New approach for early and specific Alzheimer Disease diagnosis from different plasma biomarkers

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Abstract

Background: Alzheimer Disease (AD) is a complex pathology, in which several biochemical pathways could be involved. Therefore, the development of clinical studies combining different nature biomarkers in an AD diagnosis approach is required. Specifically, the present study evaluated blood biomarkers from different molecular pathways (epigenomics, lipid metabolism, lipid peroxidation), to obtain an early and specific AD diagnosis approach.

Methods: The participants were classified into early AD (n= 53), and non-AD (healthy controls, other dementias) (n= 83). Blood samples were collected and biochemical determinations (microRNAs, lipids, lipid peroxidation compounds) were carried out by quantitative PCR and liquid chromatography coupled to mass spectrometry, respectively. Then, a logistic regression model with a Bayesian variable selection procedure was developed.

Results: The Bayesian variable selection procedure for microRNAs did not show any relevant variable. Therefore, microRNAs biomarkers were excluded. So, the developed model considered only lipids and lipid peroxidation compounds. The corresponding selected variables were age, 18:0 LysoPC, PGE_2, isoprostanes and isofurans. The validated model (by leave-one-out cross-validation) provided satisfactory diagnosis indexes (AUC 0.83, Sensitivity 87 %, Specificity 79 %).

Conclusion: The developed model included biomarkers from different pathways (lipid metabolism, oxidative stress), achieving a promising approach to early, specific and minimally invasive AD diagnosis. Nevertheless, further work to validate clinically these preliminary results with an external cohort is required. Also, the integration of different compounds coming from several biochemical pathways could constitute a relevant research field for the development of AD therapeutic targets.

Keywords: plasma, biomarker, lipid, lipid peroxidation, microRNA, Alzheimer Disease, diagnosis model, Bayesian statistics

1. Introduction

Alzheimer Disease (AD) is the main type of dementia, and its incidence is increasing due to the aging population [1]. Its early diagnosis is based on cerebrospinal fluid (CSF) biomarkers, which involves an invasive sampling and shows some side-effects. Recent research has focused on the identification of blood biomarkers [2,3]. However, the disease high complexity requires to identify biomarkers associated to the alteration of different pathways from the initial stages of AD [4]. In this sense, it would be interesting to develop clinical studies combining different types of blood biomarkers as an AD diagnosis approach.

The main histological markers in AD are the extracellular Amyloid-β42 (Aβ42) plaques, and intracellular phosphorylated Tau (p-Tau) tangles [5,6]. Among the most relevant physiopathological mechanisms involved in AD, chronic inflammation plays an important role, since it acts over the Aβ42 accumulation [7]. In addition, the lipid metabolism [8] and oxidative stress, specifically lipid peroxidation [9], could be molecular pathways involved in early AD.

Recent research suggests that a multiple-nature impairment, involving changes at metabolomics, epigenomics, transcriptomics, and proteomics levels could lead to AD [10,11]. Therefore, a multi-omic approach could improve the knowledge of the AD physiopathology [12–15]. In this sense, some works have identified different nature biomarkers, which considered simultaneously could improve their individual AD diagnosis capacity [16].

The aim of the present study is to evaluate simultaneously some biomarkers from different biochemical pathways (microRNAs, lipids, lipid peroxidation compounds) as potential early AD biomarkers in blood, and to develop a diagnosis approach.

2. Material and Methods

2.1. Participants and samples collection

The participants were patients from the Cognitive Disorders Unit in Hospital Universitari I Politècnic La Fe (Valencia, Sapin). They were clinically evaluated by neuropsychological tests (Clinical Dementia Rating (CDR) [17], Mini-Mental State Examination (MMSE) [18], Repeatable Battery for Assessment of Neuropsychological Status Delayed Memory (RBANS.DM) [19], Functionality Assessment Questionnaire (FAQ), [20]), and CSF biomarkers (Aβ42, total Tau, p-Tau181). From these tests, patients were diagnosed with non-cognitive impairment (n=49), early AD (n= 53), and other dementias (frontotemporal dementia, vascular dementia, dementia with Lewy Bodies) (n= 34). For this study, participants were classified into AD group (n= 53) and non-AD group (n= 83). The non-AD group included non-cognitive impairment patients and patients with other dementias.

Blood samples were collected from the participants in EDTA-tubes, they were centrifuged (10 min, 2000 g, room temperature) to separate plasma fractions, and they were stored at -80ºC until the analysis.

The informed consent was obtained from all participants and the Ethics Committee of the Health Research Institute of La Fe (Valencia) approved the study protocol (2019/0105).

2.2. Biomarkers determination

2.2.1. Lipid peroxidation compounds

The analytical method to determine lipid peroxidation compounds (PGE_2, $PGF_{2\alpha}$, 15-E_{2t}-IsoP, 15-F_{2t}-IsoP, 15(*R*)-15-F_{2t}-IsoP, 2,3-dinor-15-epi-15-F_{2t}-IsoP, 15-keto-15-E_{2t}-IsoP, 15-keto-15-F_{2t}-IsoP, 5-F_{2t}-IsoP, 4(*RS*)-4-F_{4t}-NeuroP, 10-epi-10-F_{4t}-NeuroP, 14(*RS*)-14-F_{4t}-NeuroP, 1a,1b-dihomo-$PGF_{2\alpha}$, Ent-7(*RS*)-7-F_{2t}-dihomo-IsoP, 17-F_{2t}-dihomo-IsoP, 17-epi-17-F_{2t}-dihomo-IsoP, 17(*RS*)-10-epi-SC-Δ^{15}-11-dihomo-IsoF, 7(*RS*)-ST-Δ^{8}-11-dihomo-IsoF) and 4 total parameters (isoprostanes, isofurans, neuroprostanes, neurofurans) in plasma samples was described in a previous work [21]. Briefly, samples treatment consisted of basic hydrolysis followed by solid-phase extraction for sample clean-up and analytes pre-concentration. Then, samples were analysed using liquid

chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS, Waters Acquity UPLC-Xevo TQD system (Milford, MA, USA)).

2.2.2. Lipids

The analytical method for targeted analysis of lipid compounds (18:1 LPE, 18:0 LPC, 16:1 SM (d18:1/16:1), 16:0 SM (d18:1/16:0), 18:0 SM (d18:1/d18:0), 18:1 (9-Cis) PE (DOPE), 24:0 SM) and LPC 17:0, 17:0 SM (d18:1/17:0), 17:0 PE) in plasma samples was described in a previous study [8]. Briefly, 150 µL of cold isopropanol were added to 50 µL of plasma, vortexed and kept at −20 °C for 30 min. Then, it was centrifuged (13000 g, 10 min, 4 °C) and 90 µL of supernatant were transferred to a 96-wells plate, where internal standard mix solution was added to each sample. Finally, the samples were analysed by liquid chromatography coupled to time-of-flight mass spectrometry (UPLC-TOF/MS-Orbitrap QExactive Plus MS, ThermoFisher Scientific (Massachusetts, USA)).

2.2.3. MicroRNAs

The analytical method based on quantitative PCR to determine some microRNAs (has-miR-92a-3p, has-miR-486-5p, has-miR-29a-3p, miR-486-3p, miR-150-5p, miR-320b, miR-483-3p, miR-342-3p) in plasma samples was described in a previous study [22]. Briefly, retrotranscription and amplification steps were carried out from the RNA extracted (TaqMan Advanced miRNA Assays) (https://tools.thermofisher.com/content/sfs/manuals/100027897_TaqManAdv_miRNA_Assays_UG.pdf). Finally, samples were diluted, and real time PCR was carried out in duplicate using the thermocycler (ViiA7, Applied Biosystems, (California, USA)).

2.3. Statistical analysis

2.3.1 Logistic regression model

A logistic regression model was developed using demographic factors (sex, age) and plasma biomarkers levels (lipid peroxidation compounds, lipids, microRNAs) as predictor variables, and participants group (AD, non-AD) as response variable, to provide the probability of developing

$y_i = \begin{cases} 1 & \text{if person } i \in AD \\ 0 & \text{otherwise} \end{cases} \rightarrow y_i \sim Ber(\pi_i)$

AD. The response variable for each person, i, was considered to follow a Bernoulli distribution with probability π_i. Then, a linear predictor was used involving the above-mentioned variables, namely $(x_{1i}, x_{2i}, x_{3i}, x_{4i} \dots)$, to explain the log of the odds for π_i:

$$\text{logit}(\pi_i) = \log\left(\frac{\pi_i}{1-\pi_i}\right) = \beta_o + \beta_1 x_{1i} + \beta_2 x_{2i} + \beta_3 x_{3i} + \beta_4 x_{4i} + \cdots$$

① To select relevant biomarkers, several strategies were performed. First, a diagnosis model was developed independently for each biomarkers' family (lipid peroxidation compounds, lipids, ② miRNAs). Secondly, a model including simultaneously all biomarkers from the families identified as relevant in the previous independent models (lipid peroxidation compounds and lipids) was ③ developed. After selecting the best model, a Bayesian variable selection strategy (see Section 2.3.2) was used to identify the most relevant biomarkers. The final model including these selected biomarkers was fitted from a Bayesian perspective using the Integrated Nested Laplace Approximation (INLA) software within R [23]. This Bayesian approach was used given its advantages in the interpretability of the results departing for the usual p-values scenario.

2.3.2 Variables selection

The predictor variables (age, sex, lipid peroxidation compounds, lipids, microRNAs) have different impact on AD development, so the first step was to select the most relevant biomarkers.

Using the logistic linear regression model above, the selection of biomarkers was performed by means of a Bayesian variable selection procedure for generalized linear models [24].

The aim of this procedure is to consider all possible combinations of biomarkers for the linear predictor. Then, using the Bayes Theorem the probability of each combination being the "true" model is estimated and, from those, an inclusion probability is provided for each biomarker. Then, the biomarkers with the larger inclusion probabilities were selected.

This analysis was performed using the bas.glm() function from the BAS package in R (Clyde, Merlise (2022) BAS: Bayesian Variable Selection and Model Averaging using Bayesian Adaptive Sampling, R package version 1.6.4, https://cran.r-project.org/package=BAS,).

2.3.3 Model Validation.

The validation procedure was carried out to assess the performance and generalizability of its predictive performance, discriminating between AD and non-AD groups. It was based on the Leave-One-Out Cross Validation (LOOCV) method, which provided a robust estimate [25].

From the model validation results a cut-off point, corresponding to the individual probability of developing AD, was set. In addition, the model performance was evaluated from different diagnosis indexes (area under the receiver operating characteristic curve (AUC-ROC), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV).

3. Results

3.1 Demographic and clinical participants description

Table 1 summarises the demographic and clinical characteristics for the participants groups (non-AD group (n= 83), AD group (n= 53)). As expected, neuropsychological variables (CDR, RBANS, FAQ, MMSE) and CSF biomarkers (Aβ42, t-Tau, p-Tau181) showed significant differences between participants groups. Also, age showed significant difference between groups, while sex not.

3.2 Plasma biomarkers determinations

The plasma biomarkers levels showed some differences between participants groups. For lipid peroxidation (Table 2), some compounds showed higher levels in the non-AD group (isofurans, isoprostanes, PGE_2, $PGF_{2\alpha}$, 15-E_{2t}-IsoP) than in the AD group. For lipids (Table 3), some compounds showed higher levels in the AD group (16:1 SM, 18:1 LysoPE, DOPE). For microRNAS,

they were determined only in a subgroup of participants (n= 43) (Table 4), some miRNAs showed higher levels in the AD group (miR-486-3p7, miR-150-5p8, miR-342-3p11).

3.3. Diagnosis model development

Plasma biomarkers levels were used to develop a diagnosis model. The Bayesian variable selection procedure did not identify any microRNA as relevant variable for the AD detection; neither considering simultaneously the 3 biomarkers families, nor considering only microRNAs biomarkers. Therefore, microRNAs were excluded as predictor variables.

From the other predictor variables, those with a posterior probability of inclusion (PPI) > 20% were selected. Specifically, they were age, 18:0 LysoPC, PGE_2, isoprostanes and isofurans (see Table S1 in Supplementary Material). The threshold for PIPs was established at 0.2 (20%) since, as observed in Figure 1, it seems to be the natural cut-off point for these probabilities with the rest of variables having a much lower one.

Then, the parameters of the model developed from the selected variables were estimated using a Bayesian approach through the R package INLA [23]. The posterior distributions for the parameters (mean, standard deviation, quartile 1, median, quartile 3, mode), are summarized in Table 5. Here, the mean can be seen as a point estimate for these parameters while the sd and the first and third quartiles represent the uncertainty around these values. As observed, a very large estimation for the coefficient associated to isofurans was obtained, with a very large posterior variance. This extreme effect may be due to the correlation between the variables isoprostanes and isofurans (0.9), both included in the model. However, the present work is focused on the model predictive performance.

In this sense, the model was validated using LOOCV. From these validation results, the optimal cut-off point was set up at 0.4, in which sensitivity plus specificity was maximum. So, a patient with an estimated probability > 0.4 would be considered with some risk of AD development.

Finally, the diagnosis indexes were obtained from the validation model results and the selected cut-off. As observed in Table 6, the AUC was 0.83, Sensitivity 87%, Specificity 79%, PPV 73%, and NPV 90.41%.

4. Discussion

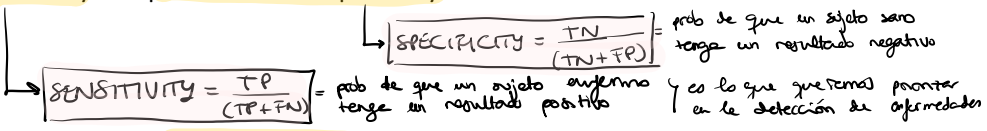
The present study provided a preliminary approach to early AD diagnosis from different biochemical compounds determined in blood samples. Actually, it was observed that the levels of some lipids and lipid peroxidation compounds could be impaired in early AD in comparison with other dementias and healthy conditions. In this sense, Nie et al. showed that lipid metabolism and oxidative stress are key mechanisms in AD, but the link between both plasma biomarker types in AD is poorly understood [26].

In general, lipid metabolism plays an important role in AD development, since brain contains a high lipid composition, and recent research has focused on the relationship between lipids and the typical AD histological impairment in brain [27]. Also, some derivative metabolites could be found in blood, considering the blood-brain barrier alteration under pathological conditions. In this sense, the peripheral study of early AD biomarkers is of high interest [28]. Specifically, the blood lipids levels have been evaluated in some previous works [29,30], observing that lipids metabolites in plasma might indirectly indicate early changes in AD (e.g. neuronal membrane impairment). In the present study, the 18:1 LysoPE showed higher levels in the AD group than in the non-AD group. Similarly, Llano et al. showed that higher serum levels of LysoPE predicted faster conversion from MCI to AD [31]. In addition, a previous lipidomics study showed statistically significant differences in the levels of lipid families (diglycerols, monoglycerides, phosphatidylethanolamines) between the MCI-AD and healthy groups [8], reflecting the relevant role of some lipid families. In addition, several fatty acids showed strong positive correlations with some microRNAs, which could regulate genes implied in fatty acids metabolism (e.g. elongation of very long-chain fatty acids, fatty acid desaturases) [12].

Oxidative stress is another pathway with an important effect over the AD development. In this sense, some oxidative metabolites have been studied (DNA, proteins, lipid peroxidation). Specifically, previous studies revealed that lipid peroxidation could play an important role in early AD development. In fact, a promising AD differential diagnosis model was developed with a validated AUC of 0.74 [32]; and some plasma isoprostanoids (15-*epi*-15-F_{2t}-IsoP, PGE_2, 15-keto-15-E_{2t}-IsoP) were identified as potential biomarkers of preclinical AD diagnosis [33].

The development of a diagnosis model from these different biomarkers could represent a useful tool in early AD screening. With this purpose and given the binary nature of the outcome, in the present work a logistic regression model was considered and fitted using a Bayesian perspective. To select the variables to be included in the linear predictor, a variable selection procedure was carried out. Among the different available approaches, the Bayesian variable selection is considered a powerful statistical technique used in the field of biomarker detection and analysis [ref??]. It offers a systematic and probabilistic approach to identify relevant variables or features from a pool of potential candidates, which can be crucial for understanding disease mechanisms, predicting outcomes, and discovering novel biomarkers. Actually, Bayesian variable selection employs the Bayes theorem to model the uncertainty associated with each variable's contribution to the observed data. The central idea is to assign a probability distribution to the inclusion or exclusion of each variable in the statistical model.

Once the model is selected and fitted, the performance metrics should align with the specific objectives and constraints of the biomarker detection study. The present work aims to advance in an early diagnosis approach. So, sensitivity was prioritized over specificity in order to ensure that cases are not missed.



Regarding the limitations of this study, there was a small sample size, but all these participants were accurately classified into AD and non-AD groups attending to the standard CSF biomarkers (Aβ42, t-Tau, p-Tau181). In addition, microRNAs were not included since they were not selected

by the selection procedure. So, only two compounds' families (lipids, lipid peroxidation compounds) were finally included in the potential diagnosis model.

5. Conclusions

Biomarkers from different biochemical pathways were evaluated to observe their involvement in early AD development, in comparison with other dementias. Specifically, lipid metabolism and lipid peroxidation could play an important role in AD, since 18_0_LysoPC, PGE2, isoprostanes and isofurans were selected as relevant variables.

The corresponding developed model showed satisfactory diagnosis indexes, constituting a promising approach to early, specific and minimally invasive AD diagnosis. Nevertheless, further work to validate clinically these preliminary results with an external cohort is required. Also, the integration of different types of biomarkers could constitute a relevant research field for the development of AD therapeutic targets.

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Figure captions

Figure 1. Elbow plot for each variable posterior probability of inclusion, ordered by its magnitude. Horizontal line is set at 0.2 (probability of a 20%). Variables 1: age, 2: isoprostanes, 3: 18_0_LysoPC, 4: PGE2, 5: isofurans, 6: 18_1_LysoPE, 7: CO5-769, 8: 8iso15RPGF2alfa, 9: 2,3-dinor-iPF2alfa, 10: neuroprostanes, 11: neurofurans, 12: 8-iso-15-keto-PGE2, 13:8-iso-15-keto-PGF2alfa, 14: 5-iPF2alfa-VI, 15: PGF2alfa, 16: 8-iso-PGE2, 17: CO5-776, 18: 16_1_SM, 19: 16_0_SM, 20: DOPE, 21: CO1-31, 22: sex, 23: ADT-207, 24: 8-iso-PGF2alfa, 25: VB559m1, 26: AG495m4R, 27: CO5-778, 28: 18_0_SM, 29: ADT-420, 30: 1a,1b-dihomo.

Table 1. Demographic and clinical variables for the participants groups.

Variable	**AD Group (n= 53)**	**Non-AD Group (n= 83)**
Gender (Female, n (%))	31 (58)	47 (57)
Age (years, median (IQR))	71 (69- 74)	63 (59.5- 68)
CSF Aβ42 (pg mL^{-1}, median (IQR))	564 (457.86-699.07)	1224.15 (954.24-1451)
CSF p-Tau181 (pg mL^{-1}, median (IQR))	82 (68-107)	32 (27-42)
CSF t-Tau (pg mL^{-1}, median (IQR))	566 (412.5-761.5)	225 (178.5-279.5)
CSF t-Tau/Aβ42 (median (IQR))	0.93 (0.69-1.38)	0.19 (0.16-0.23)
CDR (score, median (IQR)	0.5 (0.5-0.5)	0 (0-0.5)
MMSE (score, median (IQR))	24 (23-27)	29 (28-29)
RBANS-DM (score, median (IQR))	56 (43-72)	98 (94.75-102)

*IQR: Inter-quartile range

Table 2. Lipid peroxidation compounds plasma levels in the participants groups

Biomarker (nmol L^{-1})	AD Group (n= 53)		Non-AD Group (n= 83)	
	Median	**IQR**	**Median**	**IQR**
Isofurans (a.u.)	0.20	0.12-0.29	0.36	0.22-0.48
Isopostanes (a.u.)	0.38	0.30-0.61	0.81	0.50-1.39
Neurofurans (a.u.)	0.21	0.11-0.39	0.17	0.08-0.31
Neuroprostanes (a.u.)	0.01	0.00-0.10	0.08	0.00-0.56
PGE_2	0.07	0.00-0.40	0.28	0.11-0.45
$PGF_{2\alpha}$	0.58	0.07-0.90	0.70	0.32-0.98
1a,1b-dihomo-$PGF_{2\alpha}$	0.00	0.00-1.43	1.27	0.00-3.41
2,3-dinor-15-*epi*-15-F_{2t}-IsoP	0.00	0.00-0.0	0.00	0.00-0.03
5-F_{2t}-IsoP	1.27	0.48-1.98	2.08	1.09-2.89
15-keto-15-E_{2t}-IsoP	0.00	0.00-0.20	0.42	0.00-0.96
15-keto-15-F_{2t}-IsoP	0.28	0.15-0.42	0.48	0.29-0.81
15(*R*)-15-F_{2t}-IsoP	0.38	0.22-0.58	0.50	0.22-0.68
15-E_{2t}-IsoP	0.45	0.07-1.80	0.72	0.21-1.50
15-F_{2t}-IsoP	0.02	0.00-0.14	0.05	0.01-0.08
17(*RS*)-10-*epi*-SC-Δ^{15}-11-dihomo-IsoF	0.00	0.00-0.00	0.00	0.00-0.00
7(*RS*)-ST-Δ^{8}-11-dihomo-IsoF	0.00	0.00-0.15	0.00	0.00-0.20
D4-10-*epi*-10-F_{4t}-NeuroP	0.20	0.00-0.32	0.20	0.10-0.30
14(*RS*)-14-F_{4t}-NeuroP	0.75	0.00-1.75	1.15	0.29-1.80
Ent-7(*RS*)-7-F_{2t}-dihomo-IsoP	0.10	0.00-0.17	0.05	0.00-0.20

17-*epi*-17-F_{2t}-dihomo-IsoP	0.00	0.00-0.00	0.00	0.00-0.00
17-F_{2t}-dihomo-IsoP	0.00	0.00-0.00	0.00	0.00-0.00
4(*RS*)-4-F_{4t}-NeuroP	1.15	0.35-1.52	1.57	0.85-3.62

*a.u.: arbitrary units; *IQR: Inter-quartile range

Table 3. Lipids plasma levels in the participants groups

Biomarker (µg mL $^{-1}$)	AD Group (n= 53)		Non-AD Group (n= 83)	
	Median	**IQR**	**Median**	**IQR**
16:1 SM	28.578	14.68-118.29	29.511	15.06-121.67
16:0 SM	223.24	166.44-290.32	206.04	133.38-285.60
18:0 LysoPC	28.58	14.68-118.29	29.51	15.07-121.67
18:0 SM	87.77	48.69-258.73	89.25	58.40-272.02
18:1 LysoPE	1.47	1.13-2.17	1.26	0.95-1.72
DOPE	16.16	6.45-50.53	12.69	6.56-47.66

*IQR: Inter-quartile range

Table 4. MicroRNAs plasma levels in the participants groups

Biomarker (relative units)	AD Group (n= 25)		Non-AD Group (n= 18)	
	Median	**IQR**	**Median**	**IQR**
miR-92a-3p	1.28	0.67-1.62	0.83	0.63-1.92
miR486-5p	0.92	0.70-1.82	1.06	0.66-1.49
miR-29a-3p	0.88	0.55-1.25	1.07	0.81-2.12
miR-486-3p	1.33	0.54-2.12	0.99	0.71-1.92
miR-150-5p	1.28	0.62-1.82	0.99	0.60-1.26
miR-320b	1.14	0.83-1.66	0.91	0.57-1.65
miR-483-3p	1.06	0.62-1.59	0.98	0.62-1.36
miR-342-3p	1.21	0.55-2.39	0.92	0.65-1.13

*****IQR: Inter-quartile range

Table 5. Summary of the posterior distribution for the coefficients associated to the selected variables.

variable	Posterior mean	Posterior sd	Posterior Q1	Posterior Q3
(Intercept)	-15,987	3,492	-22,832	-9,142
Edad	0,244	0,050	0,146	0,343
LysoPC_18_0	0,025	0,008	0,008	0,041
PGE_2	-2,930	0,901	-4,696	-1,164
Isoprostanes	-6,121	1,714	-9,482	-2,761
Isofurans	11,310	3,785	3,891	18,73

Table 6. Diagnosis indexes for the developed model. Maximum and minimum values for the LOOCV procedure are provided in brackets.

AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
0.83 (0.77-0.89)	86.79 (74.05-94.09)	79.52 (68.96-87.29)	73.02 (60.13-83.07)	90.41 (80.67-95.73)