**High-fat diet-induced plasma protein and liver changes in obese rats can be attenuated
by melatonin supplementation**

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23 **Abbreviations:**

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25

26 APOE; apolipoprotein E

27 A1AT; Alpha-1-antiproteinase

28 BMI; body mass index

29 CRP; C-reactive protein

30 FBG; fibrinogen

31 GPx3; glutathione peroxidase 3

32 HFD; high-fat diet

33 MBP-A; mannose-binding protein A

34 PLG; plasminogen

35 TTR; transthyretin

36 T2DM; Type 2 diabetes mellitus

Abstract

Obesity triggers changes in protein expression in various organs that might participate in the pathogenesis of obesity. Melatonin has been reported to prevent or attenuate such pathological protein changes in several chronic diseases. However, such melatonin effects on plasma proteins have not yet been studied in an obesity model. Using a proteomic approach, we investigated the effect of melatonin on plasma protein profiles after rats were fed a high-fat diet (HFD) to induce obesity. We hypothesized that melatonin would attenuate abnormal protein expression in obese rats. After 10 weeks of the HFD, animals displayed increased body weight and fat accumulation as well as increased glucose levels, indicating an obesity-induced pre-diabetes mellitus like state. Two-dimensional gel electrophoresis and liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) revealed 12 proteins whose expression was altered in response to the HFD and the melatonin treatment. The altered proteins are related to the development of liver pathology, such as cirrhosis (A1AT), thrombosis (FBG, PLG), and inflammation (MBP-A, complement C4, complement factor B), contributing to liver steatosis or hepatic cell death. Melatonin treatment most probably reduced the severity of the HFD-induced obesity by reducing the amplitude of HFD-induced plasma protein changes. In conclusion, we identified several potential biomarkers associated with the progression of obesity and its complications, such as liver damage. Furthermore, our findings reveal melatonin's a beneficial effect of attenuating plasma protein changes and liver pathogenesis in obese rats.

Keywords: high-fat diet; melatonin; obesity; plasma protein; proteomics

1. Introduction

Obesity is defined as abnormal or excessive fat accumulation that negatively affects multiple metabolic pathways, thus impairing health. More than 600 million people worldwide are obese, including children [1]. Furthermore, there has been a dramatic increase in obesity in low- and middle-income countries. Obesity is linked to more deaths worldwide than malnutrition and is a major risk factor for a number of chronic diseases, including diabetes, cardiovascular diseases, and cancer. The most common cause of obesity is an increased calorie intake from high-energy foods or high dietary fat. High-fat diet (HFD) is commonly used in laboratory animals to induce obesity. These high-fat diets affect the protein expression in many insulin target tissues, such as muscles, adipose tissue, and the liver [2, 3]. Many circulating proteins have been shown to be altered in obesity and contribute to its pathogenesis. Obesity-associated changes in several circulating markers that influence body weight, insulin sensitivity, blood pressure, oxidative stress, coagulation, fibrinolysis, inflammation, and adverse levels of various adipokines lead to impairment of various organs [4, 5].

The pineal hormone melatonin is a regulator of biological rhythms, with antioxidant and anti-inflammatory properties. It also influences several hormones involved in metabolic pathways, such as insulin, glucagon, and somatostatin [6, 7]. Previous reports have shown a significant improvement in plasma liver enzymes and plasma lipids and altered circulating levels of insulin and adipokines, such as leptin, adiponectin, ghrelin, and resistin in patients with nonalcoholic fatty liver disease treated with melatonin [8, 9]. Melatonin significantly

increased glutathione and attenuated oxidative stress in rats with high-fat diet-induced fatty liver disease [10]. Chronic oral melatonin reduces liver steatosis and mitochondrial dysfunction in diabetic obese rats [11]. However, the effects of melatonin on global plasma protein levels have not yet been analyzed in HFD obese animals. We hypothesized that melatonin would improve the alteration of plasma protein in obese animals. Therefore, we investigated the effects of melatonin on various plasma protein levels in HFD-induced obese rats. We used a proteomic approach providing high-throughput quantitative proteome profiling to identify the differentially expressed plasma proteins between non-obese and obese rats with or without melatonin treatment.

2. Methods and materials

2.1. Animal treatment protocol

Twenty-four adult male Wistar rats (190-200 g) were obtained from the National Laboratory Animal Center of Mahidol University, Thailand. Animals were allowed to adjust to a 12 h light:12 h dark cycle with lights on from 6 am (Zeitgeber time (ZT) 0) at constant temperature for 1 week before experimental treatments were started. Rats were divided into three groups: a control group (n=6) was fed a standard diet of CP082 (Perfect Companion Group Co. Ltd, Bangkok, Thailand) throughout the 10-week experiment, and the two other groups were fed a high-fat diet and received melatonin (HFD+Mel) (n=9) or vehicle injections (HFD) (n=9). The composition of the standard and HFD is indicated in Table 1. The HFD used in this study was modified form the HFD2 diet of Sinitskaya et al. [12]. HFD animals were fed a HFD for 4 weeks before melatonin or vehicle treatments. This protocol

was selected in order to first establish some HFD-induced changes before testing whether melatonin can reverse or slow down the pathogenesis of obesity.

In the HFD+Mel group, 10 mg/kg body weight of melatonin (Sigma-Aldrich, St Louis, MO, USA) was intraperitoneally injected daily at ZT 9 to ZT10 for 6 weeks, starting at the 5th week of dietary feeding. The doses and the time of the injection were chosen based on previous study of protective effects against fatty liver in rats [15] and our previous study in diabetic rats [13]. Control and HFD group animals received vehicle injections at the same time. Body weight was recorded weekly. The animals were kept under controlled conditions with food and water provided *ad libitum*. Rats were treated in accordance with and approved by the Laboratory Animal Care and Use Committee of Mahidol University (COA. NO. MB-ACUC 2013/003).

2.2. Sample collection

At the end of experiment, animals were fasted for 12 h. Then, blood samples were obtained before sacrifice by tail nick, and glucose levels were measured using a glucose meter and strips (OneTouch® SureStep, LifeScan, Inc. Milpitas, CA, USA). Animals were anesthetized using isoflurane gas, and then blood samples for blood parameter analyses and proteomic analyses were collected via cardiac puncture after a cardiac injection of 750 IU of heparin. After the blood collection was completed, rats were euthanized by decapitation or by perfusion with formaldehyde fixative (see below). Plasma was obtained after centrifugation at room temperature for 15 min at 3000 g and stored at -80°C until analysis. Insulin levels were

determined using a Rat/Mouse insulin ELISA (enzyme-linked immunosorbent assay) kit (EMD Millipore Corporation, Billerica, MA, USA), following the manufacturer's instructions. Epididymal and retro-peritoneal fat pads were isolated and weighed.

2.3. Liver histology evaluations

After cardiac blood sampling, four animals from the control group and six animals each from the HFD and HFD+Mel groups were perfused with 4% phosphate-buffered formaldehyde. The liver tissues were dissected and post-fixed for 12 h in the same fixative. Fixed livers were embedded in polyethylene glycol, sectioned at 6 μ m, and mounted on glass slides. The sections were stained with hematoxylin and eosin (HE) to evaluate liver steatosis.

We designed a steatosis index similar to the one validated by Kleiner et al. [14] to evaluate the severity of liver changes in the different treatment groups. This index evaluates liver changes based on vesicular fat accumulation and the presence of granulomas, which were scored from 0-4 as follows: 0 is no steatosis, 1 is light steatosis (only some peripheral vesicles) limited to cells in some areas of the liver, 2 is moderate to strong steatosis (some eosinophilic cytoplasm without vesicles) with non-affected, intact areas in the liver, 3 is strong steatosis, with some intact cells but no unaffected areas in the liver. Generalized severe steatosis was graded as 4 and characterized by cells fully filled with vesicles with almost no visible eosinophilic cytoplasm and perivascular granulomas. The steatosis index was reduced

by -0.5 for reduced extension of the changes and increased by +0.5 for presence of granulomas in grades 1 to 3. No fibrosis was observed in our animals.

2.4. Proteomics analysis

The sample preparation and proteomics analysis procedure were performed according to our previous studies [15]. Three different pools of two or three samples from each group were prepared by combining equal volumes of plasma samples from each animal to reach the appropriate total protein sample and thus yield three independent analyses for each group. To improve the performance of the proteomic analysis, albumin and immunoglobulins in the plasma samples were depleted using trichloroacetic acid (TCA)/acetone precipitation. The proteins were dissolved in lysis buffer (30 mM Tris, 2 M thiourea, 7 M urea, 4% CHAPS (w/v) at pH 8.5). Two-dimensional gel electrophoresis (2-DE) was carried out using precast immobilized pH gradient (IPG) strips (18 cm, pH 3-10NL) on an Ettan IPGphor III system (GE Healthcare, Uppsala, Sweden) followed by 12.5% sodium SDS-PAGE. A sample containing 400 µg of total protein was mixed with 340 µl of rehydration buffer containing 1% 3-10 NL IPG buffer and 1.5% destreak reagent (GE Healthcare). Samples were run through the steps of strip rehydration and isoelectric focusing (IEF) was carried out at 20°C for a total of 36,000 volt-hours. Following IEF, the IPG strips were immediately used for the second dimension. The IPG strips were incubated in equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, and 2% (w/v) SDS) containing 2% (w/v) DTT for 15 min (twice) with gentle agitation, followed by incubation in the same equilibration buffer supplemented with 2.5% (w/v) iodoacetamide for the same time periods as above at room

temperature. The second dimension was carried out in 12.5% polyacrylamide gel at a constant power of 10 watts per gel using an Ettan™ DALTsix (GE Healthcare). To visualize the protein spots, the two-dimensional (2D) gels were stained with colloidal CBB G-250. Protein patterns in the gels were recorded using an Image Scanner III (resolution 600 dpi, GE Healthcare). Differential protein expression level analysis was performed by the ImageMaster 2D Platinum version 7.0 (GE Healthcare) software. Protein spots of interest were manually excised from preparative gels and digested using in-gel trypsin digestion. Peptides were reconstituted in 10 µl of 0.1% formic acid (FA) and identified by liquid chromatography-mass spectrometry (Dionex Ultimate 3000, Thermo Scientific, Waltham, MA, USA) in combination with an electrospray ionization-ion trap mass spectrometry (ESI-Ion trap-MS, Model Amazon SL, Bruker, Bremen, Germany). To identify proteins, all MS/MS spectra recorded on tryptic peptides derived from each spot were searched against protein sequences from SwissProt databases using the MASCOT search program (www.matrixscience.com).

2.5. Statistical analyses

A power analysis was performed with G Power version 3.1 [16] to determine an adequate sample size to study a significant difference of plasma parameters. The sample size was calculated using the power procedure for one-way analysis of variance (ANOVA), considering an alpha probability of 0.05 with a power of 0.80, and an effect size was calculated using the results from our pilot study. This power analysis determined that a sample size of six animals per group was required for studies. By this analysis, we decided to use six animals for the control group and nine animals for HFD and HFD+Mel in order to

obtain enough obese rats for the experiments. Statistical analyses were performed using SPSS version 18 (IBM, Armonk, NY, USA). Body weight, fat weight, plasma parameters, protein levels, and the steatosis index are expressed as means $\pm$ SEM. The differences among groups were determined by one-way ANOVA. The effect of dietary treatment and time point on the percentage of body weight gain was tested by two-way ANOVA. Post hoc analysis was performed by Tukey comparisons in which significant differences were found by ANOVA. Statistical significance was set at $P < .05$.

3. Results

3.1. Body weight, fat weight and glucose and insulin levels

No differences in body weight were observed between groups at the beginning of the experiment. After 14 days of the high-fat diet, the body weight of high-fat diet-fed rats was significantly increased when compared to standard diet-fed rats (Fig.1A, $P < .05$) and remained significantly elevated at all subsequent time points. Thus, at the start of the melatonin treatment, HFD and HFD+Mel rats already had a significantly higher body weight than control animals, and obesity was clearly beginning to establish itself. Both the HFD and HFD+Mel groups showed a higher percentage of body weight gain compared to the control group (Fig.1A, $P < .05$). The final body weights of the HFD and HFD+Mel groups was significantly higher than that of the control group (Fig.1B, $P < .05$). However, there was no significant difference between the final body weights of the HFD group and the HFD+Mel group. Additionally, the HFD and HFD+Mel groups had a significantly higher total fat weight (Fig.1C, $P < .05$). This increase was more pronounced for the retroperitoneal fat pads

(Fig.1E, $P < .05$) than for the epididymal fat pads (Fig.1D, $P < .05$). Fasting blood glucose levels were significantly elevated in the HFD and HFD+Mel groups compared to the control group (Fig.1F, $P < .05$). However, insulin concentrations did not differ among groups (Fig.1G).

3.2. Proteomic analysis of plasma proteins

The differentially expressed proteins in the plasma were identified using LC-MS/MS. Approximately 212 spots were detected and the obtained plasma protein maps represented patterns that were similar to previous reports in rats (Fig.2). Based on high protein score ($P < .05$) and sequence coverage, 43 spots were identified by LCMS/MS analysis. The protein identification details of 43 spots are listed in Table 2. Image analysis and statistical analysis allowed the detection and identification of 12 proteins whose expression was altered in response to a high fat diet and melatonin treatment. The 12 identified proteins were almost all produced in the liver. The analysis of the samples resulted in the identification of ten upregulated and one downregulated proteins upon high-fat feeding (Fig.3). Changes in differentially expressed proteins of each group are presented in Table 3 and Fig.4. As compared to the control group, upregulated proteins identified were glutathione peroxidase 3 (GPx3) (+1.50 fold, $P < .05$), complement C4 (+2.41 fold, $P < .05$), complement factor B (+1.65 fold, $P < .05$), mannose-binding protein A (MBP-A) (+2.37 fold, $P < .05$), serotransferrin (+1.70 fold, $P < .05$), transthyretin (TTR) (+2.30 fold, $P < .05$), gelsolin (+1.68 fold, $P < .05$), apolipoprotein E (APOE) (+1.36 fold, $P < .05$), plasminogen (PLG) (+1.78 fold, $P < .05$) and fibrinogen (FBG) β chain (+1.61 fold, $P < .05$). Alpha-1-antiproteinase

(A1AT) was downregulated 2.18 fold compared to the control group ($P < .05$). In HFD+Mel, the administration of melatonin for 6 weeks significantly reduced the amplitude of the down-regulation of GPx3, serotransferrin, FBG β chain and C-reactive protein (CRP) ($P < .05$) as well as the upregulated complement factor B and APOE as compared with HFD group ($P < .05$). Melatonin had no effect on the protein levels of PLG, gelsolin, TTR, MBP-A, and A1AT in HFD+Mel as compared to the HFD group (Fig.4).

3.3. Liver histology and steatosis index

Our results show that almost all of the plasma protein changes in HFD and HFD+Mel were proteins produced in liver. After perfusion fixation, the livers of the HFD fed animals showed a “marbled” appearance, suggesting that the animals had started to develop fatty liver disease (Fig.5B) as compared to control rats (Fig.5A). We used liver histology to further evaluate cellular damage in the liver. We observed variable extents of liver steatosis, which were more severe and more extensive in HFD animals as assessed by our steatosis index (Fig.5D). We observed only microvesicular changes in the hepatic cells. No macrovesicular accumulations of lipids were observed. We observed some granulomas around blood vessels in the most severe cases of liver steatosis. Melatonin treatment seemed to reduce or prevent liver changes (Fig.5C), but did not completely reverse them, as the HFD+Mel animals still presented a significantly elevated steatosis index (Fig.5D).

4. Discussion

In the present study, we used a high-fat and high-protein diet, with a low percentage of carbohydrates, as such a high fat to carbohydrate ratio has previously been shown to be more efficient in triggering a pre-diabetic state in Wistar rats [12]. In contrast with previous results, our HFD animals quickly showed high body weight gain, increased fat weight and high blood glucose levels when compared to control animals. Our preliminary data showed no significant differences in daily food intake between normal diet and HFD groups (data not shown) as observed in other previous studies [17, 18]. The high-fat low carbohydrate diet induced obesity in our rats. We did not observe any weight loss in the HFD+Mel rats. However, although the body weight between HFD and HFD+Mel animals was not statistically significant at the end of our experiment, the HFD+Mel animals clearly stopped gaining weight in the last 3 weeks of the experiment, while the HFD animals continued to gain weight as observed in previous studies [19, 20]. This difference between HFD and HFD+Mel might have become statistically significant upon a longer treatment duration. Insulin levels showed an increasing trend in HFD-fed rats, although the change was not significantly different. No clear insulin resistance developed in our obese rats. However, histological evaluation of the liver showed that this organ was clearly affected by the HFD. HFD rats revealed more severe steatosis in hepatic cells, which may explain the lower plasma triglyceride levels in the HFD group as observed in previous reports in HFD-induced obese rats [20, 21]. Moreover, Pan et al. also showed that the concentration of triglycerides in liver tissue of HFD rats rose remarkably compared to the control group [20]. Proteomic analysis revealed a total of 12 proteins that were significantly altered after either HFD or melatonin treatment. Almost all the altered plasma proteins are produced mainly by the liver, suggesting

that at this stage the liver is affected by this diet. The microvesicular steatosis we observed is typical of non-alcoholic fatty liver disease [22].

Serotransferrin or transferrin, is a blood plasma glycoprotein that is produced mainly in the liver. It is an iron-binding transport protein that is responsible for iron metabolism and stimulating cell proliferation. Iron can affect tissues involved in glucose and lipid metabolism such as liver and adipose tissue, by interplay mechanisms with transferrin, ferritin, insulin, and hepcidin to maintain iron homeostasis [23]. Lee et al. suggested that increased serum iron status indicators, including ferritin and soluble-form transferrin receptor levels may be related to insulin resistance and metabolic syndrome [24]. A recent study in humans also showed that elevated serum transferrin concentrations were positively correlated with the risk of metabolic syndrome and obesity [25]. Hyperferritinemia with normal or mildly elevated transferrin saturation was observed in patients with metabolic syndrome or nonalcoholic fatty liver disease [26]. Here, we observed an increase in plasma serotransferrin in HFD rats that might indicate abnormal iron metabolism related to metabolic syndrome and hepatic tissue damage. Imbalance of iron homeostatic mechanisms results in an increase of free radicals [27] and systemic and parenchymal siderosis that contribute to fibrosis in liver diseases [23]. On the other hand, serotransferrin delivers iron to cells and supports hepatic cell proliferation to compensate for liver damage induced by an HFD in obese rats [28]. Melatonin administration reduced the plasma serotransferrin levels in HFD rats to the normal levels found in control animals. Therefore, melatonin may be used to control transferrin levels to regulate the balance of iron transport into cells.

Fibrinogen (FBG) and plasminogen (PLG) are both proteins produced by the liver. FBG is required for blood clotting, and plasminogen is required for fibrinolysis. Both are increased by HFD. Proteomic analysis demonstrated differential expression of FBG (γ and β chain) in human serum [29] from obese and non-obese subjects. Type 2 diabetes mellitus (T2DM) patients have high plasma FBG levels and hyperfibrinogenemia [30]. In this study, plasma FBG levels were increased by HFD as observed by Herbert et al. [31] in genetically obese Zucker rats. An increased FBG might create a thrombosis increasing the risk of developing micro- and macro-vascular complications in metabolic diseases. Our results clearly show that melatonin treatment normalized the HFD-induced FBG increase in rats. Additionally, plasminogen is the inactive precursor of the trypsin-like serine protease plasmin, which lyses the fibrin clots to fibrin degradation products. PLG conversion to plasmin (active protease) is mediated by urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). The activity of both is regulated by specific plasminogen activator inhibitors (PAIs). In a recent study, PAI-1 levels were high in obese patients [32] whereas tPA levels were lowered in adults who reported consuming a diet high in saturated fat [33]. The elevated levels of PLG in our study might be due to reduced conversion to plasmin by tPA/uPA, either through reduced expression of these plasminogen activators or increased expression of plasminogen activator inhibitors. Alternatively, the pathological state of the liver due to the HFD might induce increased PLG production.

Glutathione peroxidase 3 (GPx3) is present in the serum to help reduce hydro- and lipoperoxides from the body. Changes in *GPx3* gene expression and protein concentration were observed in T2DM [34] and obesity [35]. In our study, the changes in GPx3 were also consistent with the diet and melatonin treatment. HFD rats showed increased plasma GPx3

levels as compared to control rats. A previous study also reported that serum GPx3 concentrations are increased in overweight or obese individuals [36]. An HFD may increase levels of peroxides and other reactive species in plasma that promote thrombosis in GPx3-deficient mice [37]. Increase plasma GPx3 might thus prevent or reduce the risk of thrombosis in HFD-induced obese rats. The reduction of GPx3 in melatonin-treated rats is potentially related to the antioxidative properties of melatonin, which might lower the requirement for other antioxidative mechanisms.

Obesity has been widely recognized as a chronic inflammatory condition with increased expression and secretion of several inflammatory indicators associated with the development of a variety of metabolic disorders [38, 39]. Here, we observed elevated immunological markers in HFD animals, such as mannose-binding protein A (MBP-A), complement C4 and complement factor B. This observation is in agreement with previous reports in overweight and obese people [40, 41]. MBP-A mediates activation of the complement system via the lectin pathway. High levels of MBP-A and complement proteins may be indicative of the inflammation state in HFD animals. The complement-mediated inflammation may contribute to the development of obesity complications such as cell death or vascular damage [38]. A previous study showed that high C-reactive protein (CRP) levels are related to body mass index (BMI) in obese patients [29, 40]. CRP is normally highly upregulated during infections and inflammation. Elevated CRP was reported in response to HFD and showed a gender difference in both control and obese rats [42]. The reduction in CRP levels by melatonin indicates an anti-inflammatory effect of melatonin that might be useful to attenuate the HFD-induced inflammatory response in obesity.

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382 Alpha-1-antiproteinase or α 1-antitrypsin (A1AT) is a protease inhibitor belonging to the
383 serpin superfamily. It is a serum trypsin inhibitor that protects tissues from the enzymes of
384 inflammatory cells, especially neutrophil elastase. An imbalance between A1AT and the
385 neutrophil elastase contributes to the development of obesity and other complications, such as
386 inflammation and liver steatosis. Our observation that plasma A1AT levels were reduced in
387 HFD rats might indicate that the liver starts to progress toward a cirrhotic state even before
388 fibrosis has been observed. A previous study in obese mice showed an increase in the activity
389 of the neutrophil elastase and decreased serum levels of the A1AT [43]. In humans, A1AT
390 concentrations were mildly lower in obese women compared with non-obese women [44].
391 However, no correlation between serum levels of alpha1-antitrypsin and IR indices was
392 observed in the obese women and the lean women [45]. A1AT and adiponectin levels
393 decrease as the BMI increases. A1AT levels correlate closely with obesity, and are related to
394 other factors such as leptin, adiponectin, and insulin. A1AT might be used as a clinical
395 biomarker and may serve as a potential target for treating obesity [46]. However, melatonin
396 treatment had no effect on plasma A1AT levels in our study.

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399 Transthyretin (TTR) is a thyroid hormone transporting protein secreted by liver. The TTR
400 level is a critical determinant of retinol-binding protein 4 (RBP4) levels. TTR circulation is
401 bound to retinol-binding protein 4 (RBP4) as a complex that reduces renal clearance of
402 RBP4. High circulating levels of TTR and RBP4 are associated with glucose intolerance,
403 obesity, T2DM, and insulin resistance [47, 48]. Here, levels of plasma TTR were increased
404 approximately two-fold in HFD rats which is in agreements with studies in HFD mice [49]
405 and also in obese and T2DM patients [47]. Many studies have attempted to test the

therapeutic potential for T2DM by targeting TTR and RBP4. TTR antisense oligonucleotides decreased TTR levels and improved glucose metabolism and insulin sensitivity in obese mice [49]. Similarly, suppression of RBP4 with RNA oligonucleotides prevented HFD-induced metabolic syndrome and non-alcoholic fatty liver disease in mice [50]. Interfering with RBP4-TTR binding enhanced insulin sensitivity in obesity and T2DM [51]. However, melatonin treatment was not able to reduce the TTR levels in HFD-induced obese rats.

Plasma gelsolin is an actin-binding protein that functions as part of the extracellular actin-scavenging system. Plasma gelsolin is used for the prognosis of various pathological conditions in which a low gelsolin level indicates a bad prognosis [52]. Blood gelsolin levels decrease markedly in a variety of clinical conditions, including liver injury [53]. However, an *in vitro* study reported that gelsolin-specific transcript levels were upregulated under conditions of acute and chronic liver damage in human and rat [54]. The high levels of plasma gelsolin in our HFD rats may indicate that the HFD has not yet exhausted the regenerative properties of the liver and other organs. Although liver steatosis was sometimes extensive in our study, we observed no fibrosis indicative of early cirrhosis. Plasma gelsolin might be increased to cope with tissue damage induced by the HFD or try to improve host defenses against infection or inflammation in organs [55]. Elevated gelsolin increases the survival rate in animal models of different acute insults [56].

The increase in APOE is consistent with the HFD. APOE is a major chylomicron apoprotein. It binds to a specific liver and peripheral cell receptor, and is required for triglyceride catabolism. Previous studies have shown that high plasma APOE levels are strongly

associated with risk of cardiovascular disease [57]. However, plasma APOE levels were lower in diabetic mice than nondiabetic and moderately diabetic mice [39]. High levels of APOE are also associated with an increase of plasma CRP, a marker of inflammation [57]. Interestingly, in our study, melatonin significantly increased APOE levels, while decreasing CRP levels below normal levels. While the decreased CRP levels might be due to the anti-inflammatory action of melatonin, the significance of the increase in APOE remains unclear.

In conclusion, HFD induced obesity and liver damage resulting in changes in plasma proteins. The proteomic analysis revealed 12 plasma proteins affected by the HFD that were previously reported as plasma markers of obesity and other metabolic syndromes. Melatonin treatment reduced the requirement for GPx3 to deal with the increased load of lipid peroxidation and also attenuated HFD-induced inflammation through reducing inflammatory markers. As we hypothesized, melatonin supplementation improved the alterations of plasma proteins in obese animals. Melatonin also counteracted HFD-induced increase in fibrinogen and might thus reduce blood clotting and thrombosis risk. Considering its very low toxicity, melatonin may be beneficial in HFD-induced obesity by attenuating changes in plasma proteins and thus reducing the severity of the associated inflammatory complications, particularly in the liver.

Obesity and T2DM are chronic diseases that often become more prevalent in the second half of human lives. Thus, studies such as ours only partially model the clinical reality of these diseases. Future studies should evaluate the changes induced by prolonged obesity and fully developed T2DM in older animals. Furthermore, the time-course with which changes, such as

456 those identified in our study, establish themselves during the development of obesity and
457 T2DM might be of importance for the therapeutic value of melatonin or other treatments.

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622

Figure captions

Fig.1. Changes in the basic physiological and biochemical responses of standard diet fed (Control), or high-fat diet (HFD) and melatonin-treated high-fat diet fed rats (HFD+Mel). (A) Body weight changes expressed as percent body weight gain. Two-way ANOVA with Tukey's post-hoc test was conducted to determine significant differences across all groups at each time point. (B) Final body weights (C) Total fat weights (D) Epididymal fat weights (E) Retroperitoneal fat weights (F) Fasting blood glucose levels (G) Insulin levels. Data are presented as means $\pm$ SEM (n=6-9). One-way ANOVA with Tukey's post-hoc test was used to determine significant differences between treatment groups. * $P < .05$ versus the control group.

Fig.2. A master map of the protein expression profile of the plasma of standard diet fed rats (Control) after albumin and immunoglobulin depletion by TCA/acetone precipitation. 400 μ g of protein were loaded on 18-cm 2-DE gel (with a non-linear pH 3-10 gradient) and stained with colloidal CBB G-250. The approximately 212 spots of protein were found and differentially regulated proteins between treatment groups are marked with numbered circles and listed in Table 2.

Fig.3. 2-DE gel image of differentially regulated proteins in rat plasma of each treatment group. 400 μ g of pooled proteins were loaded on 18-cm 2-DE gel (with a non-linear pH 3-10 gradient) and stained with colloidal CBB G-250. (A) A representative colloidal CBB G-250-

stained rat plasma 2-DE gel image of standard diet fed rats (Control), high-fat diet (HFD) and melatonin-treated high-fat diet fed rats (HFD+Mel). Differentially regulated proteins in each group are marked with a circle and character (A-L). (B) Higher resolution 2-DE gel images. The protein names mark the location of the corresponding differentially expressed proteins. The details of changes in differentially expressed proteins in HFD and HFD+Mel groups compared to the control group are summarized in Table 3.

Fig.4. Plasma protein levels in standard diet-fed rats (Control), high-fat diet-fed rats (HFD) and melatonin-treated high-fat diet rats (HFD+Mel). (A) GPx3. (B) APOE (C) Complement B (D) Plasminogen (E) Serotransferrin (F) Gelsolin (G) Fibrinogen (H) Transthyretin (I) MBP-A (J) Complement C4 (K) Alpha-1-antiproteinase (L) C-reactive protein. Data are presented as the means $\pm$ SEM of volume density (%) of the protein spot in three individual gels using pooled plasma of 6 to 9 rats per group. One-way ANOVA with Tukey's post-hoc test was used to determine significant differences between treatment groups. * $P < .05$ versus the control group, # $P < .05$ versus the HFD group.

Fig.5. Representative micrographs of liver histopathological evaluation in standard diet-fed rats (Control), high-fat diet-fed rats (HFD), and melatonin-treated high-fat diet rats (HFD+Mel). All animals were perfused for histology; thus, few erythrocytes are seen. The bar is 50 μ m. A-C) Hematoxylin and eosin (HE) staining. (A) Normal liver architecture in a control rats with almost no vesiculation of the cytoplasm of hepatic cells. (B) Severe microvesicular steatosis in a HFD rat with most cells completely filled with vesicles and displaying a “ballooned” appearance. (C) slightly attenuated steatosis in rats on HFD+Mel.

(D) The steatosis index evaluated the severity of these effects on the livers of rats in the control (n=4), HFD (n=6), and HFD+Mel (n=6). Data are presented as the means $\pm$ SEM. One-way ANOVA with Tukey's post-hoc test was used to determine significant differences within each treatment group. * $P < .05$ versus the control group.

Table captions

Table 2

A total of 43 proteins correspond to the master map in Fig.2.

- (a) Accession no. is SwissProt database accession number.
- (b) The nominal mass of an ion or molecule is calculated using the integer mass of the most abundant isotope of each element. This is the equivalent of summing the mass numbers of all constituent atoms.
- (c) Protein score is an ion score obtained from the analysis with Mascot software. Ions score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event. Protein score indicates the confidence of the protein identification; score value greater than 30 was considered significant ($P < .05$).
- (d) The percentage of the protein sequence covered by identified peptides.
- (e) The number of peptide spectrum matches (PSM). The number of PSM's is the total number of identified peptide spectra matched for the protein.

Table 3

- a) Relative intensity was averaged and expressed as a means $\pm$ SEM of three separate experiments.
- b) One-way ANOVA was used to determine significant differences within each treatment group and statistical significance was set at $P < .05$.
- c) Fold variations in spot intensity between each groups. Values reported in the table refer to spots characterized by a change in expression that exceeds a fold of variation >1.35 .
- d) Character in gel indicates character corresponding to the 2-DE image in Fig.3.

A.

Control

HFD

HFD+Mel

B.

Control

HFD

HFD+Mel

Table 1. Composition of the experimental diets

	Standard diet CP082	High-fat diet
Ingredient	g/kg	g/kg
Corn starch	594.16	-
Soy protein	306.25	498
Soybean oil	53.46	25
Lard	-	400
Cellulose	23.37	30
Dicalcium phosphate	7.12	24
Calcium carbonate	11.7	16
Sodium chloride	4.94	4
Vitamin and minerals	2	3
Energy (kcal/g diet)	3.66	5.63
% Energy from fat	13.67	68.30
% Energy from carbohydrate	59.56	0.57
% Energy from protein	26.77	31.13

Table 2. Protein profile in master map of rat plasma identified by ESI-Ion trap mass spectrometry

Spot No.	Accession no. ^(a)	Description	Nominal mass (M _r) ^(b)	Calculated pI value	Protein score ^(c)	Sequence coverage ^(d) (%)	Identified peptides number ^(e)
1	sp P13635	Ceruloplasmin	121562	5.34	129	6	4
2	sp P04186	Complement factor B	86319	7.18	260	9	4
3	sp Q68FP1	Gelsolin	86413	5.86	467	16	11
4	sp P02770	Serum albumin	70.682	6.09	1307	53	11
5	sp P12346	Serotransferrin	78512	7.14	579	25	13
6	sp Q01177	Plasminogen	93214	6.79	948	34	32
7	sp P12346	Serotransferrin	78512	7.14	1383	43	16
8	sp P36953	Afamin	71172	5.87	651	34	10
9	sp P20059	Hemopexin	52060	7.58	579	33	12
10	sp P01048	T-kininogen 1	48828	6.08	199	11	18
11	sp P05545	Serine protease inhibitor A3K	46760	5.31	559	31	10
12	sp Q9QX79	Fetuin-B	42391	6.71	303	25	9
13	sp P09006	Serine protease inhibitor A3N	46793	5.33	633	33	11
14	sp P17475	Alpha-1-antiproteinase	46278	5.70	645	45	2
15	sp P04276	Vitamin D-binding protein	55106	5.65	545	23	13
16	sp P02680	Fibrinogen gamma chain	51228	5.62	585	31	18

17	sp P06399	Fibrinogen alpha chain	87373	5.51	539	20	14
18	sp P06399	Fibrinogen alpha chain	87373	5.51	211	8	12
19	sp P14480	Fibrinogen beta chain	54828	7.90	435	36	7
20	sp P20760	Ig gamma-2A chain C region	35677	7.72	491	33	18
21	sp P60711	Actin, cytoplasmic 1	42052	5.29	86	15	4
22	sp Q63678	Zinc-alpha-2-glycoprotein	34224	5.87	196	16	6
23	sp P01026	Complement C3	187825	6.12	391	7	9
24	sp Q63041	Alpha-1-macroglobulin	168388	6.46	480	9	8
25	sp P06866	Haptoglobin	39052	6.10	648	36	19
26	sp P02767	Transthyretin	15824	5.77	174	31	3
27	sp Q63041	Alpha-1-macroglobulin	168388	6.46	276	3	16
28	sp P08649	Complement C4	193639	6.99	229	3	7
29	sp P20760	Ig gamma-2A chain C region	35677	7.72	384	32	15
30	sp P08649	Complement C4	193639	6.99	358	4	7
31	sp P01026	Complement C3	187825	6.12	286	4	7
32	sp P20767	Ig lambda-2 chain C region	11482	5.76	60	24	1
33	sp P23680	Serum amyloid P-component	26274	5.50	168	15	3
34	sp P01836	Ig kappa chain C region, A allele	11896	4.99	129	27	3
35	sp P01835	Ig kappa chain C region, B allele	11765	4.97	154	27	2
36	sp P23764	Glutathione peroxidase 3	25637	8.26	192	29	6
37	sp P23764	Glutathione peroxidase 3	25637	8.26	277	31	8

38	sp P04916	Retinol-binding protein 4	23567	5.49	108	12	3
39	sp P02767	Transthyretin	15824	5.77	155	24	3
40	sp P19999	Mannose-binding protein A	25748	7.55	280	27	5
41	sp P48199	C-reactive protein	25737	4.89	36	4	2
42	sp P14046	Alpha-1-inhibitor 3	165038	5.70	444	13	4
43	sp P02650	Apolipoprotein E	35788	5.23	754	48	16

Table 3. Changes of differentially expressed proteins of the HFD and HFD+Mel groups compared with control group

Protein	Average of intensity ^(a) (%Vol)			<i>P</i> value ^(b)	Up (+)/Down (-) regulation fold ^(c)			Character in gel ^(d)
	Control	HFD	HFD+Mel		HFD :Control	HFD+Mel :Control	HFD+Mel :HFD	
Glutathione peroxidase 3	0.052±0.003	0.078±0.002	0.065±0.006	.003358	+1.50	+1.25	-1.20	A
Apolipoprotein E	0.025±0.001	0.034±0.006	0.050±0.002	.033042	+1.36	+2.00	+1.47	B
Complement factor B	0.057±0.011	0.094±0.016	0.124±0.007	.004003	+1.65	+2.18	+1.32	C
Plasminogen	0.059±0.009	0.105±0.020	0.102±0.025	.008363	+1.78	+1.73	-1.03	D
Serotransferrin	0.040±0.006	0.068±0.009	0.050±0.005	.002477	+1.70	+1.25	-1.36	E
Gelsolin	0.047±0.010	0.079±0.011	0.074±0.004	.020053	+1.68	+1.57	-1.07	F
Fibrinogen beta chain	0.039±0.004	0.063±0.003	0.033±0.002	.045549	+1.61	-1.18	-1.91	G
Transthyretin	0.141±0.048	0.324±0.006	0.370±0.029	.000006	+2.30	+2.62	+1.14	H
Mannose-binding protein A	0.019±0.002	0.046±0.006	0.046±0.006	.014573	+2.37	+2.42	+1.02	I
Complement C4	0.078±0.021	0.188±0.041	0.232±0.017	.000086	+2.41	+2.97	+1.23	J
Alpha-1-antiproteinase	0.072±0.015	0.033±0.008	0.032±0.007	.044552	-2.18	-2.25	-1.03	K
C-reactive protein	0.034±0.005	0.033±0.004	0.020±0.002	.018609	-1.03	-1.70	-1.65	L

Highlights

- High-fat diet induced obesity, histological alterations of the liver and altered plasma protein levels in rats.
- Proteomic analyses identified most of the altered plasma proteins as related to liver pathogenesis.
- Melatonin supplementation reduced the severity and complications of high-fat diet induced obesity.