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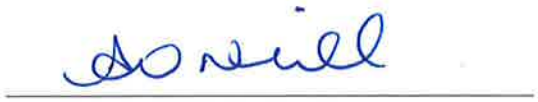
**IFN-Induced Transmembrane Protein 6 Expression in Adipose Tissue**

by

Safa Kazi

An Undergraduate Thesis Submitted to
The University Honors Program
Auburn University at Montgomery

In partial fulfillment of the requirements for the degree of
Bachelor of Science in Biology



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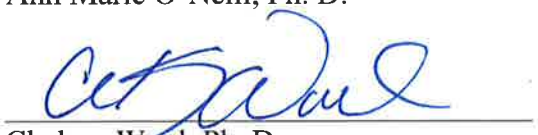

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# IFN-Induced Transmembrane Protein 6 Expression in Adipose Tissue

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**Abstract**

As of 2016, over one third of the U.S. adult population was considered obese, with numbers increasing each day. One of the characteristics of obesity is a chronic low-grade state of inflammation in adipose tissue, caused by the presence of M1 macrophages that have been implicated in tumor progression. Obesity has been linked to a worsened prognosis in a number of cancers, as it exacerbates tumor growth. In this study, the expression of a little-known marker of M1 macrophages, IFN-induced transmembrane protein (*ifitm6*), was assessed in fat depots from both lean and obese mice. We hypothesized that increased *ifitm6* gene expression in obese tissue is due to the presence of pro-inflammatory M1 macrophages. While it was found that M1 macrophages were at play, data also suggested that M1 and M2 worked simultaneously in response to different receptors.

## Introduction

One of the major factors thought to be driving the current obesity epidemic and ensuing pathologies is the western diet, characterized by high saturated fat and sugar content found in fast food (Figure 1). The United States has the highest prevalence rate of obesity, and Alabama ranks in the top five (CDC, 2016). Obesity is defined by a body mass index (BMI), which looks at weight-to-height ratio, of greater than or equal to 30 (WHO, 2018). In addition to obesity, excess consumption of unhealthy nutrients leads to inflamed adipose tissue (Serra, 2018).

Adipose tissue secretes peptide hormones such as leptin, and several types of cytokines. Inflammation and infiltration of adipose tissue by macrophages is a hallmark of obesity (Xu, 2013). In a previous study, obese mice showed an increase in inflammation- as demonstrated by macrophage infiltration- which is histologically shown through an increase in crown-like structures (Figure 2). In obesity, adipose tissue is inflamed, and it releases chemokines, such as CCL2, CCL3, CCL5, CCL7, CCL8, CCL13, CCL17 and CCL22, that attract macrophages. These macrophages surrounding the dead or dying adipocytes, as a result of apoptosis, form these crown-like structures (Luo, 2016).

Obesity results in accumulation of visceral fat, which is dangerous because of the diseases that can arise as a result, such as coronary artery disease, diabetes and even cancer (NIDDKD, 2015).

M1 macrophages are said to be the classical type of macrophages because they are involved in immune functions and M2 are thought to be related to chronic inflammation and involvement in tissue remodeling and immune suppression (Martinez, 2014). Based on cues from their microenvironment, macrophages release cytokines and other signals to carry out immune functions. M1 macrophages are activated by interferon gamma (IFN- $\gamma$) or other products such as, interleukin 12 (IL-12), inducible nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNF-α). On the other hand, M2 macrophages are stimulated by IL-4, IL-10, IL-13, immune complexes and glucocorticoids (Fraternale, 2015). Although there is a slight distinction between the M1 and M2, these macrophages are more accurately described as a continuum based on a gradual increase or decrease in their response to cytokines and expression of receptors (Mantovani, 2009).

The inflammation in obese adipose tissue is implicated in associated diseases, however the molecular mechanisms are not clear. To learn more about these mechanisms, it is necessary to compare molecular mechanisms between subjects with high and low BMI.

The *ifitm6* gene translates surface proteins that regulate cell-cell adhesion and cell differentiation induced by splenic macrophages, thioglycolate-elicited macrophages and bone marrow-derived macrophages (Han, 2011). A previous study suggests that the *ifitm6* gene is expressed on the surface of tumor associated macrophages in mice (Han, 2011). In this study, retroperitoneal white adipose tissue (RWAT), and epididymal white adipose tissue (EWAT) stores were used from the mice to analyze *ifitm6* expression. Results from the RNA *seq* analysis performed on RNA from EWAT suggested that the expression of *ifitm6* was upregulated in adipose tissue of obese mice. This study also examined the expression of *ifitm6* on M1 and M2 macrophages and confirmed higher levels of gene expression of the M1 phenotype. Altogether, data results from the study suggest that the increased expression of *ifitm6* seen in obese adipose tissue may be use due to the increased infiltration by M1 macrophages. Data obtained from RNA *seq* analysis of adipose tissue from lean mice and obese mice, indicated differential gene expression between these groups (Luo, 2016). This data indicated that *ifitm6* expression was higher in obese mice versus lean. Given the lack of previous data on this marker, further studies looking at samples of EWAT and RWAT of obese mice can clarify the role of *ifitm6* as both a

marker for inflammation and macrophage infiltration in both adipose tissue and tumors.

## Methods and Materials

**Animals.** Mice are used to study human pathologies; however, they do not have visceral fats, thus their retroperitoneal white adipose tissue (RWAT) and epididymal white adipose tissue (EWAT) stores, in males, were used to be representative of visceral fat. In Auburn University, two groups of B6 male mice (n= 6 per group) were fed either a low fat western diet (lean) or a high fat western + sugar diet (obese) for 12 weeks. Both diets included a 1:1:1 ratio of butter, Crisco and lard, but the obese group was fed three times more calories than the lean group. Sugar was provided at a concentration of 4% in drinking water, as a combination of fructose and sucrose (Luo, 2016). The Institutional Animal Use and Care Committee approved all experiments.

**Cell culture.** RAW 264.7 cells from the monocyte cell line were incubated in DMEM supplemented with 10% fetal bovine serum, 1 % L-glutamine and 1% penicillin-streptomycin in 5% $CO_2$ media at 37ºC (n=2). In order to polarize them to either an M1 or M2 phenotype, 100 ng/mL of interferon-g (IFN- $\gamma$) and lipopolysaccharide (LPS) (M1), or 20 ng/mL of

IL-4 (M2) was added to the media and cells were incubated for another 24 hours, respectively. Following this, cells were collected by cell scraping, centrifuged at 250x for five minutes, washed in PBS, pelleted by centrifuging and stored in RNA later until the RNA extraction (Davis, 2013).

**RNA isolation.** Mice were euthanized using $CO_2$ asphyxiation followed by cervical dislocation. Retroperitoneal white adipose tissue (RWAT) and epididymal white adipose tissue (EWAT) was removed. These tissues were stored in RNA Later solution at -20$^{o}$C until RNA was extracted. The EZNA total RNA kit was used to isolate total RNA from both cells and tissue. RNA samples from the EWAT were sent to Hudson Alpha, Huntsville, Alabama for RNA *seq* analysis. Sequencing RNA samples from tissue shows differential gene expression of RNA molecules between samples. RNA *seq* allows us to compare the gene expression between the two groups. The results from the RNA *seq* data alone are not sufficient to draw a significant correlation, and thus must be validated through quantitative polymerase chain reaction (qPCR) to detect, characterize and quantify nucleic acids.

**Quantitative PCR** Reverse transcription was performed using the RT2 First Strand Kit (Qiagen, Valencia, CA). QPCR reactions were performed in BioRad CFX connect system using SsoAdvanced Universal

SYBR Green Supermix (BioRad). After initial denaturation at 95ºC for 30 seconds, the following amplification cycles were used for both adipocyte and macrophage RNA: 40 cycles of 15 seconds of denaturation at 95ºC for 30 seconds annealing at 60ºC. Primers for *ifitm6*, *rn18s*, and TNF-α were used (Figure 3). Reactions were performed in triplicate and the data was calculated using the $2^{\wedge}\Delta\Delta Ct$ method. Gene expression was normalized to the average expression of *rn18s*. A t-test analyzed statistical significance of the data. Similar to standard PCR, qPCR amplifies DNA in the same steps of denaturation, annealing of primers and elongation. What sets the two apart, is that in qPCR, fluorescent labeling enables the collection of data as the PCR progresses.

**Results**

From the RNA *seq* data obtained, it was suggested that *ifitm6* expression was upregulated 8-fold in the RNA from the epididymal white adipose tissue (EWAT) of obese mice compared to the lean mice. In order to validate these results, qPCR was performed using cDNA from the EWAT of both lean and obese mice. Results from qPCR indicated that *ifitm6* expression is significantly increased by 4-fold in epididymal white adipose tissue from obese mice compared to lean. Following this, the expression of *ifitm6* in the RWAT of the same mice was then examined by qPCR. Results

indicated that *ifitm6* expression was significantly increased by 5-fold in the RWAT of obese versus lean mice (Figure 4).

A previous study implicated that *ifitm6* is expressed on macrophages (Han, 2011). We then then sought to examine expression on RAW 264.7 cells polarized to an M1 phenotype. It was first necessary to confirm that the cells were successfully polarized to an M1 phenotype by incubating them in the presence of IFN- $\gamma$ and LPS. To accomplish this, the expression of TNF-α, a marker of M1 macrophages, was assessed in both M1 and M2. The results indicated that the cells incubated with IFN- $\gamma$ and LPS expressed TNF-α at a higher rate (4.6 fold) in comparison to those incubated with IL-4. The expression of *ifitm6* was then examined on both groups of cells using qPCR. Results obtained indicated that the cells incubated with IFN- $\gamma$ and LPS and determined to be M1 by TNF-α gene expression, demonstrated a 3-fold increase of *ifitm6* expression compared to those cells incubated with IL-4 and considered to be M2 (Figure 5). The sample size was n=2, thus statistical analysis was not possible.

## Discussion

This data suggests that the significant increase of *ifitm6* expression in obese adipose tissue is likely due to the increased presence of M1 macrophages (Figure 5). The level of expression of *ifitm6* in obese adipose tissue suggested that more of the pro-inflammatory M1 macrophages were present, rather than the anti-inflammatory M2 macrophages. To further support this observation, RAW 264.7 cells that had been treated with LPS and IFN- $\gamma$ were confirmed to be of the M1 phenotype by TNF-α expression, and it was demonstrated that these cells had a 3-fold increase in *ifitm6* expression compared to M2. A former study showed that *ifitm6* gene expression was increased in tumor bearing mice, but it is still unclear what role the *ifitm6* increase in macrophages has on obese mice (Han, 2011).

The western diet, as mentioned, is one of the main concerns of the average American. It is positively correlated in linking one to obesity, which has its own concerns when looking at its long-term effects. Future studies would be beneficial in showing the importance of *ifitm6* as a marker for M1 macrophages, and also how it presents itself on the M1/M2 continuum during different periods of adipose tissue growth. Doing so, would allow insight into the mechanisms that drive *ifitm6* and macrophages as obesity progresses.

In attempts to keep up with the number of growing cases of obesity caused by unhealthy diets, research is being done to finetune the links between tumor progression and obese adipose tissue as well. Obesity is known for its aide in tumor progression. When presented with excessive amounts of calories, adipocytes begin to get inflamed and increase in size. Some effects of that are tissue hypoxia and interruptions of the protein secretory pathway (Jiyoung, 2011). The inadequate supply of oxygen can lead to damage and even recruit more macrophages as a result. Consequently, the obese adipocytes, in comparison to lean adipocytes, become incompetent and unable to store and neutralize lipids. Their inability to carry out their functions is directly associated with obesity- related disorders, such as certain cancers (Jiyoung, 2011).

The tumor microenvironment contains an abundance of cells such as adipocytes, immune cells, and tumor-associated macrophages, which all contribute to paracrine, or cell to cell, signaling in the microenvironment (Jiyoung, 2011). Adipocytes intermingle with cancer cells by releasing signaling molecules such as inflammatory cytokines (Jiyoung, 2011). These cytokines attract immune cells, M1 macrophages, to surround the dying adipocytes. While the obese adipose tissues are growing, and ultimately dying, they become unserviceable which causes another cascade of

concerns. Issues such as chronic inflammation, hormonal irregularities, and insulin resistance can then arise causing concern not only for nearby tumor cells, but also those farther away from the source caused by the altered expression of chemokines (Jiyoung, 2011).

This would be something that would be interesting to look at in future studies. In this study, the samples were not obese enough to show the link to tumor progression. Changing the diets of the mice or prolonging the time of the diets may be a defining factor of the results.

## Charts and Graphs

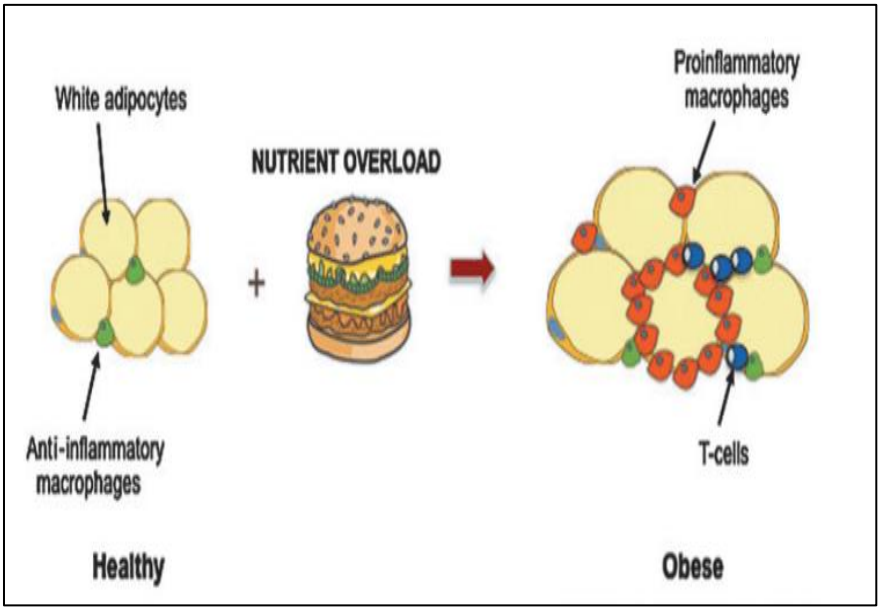


**Figure 1-** The western diet causes proinflammatory macrophage infiltration in white adipocytes (Serra, 2018).

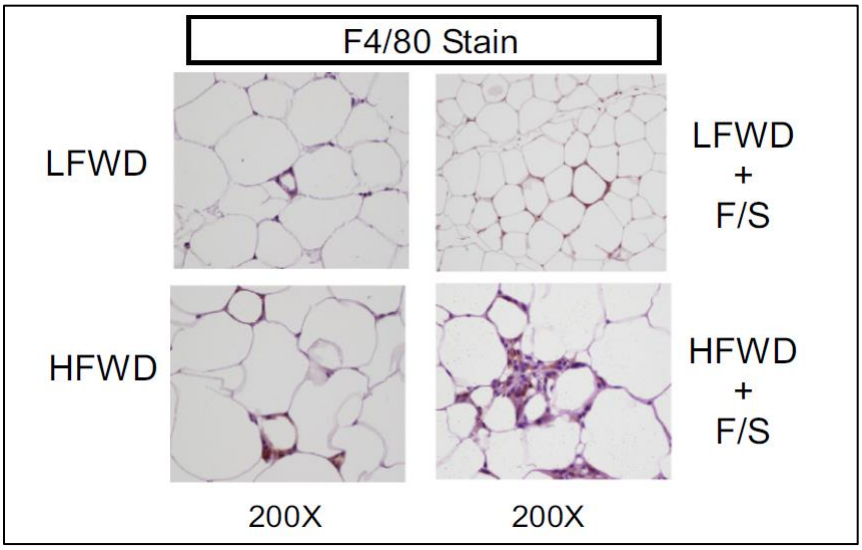


**Figure 2-** Crown-like structures, formed by macrophage infiltration, show a dramatic increase in samples from obese mice (HFWD+F/S) in comparison to lean mice (LFWD) as an indication of inflammation (Luo, 2016).

| Primer | Sequence |
|---|---|
| *ifitm6* forward primer | 5'-TACATCTACTGCGTGAAGTC-3' |
| *ifitm6* reverse primer | 5'-AAGGATGTTCAGAATCTTGG-3' |
| *rn18s* forward primer | 5'-CAGTTATGGTTCCTTTGGTC-3' |
| *rn18s* reverse primer | 5'-TTATCTAGAGTCACCAAGCC-3' |
| TNF-α forward primer | 5'-CTATGTCTCAGCCTCTTCTC-3' |
| TNF-α reverse primer | 5'-CATTGGGAACTTCTCATCC-3' |

**Figure 3-** Primer Sequences: The primer sequences used for *ifitm6*, *rn18s* and TNF-α (BioRad).

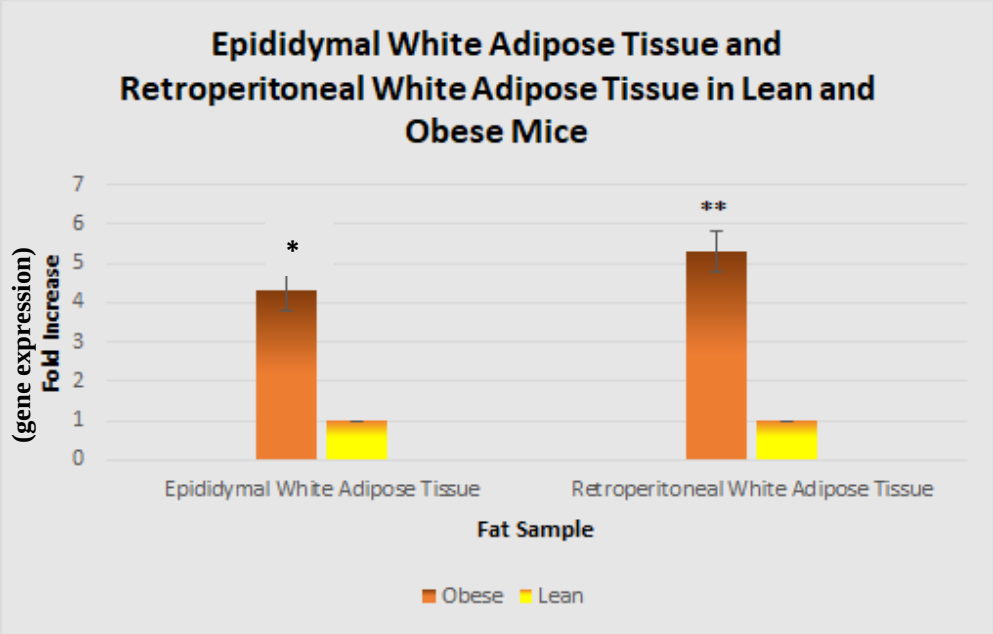


**Figure 4-** Epididymal white adipose tissue and retroperitoneal white adipose tissue in lean and obese mice. In comparison to lean mice, obese mice show an increased expression of *ifitm6* in both fat depots. (*p= 0.02, $\bar{x}$= 0.233 ± 0.4618, df= 5, ** p= 0.005, $\bar{x}$= 0.1886 ± 0.3163, df= 5).

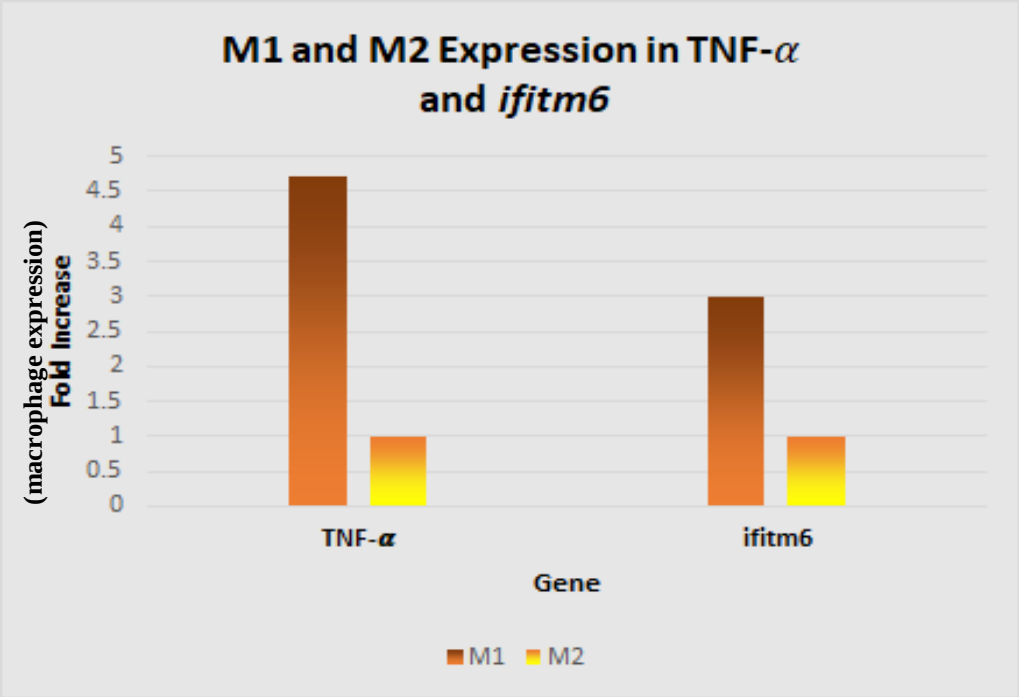


**Figure 5-** M1 and M2 Expression in TNF-α and *ifitm6*: TNF-α is expressed, showing that *ifitm6* is a marker of M1 (n=2).

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