085~positive regulation of catalytic activity	4.89E-02	56.39
	GOTERM_BP_ALL	GO:0065009~regulation of molecular function	4.97E-02	57.01
	GOTERM_BP_ALL	GO:0007189~activation of adenylate cyclase activity by G-protein signaling pathway	6.65E-02	68.00
	GOTERM_BP_ALL	GO:0010578~regulation of adenylate cyclase activity involved in G-protein signaling	6.65E-02	68.00
	GOTERM_BP_ALL	GO:0010579~positive regulation of adenylate cyclase activity by G-protein signaling pathway	6.65E-02	68.00
	GOTERM_BP_ALL	GO:0007194~negative regulation of adenylate cyclase activity	1.15E-01	86.65
	GOTERM_BP_ALL	GO:0007190~activation of adenylate cyclase activity	1.15E-01	86.65
	GOTERM_BP_ALL	GO:0031280~negative regulation of cyclase activity	1.15E-01	86.65
	GOTERM_BP_ALL	GO:0051350~negative regulation of lyase activity	1.15E-01	86.65
	GOTERM_BP_ALL	GO:0045762~positive regulation of adenylate cyclase activity	1.18E-01	87.49
	GOTERM_BP_ALL	GO:0031281~positive regulation of cyclase activity	1.21E-01	88.28
	GOTERM_BP_ALL	GO:0051349~positive regulation of lyase activity	1.29E-01	89.74
	GOTERM_BP_ALL	GO:0044092~negative regulation of molecular function	1.42E-01	92.13
	GOTERM_BP_ALL	GO:0043086~negative regulation of catalytic activity	1.69E-01	95.37
	GOTERM_BP_ALL	GO:0007186~G-protein coupled receptor protein signaling pathway	5.26E-01	100.00
	GOTERM_MF_ALL	GO:0004930~G-protein coupled receptor activity	6.25E-01	100.00

APPENDIX G

Differentially spliced genes passing the specified cutoff (p-value < 0.1 and absolute splicing index > 0.25)

No.	Gene Symbol	Description	Affected isoform*	Splicing index
1	*ARHGAP26*	*Rho GTPase activating protein 26*	NM_001135608	0.26
2	*BMX*	*BMX non-receptor tyrosine kinase*	NM_203281	> 0.25
3	*C17orf58*	*Chromosome 17 open reading frame 58*	NM_181656	0.28
			NM_181655	-0.29
4	*CACNB3*	*Calcium channel, voltage-dependent, beta 3 subunit*	NM_001206916	-0.26
			NM_001206917	0.27
5	*CHST12*	*Carbohydrate (chondroitin 4) sulfotransferase 12*	NM_001243794	-0.28
6	*CLCN2*	*Chloride channel, voltage-sensitive 2*	NM_004366	-0.30
7	*COCH*	*Coagulation factor C homolog, cochlin (Limulus polyphemus)*	NM_001135058	-0.31
			NM_004086	0.27
8	*DCLK2*	*Doublecortin-like kinase 2*	NR_036614	> 0.25
9	*DUS4L*	*Dihydrouridine synthase 4-like (S. cerevisiae)*	NR_073002	0.31
10	*FAIM3*	*Fas apoptotic inhibitory molecule 3*	NM_005449	0.40
11	*FAM109A*	*Family with sequence similarity 109, member A*	NM_001177996	0.33
12	*FCHO1*	*FCH domain only 1*	NM_001161359	-0.29
13	*GDPD1*	*Glycerophosphodiester phosphodiesterase domain containing 1*	NM_001165993	> 0.25
14	*GLRB*	*Glycine receptor, beta*	NM_000824	-0.31
15	*GRB7*	*Growth factor receptor-bound protein 7*	NM_005310	0.32
16	*IGF2BP2*	*Insulin-like growth factor 2 mRNA binding protein 2*	NM_006548	0.29
17	*IKZF3*	*IKAROS family zinc finger 3 (Aiolos)*	NR_047561	-0.32
18	*IL12RB2*	*Interleukin 12 receptor, beta 2*	NM_001559	0.44
19	*L1CAM*	*L1 cell adhesion molecule*	NM_000425	-0.26
20	*LAT2*	*Linker for activation of T cells family, member 2*	NM_032464	0.35
21	*LRRC20*	*Leucine rich repeat containing 20*	NM_001278212	-0.30
22	*MARCH1*	*Membrane-associated ring finger (C3HC4) 1, E3 ubiquitin protein ligase*	NM_017923	0.33
23	*MID1*	*Midline 1 (Opitz/BBB syndrome)*	NM_033290	> 0.25
24	*MIPOL1*	*Mirror-image polydactyly 1*	NM_138731	0.40

* Difference in medians between the obese and lean groups was analyzed using the Mann-Whitney U test; p-values for all isoforms listed are 0.06. It is possible to observe identical p-values because the Mann-Whitney U test uses rankings (instead of original values) to compute a p-value.

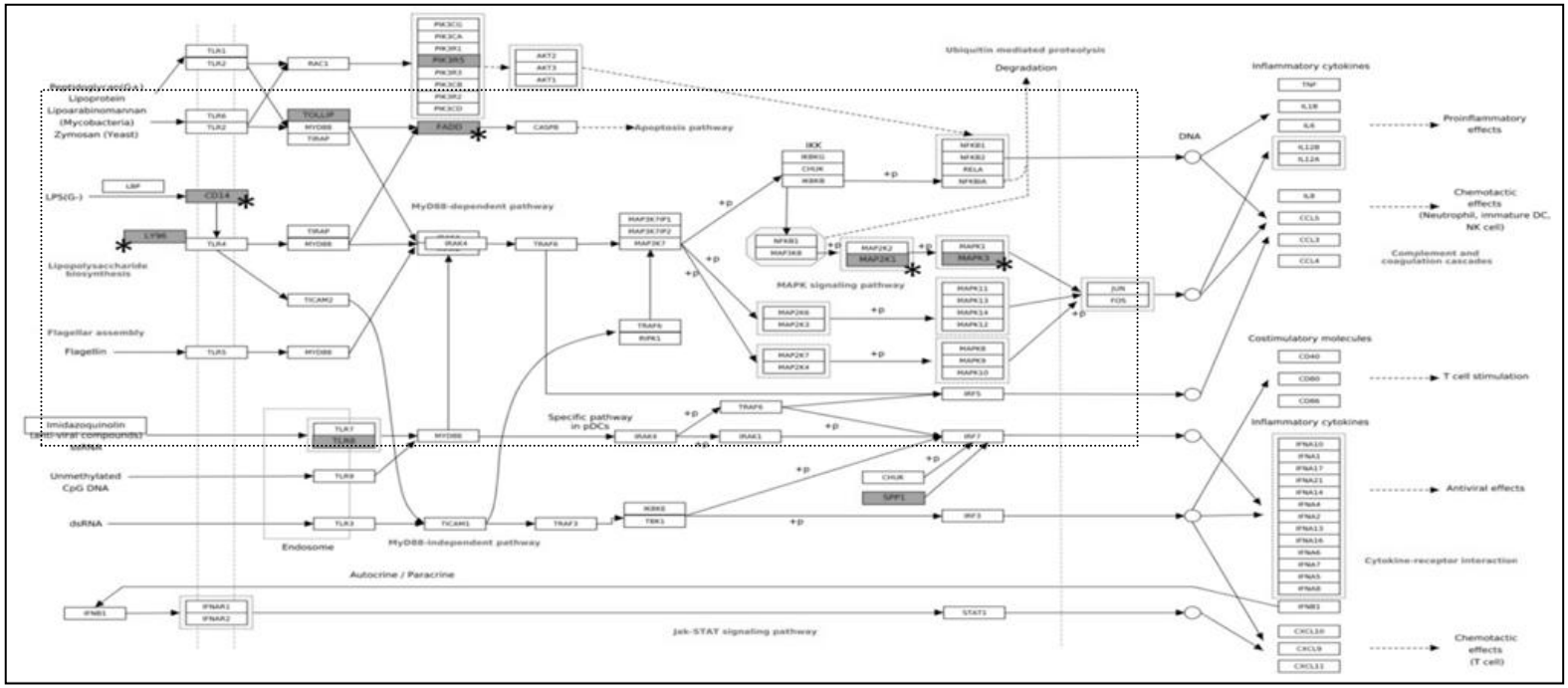
APPENDIX G, continued

No.	Gene Symbol	Description	Affected isoform*	Splicing index
25	*MRPL24*	*Mitochondrial ribosomal protein L24*	NM_024540	0.26
			NM_145729	-0.26
26	*MSANTD3*	*Myb/SANT-like DNA-binding domain containing 3*	NM_001198805	-0.36
27	*MYBL1*	*V-myb myeloblastosis viral oncogene homolog (avian)-like 1*	NM_001080416	0.39
28	*PCDH19*	*Protocadherin 19*	NM_001105243	0.27
29	*PIGC*	*Phosphatidylinositol glycan anchor biosynthesis, class C*	NM_002642	0.36
			NM_153747	-0.37
30	*PLA1A*	*phospholipase A1 member A*	NM_001206961	0.40
31	*PROX1*	*Prospero homeobox 1*	NM_002763	-0.46
			NM_001270616	0.46
32	*RFX2*	*Regulatory factor X, 2 (influences HLA class II expression)*	NM_134433	0.30
33	*SCN3A*	*Sodium channel, voltage-gated, type III, alpha subunit*	NM_001081676	-0.33
34	*SLC25A22*	*Solute carrier family 25 (mitochondrial carrier: glutamate), member 22*	NM_024698	-0.36
35	*TLR4*	*Toll-like receptor 4*	NM_003266	-0.29
36	*TMEM254-AS1*	*TMEM254 antisense RNA 1*	NR_027428	0.34
37	*TMEM51*	*Transmembrane protein 51*	NM_018022	< -0.25
38	*TNFRSF11A*	*Tumor necrosis factor receptor superfamily, member 11a, NFKB activator*	NM_001270951	-0.27
39	*TOR1AIP1*	*Torsin A interacting protein 1*	NM_015602	-0.33
			NM_001267578	0.35
40	*TRIM55*	*Tripartite motif containing 55*	NM_184086	0.36
41	*WISP1*	*WNT1 inducible signaling pathway protein 1*	NM_003882	0.32
42	*ZFAT*	*Zinc finger and AT hook domain containing*	NM_020863	-0.33
43	*ZNF155*	*Zinc finger protein 155*	NM_001260488	-0.29
44	*ZNF180*	*Zinc finger protein 180*	NM_013256	0.27
45	*ZNF273*	*Zinc finger protein 273*	NM_021148	-0.30
46	*ZNF566*	*Zinc finger protein 566*	NM_001145345	-0.42
47	*ZNF583*	*Zinc finger protein 583*	NM_001159860	< -0.25
			NM_152478	0.52
48	*ZNF707*	*Zinc finger protein 707*	NM_173831	-0.29
49	*ZNF85*	*Zinc finger protein 85*	NM_001256171	-0.30

* Difference in medians between the obese and lean groups was analyzed using the Mann-Whitney U test; p-values for all isoforms listed are 0.06. It is possible to observe identical p-values because the Mann-Whitney U test uses rankings (instead of original values) to compute a p-value.

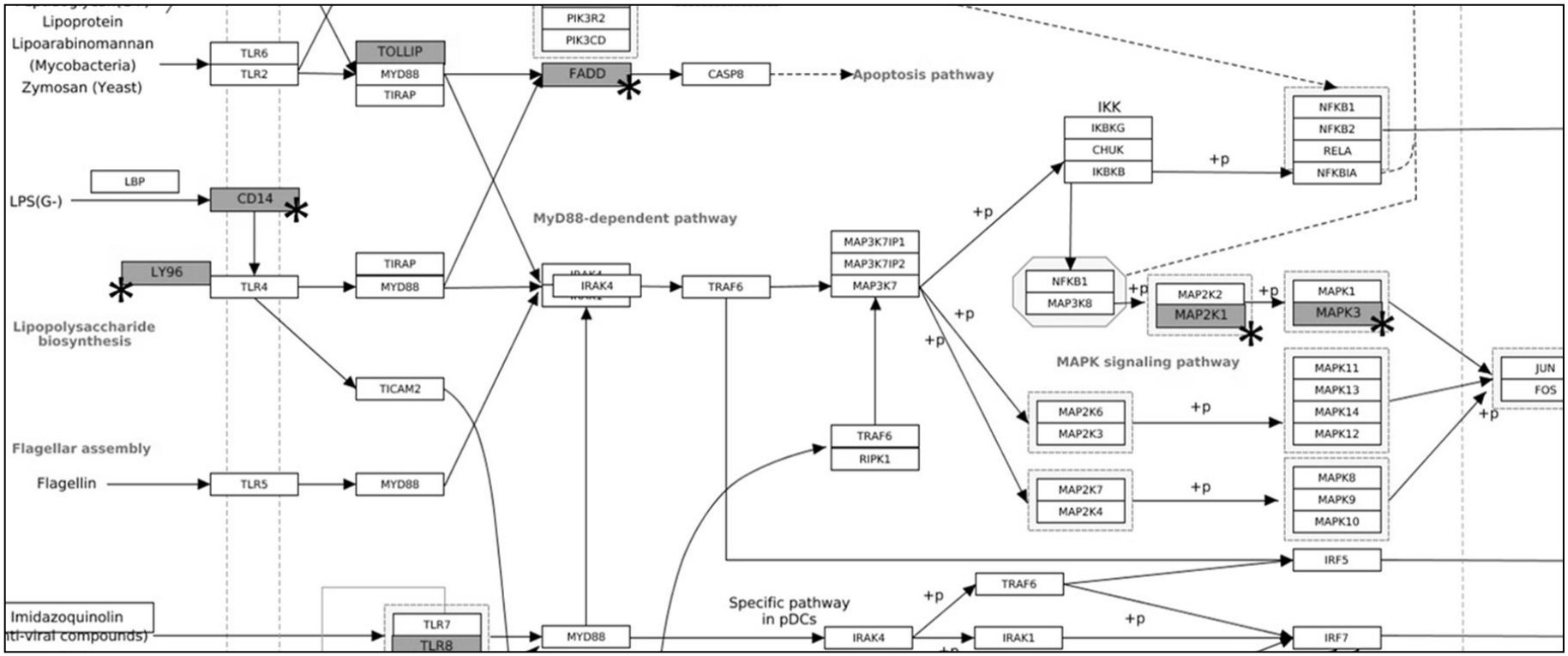
APPENDIX H

Pathway analysis of toll-like receptor signaling with differentially expressed genes (p-value < 0.05)



Differentially expressed genes involved in the TLR4 signaling are indicated by asterisks (*); the rectangular portion of this figure is enlarged for better viewing on next page.

APPENDIX H, continued



Differentially expressed genes involved in the TLR4 signaling are indicated by asterisks (*)