Circulating mesencephalic astrocyte-derived neurotrophic factor is increased in newly diagnosed prediabetic and diabetic patients, and is associated with insulin resistance

Tong Wu1), 2) *, Fang Zhang3) *, Qiu Yang4) *, Yuwei Zhang3), Qinhuai Liu2), Wei Jiang5), Hongyi Cao4), Daigang Li6), Shugui Xie7), Nanwei Tong3) and Jinhan He1), 2)

1) Department of Pharmacy, State Key Laboratory of Biotherapy, West China Hospital of Sichuan University and Collaborative Innovation Center of Biotherapy, Chengdu, Sichuan 610041, China
2) Laboratory of Clinical Pharmacy and Adverse Drug Reaction, State Key Laboratory of Biotherapy, West China Hospital of Sichuan University and Collaborative Innovation Center of Biotherapy, Chengdu, Sichuan 610041, China
3) Division of Endocrinology and Metabolism, State Key Laboratory of Biotherapy, West China Hospital of Sichuan University and Collaborative Innovation Center of Biotherapy, Chengdu, Sichuan 610041, China
4) Division of Endocrinology and Metabolism, The fifth Hospital of Chengdu, Chengdu, Sichuan 610041, China
5) Molecular Medicine Research Center, State Key Laboratory of Biotherapy, West China Hospital of Sichuan University and Collaborative Innovation Center of Biotherapy, Chengdu, Sichuan 610041, China
6) The Yinchao Community Hospital of Chengdu, Chengdu, Sichuan 610041, China
7) Chengdu Aerospace Hospital, Chengdu, Sichuan 610041, China

Abstract. Evidence has shown that endoplasmic reticulum (ER) stress was involved in the progression to type 2 diabetes mellitus (T2DM) and development of insulin resistance. Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a novel secreted protein upregulated by ER stress. This study aimed to assess serum level of MANF in normal glucose tolerance (NGT) participants and newly diagnosed prediabetic and T2DM patients. A total of 257 participants with NGT, newly diagnosed prediabetes or T2DM were recruited from Yinchao and Hangtian communities of Chengdu, Sichuan, China. Serum MANF level was quantified by enzyme-linked immunosorbent assay (ELISA). The mean age for the 257 participants (147 females) was 62±8 years (range 44-78): 71 with NGT, 115 with newly diagnosed prediabetes and 71 with T2DM. Mean serum MANF level was significantly higher with newly diagnosed prediabetes and T2DM than NGT (2.89±1.09 and 3.03±1.73 ng/mL, both p<0.001). MANF level was not correlated with insulin sensitivity indexes (homeostasis model assessment for insulin resistance [HOMA-IR], Matsuda Index and quantitative insulin sensitivity check index [QUICKI]) for NGT and T2DM participants but was correlated with such indexes for prediabetes patients. We concluded that serum MANF level was higher in patients with newly diagnosed prediabetes and T2DM than in NGT controls. MANF appears to be associated with Matsuda Index, QUICKI and HOMA-IR in prediabetes patients.

Key words: Type 2 diabetes, Insulin resistance, Mesencephalic astrocyte-derived neurotrophic factor, Endoplasmic reticulum stress

ACCUMULATING EVIDENCE has shown that endoplasmic reticulum (ER) stress was involved in the development of insulin resistance and progression to type 2 diabetes mellitus (T2DM). ER stress contributes to inflammation, steatosis and insulin resistance in liver, skeletal muscle and adipose tissue. ER stress in enlarged fat tissue modifies adipokine secretion, which may further exacerbate insulin resistance [1]. With insulin resistance in peripheral tissues, pancreatic β-cell must compensate by increasing insulin secretion, which may trigger ER stress in islets. A temporary adaptation in the ER may help restore pancreatic β-cell function. However, prolonged ER
Materials and Methods

Study population
Participants were all Han Chinese recruited from Yinchao and Hangtian communities of Chengdu, Sichuan, China. Participants with the following were excluded: history of cerebrovascular or cardiovascular events; receiving oral or intravenous corticosteroid hormone treatment; hepatic cirrhosis and ascites; or receiving antidiabetic medication. Participants were divided into 3 groups: (1) healthy controls, normal fasting glucose and normal glucose tolerance test (NGT); (2) prediabetes, impaired fasting glucose or impaired glucose tolerance, or both, or increased HbA1C level; and (3) T2DM. The diagnosis of pre-diabetes and T2DM was based on 2016 criteria of the American Diabetes Association [15]. Criteria for the diagnosis of diabetes were fasting plasma glucose (FPG) ≥126 mg/dL (7.0 mmol/L) or 2-h plasma glucose (2-h PG) ≥ 200 mg/dL (11.1 mmol/L) after a 75-g oral glucose tolerance test (OGTT) or HbA1C ≥6.5% (48 mmol/mol); criteria of prediabetes diagnosis were 100 mg/dL (5.6 mmol/L) ≤ FPG ≤ 125 mg/dL (6.9 mmol/L) or 140 mg/dL (7.8 mmol/L) ≤ 2-h PG ≤ 199 mg/dL (11.0 mmol/L) or 5.7% ≤ HbA1C ≤ 6.4% (39–46 mmol/mol). Participants were sex-, body mass index (BMI)- and age-matched among the 3 groups. The study was approved by the Biological Sciences Ethical Committee of West China Hospital of Sichuan University, China.

Blood sampling and variable assessment
All participants underwent the 75g oral glucose tolerance test (OGTT) and blood samples were collected at 0, 30 and 120min after glucose administration. Serum aliquots were stored at -80°C and were not thawed until analysis. Plasma glucose and insulin were measured at indicated time points (fasting, 30 min and 120 min points during OGTT). Fasting plasma were additionally assayed for triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), hemoglobin (Hb), glycated hemoglobin (HbA1c), uric acid (UA), creatinine (Cr), blood urea nitrogen (BUN), alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

Blood glucose was measured by a glucose oxidase method adapted to an automated analyzer (Hitachi704; Boehringer Mannheim, Germany) and serum insulin

stress impairs insulin synthesis and causes β-cell apoptosis via multiple pathways [1-3]. Eventually, β-cell proliferation capacity deteriorates, glucose tolerance worsens and fasting glucose levels increase, which culminates in overt hyperglycemia. Therefore, ER stress may play a role in the pathogenesis of diabetes.

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a novel secreted protein discovered from a rat mesencephalic type-1 astrocyte cell line [4]. Lindholm et al. showed that MANF mRNA and protein levels were relatively high in brain and non-neuronal organs such as liver and pancreas [5]. MANF is localized in the lumen of the ER and may act as both an intracellular ER stress-induced protein and a secreted cytokine. Intracellularly, MANF is upregulated by ER stress in vivo and protects several cell populations against ER stress-induced cell death [6]. Extracellularly, MANF, acting as a neurotrophic factor, plays a key role in regulating the development of the dopaminergic system in zebrafish and selectively supports and protects midbrain dopamine neurons in both mouse and rat models of Parkinson’s disease [5, 7, 8]. As well, MANF can rescue cardiomyocytes and cortical neurons from ischemia-induced injury in vivo [9-11]. MANF expression was found increased in β-cell of diabetic Akita mice with ER stress induced by the accumulation of proinsulin in the ER [12]. MANF overexpression can specifically promote β-cell proliferation and survival [13]. In contrast, MANF deficiency leads to progressive loss of β-cell, thereby resulting in diabetes mellitus due to reduced β-cell proliferation and enhanced β-cell apoptosis. Furthermore, it was reported that increased circulating MANF levels were associated with the clinical manifestation of type 1 diabetes in children 1–9 years of age [14]. These results suggest that MANF is regulated by ER stress and is involved in the pathogenesis of diabetes.

In this study, we uncovered a novel association of serum MANF level and insulin resistance in newly diagnosed prediabetic patients. The serum level of MANF was significantly higher in such patients as compared to healthy controls. Serum levels of MANF were well correlated with indices of insulin resistance, including homeostasis model assessment for insulin resistance (HOMA-IR), Matsuda Index and quantitative insulin sensitivity check index (QUICKI), in prediabetic patients.
level was measured by electrochemiluminescence (Cobase411; Roche, Switzerland). HbA$_{1C}$ level was determined by high-performance liquid chromatography (Bio-Rad D-10 hemoglobin A1C radiometer). Levels of TG, TC, LDL-C, and HDL-C were determined by enzymatic methods with commercial reagent kits (Boehringer Mannheim). Levels of UA, Cr, BUN, ALT and AST were measured as described [16]. Serum MANF level in blood samples was measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (cat: SEC300Hu, Uscn, Life Science, Inc., China). The detection range of the assay was 15.6-1,000 pg/mL. Intra- and interassay coefficients of variation, according to the manufacturer, were 10% and 12%, respectively.

Assessment of insulin resistance
The HOMA-IR, Matsuda Index and QUICKI were used to estimate insulin sensitivity and were calculated as follows: HOMA-IR = fasting serum insulin (FINS, mIU/L) × fasting plasma glucose (FPG, mmol/L) / 22.5; QUICKI = 1 / [log FPG (mg/dL) + log FINS (mmol/L)]; and Matsuda Index = 10,000 / √[FPG (mg/dL) × FINS (mIU/L) × mean OGTT glucose concentration × mean OGTT insulin concentration] [17-19].

Statistical analysis
Kolmogorov-Smirnov test was used to test continuous variables for normal distribution. Continuous data with normal distribution are presented as mean ± SD and were analyzed by one-way ANOVA followed by least significant difference (LSD) tests. Non-normally distributed continuous data are presented as median (interquartile range [IQR]) and were analyzed by Mann-Whitney U test. Categorical data are shown as frequency (%) and were analyzed by chi-square test. Correlation of serum MANF level with the different variables was tested by Pearson (for normally distributed variables) or Spearman (for non-normally distributed variables) correlation analysis, as appropriate. Two-sided $p<0.05$ was considered statistically significant. All statistical analyses involved use of SPSS 22.0 (SPSS Inc., Chicago, IL).

Results

Characteristics of the study population
The 257 participants had mean age 62±8 years (range 44-78): 71 with NGT, 115 with newly diagnosed prediabetes and 71 with T2DM. Age, male/female ratio, BMI, waist-to-hip ratio and levels of Hb, I$_{0}$, I$_{30}$, I$_{120}$, HDL-C, LDL-C, Cr, BUN and ALT did not differ among the 3 groups (Table 1). Levels of HbA$_{1C}$, FPG, G$_{30}$, G$_{120}$, TG and TC were higher for prediabetes and T2DM than NGT participants. As well, HbA$_{1C}$, FPG, G$_{30}$ and G$_{120}$ levels were higher for T2DM than prediabetes patients.

As expected, the groups differed in insulin resistance-related indices (Table 1). HOMA-IR score was higher for prediabetes and T2DM than NGT participants and the Matsuda Index and QUICKI were lower, which indicates impaired insulin sensitivity in these participants.

Serum MANF levels
The serum MANF level was 2.13±1.37, 2.89±1.09 and 3.03±1.73 ng/mL for NGT, prediabetes and T2DM participants (Table 1). MANF level was sharply elevated with newly diagnosed prediabetes and T2DM ($p<0.001$) but was comparable between prediabetes and T2DM participants ($p=0.486$) (Fig. 1).

Correlations between MANF levels and measured variables
The increased MANF level in prediabetic and diabetic patients led us to investigate a possible association between MANF level and diabetes-related indices for all participants. We did not find a significant correlation between MANF level and serum insulin or glucose levels or insulin sensitivity indexes (HOMA-IR, Matsuda Index and QUICKI) for all participants. On subgroup analysis (Table 2), MANF level was not associated with insulin sensitivity indexes (including HOMA-IR, QUICKI and Matsuda Index) for NGT participants. On correlation analysis of prediabetes patients, serum MANF level was positively correlated with I$_{0}$ ($r=0.217$, $p=0.020$) and HOMA-IR ($r=0.188$, $p=0.045$) (Fig. 2), indexes of insulin resistance, and negatively correlated with Matsuda Index ($r=-0.185$, $p=0.048$) and QUICKI ($r=-0.212$, $p=0.023$) (Fig. 2), indexes of insulin sensitivity. Therefore, MANF level was well associated with insulin resistance in prediabetic patients. For T2DM patients, MANF level was not correlated with I$_{0}$, Matsuda Index, QUICKI or HOMA-IR (Table 2).
## Table 1  Characteristics of participants with normal glucose tolerance (NGT), prediabetes and type 2 diabetes mellitus (T2DM)

| Demographic characteristics | NGT (n=71) | Prediabetes (n=115) | T2DM (n=71) | p-value |
|-----------------------------|------------|---------------------|-------------|---------|
| Age (years)                 | 60.54 ± 8.64 | 62.01 ± 8.43 | 63.28 ± 8.35 | 0.156 |
| Female (%)                  | 46 (64.8%) | 59 (51.3%) | 42 (59.2%) | 0.181 |
| Anthropometric characteristics |          |                      |             |         |
| BMI (kg/m²)                 | 24 (23, 26) | 25 (23, 27) | 25.03 ± 2.89 | 0.179 |
| W/H ratio                   | 0.89 (0.85, 0.93) | 0.90 (0.86, 0.93) | 0.89 (0.87, 0.94) | 0.620 |
| HbA1C (%)                   | 138.25 ± 16.66 | 141.03 ± 22.57 | 140.49 ± 16.24 | 0.450 |
| HbA1C (%)                   | 5.20 (5.00, 5.30) | 5.60 (5.12, 5.87) | 6.30 (5.77, 6.80) | <0.001 |
| Hormonal factors            |           |                      |             |         |
| I0 (mIU/L)                  | 7.36 ± 3.72 | 7.42 (5.00, 10.80) | 8.10 (5.70, 11.90) | 0.082 |
| I30 (mIU/L)                 | 52.50 (36.41, 78.10) | 50.60 (31.82, 81.20) | 42.64 (24.19, 86.10) | 0.603 |
| I120 (mIU/L)                | 41.19 (24.47, 69.30) | 45.70 (24.83, 75.75) | 49.80 (29.68, 73.50) | 0.219 |
| MANF (ng/mL)                | 2.13 ± 1.37 | 2.89 ± 1.09 a | 3.03 ± 1.73 a | <0.001 |
| Metabolic factors           |           |                      |             |         |
| FPG (mM)                    | 4.80 (4.50, 4.90) | 5.70 (5.50, 5.90) a | 7.10 (6.30, 7.80) a,b | <0.001 |
| G30 (mM)                    | 8.30 (7.30, 9.30) | 10.52 ± 2.01 a | 13.98 ± 3.77 a,b | <0.001 |
| G120 (mM)                   | 1.30 (1.00, 2.00) | 1.50 (1.10, 2.20) a | 1.60 (1.10, 2.50) a | 0.044 |
| TC (mM)                     | 4.86 ± 0.86 | 4.93 ± 0.89 | 5.21 ± 1.00 a | 0.049 |
| HDL-C (mM)                  | 1.40 (1.30, 1.70) | 1.40 (1.20, 1.60) | 1.40 (1.20, 1.60) | 0.206 |
| LDL-C (mM)                  | 2.75 ± 0.63 | 2.80 (2.40, 3.30) | 2.90 (2.50, 3.40) a | 0.157 |
| UA (µM)                     | 308.41 ± 75.83 | 343.38 ± 83.10 a | 304.00 (272.00, 375.00) b | 0.007 |
| Cr (µM)                     | 69.69 ± 16.30 | 70.12 ± 17.77 | 68.44 ± 17.94 | 0.815 |
| BUN (mM)                    | 5.00 ± 1.30 | 4.90 (4.37, 6.22) | 5.43 ± 1.20 | 0.127 |
| ALT (U/L)                   | 23.00 (16.75, 29.00) | 25.00 (19.00, 37.00) a | 21.00 (17.00, 33.00) | 0.059 |
| AST (U/L)                   | 24.00 (20.00, 28.00) | 25.00 (21.00, 30.00) | 21.00 (19.00, 26.00) b | 0.040 |

Data are mean ± SD or median (interquartile range) or number (%). BMI, body mass index; W/H, waist-to-hip ratio; QUICKI, quantitative insulin sensitivity check index; HOMA-IR, homeostasis model assessment of insulin resistance; FPG, fasting plasma glucose; Hb, hemoglobin; HbA1C, glycosylated hemoglobin; TG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; UA, uric acid; Cr, creatinine; BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase. * p<0.05 vs NGT; b p<0.05 vs prediabetes.

## Table 2  Correlations between serum level of mesencephalic astrocyte-derived neurotrophic factor (MANF) and indices of insulin sensitivity in participants

|                  | NGT (0.03, 5.93) a | Prediabetes (0.30, 5.82) a | T2DM (0.15, 8.86) a |
|------------------|-------------------|---------------------------|-------------------|
| r                |                   |                           |                   |
| p                |                   |                           |                   |
| I0               | -0.052            | 0.67                      | 0.217 * <0.05     |
| HOMA-IR          | -0.051            | 0.67                      | 0.188 * <0.05     |
| Matsuda Index    | 0.015             | 0.90                      | -0.185 * <0.05    |
| QUICKI           | 0.047             | 0.70                      | -0.212 * <0.05    |

* MANF concentration (ng/mL) (minimum, maximum). Pearson (for normally distributed variables) or Spearman (for nonnormally distributed variables) correlation analysis were used to test correlation of serum MANF level with the different variables. * p<0.05.

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** Fig. 1  Serum level of mesencephalic astrocyte-derived neurotrophic factor (MANF) in participants with normal glucose tolerance (NGT), prediabetes and type 2 diabetes mellitus (T2DM)**

The results were expressed as the mean±SEM. ** p<0.01 as indicated.
Discussion

In this study, we analyzed serum MANF levels and found that circulating MANF concentrations were significantly elevated in newly diagnosed prediabetic and diabetic patients from Yinchao and Hangtian communities of Chengdu, Sichuan, China. In prediabetes patients, serum MANF level was associated with indexes of insulin resistance, including HOMA-IR, Matsuda Index and QUICKI. To our knowledge, this study is the first to link serum MANF level and insulin resistance in patients of type 2 diabetes. However, the source of circulating MANF was not clear. MANF is widely expressed in mammals, including brain (hippocampus, cortex and midbrain), liver, testis and so on [5]. It’s not known which organ contributes the most to this induction on the basis of our research.

The ER is an important site for protein synthesis, folding, processing and quality control. Under normal physiological conditions, proteins are correctly folded and transported and misfolded proteins are degraded. Cellular insult can cause accumulation of unfolded or misfolded proteins in the ER, triggering ER stress. With ER stress, cells activate complementary adaptive mechanisms to restore ER homeostasis, known as the unfolded protein response (UPR) [20, 21].

Much evidence shows that ER stress plays a role in the pathogenesis of T2DM and insulin resistance. First, obese mice and human show increased levels of ER stress factors in liver and adipose tissues [22, 23]. Second, recent evidence suggests that the UPR might participate in hepatic insulin resistance in different ways [24, 25]. Third, ER stress is involved in adipose insulin resistance [26]. Finally, ER stress also leads to muscle insulin resistance [27].

Previous evidence suggested that MANF is a UPR-upregulated protein and acts as a protective factor against ER stress [6]. MANF is a downstream target of activating transcription factor 6α (ATF6α), activating transcription factor 6β (ATF6β) and X-box binding protein 1 (Xbp1s) and mediated by 5’-flanking region of ER stress-response element II [12, 28]. Our finding...
of MANF levels higher in prediabetes and T2DM than NGT participants could be explained by ER stress in prediabetes and T2DM patients.

The HOMA-IR, Matsuda Index and QUICKI tests are widely used in the clinic for assessing insulin resistance with fasting glucose and insulin or C-peptide concentrations. When used appropriately, the indexes can yield valuable data [29]. Therefore, we used these indexes to assess insulin sensitivity in our study. Serum MANF level was independently correlated with all indexes of insulin sensitivity in newly diagnosed prediabetes patients.

Several reasons were postulated for the discrepant association of MANF level in different groups. MANF is expressed in multiple tissues, including brain and heart, which may affect its level as well. In patients with prediabetes, insulin resistance in liver, skeletal muscle and adipose tissue will cause ER stress in these tissues, to induce MANF expression. The increased MANF level will help restore ER homeostasis and prevent ER stress-induced cellular damage. However, with the development of prediabetes, ER stress is prolonged and is further elevated by overt hyperglycemia [30]. Chronic and overwhelming ER stress disrupts protein folding in the ER, reduces insulin secretion to invoke oxidative stress, and activates cell death pathways [31]. The loss of adaption in the ER prevents further MANF induction and may disrupt the association of MANF level and insulin resistance we observed in prediabetic patients.

A Clinical exome sequencing (CES) study in Middle Eastern patients with suspected Mendelian disorders showed that MANF was one of novel candidate disease genes [32]. MANF gene is mutated in intron regions in a 22-year-old woman presented with T2DM, obesity and other disease phenotypes. However, the study didn’t present the change of MANF levels resulted from this mutation. The young woman has many diseases besides diabetes. In our study, we analyzed serum MANF concentration of 257 participants directly, finding that MANF levels were significantly increased in prediabetic and diabetic patients. The induction may be the result of MANF gene mutation. At the same time, we averted influences of diseases included in CES study.

The neurotrophic factor cerebral dopamine neurotrophic factor (CDNF), found in 2007 by bioinformatics and biochemical approaches [33] is paralogous to MANF, members of a novel evolutionary conserved neurotrophic factor (NTF) family in vertebrates. Human CDNF and MANF show 59% amino acid identity. Both the sequence and 3D structure of the two are very similar but differ greatly from other growth factors and NTFs [34]. CDNF also protects and rescues midbrain dopamine neurons on the 6-OHDA-induced rat model of Parkinson’s disease [33]. In addition, CDNF is a new ER stress-response protein, like MANF [35]. Cheng et al. showed that overexpression of CDNF in astrocytes could decrease lactate dehydrogenase proportion and suppress the secretion of inflammatory cytokines induced by ER stress [36]. Therefore, CDNF may also play a role in the progression of T2DM and be related to β-cell function and insulin sensitivity. However, few studies have focused on the relationship of CDNF and the above parameters. This assumption needs to be validated.

Our study has some limitations. First, the number of subjects was relatively limited, and the clinical features were not totally matched among the 3 groups, which may imply biased results. Second, participants were newly diagnosed as having prediabetes or diabetes, so we can exclude the effect of antidiabetic drugs, but the effects of other drugs were not considered. Finally, our study was conducted in a particular population and restricted to 2 sites in China, so a large cohort is needed from the same or different ethnic populations and from different locations for study.

In conclusion, we revealed an association of MANF level with diabetes-related parameters. Serum MANF level was significantly higher with newly diagnosed prediabetes and T2DM patients than non-diabetic controls in China and appears to be associated with Matsuda Index, QUICKI and HOMA-IR in prediabetes but not T2DM patients in this population. However, the mechanism behind the induction remains unclear and need be analyzed in future.

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Disclosure

The authors have nothing to disclose.
Serum MANF and diabetes

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