***Regular Article***

# Evaluation of Two Commercial Enzyme-Linked Immunosorbent Assay Kits for the Detection of Human Circulating Metrnl

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Received October 19, 2017; accepted December 22, 2017; advance publication released online February 2, 2018

**Metrnl is a newly discovered secreted protein with neurotrophic activity and metabolic effect, while in earlier studies its circulating level in human was not explored. We evaluated two commercial enzyme-linked immunosorbent assay kits (DY7867-05, R&D Systems and SK00478-02, Aviscera Bioscience) for the detection of human circulating Metrnl. The DY7867-05 kit showed superiority over the SK00478-02 kit since it generated better curve fitting degree, smaller variation among tests, higher inter-assay reproducibility and better specificity, and could effectively detect human Metrnl in six types of blood samples. Subsequent analysis was performed using the DY7867-05 kit. Sample storage conditions were investigated. No gender difference in circulating Metrnl levels was found, while people with newly diagnosed type 2 diabetes mellitus (T2DM) had significantly lower Metrnl levels compared to the healthy controls.**

**Key words** circulating Metrnl; enzyme-linked immunosorbent assay (ELISA); blood sample type; sample storage; gender difference; type 2 diabetes mellitus (T2DM)

The recently discovered protein Metrnl is a secreted protein.[1–4] *Metrnl* gene is located on human chromosome 17q25.3 and the protein is predicted to contain 311 amino acids, with a signal peptide of 45 amino acids and without any transmembrane region, suggesting a mature protein of 266 amino acids (*ca.* 30 kDa) when secreted.[1]

First described in 2012,[2] Metrnl is soon being recognized as serving important roles in neural development,[2,5] white adipose browning[6] and insulin sensitization.[7] Besides, it might be involved in both innate and acquired immune response,[8] in maintaining gut antimicrobial peptides[4] as well as in osteoblast function.[9] Our previous studies showed that Metrnl is highly expressed in white adipose tissue in both rodents and humans and plays important roles in adipose biology.[3,7] Recently, in line with our animal results, Löffler *et al.*[10] showed that Metrnl expression is higher in subcutaneous adipocytes of obese children when compared with lean children and is associated with adipocyte size.

As Metrnl is a secreted protein, the measurement of human circulating Metrnl is of great value to further explore its roles in physiological and pathological processes as well as the mechanisms, just as in the case of another adipokine nicotinamide phosphoribosyltransferase (NAMPT)/Visfatin.[11] However, due to lack of reliable methods, very little is known about Metrnl level in human circulation. Recently, there are two commercially available enzyme-linked immunosorbent assay (ELISA) kits for human Metrnl detection, but the usability of the kits is not validated.

In this study we first performed some necessary study of the detection method including testing the two commercial ELISA kits for human Metrnl detection and comparing their usability. Then, influencing factors of detection such as different blood collection tubes and sample storage conditions were tested. Further, Metrnl levels between male and female people as well as between the healthy people and people with newly diagnosed T2DM were compared.

## Results

**Standard Curves and the Uncertainty** Typical standard curves of the two kits were generated as shown in Fig. 1. The four parameter logistic (4PL) nonlinear regression model was applied for curve-fitting analysis, resulting a near perfect $R^2$ from 0.99996 to 0.99999 for standard curves of the DY7867-05 kit, while a respectable $R^2$ value from 0.9903 to 0.9984 for those of the SK00478-02 kit.

For ELISA methods, the standard curves greatly affect the entire uncertainty of the assays,[12] and much of the uncertainty of concentration estimation is a result of the highly uncertain standard curves developed in separate tests.[13] Since fitted standard curves vary from assay to assay, which can lead to different concentration estimation of a same sample, we compared individually fitted standard curves of separate assays performed on different days. The standard curves of the DY7867-05 kit (Fig. 2b) had a less variation among tests than the SK00478-02 kit (Fig. 2a).

**Reproducibility** The standard points were assayed to calculate the inter-assay coefficient of variation (CV) for inter-assay reproducibility. As summarized in Table 1, the inter-assay CV decreased as the concentration of Metrnl increased in both cases. The DY7867-05 kit obviously obtained a better inter-assay reproducibility with lower CV ranging from 0.18 to 5.73% compared to 1.38 to 116.83% with SK00478-02 assay.

**Specificity** To assess the specificity of the two ELISA kits, we tested serum samples from one mouse and one rat. The SK00478-02 kit detected circulating Metrnl of both species with concentrations more than 10 ng/mL (data not shown), while the DY7867-05 kit exhibited no cross-reactivity.

**Metrnl Detection in Six Types of Blood Samples** We

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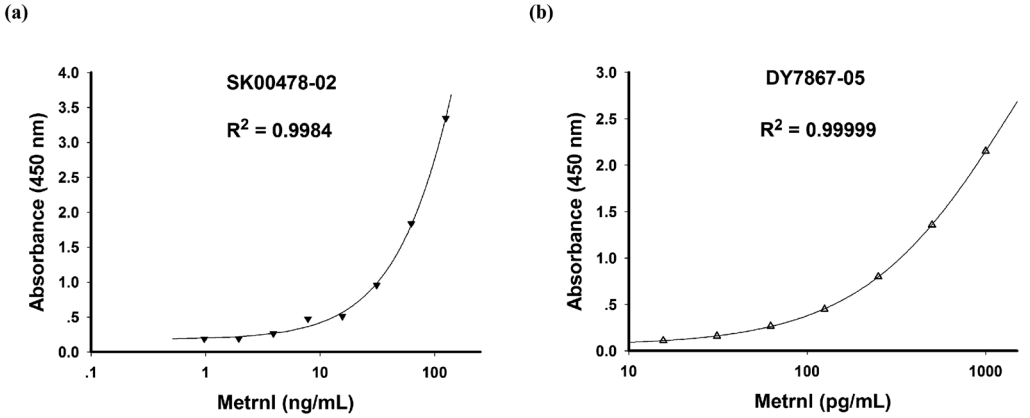


Fig. 1. Typical Standard Curves Generated by SK00478-02 Kit (a) and DY7867-05 Kit (b)

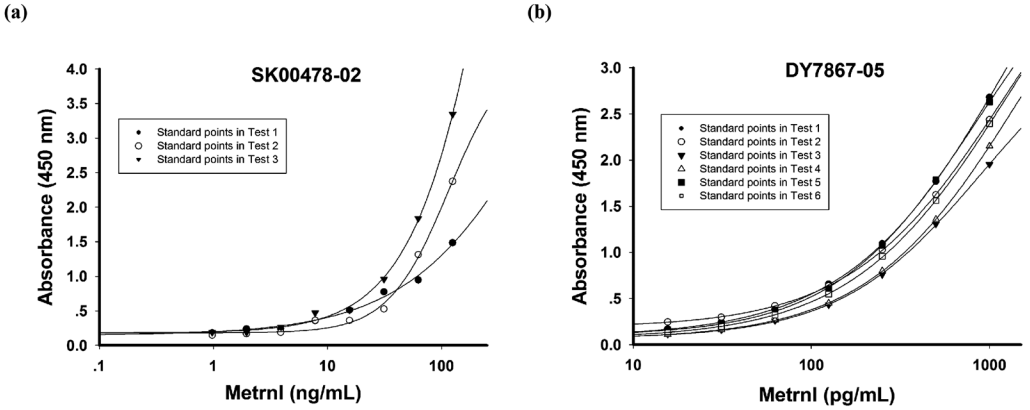


Fig. 2. Individually Fitted Standard Curves in Separate Assays by SK00478-02 Kit (a) and DY7867-05 Kit (b)

Table 1. Inter-assay Reproducibility of the Standard Points

| SK00478-02 ($n$=3) | | DY7867-05 ($n$=6) | |
|---|---|---|---|
| Concentration (ng/mL) | Inter-assay (CV, %) | Concentration (pg/mL) | Inter-assay (CV, %) |
| 0.977 | 116.83 | 15.625 | 5.73 |
| 1.953 | 107.33 | 31.25 | 4.45 |
| 3.906 | 35.90 | 62.5 | 3.16 |
| 7.813 | 24.63 | 125 | 1.65 |
| 15.625 | 15.78 | 250 | 1.24 |
| 31.25 | 15.32 | 500 | 0.80 |
| 62.5 | 8.13 | 1000 | 0.18 |
| 125 | 1.38 | | |

first analyzed 15 serum (additive) samples (collected using tubes with the additive of clot activator) from 15 healthy volunteers using both kits. In the case of SK00478-02 kit, only six samples were detectable, among which two estimated concentrations were smaller than the lowest standard point 0.977 ng/mL (Fig. 3a). In the case of DY7867-05 kit, all samples were detected within the standard range (Fig. 3b). Interestingly, for each of the six samples detectable in both cases, the estimated two concentrations from the two kits highly differed and the data obtained from the two kits showed no correlation (Fig. 3c).

We next investigated if Metrnl level in serum differs from that in plasma. Both serum (additive) samples and plasma (ethylenediaminetetraacetic acid (EDTA)) samples (collected using tubes with spray-coated K2 EDTA) from another healthy 11 volunteers were tested using the two kits. In the case of SK00478-02 kit, as similar to the above mentioned results (Fig. 3a), all serum samples were undetectable, while all plasma samples were detectable (Fig. 4a). In contrast, by DY7867-05 kit, Metrnl was detected in all samples, and no concentration difference was found between the two sample types (Fig. 4b).

Using the SK00478-02 kit, we further assayed three types of plasma samples (collected using tubes added with EDTA, lithium heparin and sodium citrate, respectively) from ten volunteers to figure out if SK00478-02 kit was suitable to detect Metrnl concentrations in other plasma types. As is shown in Fig. 4c, EDTA plasma samples were all detectable as before, while as for other two types of plasma samples, only 3 and 2 samples were detectable, respectively.

Meanwhile, as both serum (additive) and plasma (EDTA) samples could be detected by the DY7867-05 kit, we also tested other types of serum and plasma samples using this kit. For each of the 13 volunteers, blood was collected to produce three types of serum samples and three types of plasma samples. Restricted by the limited 96 wells per microplate, we performed the assays of serum and plasma samples in two separate microplates (Figs. 5a and b). The results showed that mean values of Metrnl concentrations in serum (nonadditive) samples (collected using plain tubes without additive), serum (additive) samples and serum (gel separation) samples (collect-

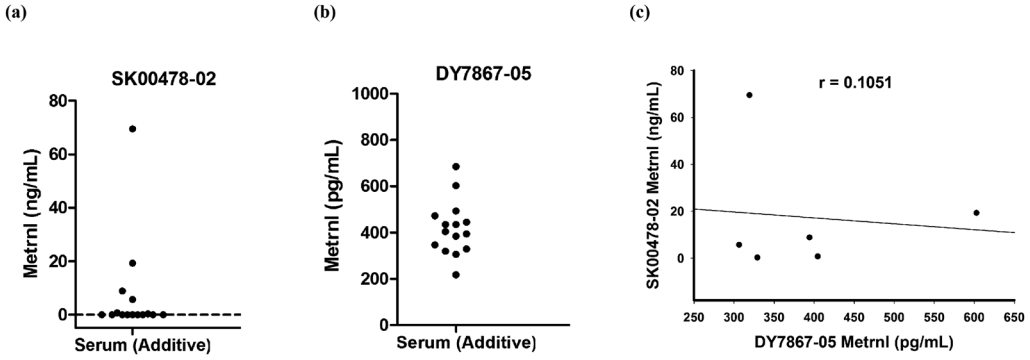


Fig. 3. Metrnl Detection in 15 Serum (Additive) Samples Using Both SK00478-02 Kit (a) and DY7867-05 Kit (b)

The concentrations of Metrnl in all samples were plotted. For the six samples detectable in both cases, the correlation coefficient ($r$) between the estimated concentrations from the two kits was shown (c).

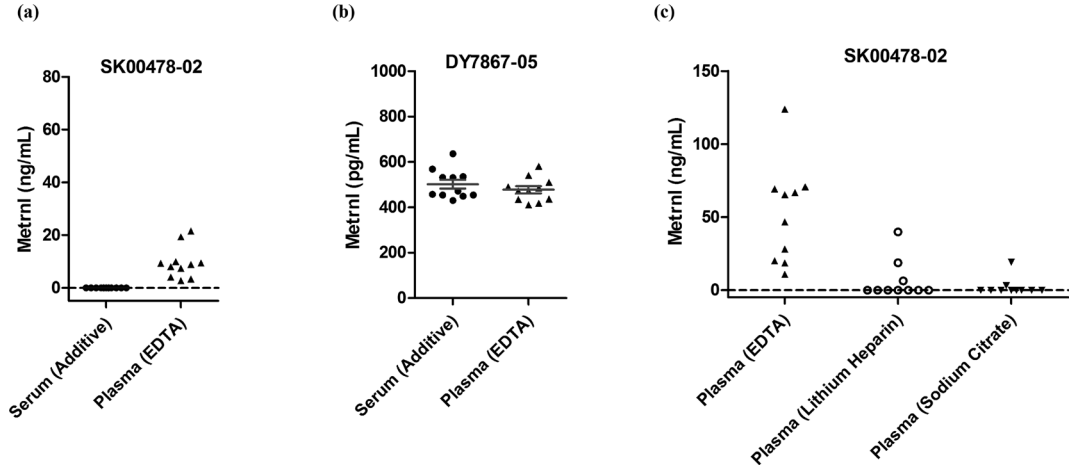


Fig. 4. Metrnl Detection in Serum (Additive) and Plasma (EDTA) Samples from 11 People Using Both SK00478-02 Kit (a) and DY7867-05 Kit (b), and in Three Types of Plasma Samples from 10 People Using SK00478-02 Kit (c)

The concentrations of Metrnl in all samples were plotted. For estimated concentrations below the limit of detection in assays by SK00478-02 kit, dots represented 0 ng/mL were plotted (a, c). Data were represented as means±S.E.M. (b). Two-tailed paired Student's $t$-test was used to compare difference between serum (additive) group ($n$=11) and plasma (EDTA) group ($n$=11) (b).

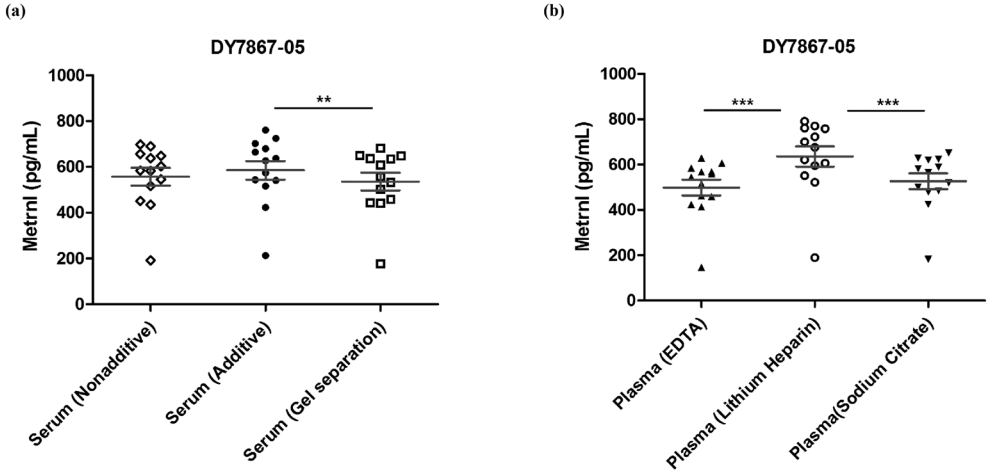


Fig. 5. Metrnl Concentrations in Three Types of Serum Samples (a) and in Three Types of Plasma Samples (b) Detected by DY7867-05 Kit

The concentrations of Metrnl in all samples were plotted. Two-way ANOVA was used to compare differences among three groups, followed by pairwise comparisons subject to Bonferroni corrections for multiple tests. Data were presented as mean±S.E.M. ** $p<0.01$. *** $p<0.001$.

ed using tubes with the additive of clot activator and serum separation gel) were 557, 585 and 536 pg/mL, respectively, with a statistic difference between serum (additive) and serum (gel separation) samples (Fig. 5a). As for the three types of plasma samples, Metrnl concentrations in lithium heparin plasma were significantly higher than those in EDTA plasma

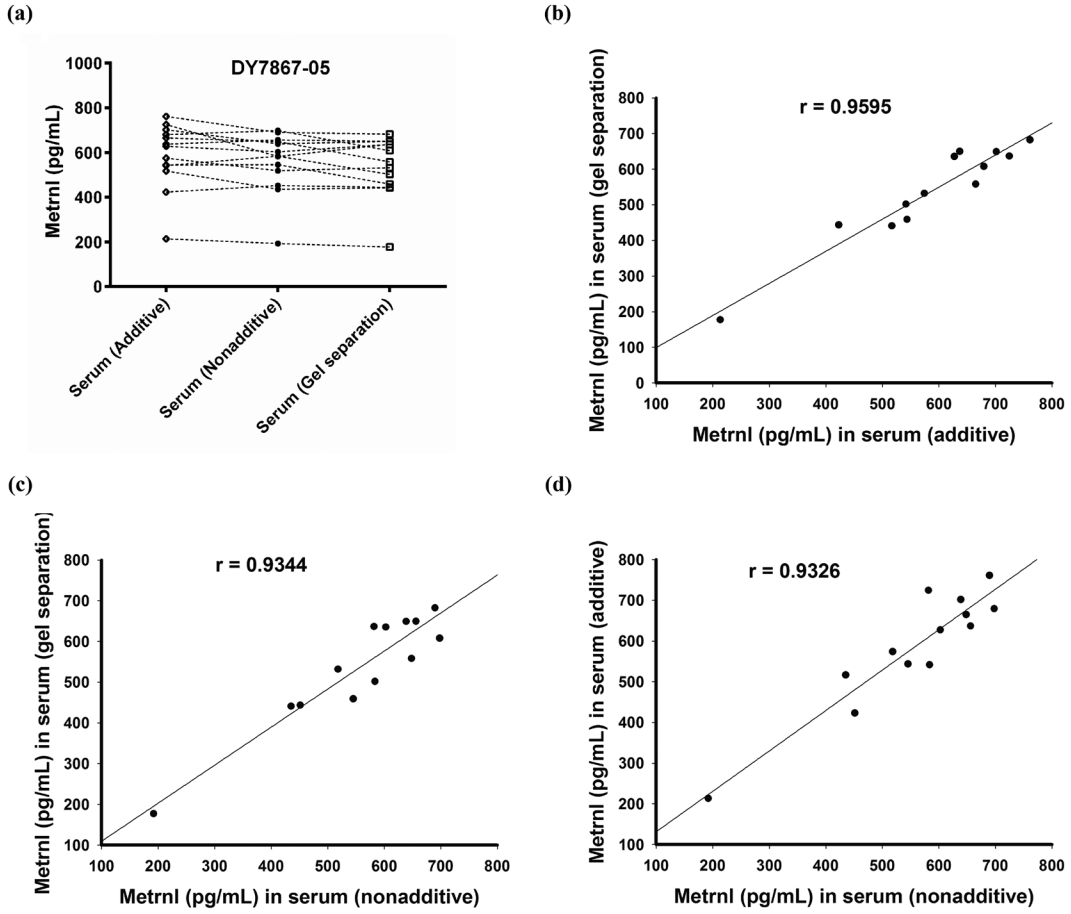


Fig. 6. Metrnl Concentrations in Three Types of Serum Samples Detected by DY7867-05 Kit

Every three concentration dots from the same volunteer were connected by dashed line (a). The correlation coefficients ($r$) among groups were shown (b–d).

and in sodium citrate plasma, with mean concentrations 635, 499 and 527 pg/mL respectively (Fig. 5b). Besides, data analysis showed good correlations among serum sample types (Fig. 6) and among plasma sample types (Fig. 7).

**Sample Storage** To investigate the effect of temperature and duration of blood sample storage on detection, five time points for samples to transfer to freezing storage were set (Fig. 8). The result showed that Metrnl concentrations varied in different time point groups. Besides, Metrnl concentration of one sample decreased significantly after storage at room temperature (r.t.) for 2 d and at 4°C for 4 d.

**Gender Effect** we analyzed data in Fig. 4b which included 5 male and 6 female participants and found no difference in Metrnl levels between the two groups. This result was confirmed by the next experiment in which we included another 13 male and 10 female participants and detected their Metrnl levels in serum samples (Fig. 9).

**Circulating Metrnl in People with Newly Diagnosed T2DM** General physiological characteristics and metabolic parameters of people with newly diagnosed T2DM and their matched controls were presented in Table 2. Compared with the healthy controls, patients with T2DM had higher body weight, BMI, waist circumference, waist hip ratio (WHR), systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting serum glucose (FSG), blood triglycerides (TG), low density lipoprotein cholesterol (LDL-c), haemoglobin $A_{1c}$ ($HbA_{1c}$), fasting serum insulin (FSI) and homeostasis model assessment for insulin resistance (HOMA-IR), while serum Metrnl concentrations in the patients were lower ($p<0.05$) (Fig. 10).

## Discussion

Based on our results, we consider the SK00478-02 kit for human Metrnl detection is inappropriate. First, the fitted standard curves from separate assays on different days displayed larger variation among tests when compared to the DY7867-05 kit, indicating SK00478-02 kit with higher uncertainty. Second, the inter-assay reproducibility assessed by calculating the inter-assay CV of the standard points was unsatisfactory. Third, as for blood sample types, only EDTA plasma sample is detectable by SK00478-02 kit. At the beginning of using this kit to examine samples (Fig. 3a), we once considered that the very low detection rate in serum samples by the SK00478-02 kit was due to its low sensitivity, since this kit was designed to detect Metrnl concentrations at ng/mL levels, with the lowest point of standards at 0.977 ng/mL. However, this is obviously unable to explain why the SK00478-02 kit could detect Metrnl in EDTA plasma, but could not recognize Metrnl in serum samples and other plasma samples (lithium heparin plasma and sodium citrate plasma). Forth, to our surprise, although the SK00478-02 kit failed to detect Metrnl in human serum, it detected Metrnl in mouse serum and rat serum. These results further suggest it was other issues rather than the sensitivity that made SK00478-02 kit unable to detect

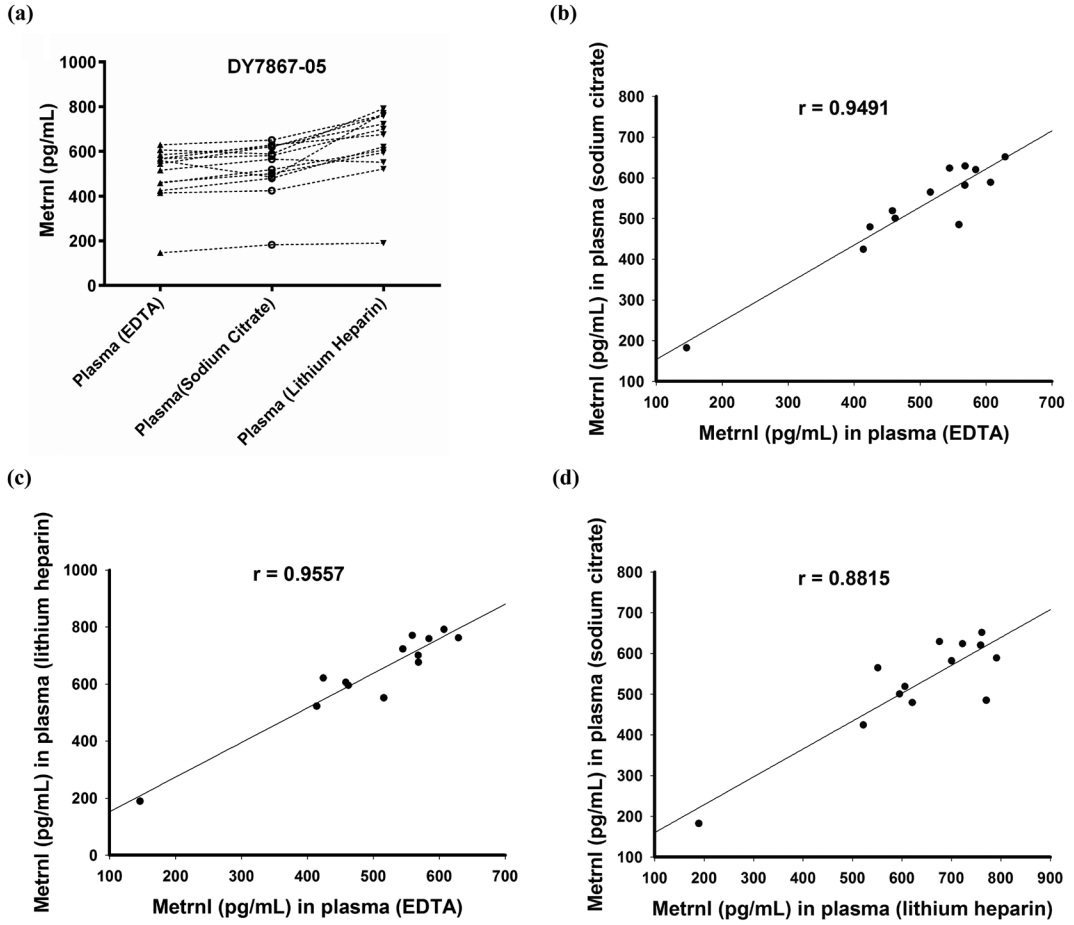


Fig. 7. Metrnl Concentrations in Three Types of Plasma Samples Detected by DY7867-05 Kit

Every three concentration dots from the same volunteer were connected by dashed line (a). The correlation coefficients (*r*) among groups were shown (b–d).

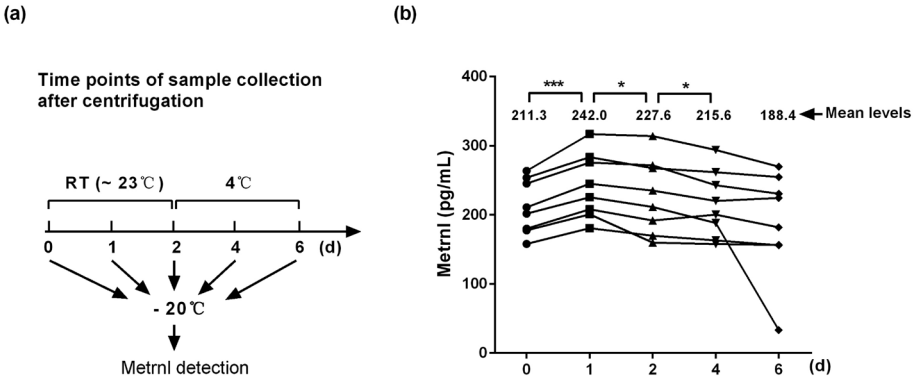


Fig. 8. Five Time Points of Serum Collection Were Setted after Centrifugation, with the Colleted Samples Transferred to −20°C Storage until Detection by DY7867-05 Kit (a); The Concentrations of Metrnl in All Samples Were Plotted with Mean Metrnl Levels of Five Groups Represented; Every Five Concentration Dots from the Same Participant Were Connected by Solid Line; Two-Tailed Paired Student's *t*-Test Was Used to Compare Difference between Two Groups (b)

$n$=8. *$p$<0.05. ***$p$<0.001.

human Metrnl.

On the whole, we consider the DY7867-05 kit suitable for human Metrnl detection. For standards assay, this kit exhibited good curve fitting degree provided by 4PL nonlinear regression model, small variation among tests and high inter-assay reproducibility. For samples assay, the kit could effectively detect human Metrnl in six types of blood samples and up to now, all the estimated concentrations were within the standard range. Moreover, the kit only detects human Metrnl, rather than rat and mouse Metrnl.

The six types of blood samples involved in our study are commonly used in clinic and our results show good correlations among these sample types in Metrnl concentrations. Our assays also provide a typical example, in which one person

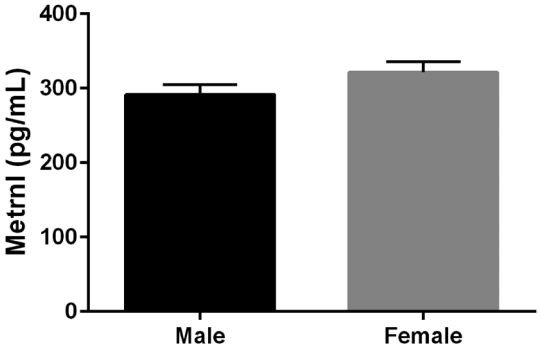


Fig. 9. Metrnl Concentrations in Male Participant Group ($n$=13) and Female Participant Group ($n$=10) Were Assayed by DY7867-05 Kit

Data were represented as means±S.E.M. Two-tailed unpaired Student's $t$-test was used to compare difference between the two groups.

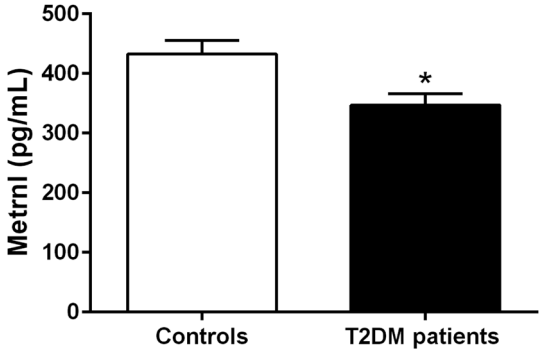


Fig. 10. Metrnl Concentrations in Patients with T2DM ($n$=9) and the Healthy Controls ($n$=11) Were Assayed by DY7867-05 Kit

Data were represented as means±S.E.M. Two-tailed unpaired Student's $t$-test was used to compare difference between the two groups. *$p<0.05$.

Table 2. General Physiological Characteristics and Metabolic Parameters of Patients with Newly Diagnosed T2DM and Their Matched Healthy Controls

| | Healthy controls ($n$=11) | Patients with T2DM ($n$=9) |
|---|---|---|
| Gender (M/F) | 6/5 | 5/4 |
| Age (years) | 34.55±2.04 | 37.33±4.55 |
| Body weight (kg) | 63.99±3.14 | 76.33±4.42* |
| Body height (cm) | 168.91±1.96 | 172.67±1.87 |
| BMI (kg/m$^2$) | 22.31±0.72 | 25.46±1.07* |
| Waist circumference (cm) | 77.77±2.32 | 90.44±4.05* |
| Hip circumference (cm) | 92.68±1.38 | 92.33±4.28 |
| WHR | 0.84±0.02 | 1.00±0.03* |
| SBP (mmHg) | 111.36±4.32 | 127.78±2.65* |
| DBP (mmHg) | 66.36±1.80 | 72.78±2.06* |
| FSG (mmol/L) | 4.80±0.17 | 8.97±0.90* |
| TC (mmol/L) | 4.85±0.39 | 6.07±0.14 |
| TG (mmol/L) | 1.15±0.14 | 2.79±0.82* |
| HDL-c (mmol/L) | 2.38±0.48 | 1.31±0.33 |
| LDL-c (mmol/L) | 2.30±0.40 | 3.70±0.44* |
| $HbA_{1c}$ (mmol/mol) | 32±1 | 90±10* |
| $HbA_{1c}$ (%) | 5.1±0.1 | 10.4±1.0* |
| FSI (mIU/L) | 7.78±0.99 | 45.06±17.48* |
| HOMA-IR | 1.66±0.21 | 19.51±7.70* |

BMI: body mass index; WHR: waist hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure. FSG: fasting serum glucose; TC: total cholesterol; TG: triglycerides; HDL-c: high density lipoprotein cholesterol; LDL-c: low density lipoprotein cholesterol; $HbA_{1c}$: plasma glycated haemoglobin $A_{1c}$; FSI: fasting serum insulin; HOMA-IR: homeostasis model assessment for insulin resistance. Data are presented as means±standard deviation (S.D.) or number. *$p<0.05$ *vs.* healthy controls.

had much lower concentration of Metrnl compared with other people in all sample types (Figs. 6, 7), further supporting that the kit is reliable. Though statistic significance was found among Metrnl concentrations in different sample types using paired $t$-test, we consider only higher concentrations in lithium heparin plasma should be noticed. Therefore, although we recommend using the same type of blood samples for Metrnl detection in comparison study, it appears acceptable when using different types of serum and plasma samples except lithium heparin plasma, which would be convenient for clinical examination, promoting Metrnl study in humans.

The DY7867-05 kit recommends standards ranging from 15.625 to 1000 pg/mL to generate a standard curve. In the present study all detected concentrations were within this range, with the maximum 770.5 pg/mL. Considering clinical samples taken from people under various physiological and pathological states, the estimated concentrations may exceed 1000 pg/mL. Thus, we tested if the maximum standard point can be extended. As a result, our subsequent study demonstrated that an extended standard curve with one additional point of 2000 pg/mL was workable (data not shown).

In this study, we used two DY7867-05 kits (each kit contains sufficient materials to run ELISAs on at least five 96-well plates), with data in Figs. 1–7 and 10 from the first kit and data in Figs. 8 and 9 from the second kit. We noticed that data from the second kit dropped compared to those from the first kit, suggesting that assays within the same kit are stable but unstable between different kits. Therefore, the comparison of circulating Metrnl needs to be performed in the same plate using the same kit. This limitation of Metrnl detection should be improved in the next generation method for detection of this novel secreted protein.

In clinical laboratory, serum or plasma samples are usually obtained in time for target analysis after blood collection. In general situation, the samples are stored at r.t. for the first day to get assayed and will then be moved to 4°C storage for one week. To investigate the effect of temperature and duration of blood sample storage on detection, five time points for samples to transfer to freezing storage were set. Our results indicated that samples in the same experiment should be stored in same condition and severe degradation could happen within 6 d of r.t. or 4°C storage. Thus, we recommend immediate freezing storage of serum and plasma samples after blood centrifugation.

The study of the small population of newly diagnosed T2DM patients and healthy controls within this work showed lower Metrnl levels in the patient group. The result, consistent with the conclusion from our previous studies that adipose Metrnl is an insulin sensitizer against insulin resistance induced by a high-fat diet,[7] indicated that Metrnl might be related to the pathophysiology of insulin resistance in the patients. However, this experiment has some limitations such as the numbers of two groups are small. Future studies with large population size are needed to further confirm the role of circulating Metrnl in the development of metabolic syndrome.

## Conclusion

In this study, we evaluated two ELISA kits (DY7867-05 and

SK00478-02) for the detection of human circulating Metrnl. The DY7867-05 kit is considered superior in Metrnl detection, suitable for various kinds of blood sample types. For good comparison in an assay, immediate freezing storage of serum and plasma samples are recommended after blood centrifugation and samples should be assayed in the same plate using the same DY7867-05 kit. There was no gender difference in circulating Metrnl level. In patients with T2DM, Metrnl levels were significantly lower compared to the healthy controls.

## Experimental Methods

**Two Commercially Available ELISA Kits for Human Metrnl Detection** One ELISA kit was purchased from R&D Systems Inc. (Minneapolis, MN, U.S.A.) with Catalogue no. DY7867-05. Another ELISA kit was purchased from Aviscera Bioscience Inc. (Suite C Santa Clara, U.S.A.) with Catalogue no. SK00478-02.

Both kits were used following the manufacturers' instructions. All standards and blood samples were assayed in two duplicates. The absorbance results were read by a microplate reader (Tecan Infinite M200, Tecan Austria GmbH, Grödig, Austria).

**Six Commonly Used Tubes for Collection of Human Serum and Plasma Samples** Three kinds of tubes were used to obtain three types of serum samples. Plain tubes without additive (Catalogue No. 25012101) and pro-coagulation tubes with the additive of clot activator (Catalogue No. 230611) were purchased from Shanghai Kehua Bioengineering Co., Ltd. (Shanghai, China), with the corresponding serum samples referred to as serum (nonadditive) and serum (additive) respectively. Separation gel tubes with the additive of clot activator and serum separation gel (Catalogue No. 367955) were purchased from Becton Dickinson (BD, Franklin Lakes, NJ, U.S.A.), and the corresponding serum samples were referred to as serum (gel separation).

In addition, three types of plasma samples were obtained by using three kinds of tubes all purchased from Becton Dickinson (BD, Franklin Lakes, NJ, U.S.A.). Plasma samples obtained by using K2 EDTA tubes with spray-coated K2 EDTA (Catalogue No. 367863), lithium heparin tubes with 75 USP units of lithium heparin spray-coated (Catalogue No. 367884), and sodium citrate tubes with 9 NC 0.109 M buffered sodium citrate (Catalogue No. 363095), were referred to as plasma (EDTA), plasma (lithium heparin), and plasma (sodium citrate), respectively.

Blood was collected through vein puncture directly into the tubes. Serum and plasma samples were obtained by allowing the tubes to stand at r.t. for 1 h, after which the tubes were centrifuged at 3000×***g*** for 15 min at 4°C and the supernatant of each sample was then aliquoted into labeled sterile EP tubes and stored at −20°C. In the investigation of sample storage condition, the storage process after centrifugation was modified as shown in Fig. 8(a), in which five time points of serum collection were set as follows: aliquots of each serum sample of the 8 participants were collected immediately as well as after 1 and 2 d storage at r.t.; then the 8 samples were steadily moved to 4°C, and aliquots of each sample were collected after 2 and 4 d storage at 4°C. Every aliquot was stored at −20°C until used for only once.

**Human Participants and Study Design** The study was approved by the medical ethical committee of the Second Military Medical University. The detection was fully explained, and the written informed consents were obtained from all participants. All participants were fasting overnight (no food, coffee or tea from 21:00 to 9:00) prior to blood collection.

To study the detection method of circulating Metrnl, a total of 80 volunteers were included. They were 26 staff members (13 women and 13 men) aged 23–50 years in our lab and 54 students aged 20–25 years from the Second Military Medical University. All of them were in normal figure (18.0<body mass index (BMI)<24.0), had a good general health, with no history of T2DM or other chronic diseases, and were not taking any medication. Of the 26 staff members, 15 were involved in serum (additive) sample collection and the other 11 were collected both serum (additive) and plasma (EDTA) samples. All these samples were detected using both ELISA kits. Ten of the students were collected blood to produce three types of plasma samples which were subsequently analyzed by SK00478-02 kit, and 13 of them were collected blood to produce six types of blood samples which were subsequently analyzed by DY7867-05 kit. The rest 31 students were collected serum (nonadditive) samples for Metrnl detection by DY7867- kit, with 23 of them involved in the investigation of gender effect on Metrnl level while samples of the other 8 students used to explore sample collection condition.

To explore the circulating Metrnl level in people with T2DM, 9 drug-naïve people with newly diagnosed T2DM (5 men and 4 women, age ranging from 21 to 62 years) were recruited from outpatient units of Changhai Hospital. The diagnosis of T2DM was made according to WHO criteria (1999).[14] People with known coronary heart disease, hypertension and previously diagnosed diabetes were excluded. Eleven healthy people (6 men and 5 women, age ranging from 26 to 50 years) were recruited as controls. Serum (additive) samples were collected from them to detected Metrnl using DY7867-05 kit.

**Clinical Examination** For people with newly diagnosed T2DM and their matched controls, clinical metabolic parameters were measured. FSG, TG, total cholesterol (TC), HDL-c and LDL-c were quantified by automatic biochemical analyzer (Hitachi High-Technologies, Tokyo, Japan). Plasma glycated $HbA_{1c}$ was determined by HPLC with automated $HbA_{1c}$ analyzer (Bio-Rad Laboratories, Hercules, CA, U.S.A.). FSI was analyzed by an ELISA kit (Shibayagi Co., Ltd., Shibukawa, Japan). HOMA-IR was determined using the following simplified equation: HOMA-IR=FSI (mIU/L)×FSG (mmol/L)/22.5. Besides, general physiological characteristics including age, gender, body weight, body height, waist circumference, hip circumference, SBP and DBP were recorded. BMI and WHR were calculated.

**Statistical Analysis** In each assay, the duplicate readings for each standard and sample were averaged and a standard curve with mean values of Absorbance *vs.* Concentration (in log scale) was plotted. A four parameter logistic (4PL) nonlinear regression model offered by SigmaPlot software (version 10.0) was applied for standard curve-fitting analysis as well as sample concentration calculations. The inter-assay coefficient of variation (CV) was calculated from the formula: CV=(Standard Deviation of the means of the duplicates)/(Grand Mean of the Duplicates)×100%. Two-tailed paired and unpaired Student's *t*-tests were used properly to compare Metrnl levels in two groups. Two-way ANOVA was used to compare differences among three groups, followed by pair-

wise comparisons subject to Bonferroni corrections for multiple tests. All statistical tests were conducted by SPSS software (version 22.0; SPSS Inc., Chicago, IL, U.S.A.), with $p<0.05$ considered significantly different.

**Acknowledgments** This work was supported by Grants from the National Natural Science Foundation of China (81730098, 81130061, and 16JC1405100) and the Military Medicine Project of PLA (16QNP087).

**Conflict of Interests** The authors declare no conflict of interest.

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