



## Research Paper

# Biomarkers for Early Diagnostic of Mild Cognitive Impairment in Type-2 Diabetes Patients: A Multicentre, Retrospective, Nested Case–Control Study



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## ABSTRACT

**Background:** Both type 2 diabetes mellitus (T2DM) and Alzheimer's disease (AD) are common age-associated disorders and T2DM patients show an increased risk to suffer from AD, however, there is currently no marker to identify who in T2DM populations will develop AD. Since glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) activity, ApoE genotypes and olfactory function are involved in both T2DM and AD pathogenesis, we investigate whether alterations of these factors can identify cognitive impairment in T2DM patients.

**Methods:** The cognitive ability was evaluated using Minimum Mental State Examination (MMSE) and Clinical Dementia Rating (CDR), and the mild cognitive impairment (MCI) was diagnosed by Petersen's criteria. GSK-3 $\beta$  activity in platelet, ApoE genotypes in leucocytes and the olfactory function were detected by Western/dot blotting, the amplification refractory mutation system (ARMS) PCR and the Connecticut Chemosensory Clinical Research Center (CCCRC) test, respectively. The odds ratio (OR) and 95% confidence intervals (95% CI) of the biomarkers for MCI diagnosis were calculated by logistic regression. The diagnostic capability of the biomarkers was evaluated by receiver operating characteristics (ROC) analyses.

**Findings:** We recruited 694 T2DM patients from Jan. 2012 to May. 2015 in 5 hospitals (Wuhan), and 646 of them met the inclusion criteria and were included in this study. 345 patients in 2 hospitals were assigned to the training set, and 301 patients in another 3 hospitals assigned to the validation set. Patients in each set were randomly divided into two groups: T2DM without MCI (termed T2DM-nMCI) or with MCI (termed T2DM-MCI). There were no significant differences for sex, T2DM years, hypertension, hyperlipidemia, coronary disease, complications, insulin treatment, HbA1c, ApoE  $\epsilon$ 2, ApoE  $\epsilon$ 3, tGSK3 $\beta$  and pS9GSK3 $\beta$  between the two groups. Compared with the T2DM-nMCI group, T2DM-MCI group showed lower MMSE score with older age, ApoE  $\epsilon$ 4 allele, higher olfactory score and higher rGSK-3 $\beta$  (ratio of total GSK-3 $\beta$  to Ser9-phosphorylated GSK-3 $\beta$ ) in the training set and the validation set. The OR values of age, ApoE  $\epsilon$ 4 gene, olfactory score and rGSK-3 $\beta$  were 1.09, 2.09, 1.51, 10.08 in the training set, and 1.06, 2.67, 1.47, 7.19 in the validation set, respectively. The diagnostic accuracy of age, ApoE  $\epsilon$ 4 gene, olfactory score and rGSK-3 $\beta$  were 0.76, 0.72, 0.66, 0.79 in the training set, and 0.70, 0.68, 0.73, 0.79 in the

**Abbreviations:** T2DM, type 2 diabetes mellitus; AD, Alzheimer's disease; GSK-3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; MMSE, minimum mental state examination; CDR, clinical dementia rating; MCI, mild cognitive impairment; ARMS, amplification refractory mutation system; CCCRC, Connecticut Chemosensory Clinical Research Center; OR, odds ratio; CI, confidence intervals; ROC, receiver operating characteristics; AUC, the area under the curve; ApoE, apolipoprotein E; HbA1c, hemoglobin A1c.

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validation set, respectively. These four combined biomarkers had the area under the curve (AUC) of 82% and 86%, diagnostic accuracy of 83% and 81% in the training set and the validation set, respectively.

*Interpretation:* Aging, activation of peripheral circulating GSK-3 $\beta$ , expression of ApoE  $\epsilon$ 4 and increase of olfactory score are diagnostic for the mild cognitive impairment in T2DM patients, and combination of these biomarkers can improve the diagnostic accuracy.

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## 1. Introduction

Type 2 diabetes mellitus (T2DM) and Alzheimer's disease (AD) are the most common age-associated disorders and the prevalence of the diseases is increasing with population aging (Biessels et al., 2008; Exalto et al., 2012; Stumvoll et al., 2005; Umegaki, 2014). The epidemiological study (Strachan et al., 2011) shows that T2DM patients show a 1.5–2.0-fold increased risk of AD. In a longitudinal cohort study lasting up to 9 years, the risk of developing AD was 65% higher in persons with diabetes than in non-diabetic controls (Arvanitakis et al., 2004). A community-based controlled study suggests that frank diabetes (35%) or glucose intolerance (46%) may be present in up to 80% of AD patients (Janson et al., 2004). Over a maximum 11 years of follow-up, diabetic patients experienced a higher incidence of AD than non-diabetic subjects (0.48% vs. 0.37%) (Huang et al., 2014). Diabetes is associated with a 50–100% increased risk of AD (Biessels et al., 2008).

The long preclinical phase of AD provides opportunity for intervention (Ballard et al., 2011). Compared to healthy individuals, the patients with mild cognitive impairment (MCI) have an increased risk to develop AD (Petersen et al., 1999). Therefore, early diagnosis of MCI is critical for developing efficient interventions to postpone or prevent AD. This requires developing new diagnostic tools to predict dementia among the high risk people with MCI.

Since the pathophysiological events leading to dementia precede the clinical symptoms, biomarkers for MCI have become an area of great interest for both researchers and clinicians (Tan et al., 2014). The biomarker measurements are principally of brain amyloidosis (amyloid positron emission tomography, cerebrospinal fluid A $\beta$ 42) and neurodegeneration (medial temporal atrophy on MR, fluorodeoxyglucose positron emission tomography, CSF tau) (Bocchetta et al., 2015). However, these widely validated biomarkers are hampered by practical difficulty that severely limit their application in large populations. Thus, finding non-invasive and cost-effective biomarkers is of great importance for the early diagnosis.

Olfactory dysfunction is an early clinical feature in AD patients (Devanand et al., 2010; Graves et al., 1999; Naka et al., 2010), which can predict the incidence of MCI and its conversion into AD (Rahayel et al., 2012). The apolipoprotein E (ApoE)  $\epsilon$ 4 allele is a dose-dependent risk factor for AD (Farrer et al., 1997; Weisgraber, 1994), and AD patients with ApoE  $\epsilon$ 4 usually show an earlier age of onset and a more rapid progression of the disease (Corder et al., 1993). Olfactory impairment together with ApoE  $\epsilon$ 4 genotype can be a marker for cognitive decline (Graves et al., 1999), although it is also reported that the ApoE gene plays a role in olfactory functioning that is independent of dementia conversion within 5 years (Olofsson et al., 2010). Glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) is a serine/threonine kinase that regulates diverse cellular functions ranging from glycogen metabolism to gene transcription and cell survival (Grimes and Jope, 2001; Woodgett, 1990). The GSK-3 $\beta$  activity is increased in AD and MCI patients, and activation of GSK-3 $\beta$  causes tau hyperphosphorylation and A $\beta$  overproduction (Hye et al., 2005; Pei et al., 1997; Plattner et al., 2006). These studies suggest that the olfactory dysfunction, GSK-3 $\beta$  activation and ApoE  $\epsilon$ 4 expression are predicting factors of cognitive impairments.

It is very likely that the future diagnosis and treatment of MCI will rely on the combination of clinical and laboratory data. To our knowledge, no associations with potential biomarker profiles or cognitive function in T2DM patients have been published. In this study, we aim

to establish diagnostic accuracy of the peripheral platelet GSK-3 $\beta$  activity, the ApoE gene polymorphisms and the olfactory score in directing MCI from T2DM patients.

## 2. Methods

### 2.1. Study Design and Participants

We recruited T2DM patients from five hospitals in China between January 2012 and May 2015, and the T2DM patients were diagnosed by the World Health Organization (WHO) National Diabetic Group Criteria of 2006 (Olokoba et al., 2012). The inclusion criteria were as follows: T2DM patients aged over 50 years who are literate, able to complete the neuropsychological test and volunteer to participate in this study. The exclusion criteria include history of head trauma, brain tumor, epilepsy, stroke, transient ischemic attack, coma, or presence of dementia before T2DM; previous thyroid disease; drug abuse, substance abuse, and alcohol addiction; depression, schizophrenia and other psychiatric disorders. According to Petersen's MCI criteria (Petersen et al., 1999), T2DM patients were classified into two groups: T2DM with MCI (T2DM-MCI), which met criteria for subjective and objective cognitive difficulties in the absence of significant functional loss, and T2DM without MCI (T2DM-nMCI). AD diagnosis is based on the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria (McKhann et al., 1984).

All participants gave written informed consent and were treated in accordance with the Declaration of Helsinki. The study was approved by Tongji Medical School Ethics Committee.

### 2.2. Procedures

All participants underwent a complete assessment as the following: neuropsychological evaluation, olfactory score test, platelet GSK-3 $\beta$  activity measurement and ApoE genotyping. The neuropsychological evaluations mainly included the Minimum Mental State Examination (MMSE) and the Clinical Dementia Rating (CDR), which were done by two examiners who had neurology training and experience with neurophysiologic techniques. The MMSE scale, one of the best-known and the most widely used brief screening instruments for cognitive impairment, provides a total score ranging from 0 to 30, with lower scores indicative of greater cognitive impairment. The CDR scale is a numeric scale used to quantify the severity of symptoms of dementia from six areas: memory, orientation, judgment and problem solving, community affairs, home and hobbies, and personal care. In this study, we used the Petersen's MCI criteria as the reference standard. In addition, MMSE and CDR scores for MCI diagnosis were 24–27 and 0.5, respectively.

The Connecticut Chemosensory Clinical Research Center (CCCC) test was used to measure the olfactory score, which was done blind to the other measures by the examiners. A stock solution with 4% butanol was diluted with distilled water as one third ratio to eight dilutions in eight flasks. The participants were exposed to the flasks from low to high concentrations of butanol along with a blank, and were asked to identify which one smelled stronger. Incorrect choices triggered another blank paired with the next higher concentration, whereas correct choices led to another presentation of the same concentration in another bottle and a blank. Four correct choices in a row led to the end of the

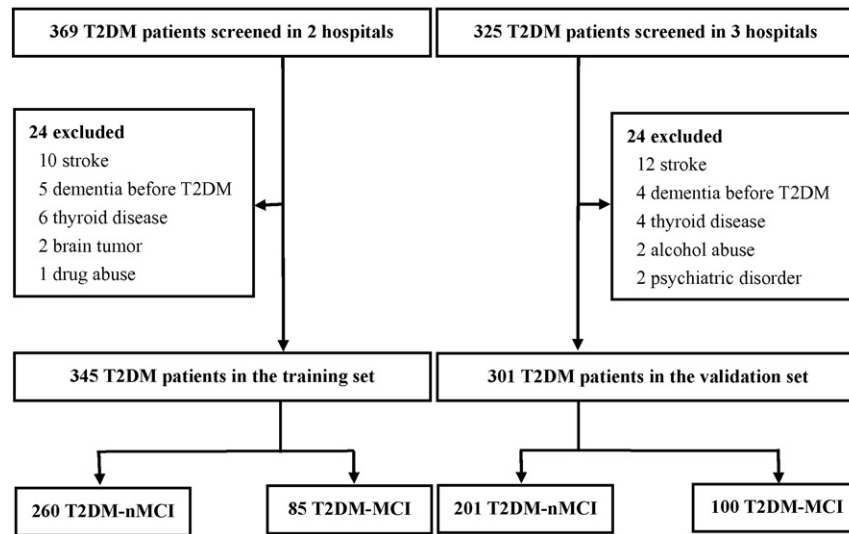


Fig. 1. Study profile.

test, and the number of this concentration marked the score. The higher scores indicated greater olfactory impairment.

For biochemical indicators, all participants were asked to measure fasting blood glucose, postprandial blood glucose, serum insulin, hemoglobin A1c (HbA1c) and serum magnesium the next morning. On receipt, serum, leukocytes and platelet samples were separated from EDTA whole blood by centrifugation, which were collected and stored at  $-80^{\circ}\text{C}$  until analysis. The total GSK-3 $\beta$  (tGSK-3 $\beta$ ) and serine-9 phosphorylated GSK-3 $\beta$  (pS9GSK-3 $\beta$ , the inactive form of the kinase) in serum or platelet were measured by ELISA (CUSABIO BIOTECH CO., Ltd., China), Western blotting or dot blotting. The ratio of tGSK-3 $\beta$ /pS9GSK-3 $\beta$  was used as a measure of GSK-3 $\beta$  activity. In dot blotting, the levels of tGSK-3 $\beta$  and pS9GSK-3 $\beta$  were normalized by the same control people (non-T2DM). In addition, the biochemical activity of GSK-3 $\beta$  in platelet was measured by using enzyme activity assay kit according to the manufacturer's guidance (Genmed, MA, USA).

For ApoE genotyping, the Gerard's method with modifications in the multiplex amplification refractory mutation system PCR (ARMS) was applied (Donohoe et al., 1999; Kim et al., 2000). Genomic DNA was extracted from peripheral blood leukocytes using DNA extraction kit (Aidlab Biotechnologies Co., Ltd., China). The primers were designed and synthesized by Invitrogen Life Technologies (Shanghai, China) for ApoE with specific Cys primers (Cys112:5'-CGCGGACATGGAGGACGTTT-3', and Cys158:5'-ATGCCG ATGACTGCAGAAATT-3'), Arg primers (Arg112:5'-CGCGGACATGGA

GGACGTC-3', and Arg158:5'-ATGCCGATGACCTGCAGAATC-3'), and a common reverse primer (5'-GTTTCAGTGATTGCTGCTGGGCA-3'). The PCR protocol was performed with 12.5  $\mu\text{l}$  Taq MasterMix, 50 ng genomic DNA, 0.4  $\mu\text{M}$  Cys112/Arg112 primers or 0.8  $\mu\text{M}$  Cys158/Arg158 primers, 0.8  $\mu\text{M}$  common reverse primers, and 2  $\mu\text{l}$  DMSO in a total of 25  $\mu\text{l}$  reaction. PCR was initiated with denaturation at  $94^{\circ}\text{C}$  for 2 min, amplification by 36 cycles of  $94^{\circ}\text{C}$  for 45 s,  $64^{\circ}\text{C}$  for 45 s and  $72^{\circ}\text{C}$  for 45 s, and a final extension at  $72^{\circ}\text{C}$  for 5 min. Amplicons were resolved by 1.8% agarose gel electrophoresis and stained with GoldView Dye.

### 2.3. Statistical Analysis

Data analysis was performed by separate raters who are experienced university teachers in statistics using SPSS 19.0 window software (statistical package for the Social sciences, SPSS Inc). The continuous variables and the categorical variables were presented respectively as mean  $\pm$  SD and frequency (%). Chi square and t-test were used to compare case versus controls for categorical variables and continuous variables, respectively. All *P* values reported were considered statistically significant if *P* < 0.05.

To test whether the individual biomarker was independently associated with MCI diagnosis in T2DM patients, we constructed logistic regression models. We calculated the odds ratio (OR) and 95% confidence intervals (95% CI) for MCI of these biomarkers. A cut-off value of single biomarker reflecting the best combination of sensitivity and specificity

Table 1

Baseline characteristics of T2DM patients in the training set and the validation set.

Characteristic	The training set		The validation set	
	T2DM-nMCI (n = 260)	T2DM-MCI (n = 85)	T2DM-nMCI (n = 201)	T2DM-MCI (n = 100)
Age (years)	60.31 $\pm$ 6.34	65.34 $\pm$ 8.37*	63.24 $\pm$ 8.00	67.91 $\pm$ 8.68*
Male (%)	42.31%	37.65%	46.77%	35.00%
T2DM years	7.29 $\pm$ 5.27	8.49 $\pm$ 6.86	8.45 $\pm$ 6.89	8.80 $\pm$ 7.85
Hypertension	45.38%	37.65%	53.23%	62.00%
Hyperlipidemia	6.54%	4.71%	8.96%	11.00%
Coronary disease	8.85%	3.53%	8.46%	15.00%
Complication	64.23%	58.83%	47.26%	42.00%
Insulin treatment	47.69%	44.71%	41.79%	36.00%
MMSE	29.05 $\pm$ 0.85	26.00 $\pm$ 1.13*	29.10 $\pm$ 0.80	24.94 $\pm$ 2.94*

Data are mean  $\pm$  SD or n (%). T2DM = type 2 diabetes mellitus. MCI = mild cognitive impairment. T2DM-nMCI = T2DM without MCI group. T2DM-MCI = T2DM with MCI group. MMSE = the Minimum Mental State Examination.

\* *P* < 0.05 versus the T2DM-nMCI group.

Table 2

Potential markers of T2DM patients in the training set and the validation set.

Characteristic	The training set		The validation set	
	T2DM-nMCI (n = 260)	T2DM-MCI (n = 85)	T2DM-nMCI (n = 201)	T2DM-MCI (n = 100)
Olfactory	6.31 $\pm$ 1.33	7.37 $\pm$ 1.91*	6.76 $\pm$ 1.48	8.02 $\pm$ 1.82*
HbA1c	8.19 $\pm$ 2.12	8.27 $\pm$ 2.09	7.82 $\pm$ 1.81	8.20 $\pm$ 1.89
ApoE $\epsilon$ 2	11.92%	11.76%	23.88%	21.00%
ApoE $\epsilon$ 3	96.92%	94.12%	93.03%	87.00%
ApoE $\epsilon$ 4	13.08%	27.06%*	10.45%	25.00%*
tGSK3 $\beta$	1.34 $\pm$ 1.93	1.92 $\pm$ 3.16	1.85 $\pm$ 3.62	2.74 $\pm$ 3.97
pS9GSK3 $\beta$	2.13 $\pm$ 4.00	2.94 $\pm$ 6.53	3.02 $\pm$ 3.92	2.64 $\pm$ 4.05
rGSK3 $\beta$	0.76 $\pm$ 0.28	1.19 $\pm$ 0.67*	0.70 $\pm$ 0.46	1.66 $\pm$ 1.04*

Data are mean  $\pm$  SD or n (%). T2DM = type 2 diabetes mellitus. MCI = mild cognitive impairment. T2DM-nMCI = T2DM without MCI group. T2DM-MCI = T2DM with MCI group. HbA1c = hemoglobin A1c. ApoE = Apo lipoprotein E. GSK-3 $\beta$  = glycogen synthase kinase-3 $\beta$ . GSK-3 $\beta$  ratio = total GSK-3 $\beta$ /Ser9-GSK-3 $\beta$ .

\* *P* < 0.05 versus the T2DM-nMCI group.

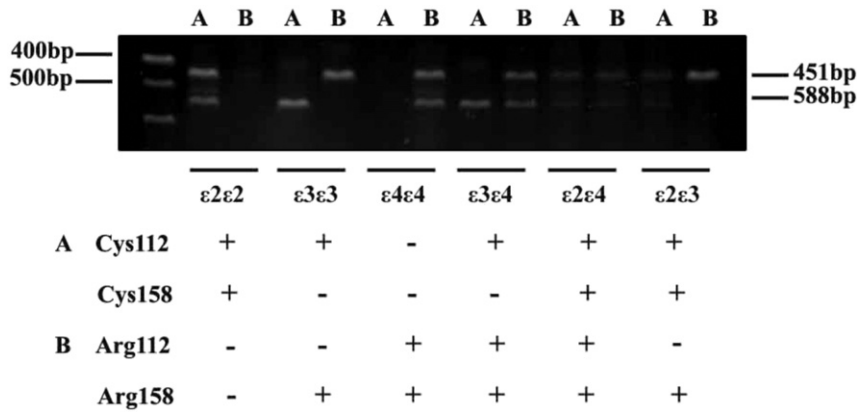


Fig. 2. Six ApoE genotypes detected in this study. We detected six ApoE genotypes: ε2ε2, ε3ε3, ε4ε4, ε2ε3, ε2ε4, and ε3ε4 in T2DM patients.

was ascertained, and the area under the curve (AUC) and diagnostic accuracy were calculated. Receiver operating characteristic curves (ROC) were plotted to assess the diagnostic accuracy of GSK-3β and the combined biomarkers.

### 3. Results

We screened 694 T2DM patients between Jan. 2012 and May 2015, and 646 of them met the inclusion criteria, thus were enrolled for the study (Fig. 1). Based on the recruiting time and the hospitals, 345 patients in 2 hospitals were assigned to the training set, and 301 of them in 3 hospitals were assigned to the validation set. According to Petersen's MCI criteria, the T2DM patients were divided into 85 T2DM with MCI

(T2DM-MCI) and 260 T2DM without MCI (T2DM-nMCI) in the training set, and 201 T2DM-nMCI and 100 T2DM-MCI in the validation set.

#### 3.1. Demographics and Clinical Characteristics of the Participants

Table 1 shows the demographic and clinical data of all the participants in the training set and the validation set. T2DM-MCI group had older age ( $65.34 \pm 8.37$  vs  $60.31 \pm 6.34$  and  $67.91 \pm 8.68$  vs  $63.24 \pm 8.00$ ) and lower MMSE score ( $26.00 \pm 1.13$  vs  $29.05 \pm 0.85$  and  $24.94 \pm 2.94$  vs  $29.10 \pm 0.80$ ) than T2DM-nMCI group in the training set and the validation set, respectively. There were no significant differences for sex, T2DM years, hypertension, hyperlipidemia, coronary disease, complications and insulin treatment. T2DM-MCI participants had

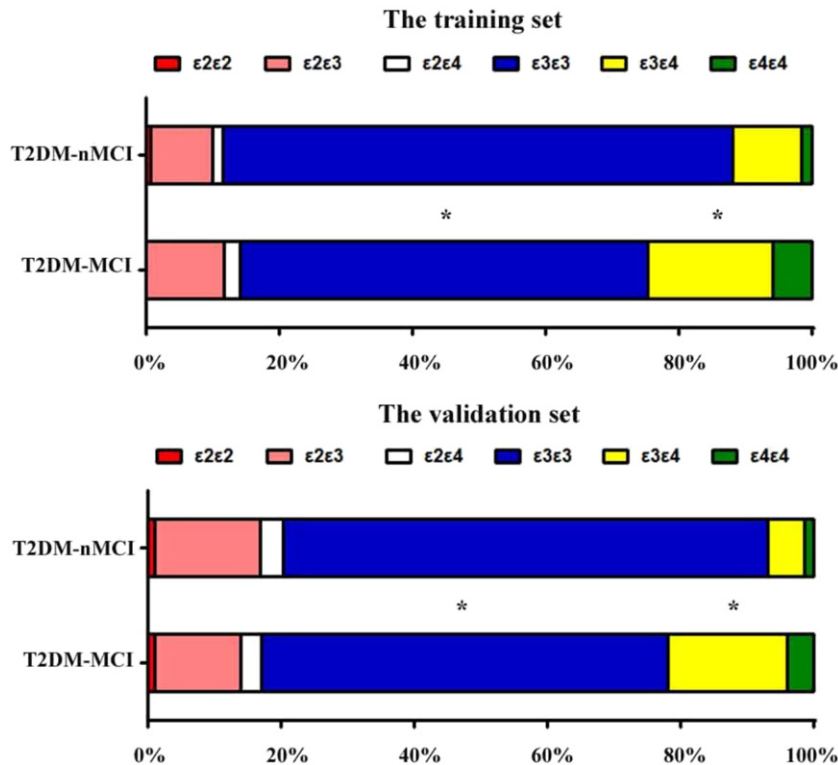
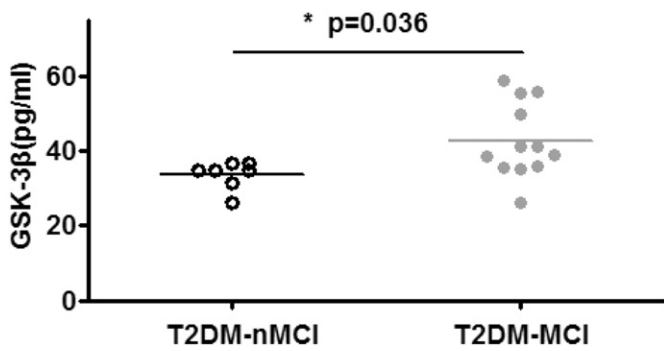
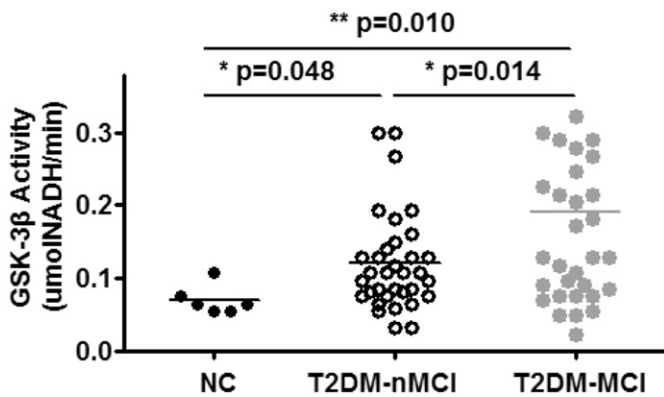


Fig. 3. The differences of ApoE between T2DM-nMCI group and T2DM-MCI group in the training set and the validation set. The ε3ε4 genotype in T2DM-MCI group was higher than that in T2DM-nMCI group, and the ε3ε3 genotype in T2DM-MCI group was lower than that in T2DM-nMCI. \*P < 0.05 versus the T2DM-nMCI group.





**Fig. 4.** The difference of GSK-3 $\beta$  expressions in serum between T2DM-nMCI group (n = 7) and T2DM-MCI group (n = 12). The expressions of GSK-3 $\beta$  in serum of T2DM-MCI group were higher than those in T2DM-nMCI group \* $P < 0.05$  versus the T2DM-nMCI group.



**Fig. 5.** The differences of platelet GSK-3 $\beta$  activity between control group (n = 6), T2DM-nMCI group (n = 34) and T2DM-MCI group (n = 33). T2DM-MCI group had higher GSK-3 $\beta$  activity than T2DM-nMCI group, and T2DM-nMCI group had higher GSK-3 $\beta$  activity than control group in the platelet. \* $P < 0.05$ , \*\* $P < 0.01$  versus the NC group or T2DM-nMCI group.

a CDR of 0.5, and T2DM-nMCI had a CDR of 0. In addition, MMSE score was negatively correlated with the ages in T2DM patients ( $P < 0.001$ , Fig. S1).

### 3.2. Comparison of Olfactory Score and ApoE Genotypes between T2DM-MCI and T2DM-nMCI Patients

We tested all 646 participants with olfactory score, HbA1c level and ApoE genotypes. The olfactory score was higher in T2DM-MCI group than the T2DM-nMCI group (Table 2). A strong and significant negative correlation was detected between olfactory score and MMSE score in T2DM patients ( $P < 0.001$ , Fig. S2). By ARMS-PCR, six ApoE genotypes including  $\epsilon 2\epsilon 2$ ,  $\epsilon 3\epsilon 3$ ,  $\epsilon 4\epsilon 4$ ,  $\epsilon 2\epsilon 3$ ,  $\epsilon 2\epsilon 4$ , and  $\epsilon 3\epsilon 4$  were detectable in T2DM patients (Fig. 2). Quantitative data demonstrated that the frequency of  $\epsilon 3\epsilon 4$  genotype was higher while  $\epsilon 3\epsilon 3$  was lower in T2DM-MCI group

than T2DM-nMCI group, and no differences of  $\epsilon 2\epsilon 2$ ,  $\epsilon 4\epsilon 4$ ,  $\epsilon 2\epsilon 3$  and  $\epsilon 2\epsilon 4$  were detected between the two groups (Fig. 3). Further analyses showed that percentage of  $\epsilon 4$  allele was higher in T2DM-MCI group than the T2DM-nMCI group in both the training set and the validation set (Table 2). Moreover, the ApoE  $\epsilon 4$  (+) group had lower MMSE scores than ApoE  $\epsilon 4$  (–) group in T2DM patients ( $P < 0.01$ , Fig. S3).

### 3.3. Comparison of Platelet GSK-3 $\beta$ Activity Between T2DM-MCI and T2DM-nMCI Patients

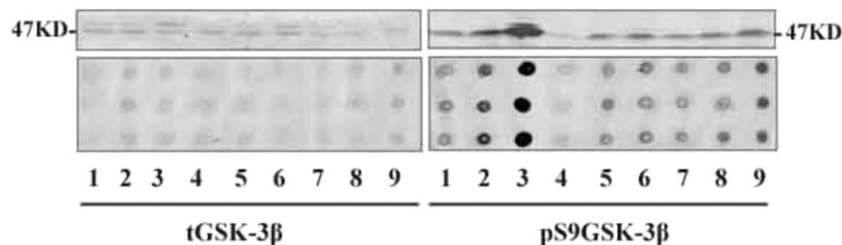
By ELISA, we found that serum level of GSK-3 $\beta$  protein was higher in T2DM-MCI group (n = 12) than T2DM-nMCI group (n = 7, Fig. 4). By using the enzyme activity assay kit, we confirmed that T2DM-MCI group (n = 33) had higher platelet GSK-3 $\beta$  activity than T2DM-nMCI group (n = 34), and the T2DM-nMCI had higher GSK-3 $\beta$  activity than control group (n = 6, Fig. 5). To further verify whether the level of GSK-3 $\beta$  in platelet can be used as a clinical diagnostic biomarker to identify MCI in T2DM patients, we analyzed the total and pS9GSK-3 $\beta$  by Western blotting and dot blotting. We first verified that results of dot blots were consistent with those of Western blots (Fig. 6). As the procedure of dot blotting is much simpler than that of Western blotting, we used dot blotting for the analyses of tGSK-3 $\beta$  and pS9GSK-3 $\beta$  proteins in all 646 participants. The ratio of tGSK-3 $\beta$ /pS9GSK-3 $\beta$  (rGSK-3 $\beta$ ) was used to represent platelet GSK-3 $\beta$  activity, and higher ratios indicated higher GSK-3 $\beta$  activity. We found that levels of pS9GSK-3 $\beta$  (the inactive form of the kinase) and tGSK-3 $\beta$  were not significantly changed, while the level of rGSK-3 $\beta$  was significantly increased in T2DM-MCI patients compared to T2DM-nMCI patients (Fig. 7, Table 2). In addition, T2DM-AD group had higher rGSK-3 $\beta$  than T2DM-MCI group, and T2DM-MCI group had higher rGSK-3 $\beta$  than T2DM-nMCI group in the platelets (Fig. 8). A negative correlation between platelet rGSK-3 $\beta$  and MMSE score was seen in T2DM patients ( $P < 0.001$ , Fig. S4).

### 3.4. Potential Diagnostic Biomarkers for Identifying MCI in T2DM Patients

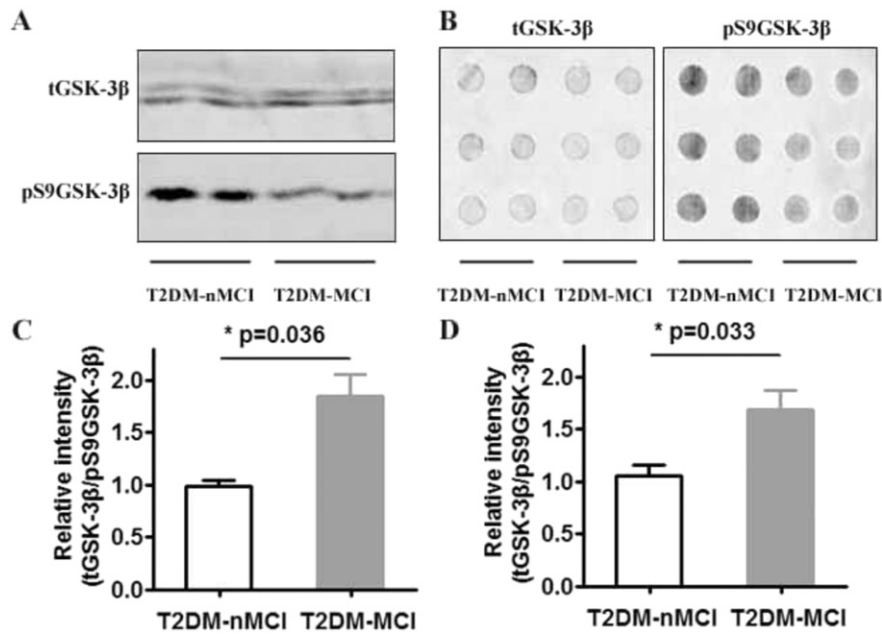
We used logistic regression models to calculate the odds ratio (OR) and 95% confidence intervals (95% CI) for MCI of all biomarkers. We found that age, ApoE  $\epsilon 4$  gene, olfactory score and platelet GSK-3 $\beta$  activity were strongly associated with MCI in T2DM patients (Table 3). In the training set, the OR values (95% CI) of age, ApoE  $\epsilon 4$  gene, olfactory score and rGSK-3 $\beta$  were 1.09 (1.05–1.14), 2.09 (1.00–4.35), 1.51 (1.24–1.85) and 10.80 (4.49–26.00), respectively. In the validation set, the OR values (95% CI) of age, ApoE  $\epsilon 4$  gene, olfactory score and rGSK-3 $\beta$  were 1.06 (1.02–1.10), 2.67 (1.21–5.90), 1.47 (1.21–1.79) and 7.19 (3.92–13.16), respectively (Table 3). These data suggest that increases of age, ApoE  $\epsilon 4$  gene, olfactory score and platelet rGSK-3 $\beta$  may be diagnostic for MCI in T2DM patients.

### 3.5. Diagnostic Efficacy of Single Biomarker

We applied ROC models to calculate the area under the curve (AUC), the accuracy, the specificity and sensitivity of single

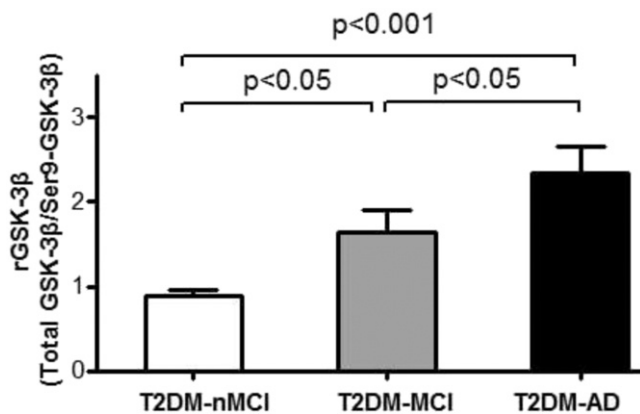


**Fig. 6.** The comparison of GSK-3 $\beta$  expressions in the platelets of different participants by Western blot and Dot blot. The results of Western blot analysis were in accordance with those of Dot blot.



**Fig. 7.** The differences of platelet GSK-3 $\beta$  activity between T2DM-nMCI group and T2DM-MCI group by Western blot and Dot blot. (A) The expressions of total GSK-3 $\beta$  and p-GSK-3 $\beta$  (ser9) in the platelet by Western blot; (B) the expressions of total GSK-3 $\beta$  and p-GSK-3 $\beta$  (ser9) in the platelet by Dot blot; (C) quantitative analysis of Western blot; (D) Quantitative analysis of Dot blot. \* $P < 0.05$  versus T2DM-nMCI group.

biomarkers in the training set and the validation set. We used the following formulas for the calculations: % sensitivity = [true-positive / (true-positive + false-negative)]  $\times$  100; % specificity = [true-negative / (true-negative + false-positive)]  $\times$  100. Age had a maximal AUC of 68.0% and an accuracy of 76.0% in the training set, and a maximal AUC of 65.0% and an accuracy of 70.0% in the validation set (Table 4). The diagnostic value of olfactory score for MCI revealed a AUC of 72.0% and an accuracy of 66.0% against controls. In ApoE gene polymorphisms,  $\epsilon 4$  allele showed accuracy of 72.0% and 68.0% in the training set and the validation set, respectively. The rGSK-3 $\beta$  for MCI diagnosis resulted in an AUC of 69.0% and an accuracy of 79.0% in the training set, and an AUC of 81.0% and an accuracy of 79.0% in the validation set. Therefore, the remaining analyses focused on age, platelet rGSK-3 $\beta$ , ApoE  $\epsilon 4$ , olfactory score and the combinations.



**Fig. 8.** The differences of platelet rGSK-3 $\beta$  between T2DM-AD group ( $n = 15$ ), T2DM-nMCI group ( $n = 15$ ) and T2DM-MCI group ( $n = 15$ ). T2DM-AD group had higher rGSK-3 $\beta$  than T2DM-MCI group, and T2DM-MCI group had higher rGSK-3 $\beta$  than T2DM-nMCI group in the platelet.

### 3.6. Diagnostic Capability of the Combined Biomarkers

The diagnostic capability of ApoE  $\epsilon 4$  and olfactory score were combined, generating ROC curves with AUC of 72.0% and 73.0%, accuracy of 78.0% and 73.0% in the training set and the validation set, respectively (Table 5). By combining rGSK-3 $\beta$  and ApoE  $\epsilon 4$ , we generated ROC curves with AUC of 77.0% and 80.0%, accuracy of 72.0% and 83.0% in the training set and the validation set, respectively. Using only rGSK-3 $\beta$  and olfactory score from the biomarker profile, it yielded AUC of 79.0% and 84.0%, accuracy of 77.0% and 80.0% in the training set and the validation set, respectively. The addition of rGSK3 $\beta$ , olfactory score and ApoE  $\epsilon 4$  generated ROC curves with AUC of 79.0% and 85.0%, accuracy of 74.0% and 80.0% in the training set and the validation set, respectively (Table 5).

By combining age, platelet rGSK-3 $\beta$ , ApoE  $\epsilon 4$  and olfactory score, we generated a ROC curve with an AUC of 82.0% (95% CI 0.76–0.87) and an accuracy of 83.0% in the training set (Model 1) (Table 5, Fig. 9). In the validation set (Model 2), an increased AUC of 86.0% and a decreased

**Table 3**  
Logistic regression models for MCI in T2DM patients.

Variable	B	S.E.	Wald	Sig.	Exp(B)	95% CI	
						Lower	Upper
<i>The training set</i>							
Age	0.09	0.02	17.31	<0.001	1.09	1.05	1.14
ApoE $\epsilon 4$	0.74	0.37	3.87	0.049	2.09	1.00	4.35
Olfactory	0.41	0.10	16.14	<0.001	1.51	1.24	1.85
rGSK-3 $\beta$	2.38	0.45	28.19	<0.001	10.80	4.49	26.00
<i>The validation set</i>							
Age	0.06	0.02	10.02	0.002	1.06	1.02	1.10
ApoE $\epsilon 4$	0.98	0.40	5.93	0.015	2.67	1.21	5.90
Olfactory	0.39	0.10	15.28	<0.001	1.47	1.21	1.79
rGSK-3 $\beta$	1.97	0.31	40.82	<0.001	7.19	3.92	13.16

B = the estimated logit coefficient. S.E. = the standard error of the coefficient. Wald = B/S.E. Sig. = the significance level of the coefficient. Exp(B) = the odds ratio of the individual coefficient. CI = confidence interval. ApoE = Apo lipoprotein E. GSK-3 $\beta$  = glycogen synthase kinase-3 $\beta$ . GSK-3 $\beta$  ratio = total GSK-3 $\beta$ /Ser9-GSK-3 $\beta$ .

**Table 4**  
Diagnostic efficacy of single biomarker in the training set and the validation set.

Variables	Cutoff	Specificity	Sensitivity	AUC (95% CI)	Accuracy
<i>The training set</i>					
Age	65.50	0.85	0.49	0.68 (0.61,0.75)	0.76
HbA1c	7.15	0.62	0.42	0.49 (0.42,0.56)	0.57
Olfactory	6.75	0.65	0.71	0.72 (0.66,0.79)	0.66
ApoE $\epsilon$ 2	–	0.88	0.12	–	0.69
ApoE $\epsilon$ 3	–	0.03	0.94	–	0.26
ApoE $\epsilon$ 4	–	0.87	0.27	–	0.72
tGSK3 $\beta$	1.03	0.73	0.39	0.50 (0.42,0.58)	0.64
pS9GSK3 $\beta$	0.40	0.89	0.33	0.59 (0.52,0.67)	0.75
rGSK3 $\beta$	1.05	0.90	0.48	0.69 (0.62,0.76)	0.79
<i>The validation set</i>					
Age	68.50	0.80	0.49	0.65 (0.59,0.72)	0.70
HbA1c	7.05	0.43	0.74	0.57 (0.51,0.64)	0.53
Olfactory	7.75	0.77	0.64	0.72 (0.66,0.79)	0.73
ApoE $\epsilon$ 2	–	0.76	0.21	–	0.58
ApoE $\epsilon$ 3	–	0.07	0.87	–	0.34
ApoE $\epsilon$ 4	–	0.90	0.25	–	0.68
tGSK3 $\beta$	1.29	0.69	0.61	0.65 (0.59,0.72)	0.66
pS9GSK3 $\beta$	0.49	0.83	0.36	0.59 (0.52,0.66)	0.67
rGSK3 $\beta$	1.29	0.91	0.55	0.81 (0.76,0.86)	0.79

ROC = receiver operating characteristics. AUC = the area under the curve. CI = confidence interval. GSK3 $\beta$  = glycogen synthase kinase-3 $\beta$ , tGSK3 $\beta$  = total GSK3 $\beta$ , pS9GSK3 $\beta$  = serine-9 phosphorylated pS9GSK3 $\beta$ , rGSK3 $\beta$  = ratio of tGSK3 $\beta$ /pS9GSK3 $\beta$ .

accuracy of 81.0% were observed but the difference was not statistically significant from Model 1 (Table 5, Fig. 9).

#### 4. Discussion

Both AD and T2DM are age-associated disorders that affect millions of people around the world. The incidence of AD increases to 14.2% in diabetic patients, and over 80% of AD patients have T2DM or show abnormal blood glucose levels. Therefore, searching for biomarkers that can diagnose or predict conversion of T2DM into AD makes it possible to start an early intervention to prevent an outbreak of AD. In the present study, we investigated what could be the biomarkers for the cognitive impairments in T2DM patients, and validated the diagnostic accuracy of these biomarkers. According to Petersen's MCI criteria, we divided

T2DM patients into two groups, i. e., T2DM-MCI and T2DM-nMCI. As predicted, age is a strong factor for cognitive decline in T2DM patients. Furthermore, we first demonstrated that age, peripheral circulating GSK-3 $\beta$  activation, ApoE  $\epsilon$ 4 expression and olfactory score are diagnostic for the mild cognitive impairment in T2DM patients, and combination of these biomarkers can improve the diagnostic accuracy.

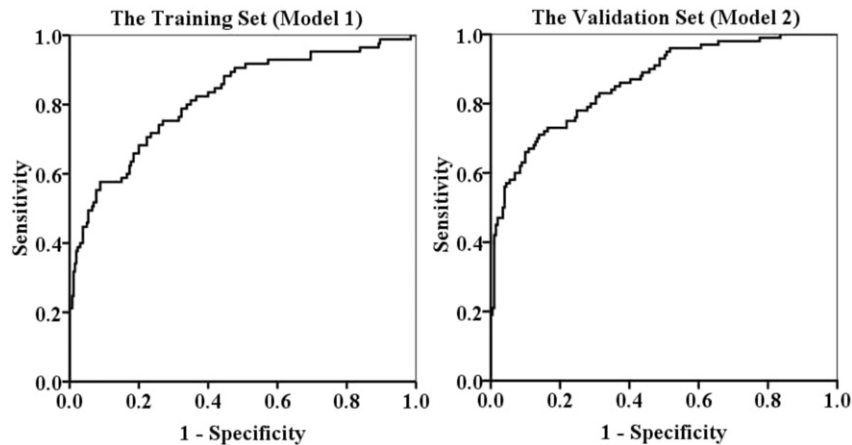
Firstly, we find that activation of GSK-3 $\beta$  in peripheral circulating platelet is diagnostic of cognitive impairment in T2DM patients. Accumulating laboratory evidence suggest that GSK-3 $\beta$  activation is involved in both T2DM and AD, such as disturbed glycogen metabolism (Rayasam et al., 2009), tau hyperphosphorylation (Martin et al., 2013), A $\beta$  production (Phiel et al., 2003), and neurodegeneration (Hooper et al., 2008). Clinical studies also show that level of GSK-3 $\beta$  mRNA and protein in leucocytes is increased (Hye et al., 2005; Sheng et al., 2012), and the GSK-3 $\beta$  gene variants are associated with cognitive function in AD patients (Kettunen et al., 2015). However, it is not reported whether GSK-3 $\beta$  activity is different in human T2DM patients with or without cognitive impairment. In the present study, we found that the GSK-3 $\beta$  activity was much higher in T2DM-MCI patients than the cognitively healthy T2DM patients, indicating that GSK-3 $\beta$  activation could be an indicator of cognitive impairment in T2DM patients. This finding is of great significance because it provides a feasible tool to screen among the rapidly increasing T2DM populations who may develop into AD, by which we can also establish efficient strategies to arrest the conversion of T2DM into AD.

Secondly, we find that expression of ApoE  $\epsilon$ 4 gene is strongly associated with the cognitive impairment in T2DM patients. ApoE gene has three major alleles ( $\epsilon$ 2,  $\epsilon$ 3 and  $\epsilon$ 4) based on the amino-acid substitutions (Arg or Cys) at 112 and 158 of the protein (Sutton et al., 2015), in which the ApoE  $\epsilon$ 4 allele has a reduced ability to repair neuronal damage and a decreased antioxidant activity compared with the  $\epsilon$ 2 and  $\epsilon$ 3 (Ma et al., 1994). Growing genetic evidence suggests that ApoE  $\epsilon$ 4 allele is the major genetic risk factor for AD (Bertram et al., 2010) and is associated with an increased risk of MCI conversion into AD (Devanand et al., 2005). ~70% of AD patients have one or two copies of ApoE  $\epsilon$ 4. The presence of ApoE  $\epsilon$ 4 allele confers greater amyloid burden (Fleisher et al., 2011), changes of amyloid and tau in cerebrospinal fluids (Vemuri et al., 2010), higher degree of neurodegeneration (Caroli et al., 2010), and alterations in brain function and glucose metabolism (Langbaum

**Table 5**  
Diagnostic efficacy of the combined biomarkers.

Variables	Specificity	Sensitivity	AUC (95% CI)	Accuracy
<i>The training set</i>				
ApoE $\epsilon$ 4 + olfactory	0.85	0.54	0.72 (0.65,0.79)	0.78
ApoE $\epsilon$ 4 + rGSK3 $\beta$	0.83	0.58	0.72 (0.65,0.79)	0.77
ApoE $\epsilon$ 4 + age	0.76	0.65	0.71 (0.64,0.78)	0.73
rGSK3 $\beta$ + age	0.72	0.69	0.77 (0.71,0.83)	0.71
Olfactory + age	0.76	0.73	0.78 (0.72,0.83)	0.75
rGSK3 $\beta$ + olfactory	0.78	0.73	0.79 (0.73,0.85)	0.77
ApoE $\epsilon$ 4 + olfactory + rGSK3 $\beta$	0.73	0.74	0.79 (0.73,0.85)	0.74
ApoE $\epsilon$ 4 + olfactory + age	0.71	0.74	0.77 (0.72,0.83)	0.72
ApoE $\epsilon$ 4 + rGSK3 $\beta$ + age	0.78	0.66	0.78 (0.72,0.84)	0.75
Olfactory + rGSK3 $\beta$ + age	0.80	0.72	0.82 (0.77,0.87)	0.78
rGSK3 $\beta$ + olfactory + ApoE $\epsilon$ 4 + age	0.91	0.58	0.82 (0.76,0.87)	0.83
<i>The validation set</i>				
ApoE $\epsilon$ 4 + olfactory	0.80	0.59	0.73 (0.67,0.79)	0.73
ApoE $\epsilon$ 4 + rGSK3 $\beta$	0.88	0.63	0.83 (0.78,0.88)	0.80
ApoE $\epsilon$ 4 + age	0.74	0.64	0.69 (0.62,0.75)	0.70
rGSK3 $\beta$ + age	0.86	0.70	0.83 (0.78,0.88)	0.81
Olfactory + age	0.75	0.73	0.77 (0.71,0.82)	0.74
rGSK3 $\beta$ + olfactory	0.90	0.60	0.84 (0.79,0.88)	0.80
ApoE $\epsilon$ 4 + olfactory + rGSK3 $\beta$	0.86	0.69	0.85 (0.80,0.89)	0.80
ApoE $\epsilon$ 4 + olfactory + age	0.69	0.76	0.78 (0.72,0.83)	0.71
ApoE $\epsilon$ 4 + rGSK3 $\beta$ + age	0.79	0.78	0.84 (0.79,0.89)	0.79
Olfactory + rGSK3 $\beta$ + age	0.87	0.67	0.86 (0.81,0.90)	0.80
rGSK3 $\beta$ + olfactory + ApoE $\epsilon$ 4 + age	0.86	0.71	0.86 (0.82,0.91)	0.81

AUC = the area under the curve. CI = confidence interval.



**Fig. 9.** ROC (receiver operating curves) show diagnostic efficacy of the combined biomarkers (age, ApoE  $\epsilon$ 4 gene, olfactory threshold and GSK-3 $\beta$  ratio) in the training set and the confirmation in the validation set.

et al., 2009). The T2DM patients carrying ApoE  $\epsilon$ 4 allele had a higher number of hippocampal neuritic plaques and neurofibrillary tangles in the cortex and hippocampus, and they had a higher risk of cerebral amyloid angiopathy (Peila et al., 2002). In the current study, we are the first to show that the expression of ApoE  $\epsilon$ 4 allele is strongly associated with the cognitive impairment in T2DM patients, which provides a viable genetic factor for screening the high AD risk individuals in T2DM patients.

Thirdly, we find that reduction of olfactory score is indicative for cognitive impairment in T2DM patients. Olfactory dysfunction is a common and early symptom of many neurodegenerative diseases, particularly of AD and MCI (Attems et al., 2014; Lehrner et al., 2009; Christen-Zaech et al., 2003). ~86% of normal elderly participants present with Alzheimer-type neurofibrillary tangles in the olfactory bulbs, and one third of them also have amyloid deposition (Kovacs, 2004). Studies show that the identification of olfactory impairment has clinical utility as an early diagnostic biomarker for MCI and AD (Devanand et al., 2000; Djordjevic et al., 2008; Vyhnaek et al., 2015). In the present study, we find a strong association of smell identification deficit with cognitive impairment in T2DM patients, which provides an applicable clinical tool in screening the potential AD conversion in T2DM patients. We also find that combination of ApoE  $\epsilon$ 4 gene, olfactory score and the ratio of tGSK-3 $\beta$ /pS9GSK-3 $\beta$  can improve the AUC and accuracy of MCI diagnosis in T2DM patients.

Additionally, we developed a simple protocol (dot blot) for measurement of total and pS9GSK-3 $\beta$  in human platelets. As this method is simple handling and has good reproducibility, we believe that the method may be suitable for application in clinical laboratories. Platelets and neurons have several homeostatic functions in common, such as accumulation and release of neurotransmitters, expression of membrane-bound compounds, and responsiveness to variations in calcium concentration (El Haouari and Rosado, 2009).

The major limitation of the current study is the follow-up investigation to the same participants, although we have designed training set and validation set to redeem this weakness. Further multi-center and longitudinal studies will confirm how informative these biomarkers in predicting the conversion of T2DM into AD.

In conclusion, we show in the present study that the alterations and the combined application of age, ApoE  $\epsilon$ 4 gene, olfactory score and tGSK-3 $\beta$ /pS9GSK-3 $\beta$  ratio are diagnostic for mild cognitive deficits in T2DM patients, which shed light on developing preventive strategies to arrest conversion of T2DM into AD.

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The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

#### Author Contributions

ZPX and JZW initiated and designed the study. ZPX, SZ, LFZ, CHZ, HHL, HJR, YZ, PYZ, LD, NL, CXK, QW, DK and JRL recruited the patients, collected blood samples. ZPX, RXH, MZL, YG, SJZ, YCL, JWY, XW, LF, QZZ and GPL performed experiments. SLY, SW and HWJ performed the data analysis, ZPX and JZW wrote the manuscript.

#### Conflicts of Interest

The authors have no conflict of interest.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2016.02.014>.

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