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Strain and Depot-specific Differences in Adipose Tissues of Obese BFMI Mice

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Abstract. *Background/Aim: Obesity is associated with the structural and functional disorders related to the molecules of the tissues, cells, and membranes. This study aimed to examine the alterations in the secretion of inflammatory cytokines and metabolic factors and structural changes in inguinal (IF) and gonadal (GF) adipose tissues at the molecular level. Materials and Methods: The IF and GF tissues of Berlin Fat Mouse Inbred (BFMI) lines namely BFMI852, BFMI856, BFMI860, BFMI861 obese and DBAJ control mouse lines were used for mRNA expression and Attenuated Total Reflection – Fourier Transform Infrared Spectroscopy (ATR-FTIR) studies. The mRNA levels of inflammatory cytokines including leptin, interleukin 6 (IL-6), tumor necrosis factor-alpha (Tnf-α), and insulin-like growth factor-1 (Igf-1), and peroxisome proliferator-activated receptor gamma 2 (Pparγ-2), were investigated using quantitative reverse transcriptase real-time PCR (qRT-PCR). Infrared spectroscopy does not provide information about specific proteins, instead, it gives information about overall (total) proteins, which is called global information. Therefore, in the current study, adequate information about secondary structures of adipose tissues proteins was obtained using artificial neural network (ANN) and secondary derivative-vector normalization methods based on the spectral profiles. Results: According to the mRNA expression studies, high leptin resistance was found in all BFMI lines. Differences were observed in the levels of measured factors except for Igf-1 among BFMI lines. Protein secondary structure studies showed an increase in random coil contents, especially for BFMI860, which indicates denaturation of the proteins. Conclusion: Among the spontaneous obese BFMI mouse lines, the BFMI860 line is the most suitable for obesity studies. Obesity-induced effect on the adipose tissues varies considerably with location, type of adipose tissue, and animal line.*

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Excessive body fat accumulation is associated with elevated levels of free fatty acids and triglycerides in the blood, contributing to the development of obesity-related metabolic disturbances, especially insulin resistance (1). Since adipokines, which are secreted from adipose tissue, play a role in homeostasis, regulation of blood pressure, lipid and glucose metabolism, and inflammation, adipose tissue is considered as an endocrine organ. Therefore, it has an important role in the emergence of obesity-related pathologies such as insulin resistance, diabetes, and cardiovascular disease. Increased circulating levels of adipokines are observed during obesity. For example, interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) contribute to the induction of insulin resistance (2) and are associated with altered glucose and lipid metabolism (3). Leptin regulates energy homeostasis and endocrine functions, affects glucose, lipid, and bone metabolism, and shows proinflammatory activity (4, 5). High level of leptin was reported as a marker of cardiovascular disease (6) and leptin resistance (7). PPARγ2 regulates adipogenesis, affects adipose tissue lipid storage, exhibits metabolic inflexibility, and is associated with whole-body and adipose tissue insulin sensitivity (8).

There are many studies on gene expression and the levels of different proteins in metabolic diseases (9-12). The functional changes of proteins are not only due to variations in gene expression or protein synthesis but also to their structure. It is known that changes in their three-dimensional structure, which is also called functional structure or native structure of the protein, also affect the ability of proteins to perform their tasks (13). Any variation in the regular folding stage of the proteins leads to changes in secondary, tertiary, or quaternary structures of proteins, which causes the loss of their regular function or the gain of different functions (14, 15). Therefore, alterations in the structures of existing proteins without alterations in their expression can also lead to dysfunction of the proteins.

The goals of the present study were, first, to detect differences in the immune response to body fat storage by analyzing mRNA expression levels of *Pparγ2, Leptin, TNF-A*, and *Igf-1* genes between BFMI and DBAJ mice (line effect) and, second, to investigate the alteration in the secondary structure of protein content in gonadal and inguinal fat tissues of obese male BFMI lines, which are new animal models for obesity.

Materials and Methods

Animals. In the present study, 10-week-old males of the Berlin Fat Mouse Inbred (BFMI) line derivatives including BFMI852, BFMI856, BFMI860, and BFMI861 were used. At the age of 3 weeks, the animals were fed with a rodent standard breeding diet (SBD, V1534-000, ssniff R/M-H, Ssniff Spezialdiäten GmbH, Soest, Germany). Each line group comprised 6 mice. The mice were decapitated at the age of 10 weeks and their inguinal and gonadal adipose tissues were dissected. All animals were grown, and their tissues were prepared as described in Baloğlu *et al*. (16). Research ethics approval for all animal research procedures was obtained by the German Animal Welfare Authorities (approval no. G0171/10).

RNA isolation and qRT-PCR. Total RNA isolation was performed using the NucleoSpin RNA®II kit (Macherey-Nagel, Düren, Germany). However, a sufficient amount of RNA could not be obtained since the amount of fat in adipose tissue was too high compared to that in normal tissues. Therefore, total RNA was isolated using Trizol as previously described (17-19) with modifications.

Attenuated Total Reflection – Fourier Transform Infrared (ATR-FTIR) Spectroscopy:
Data collection and spectral analysis. IR spectra of inguinal fat (IF) and gonadal fat (GF) samples were obtained using a Perkin–Elmer spectrum 100 FTIR spectrometer (Perkin Elmer Inc., Boston, MA, USA). Tissue samples were mounted on the diamond/zinc-selenite (Diamont/ZnSe) crystal of the ATR unit without any pretreatment. Spectra were obtained in the 4000 and 650 cm^{-1} wavenumber region at a spectral resolution of 4 cm^{-1} with 64 scans co-added. Each tissue sample was scanned three times using three randomly taken samples of IF and GF tissues. The average spectrum was obtained from these three replicate spectra and used for detailed data and statistical analysis. All spectral analyses were performed using Spectrum One software (Perkin Elmer).

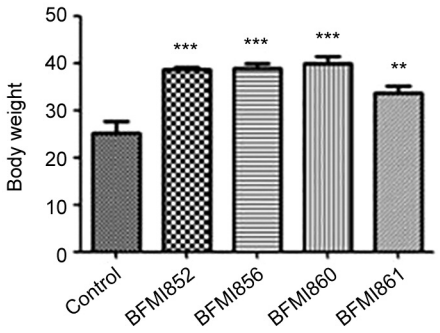

Figure 1. *Body weight of Berlin Fat Mouse Inbred (BFMI) and control (DBA/2J) male mice fed standard breeding diet (SBD) at the end of 10 weeks*.

Protein secondary structure analysis by artificial neural networks (ANNs). Protein secondary structure changes were determined in obese mouse adipose tissues by applying bioinformatics methods such as ANNs as well as the second derivative-vector normalization method. For this purpose, the amide I band located in the 1700-1600 cm^{-1} wavenumber range of FTIR spectra was used.
In this study, a form of the Bayesian regulated backpropagation algorithm that we developed in our previous studies was used (20). Neural networks were initially trained using a data set containing FTIR spectra of a number of water-soluble proteins dissolved in water (21). The secondary structures of these proteins are known from X-ray crystallographic analysis. Since the spectra of proteins with known structures required for ANN training were very limited, to improve the training of neural networks, the size of the data set was increased by generating new spectra from the pairwise averages of the available spectra (20). Secondary structures of the generated spectra are calculated by averaging the corresponding secondary structures of the original spectra. Before starting the training procedure, FTIR spectra of the Amide I band were normalized and then their discrete cosine transforms (DCT) were obtained. The first 13 DCT coefficients were input to the ANN. In this ANN study, each protein's secondary structure elements namely, the percent content of alpha-helix, beta-sheet, and turns were obtained. Standard prediction errors of trained ANNs were obtained as 4.19% for α-helix, 3.49% for β-layer, and 3.15% for turns.

Statistics. The results were calculated as 'mean±standard error of the mean (SE)'. The differences in variance were analyzed using one-way ANOVA and Dunnett's test in GraphPad Prism V.6.00 (GraphPad Software, La Jolla, CA, USA). *p*-Values less than or equal to 0.05 were considered statistically significant (such as *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). Statistical significance was demonstrated with # for BFMI lines and with * for the DBA/J2 control line.

Results and Discussion

Changes in phenotype and gene expression. There are major differences in the phenotypes of BFMI lines including body

Table I. *Differences in mRNA expression levels of the leptin, Pparγ-2, Igf-1, Tnf-α, and IL-6 genes in gonadal fat (GF) tissue of Berlin Fat Mouse Inbred (BFMI) and control mice lines.*

GF	Control	BFMI852	BFMI856	BFMI860	BFMI861
Leptin	1	17.13±3.30**	5.21±0.73	7.32±0.28	11.18±3.72*
Pparγ-2	1	1.12±0.30	0.73±0.11	1.03±0.37	0.55±0.17
Igf-1	1	0.70±0.25	0.52±0.05	1.02±0.50	0.24±0.04
Tnf-α	1	0.63±0.20	0.49±0.12	0.59±0.27	0.33±0.06
IL-6	1	0.58±0.18	0.43±0.14	0.51±0.22	0.30±0.04

The values are the mean±standard error of the mean for each group. The degree of significance is denoted with asterisks (*) for the comparison of the control group with other BFMI lines. (*$p<0.05$; **$p<0.01$).

Table II. *Differences in mRNA expression levels of the leptin, Pparγ-2, Igf-1, Tnf-α, and IL6 genes in inguinal fat (IF) tissue of Berlin Fat Mouse Inbred (BFMI) and control mice lines.*

GF	Control	BFMI852	BFMI856	BFMI860	BFMI861
Leptin	1	17.13±3.30**	5.21±0.73	7.32±0.28	11.18±3.72*
Pparγ-2	1	1.12±0.30	0.73±0.11	1.03±0.37	0.55±0.17
Igf-1	1	0.70±0.25	0.52±0.05	1.02±0.50	0.24±0.04
Tnf-α	1	0.63±0.20	0.49±0.12	0.59±0.27	0.33±0.06
IL-6	1	0.58±0.18	0.43±0.14	0.51±0.22	0.30±0.04

The values are the mean±standard error of the mean for each group. The degree of significance is denoted with asterisks (*) for the comparison of the control group with other BFMI lines (*$p<0.05$; **$p<0.01$).

fat mass, body fat percentage, weights of dissected fat pads, inner organs, and glucose concentrations depending on the selection process for high fatness and low protein content during the establishment of BFMI (22). However, features of metabolic syndrome and reduced insulin sensitivity were only seen in 860 and 861 lines. Hantschel *et al*. (23) reported that the BFMI860 line has higher serum triglyceride levels when fed both the standard breeding diet and high-fat diet. In support, an infrared and chromatographic study from our group reported that among the BFMI lines, the BFMI860 and BFMI861 lines showed marked obesity-induced alterations in the structure and content of lipids and glycogen of adipose and skeletal muscle tissues (24). The male DBA/J2 mice line is often used as a control showing a wild type-like phenotype (16, 25).

In order to characterize the BFMI mouse models at the molecular level, mRNA expression of *leptin, Igf-1, Tnf-α,* and *Pparγ-2*, which are associated with obesity and accepted as markers of inflammation and/or obesity, were analyzed using qRT-PCR.

The Berlin Fat Mouse is characterized by a high body fat mass with altered lipid metabolism but normal lean mass (22), and impaired fat oxidation (26). The study was performed with males because previous studies have shown that diet-induced changes in body composition were more apparent in males than females. BFMI males were highly sensitive to increased fat deposition with respect to affected adipokine release and glucose tolerance (23). They also showed altered structure and content of lipids (22). At 10 weeks, the body weight of all BFMI males was higher than that of the control (Figure 1). Body weight gain was markedly highest in the males of BFMI 860 lines. In addition, BFMI 861 mice accumulated proportionally less fat among all BFMI lines.

We studied obesity-induced changes in the expression of *Igf-1, Tnf-α,* and *Pparγ-2* genes in IF and GF tissues. The results of mRNA expression in GF showed that leptin level was significantly increased in the BFMI852 and BFMI861 lines relative to the control group (Table I). There were no marked alterations in the expression levels of the other studied genes. In the IF tissue, leptin mRNA levels were significantly higher in all BFMI lines (Table II). The mRNA expression level of *Pparγ-2* was significantly decreased in the BFMI852, 856, and 861 lines. *Tnf-α* and *IL-6* expression also showed a decrease in BFMI856 and 860 lines. However, no statistically significant changes were detected in the mRNA expression level of the *Igf-1* gene in all lines. It was observed that despite the similar body weight of BFMI 852, 856, and 860, there were slight differences in their response to the storage of high amount of fat.

Leptin is a critical hormone for the regulation of body weight, and food intake by suppressing appetite and

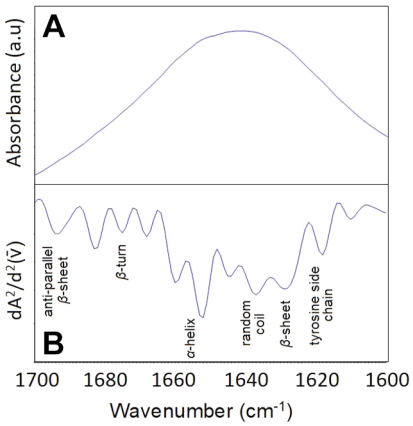

Figure 2. *Representative Attenuated Total Reflection – Fourier Transform Infrared Spectroscopy (ATR-FTIR) spectrum (A) and second derivative spectrum of gonadal fat (GF) tissue of BFMI860 line (B) in the 1700-1600 cm^{-1} region.*

increasing energy expenditure (4, 27). It also controls immunity and inflammation (5). It is known that leptin levels are positively correlated with BMI (28) and high level of leptin is a marker of leptin resistance. It may also be linked to an increased risk of cardiovascular disease (27). In obese individuals, excessive fat accumulation leads to secretion of high levels of leptin and leptin resistance and consequently loss of regulation of satiety, energy expenditure and body weight/adiposity (29). Thus, elevated leptin expression in GF tissues of BFMI852 and BFMI861 lines and IF tissues of all BFMI lines indicated that obesity is in progress in these animals.

TNF-α is a multifunctional proinflammatory cytokine, which regulates immune function, cell differentiation and proliferation, apoptosis, and energy metabolism. It functions in lipolysis stimulation, regulation of adipokines production, insulin resistance, and inhibition of adipose differentiation (2, 3). It is considered a likely mediator of insulin resistance and type II diabetes associated with high visceral adiposity (30). IL-6 is a proinflammatory mediator and a stress-induced cytokine with multiple effects including regulation of inflammatory response, enhancement of adipose tissue lipolysis, and stimulation of acute phase protein synthesis (31, 32). An elevated level of IL-6 is also a predictor for the development of type II diabetes and cardiovascular disease (33). The low expression levels of TNF-α and IL-6 in spite of high fat storage in BFMI 856 and BFMI 860 mouse lines indicate that neither inflammation nor insulin resistance were in question. Furthermore, no inflammatory response except leptin resistance appeared as indicated by marked changes in the expression levels of leptin in all BFMI lines.

Pparγ-2 plays a role in adipocyte differentiation, lipid and glucose metabolism, inflammation, and maintenance of metabolic homeostasis (34). It inhibits the production of pro-inflammatory factors such as TNF-α, IL-6, and IL-1β (35). Down-regulation of Pparγ-2 expression in BFMI 852, 856, and 861 lines may indicate that the inflammatory response in these lines was not yet developed well or given high leptin expression, it may indicate that it is at a very early stage for detection.

Protein secondary structure analysis. The regular protein structure is critical to perform their function (13). ATR-FTIR spectroscopy provides direct and rapid spectral acquisition to obtain data about the secondary structure of protein. Figure 2A shows a representative ATR–FTIR spectrum of the protein amide I band of the control line located in the 1700-1600 cm^{-1} region at room temperature (20˚C). This well-known amide I band originates mainly from the C=O stretching vibration (80%) of the peptide bonds (36). This band contains unresolved bands belonging to the secondary structures, which can be resolved and easily observed in the second derivative spectrum of the protein (Figure 2B). The bands at 1653 cm^{-1} and 1632 cm^{-1} are assigned to an α-helix and β-sheet, respectively, and the band at 1678 cm^{-1} is attributed to a β-turn. The band located at 1670-1695 cm^{-1} represents anti-parallel β-sheet structure whose certain assignment is difficult because of the overlapping absorptions from β-turns and unordered structures (36). The bands related to a random coil and a tyrosine side chain were observed at 1639 cm^{-1} and 1617 cm^{-1}, respectively.

The alterations in the protein secondary structures in inguinal and gonadal adipose tissues of male BFMI mice were examined using artificial neural networks and secondary derivative-vector normalization methods. Changes in protein secondary structures observed in GF and IF tissues of male mice obtained using ANNs are shown in Figure 3 and Figure 4, respectively, and using second derivative vector normalization approach in Table III and Table IV, respectively.

In addition to the alterations in protein structure, dysregulations of adipokines are also observed in metabolic diseases (2, 10, 11). In the present study, differences in the mRNA expression levels of *Pparγ2*, *leptin*, *TNF- α*, and *IL-6* genes between BFMI and DBAJ mice were determined among obese BFMI lines. The qRT-PCR studies showed increased levels of leptin in the GF tissues of all male BFMI mice, which implies that obesity has just begun to develop in these animals, and leptin resistance may be developing. Taken together with the expression results in GF and IF tissues, the fat storage location is critical in inducing measured changes in inflammatory factors.

Leptin is associated with post-feeding satisfaction. It is well known that leptin resistance is correlated with the increase of adipose tissue in the body. This condition is believed to be related to the occurrence and/or continuation of obesity. The increased expression profiles

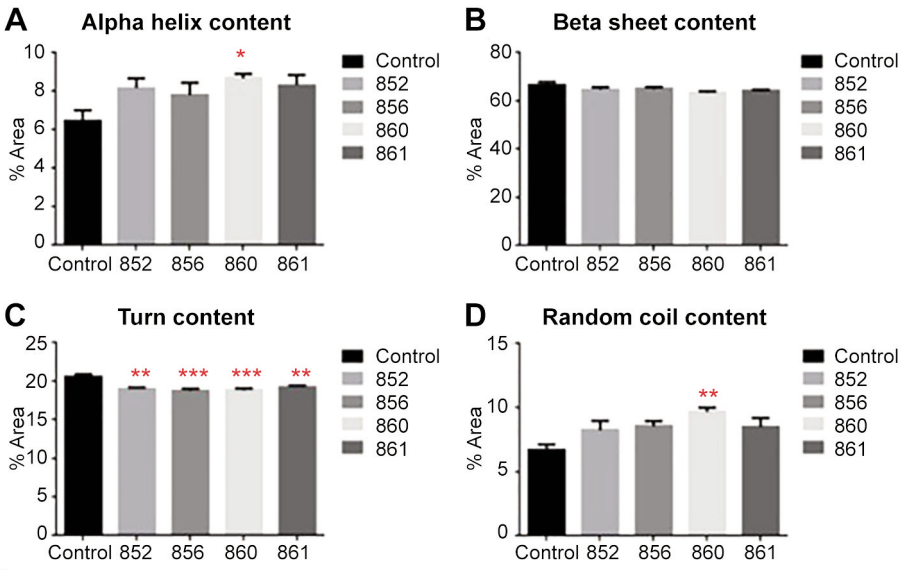

Figure 3. *Alteration in the content of A) alpha helix B) beta sheet C) turn D) random coil in male gonadal fat tissue using artificial neural network (ANN) analysis.*

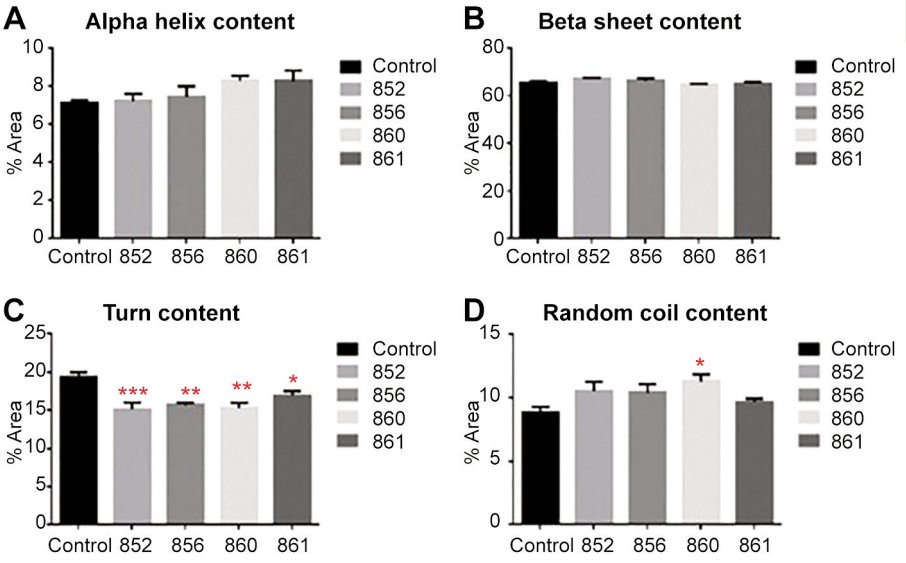

Figure 4. *Alteration in the content of A) alpha helix, B) beta sheet, C) turn, D) random coil in male inguinal fat tissue using artificial neural network (ANN) analysis.*

of leptin indicate that these animals are hyperphagic. This finding demonstrates the suitability of the BFMI models for obesity studies.

No significant changes in the gene expression levels of *TNF-α* and *IL-6* cytokines were found in GF and IF tissues of BFMI lines. According to these results, obesity-induced inflammation may be in the very early stage to observe significant changes in mRNA expression levels of these cytokines in the studied BFMI mice fed with a standard diet (SBD) for 10 weeks. The marked alterations in the secondary structure of IF and GF proteins together with the increased leptin levels were observed may indicate the triggering of the immune response. In addition, Baloglu *et al*. (16) reported the structural changes in lipids and proteins at inguinal and gonadal fat tissues of obese 10 weeks old BFMI lines at and showed that changes in inguinal fat were more dramatic,

Table III. *Changes in the protein secondary structure in male Berlin Fat Mouse Inbred (BFMI) gonadal fat (GF) tissue using signal intensity values of second derivative infrared spectra.*

	Control	852	856	860	861
Alpha helix	–0.17±0.02	–0.24±0.02↑	–0.23±0.01↑	–0.22±0.02↑	–0.19±0.02↑
Beta sheet	–0.20±0.02	–0.17±0.01↓	–0.18±0.01↓	–0.17±0.02↓	–0.16±0.02↓
Turn	–0.09±0.02	–0.06±0.01↓	–0.05±0.01↓	–0.05±0.00*↓	–0.03±0.00*↓
Random coil	–0.08±0.01	–0.09±0.02↓	–0.09±0.01↑	–0.11±0.01*↑	–0.09±0.02↑

The values are the mean±standard error of the mean for each group. The degree of significance is denoted with asterisks (*) for the comparison of the control group with other BFMI lines (*$p<0.05$).

Table IV. *Changes in the protein secondary structure in male Berlin Fat Mouse Inbred (BFMI) inguinal fat (IF) tissue using signal intensity values of second derivative infrared spectra.*

	Control	852	856	860	861
Alpha helix	–0.16±0.01	–0.19±0.01↑	–0.18±0.01↑	–0.17±0.02↑	–0.20±0.02↑
Beta sheet	–0.17±0.02	–0.13±0.01↓	–0.120.01↓	–0.10±0.01*↓	–0.13±0.02↓
Turn	–0.10±0.00	–0.04±0.01↓	–0.01±0.00*↓	–0.02±0.01*↓	0.02±0.01*↓
Random coil	–0.05±0.01	–0.06±0.01↑	–0.08±0.01↑	–0.09±0.00*↑	–0.09±0.02↑

The values are the mean±standard error of the mean for each group. The degree of significance is denoted with asterisks (*) for the comparison of the control group with other BFMI lines (*$p<0.05$).

obesity was in progress, and insulin resistance and therefore for type 2 diabetes was in question.

However, stromal cells may be the reason for not observing significant alterations in mRNA expression levels of inflammation markers in the studied BFMI lines. Stromal cells (macrophages) are associated with white adipose tissue and are responsible for the release of inflammatory cytokines. For this reason, the use of advanced techniques such as separation of stromal cells from adipose tissue to determine the cytokine expression levels may provide additional information on obesity-associated inflammation. In addition, since the cytokines studied here are generally released and act as paracrine factors, there may be a post-transcriptional regulation, *i.e.*, translation, or release steps.

In protein secondary structure analysis studies, ANN and the second derivative vector normalization methods showed similar results. A marked decrease in beta-turn content and a significant increase in random coil content were observed especially in BFMI860 male mice, indicating that there is a structural change in adipose tissue proteins, especially in inguinal fat tissues. This finding is consistent with the results of Baloglu *et al.* (16) who provided evidence of structural changes in proteins based on the variations in the band area ratio of amide I to amide I. The amide I band indicates C=O stretching/NH bending, and the amide II band corresponds to the NH bending/CN stretching vibrations. Therefore, the changes in the of amide I and amide II band areas reflect alterations in protein structure. Although no significant changes were observed in proinflammatory cytokines, the detected changes in protein structure imply that BFMI860 mice show a more severe response to excess fat storage than other BFMI lines. These alterations may contribute to adipose tissue dysfunction in obesity due to the structure-function relationship. In other words, these lines could be considered more sensitive to obesity relative to other BFMI lines.

Determination of changes in protein secondary structures in adipose tissues examined as in our study is necessary to detect changes in macromolecular structure and functions for phenotypic characterization of spontaneous obesity in BFMI mice. In addition, this is the first report of secondary structural changes of proteins in adipose tissues and BFMI lines.

Conclusion

Our results clearly showed that increased fat deposition in adipose tissues causes significant increases in the mRNA expression level of leptin that led to leptin resistance in all studied BFMI mice. The results also showed that the IF tissue is more sensitive to the effect of fat storage in the lines studied. BFMI860 strain responds to obesity more strongly than the other lines and shows the most significant alterations in protein secondary structure. Considering all studied male BFMI lines, BFMI860 is a suitable model for spontaneous obesity studies.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization and methodology: F.S., M.S. and G.B.; formal analysis: I.S., and M.S.; investigation: I.S..; resources: F.S. and G.B.; writing—original draft preparation: A.D.; writing—review and editing: A.D., and F.S.; supervision: F.S. All Authors have read and agreed to the published version of the manuscript.

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