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ORIGINAL RESEARCH

Novel Biomarkers as Non-Invasive Diagnostic Tools in IgA Nephropathy: A Comparative Study with Lupus Nephritis and Membranous Nephropathy

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Rationale: The diagnostic value of endothelial-associated biomarkers in IgAN and their ability to differentiate it from other kidney diseases have not yet been clarified.

Objective: This study aimed to investigate the diagnostic value of endothelial-associated biomarkers in IgAN patients.

Methods and Results: This is a cross-sectional study involving 96 participants, with IgAN, LN, MN, and healthy subjects recruited in a 1:1:1:1 ratio. Seventy-five percent of the sample was used for developing a classification model, and the remaining 25% was used for constructing a validation cohort. Plasma levels of 12 endothelial-associated biomarkers were detected using multiplex immunoassay technology. Among all the biomarkers evaluated, VLA-4 and VEGFD were prioritized for distinguishing IgAN from other groups ($p<0.001$), with 85% classification accuracy. These two biomarkers also showed significant correlation with eGFR (VLA-4: $r = -0.291$, $P = 0.021$; VEGFD: $r = -0.271$, $P = 0.031$) and Gd-IgA1 (VLA-4: $r = 0.403$, $P = 0.003$; VEGFD: $r = 0.412$, $P = 0.002$). These two biomarkers also showed superior diagnostic efficacy (AUC=0.952 and 0.945) compared to Gd-IgA1 (AUC=0.736). Subgroup analysis of IgAN patients revealed clinically relevant effect sizes for the IgA and IgA/C3 ratios between high- and low-VLA-4 and VEGFD groups, with Hedges' g values of 0.962 and 0.819, respectively. The diagnostic efficacy of VLA-4 and VEGFD levels in IgAN was further validated in an independent cohort comprising 24 participants.

Conclusion: VLA-4 and VEGFD emerge as robust, non-invasive biomarkers for IgAN diagnosis and may play significant roles in the pathogenesis of IgAN.

Plain Language Summary: We investigated the diagnostic value of endothelial-associated biomarkers in Immunoglobulin A nephropathy patients. We identified two endothelial-associated biomarkers, VLA-4 and VEGFD, that could be used as biomarkers to distinguish IgAN patients from individuals with other types of glomerulonephritis and healthy subjects. We consider VLA-4 and VEGFD may play a significant role in mediating vascular endothelial damage and can serve as potential diagnostic biomarkers in patients with IgAN.

Keywords: IgA nephropathy, vascular injury, endothelial-associated biomarkers, machine learning

Introduction

Immunoglobulin A nephropathy (IgAN) is one of the most prevalent primary glomerulonephritis, with a higher disease incidence and more severe disease phenotype especially in Asian patients.[1,2] IgAN is characterized by the presence of

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mesangial galactose-deficient immunoglobulin A1 (Gd-IgA1)-containing immune-deposits,[3] albeit their efficacy as diagnostic biomarkers for IgAN has not been shown with certainty.[4,5] Kidney biopsy remains the gold-standard diagnostic test for IgAN and histological features (ie MEST-C score) have been applied to predict outcome. However, its application in early screening of high-risk patients and modifying therapy efficacy is limited. Thus, finding non-invasive indicators for the prevention and treatment of IgA nephropathy is crucial.

Gd-IgA1 deposition in the mesangial region was the initiating factor of kidney injury,[4] while its mechanism is still unclear. It was reported that Gd-IgA1-containing immune complexes exhibit a preference for glomerular endothelial cells, which cause endothelial cells to produce inflammatory cytokines and adhesion molecules.[6] Several endothelial injury biomarkers, such as von Willebrand Factor (vWF),[7,8] vascular endothelial growth factor/Soluble fms-like tyrosine kinase-1 (VEGF/sFlt-1),[9] vascular cell adhesion molecule-1 (VCAM-1),[10] endostatin,[11] platelet-derived MPs (PMPs) and endothelial-derived microparticles (EnMPs),[12] have been investigated as potential biomarkers associated with IgAN severity and prognosis. Thus, the vascular endothelial cells injury might be the earlier sign exposing to the damage induced by Gd-IgA1-containing ICs. Noteworthy, microangiopathy lesions which are frequent in IgAN were found even in patients with near-normal renal histology.[13] Glomerular endothelial cells regulate vascular functions, such as controlling the permeability of small solutes, proteins and inflammatory cells.[14] It is thereby linked to the two validated biomarkers for prognosticating kidney failure and assessing treatment efficacy in IgAN, ie proteinuria and estimated glomerular filtration rate (eGFR).[15,16] In the existing literature, studies on endothelial injury biomarkers for IgA nephropathy are fragmented and have not yet yielded a comprehensive profile.

We conducted this study to explore the role of endothelial-associated biomarkers among IgAN patients and explored the potential relationships of linkage between endothelial-associated biomarkers and IgAN patients' outcomes.

Material and Methods

Study Design and Population

This is a cross-sectional study performed at the Department of Nephrology, First Medical Center of Chinese PLA General Hospital. We recruited 24 patients for each of the three types of glomerulonephritis, including IgA nephropathy, lupus nephritis (LN), and membranous nephropathy (MN). At the same time, individuals in good health without any pre-existing medical conditions or current medication usage were recruited as the healthy control group (n=24). After explaining the benefits and risks of the study, written informed consent was obtained from participants. All participants were matched for age, gender, body mass index (BMI), smoking and alcohol consumption. A random number method was employed to randomly select 18 cases from each group to form the classification group, with the remaining 6 cases serving as the validation group. However, two healthy volunteers from classification group were excluded due to infections before sampling. At last, sixteen healthy participants are included in classification group.

Participants Screening and Blood Sampling

We screened patients based on the following inclusion criteria: (1) All patients were diagnosed by renal biopsy. (2) Resting systolic blood pressure was less than 140 mmHg and diastolic blood pressure less than 90 mmHg. The exclusion criteria are: (1) eGFR < 30 mL/min per $1.73m^2$; (2) Any kind of current infection or white blood cell count greater than 1×10^9/L; (3) Patients with malignant tumor; (4) pregnant or lactating; (5) unwillingness to provide informed consent.

Two milliliter of fasting venous blood was collected with anticoagulant tube, centrifuged at 4°C and 3000g for 10 minutes to collect the supernatant, and then stored at −80°C after subsuming. All samples were kept frozen until use.

Patient Demographics and Clinical Data Collecting

We obtained clinical characteristics including age, gender, BMI, blood pressure, pathological types of renal biopsy, comorbidity, and drug prescription within one month from our electronic medical record system. At the same time as sample collecting, level of serum creatinine, serum IgA, urea nitrogen, 24-hour urinary total protein (24h UTP) and eGFR were obtained from clinical laboratory information system.

Multiplex Immunoassay

Concentrations of endothelial-associated biomarkers were determined using a multiplexed immunoassay kit according to manufacturer's instructions: Endothelial Injury Marker 12-Plex Human ProcartaPlex™ Panel (EPX120-15,849-901Thermo Fisher Scientific), including 12 target proteins: VEGFA, VEGFD, VEGF-R2, VEGF-R3, CD62P, CD62E, PECAM-1, ANGPT1, MMP1, TM, SDC1 and VLA-4, functions related to angiogenesis and endothelium are listed in Supplemental Table 1. All samples were processed consistently to ensure that there was no bias in the use of immunoassay kits among groups. The upper and lower limit of quantification (ULQ; LLQ) and the average intra-assay %CV for each biomarker is stated in the manufacturer's Certificate of Analysis (Supplemental Table 2).

Detection of Circulating Gd-IgA1

Circulating Gd-IgA1 was quantified using a commercial enzyme-linked immunosorbent assay (ELISA) kit precoated with KM55 (27600; IBL, Fujioka, Japan). Plasma specimens (EDTA anticoagulated) were diluted 300-fold with EIA buffer, and other procedures were performed in accordance with the manufacturer's instructions.

Machine Learning

In the field of machine learning, we used a random forest classifier to classify patients in the lgAN group and non-lgAN group (including LN, MN and healthy control subjects). In order to find the optimal combination of hyperparameters, we used Grid Search technology to construct a grid containing multiple combinations of hyperparameters, the GridSearchCV object is utilized to search through a grid of parameters (including crucial hyperparameters such as the number of trees and the maximum depth of subtrees in a Random Forest classifier). This search is conducted using 5-fold cross-validation, with accuracy serving as the evaluation metric, to discover the optimal parameters. Through systematic training and evaluation of each combination, we ultimately determined the optimal hyperparameter combination, which has 30 decision trees and a maximum depth of 2. After determining the optimal hyperparameters, we further calculated the Variable Importance Measures (VIM) based on the Gini index, which aims to identify the evaluation indicators that have the greatest impact on the classification results. To present these important evaluation metrics more intuitively, we adopted the t-distributed random nearest neighbor embedding (t-SNE) algorithm. This algorithm can nonlinearly reduce the dimensionality of important variables while preserving the similarity relationship between sample points and converting it into a probability distribution, thereby achieving visualization of evaluation indicators. Machine learning was conducted using Python (version 3.8). Based on the optimal parameters obtained from the above methods, we performed leave-one-out cross-validation (LOOCV) on the classification and validation set with caret and pROC packages in R (version 4.12). Each sample in both model was sequentially used as the validation set, with the remaining samples serving as the training set for model training and validation. Area under curve (AUC), specificity and sensitivity were calculated in each LOOCV loop, The final display shows the average parameter values.

Statistical Analysis

For numerical variables that conform to a normal distribution, Analysis of Variance (ANOVA) test was employed to assess differences among 4 groups. Otherwise, non-parametric Kruskal–Wallis *H*-test was employed, and the Dunn's Test method was used for post-hoc pairwise comparisons, with the significance level adjusted to control *Type I error rate*. As for comparison between 2 groups, the *t*-test is used for normally distributed data, while the Mann–Whitney *U*-test is used for non-normally distributed data. For categorical variables, the *chi-square* test is commonly used to assess independence. However, when the expected frequency in any cell is less than 5, especially when the total sample size n is less than 5, *Fisher's* exact test should be used. The significance level α was typically set at 0.05, and a *P*-value less than this threshold was considered to indicate a statistically significant difference. Commercially available SPSS software (version 26.0; SPSS, Inc, Chicago, Illinois, USA) and GraphPad Prism (version 10.1.2; GraphPad Software Inc, San Diego, California, USA) were used for data analyses and visualization. Correlation analysis was conducted using the ggcorrplot package in R, employing the Spearman method.

Results

Participants Characteristics

The baseline characteristics in classification set of the participants in the four groups are listed in Table 1. No significant differences were observed among the four groups in terms of age, gender, BMI, smoking status, alcohol consumption, history of diabetes for all subjects. IgAN patients showed significantly higher levels of Gd-IgA1 ($P = 0.001$) and serum IgA ($P < 0.001$). MN patients were found to have more severe proteinuria ($P = 0.008$), higher levels of C3 ($P < 0.001$) and C4 ($P < 0.001$). No significant intergroup differences were observed in the administration of angiotensin receptor blockers (ARBs) among the three nephropathy groups. However, distinct therapeutic patterns were evident based on disease-specific characteristics. Patients with LN and MN universally received immunosuppressive agents or immunomodulators, including during the stable disease phase, resulting in a significant intergroup therapeutic variations. The medication profiles revealed disease-specific treatment patterns: glucocorticoids were exclusively utilized in LN (72.2%) and MN (38.9%) patients. Hydroxychloroquine utilization was restricted to patients with IgAN (55.5%) and LN (50.0%). Mycophenolate mofetil was predominantly used in LN (38.9%) compared to MN (5.6%) patients. Tacrolimus administration showed preferential use in MN (50.0%) over LN (5.6%) patients.

Table 1 Clinical and Immunological Features of Participants Across Different Groups in Classification Model

	NC (n=18)	LN (n=18)	MN (n=18)	IgAN (n=18)	*P* value
Male sex, no. (%)	8 (44.4)	7 (38.9)	10 (55.6)	9 (50.0)	0.788
Age, year	36.88 ± 10.46	38.06 ± 11.79	39.78 ± 10.84	44.39 ± 6.86	0.156
BMI, kg/m^2	22.75 ± 2.58	22.38 ± 2.50	23.08 ± 2.32	24.84 ± 4.02	0.073
Smoking, n (%)	4 (22.2)	3 (16.7)	5 (27.8)	4 (22.2)	0.873
Alcohol use, n (%)	3 (16.6)	2 (11.1)	4 (22.2)	3 (16.7)	0.837
Hypertension, n (%)	0 (0)	6 (33.3)	9 (50.0)	8 (44.4)	0.001
Diabetes, n (%)	0 (0)	2 (11.1)	3 (16.7)	5 (27.8)	0.061
24h UTP, g/d	NA	1.70 [0.53, 2.86]	3.33 [1.53, 5.14] *	0.94 [0.46, 1.42]	0.008
Scr, μmol/L	55.82 ± 7.27	116.88 ± 98.75	108.83 ± 93.23	108.28 ± 45.41	0.061
eGFR, mL/min per $1.73m^2$	117.03 ± 16.12*	80.85 ± 38.77	95.77 ± 35.54	77.18 ± 29.05	0.002
Gd-IgA1, ug/mL	4105.81 [2853.88, 5357.75]	4105.65 [2815.09, 5396.21]	4186.12 [2501.80, 5870.43]	8483.61 [6037.09, 10,930.13]	0.001
C3, g/L	NA	70.71 ± 36.20	149.00 ± 23.94	104.70 ± 19.50	<0.001
C4, g/L	NA	15.11 ± 11.22	36.77 ± 10.24	23.59 ± 6.76	<0.001
IgA, g/L	NA	254.29 ± 132.53	256.60 ± 103.34	458.07 ± 163.82	<0.001
Drug-used, n (%)					
ARB	NA	14 (77.8)	12 (66.7)	12 (66.7)	0.701
Glucocorticoid	NA	0	13 (72.2)	7 (38.9)	<0.001
Hydroxychloroquine	NA	10 (55.6)	9 (50.0)	0	0.001
Mycophenolate mofetil	NA	0	7 (38.9)	1 (5.6)	0.002
Tacrolimus	NA	0	1 (5.6)	9 (50.0)	<0.001

Notes: *post-hoc pairwise comparison shows statistically significant difference compared with other groups ($p < 0.05$).
Abbreviations: NC, normal control; LN, lupus nephritis; MN, membranous nephropathy; IgAN, IgA nephropathy; BMI, Body-mass index; 24h UTP, 24-hour urinary total protein; Scr, Serum creatinine; eGFR, estimated glomerular filtration rate; ARB, Angiotensin receptor blocker.

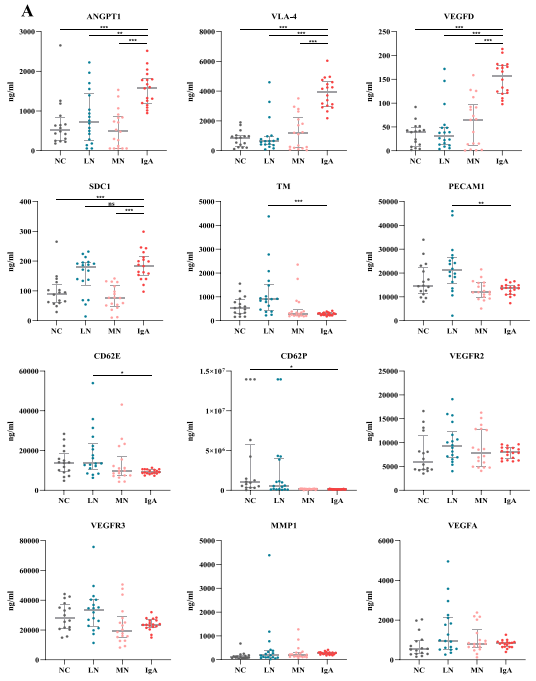
Identification of Endothelial-Associated Biomarkers Distinguishing IgAN Patients with Machine Learning Models

We measured 12 endothelial-associated biomarkers using multiplex immunoassay technology mentioned above. Among 12 biomarkers, ANGPT1 ($P < 0.001$), VLA-4 ($P < 0.001$) and VEGFD ($P < 0.001$) were able to distinguish IgAN from the other three groups, while other biomarkers could not differentiate IgAN from other 3 groups (Figure 1A and Table 2). We considered individuals with LN, MN, and healthy controls as the non-IgAN group and evaluated the efficacy of VLA-4 and VEGFD as diagnostic markers. Among all detected biomarkers, VLA-4 and VEGFD were at the top of the list generated by random forest, ranking 23.88% and 21.56% separately (Figure 1B). A t-SNE plot derived from 12 biomarkers failed to distinguish IgAN patients from the non-IgAN group. Subsequently, a refined biomarker model incorporating Boruta feature reduction was developed, identifying VLA-4 and VEGFD as key markers. This streamlined model, utilizing solely VLA-4 and VEGFD, generated a t-SNE plot that more clearly delineated the groups, achieving an almost perfect segregation of IgAN patients (Figure 1C; classification accuracy of 85%).

The diagnostic efficacy of VLA-4 and VEGFD was further evaluated utilizing the LOOCV method. The AUC, specificity, and sensitivity were determined. Both biomarkers, VLA-4 (AUC: 0.952; specificity: 92.5%; sensitivity: 87.5%) and VEGFD (AUC: 0.945; specificity: 90.7%; sensitivity: 84.7%), emerged as potential discriminators for the identification of IgAN patients. These markers exhibited enhanced diagnostic performance relative to Gd-IgA1, which represented an AUC of 0.736, a specificity of 88.5%, and a sensitivity of 77.3% (Figure 1D).

Associations Between Leading Biomarkers and Clinical Indicators in Classification Set

Since Gd-IgA1 is a specific indicator for IgAN, we conducted a further analysis to explore the correlations among the leading endothelial-associated biomarkers—VLA-4 and VEGFD—and circulating Gd-IgA1. Our analysis revealed that VLA-4 and VEGFD showed significant correlation with circulating Gd-IgA1 (VLA-4: $r = 0.403$, $P = 0.003$; VEGFD: $r = 0.412$, $P = 0.002$)



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	Feature	%Importance
1	VLA-4	23.88
2	VEGFD	21.56
3	CD62P	15.59
4	ANGPT1	8.44
5	CD62E	8.01
6	SDC1	6.95
7	MMP1	5.9
8	TM	4.97
9	PECAM1	3.42
10	VEGFR3	1.17
11	VEGFR2	0.11
12	VEGFA	0

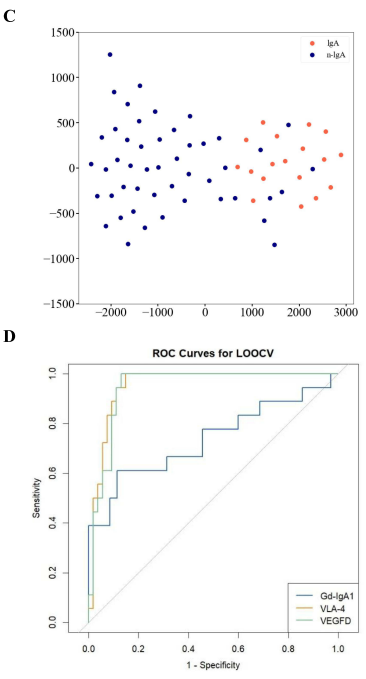

Figure 1 Identification of distinguished endothelial-associated biomarkers in IgAN patients. (**A**) Multiple comparisons of 12 endothelial-associated biomarkers among groups. VLA-4 and VEGFD were able to distinguish IgAN from the other three groups (NC, normal control; LN, lupus nephritis; MN, membranous nephropathy; IgAN, IgA nephropathy; ***, $P<0.001$; **, $P<0.01$; *, $P<0.05$). (**B**) List generated using a Random Forest algorithm delineated the relative importance of 12 endothelial-associated biomarkers in distinguishing between subject groups. (**C**) Two-dimensional plot of subjects following t-SNE dimensionality reduction of the two leading biomarkers, VLA-4 and VEGFD, demonstrating a clear distinction and clustering of IgAN patients from non-IgAN subjects. (**D**) The diagnostic effectiveness of VLA-4 (yellow line) and VEGFD (green line) assessed with ROC curves using the LOOCV method.

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Table 2 Comparative Levels of Endothelial-Associated Cytokines in Participants Across Test Model Groups

	NC (n=16)	LN (n=18)	MN (n=18)	IgAN (n=18)	*P* value
ANGPT1	1584.50[1185.75, 1831.00]	721.78[257.89, 1433.25]	502.15[55.87, 855.17]	528.6[261.65, 824.47]	<0.001
SDC1	185.33[150.16, 216.81]	178.64[117.54, 195.78]	76.39[49.22, 118.84]	91.04[60.13, 122.51]	<0.001
VEGFR2	7934.50[6571.75, 8894.50]	9182.50[6813.00, 12,302.50]	7838.00[4894.00, 12,845.25]	5991.50[4359.32, 11,430.25]	0.204
VLA-4	3931.00[2995.00, 4638.00]	638.21[403.41, 979.04]	1154.5[182.08, 2234.50]	860.92[271.78, 1016.85]	<0.001
VEGFD	157.56[121.12, 179.83]	31.87[12.50, 49.75]	65.50[10.83, 97.10]	39.91[9.46, 49.46]	<0.001
CD62P	105557.00[93,133.25, 132,520.25]	563,462.00[117,511.75, 3,963,082.25]	159,620.00[120,305.50, 176,838.25]	1,017,003.00[372,477.75, 5,797,767.00]	<0.001
VEGFR3	23624[21,968.75, 26,767.25]	33,212.5[22,424.25, 40,337]	19,476.5[14,647, 29,170]	28,098.5[20,980, 37,249.71]	0.045
MMP1	276.03[223.35, 306.55]	181.13[93.89, 387.32]	192.64[128.50, 306.36]	112.09[72.54, 183.63]	0.003
PECAM1	13678.50[11,159.75, 14,886]	21,141.00[15,564.50, 26,591]	12,018.50[9835.75, 15,839.50]	14,584.50[11,424.15, 22,445.89]	0.001
TM	284.76[230.70, 327.21]	898.88[442.84, 1526.50]	300.36[209.37, 489.24]	547.20[295.09, 883.75]	<0.001
CD62E	9007.00[8455.00, 10,553.25]	13,483.50[10,412.75, 23,456.00]	9837.00[7367.25, 17,205.25]	13,815.18[9247.26, 18,730.75]	0.014
VEGFA	827.70[647.22, 898.11]	965.04[543.23, 2144.25]	807.54[617.3, 1517.75]	538.23[298.9, 994.83]	0.123

Note: The units of the above markers' concentration are ng/mL.
Abbreviations: NC, normal control; LN, lupus nephritis; MN, membranous nephropathy; IgAN, IgA nephropathy.

(Figure 2). We further explored the relationships between the selected biomarkers and kidney function. We found that VLA-4 and VEGFD have significant correlations with eGFR (VLA-4: r = - 0.291, P = 0.021; VEGFD: r = - 0.271, P = 0.031). However, there is no significant correlation between 24-hour UTP and endothelial-associated biomarkers.

In IgAN group, 18 patients were stratified into two groups of equal size according to their VLA-4 concentration: a high-concentration group (VLA4 -H) and a low-concentration group (VLA4 -L). This stratification similarly categorized VEGFD into high- and low-concentration subgroups (Table 3). Analysis of demographic and physiological parameters, including gender, age, BMI, SBP, and DBP, revealed no significant differences between the two groups. Furthermore, renal function indicators, such as 24-hour UTP, serum creatinine, and eGFR, showed no significant differences. Similarly, immunological markers such as Gd-IgA1, complement C3 and C4, and immunoglobulins, demonstrated no significant variations. Although a notable difference in mean IgA levels was observed between the two groups, it did not achieve statistical significance. To assess the magnitude of these differences, Hedges' g, an effect size measure particularly appropriate for small sample sizes, was employed. The Hedges' g values for IgA levels and the IgA/C3 ratio were 0.962 and 0.819, respectively. These results suggest that the observed differences may hold clinical relevance, highlighting the potential diagnostic value of VLA4 and VEGFD in IgAN.

Validation of Selected Endothelial-Associated Biomarkers for the Diagnosis of IgAN

The endothelial-associated biomarkers identified above were further validated in a cohort of 24 participants (six subjects per group), with clinical specimens collected and examined during the same period. The baseline characteristics were similar to those of the original test population, with matching in age, gender, BMI, smoking status, and alcohol consumption (Table 4). Notably, within the validation group, the levels of VLA-4 and VEGFD in IgA patients exhibited significant discrepancies compared to the other three groups, as graphically represented in Figure 3A and B. ROC curve analysis was conducted using LOOCV method, and the AUC, specificity and sensitivity were determined. Both VLA-4 (AUC: 0.991; specificity: 94.4%; sensitivity: 92.0%) and VEGFD (AUC: 1.000; specificity: 94.4%; sensitivity: 95.8%) demonstrated exceptional diagnostic efficacy, as illustrated in Figure 3C and D.

Discussion

By analyzing endothelial-related biomarkers, this study for the first time reveals the significant potential of VLA-4 and VEGFD in the diagnosis of IgAN. Their diagnostic efficacy is notably superior to that of Gd-IgA1, and are further

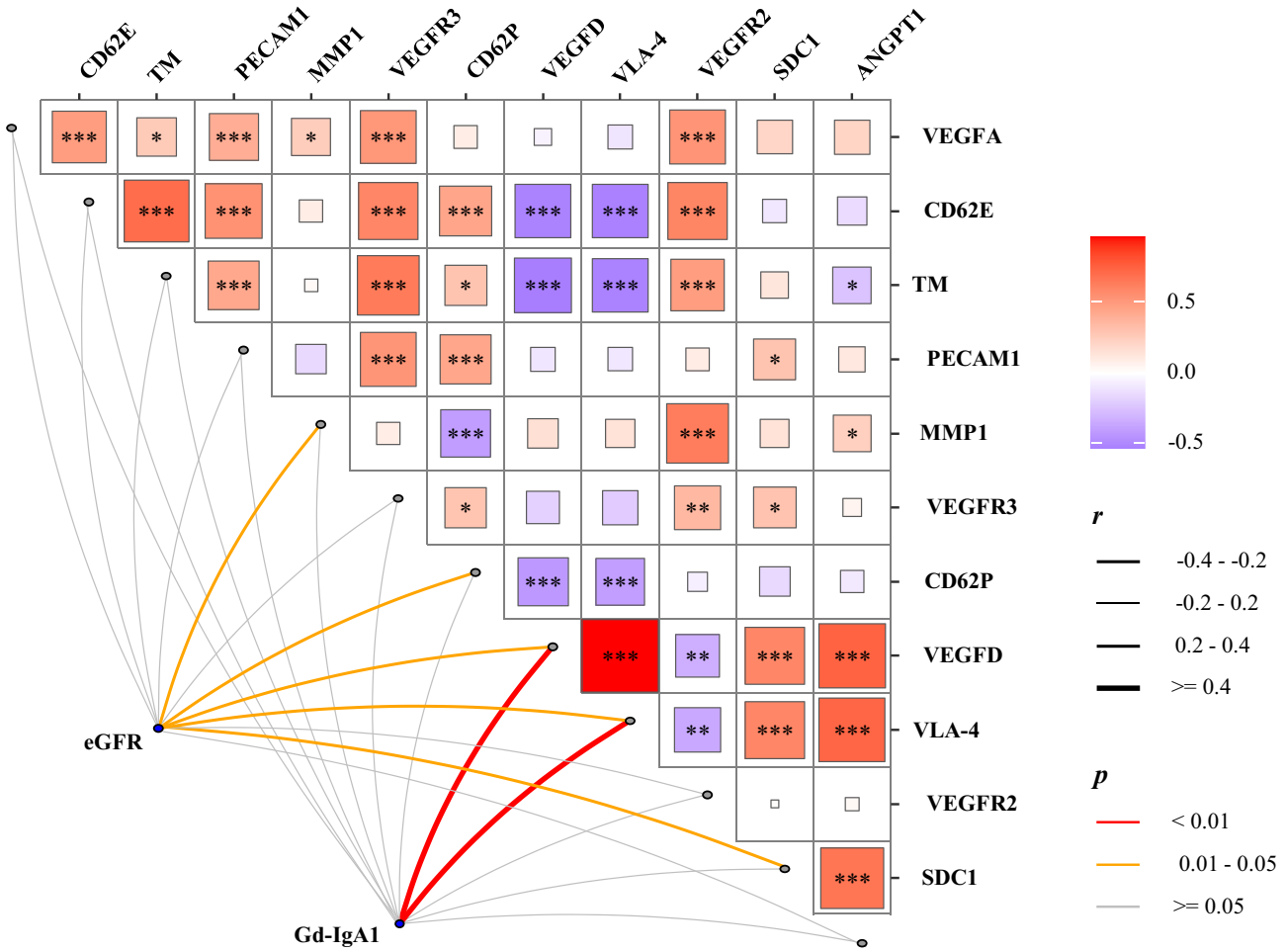

Figure 2 Correlations between biomarkers and clinical indicators in classification set. Correlations between the leading endothelial-associated biomarkers—VLA-4 and VEGFD—and circulating Gd-IgA1 and eGFR. VLA-4 and VEGFD showed significant correlation with circulating Gd-IgA1 (red lines and yellow lines) while others were not (gray lines). In addition to VLA-4 and VEGFD, other endothelial-associated cytokines including SDC1, CD62P, and MMP1 also have significant correlations with eGFR (yellow lines). In each small square, the *, **, and *** represent the P-values of the correlation between the horizontal and vertical axis indicators. Specifically, *** indicates $P < 0.001$; ** indicates $P < 0.01$; * indicates $P < 0.05$. The absence of a * mark indicates that $P > 0.05$.

validated the preliminary results in a validation cohort. Considering their roles in immune and endothelial function, these two biomarkers may serve as potential diagnostic and therapeutic targets for IgAN.

VLA-4 is a cell surface molecule belonging to the integrin family. It plays an important role in mediating the adhesion and interactions across cells. VLA-4 has a high affinity for VCAM-1, and this interaction is particularly important in inflammation and immune responses.[17,18] Previous study has demonstrated that IgA glycation plays a critical role in

Table 3 Clinical and Immunological Features of Participants in IgAN Subgroups with Different Levels of VLA-4

	VLA4 -L (n=9)	VLA4 -H (n=9)	*P* value
Male sex, n (%)	6 (66.7)	4 (44.4)	0.635
Age, year	43.00 ± 5.17	45.78 ± 8.30	0.407
BMI, kg/m^2	24.63 ± 3.33	24.70 ± 3.22	0.967
SBP, mmHg	122.89 ± 12.05	130.78 ± 10.56	0.159

(*Continued*)

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Table 3 (Continued).

	VLA4 -L (n=9)	VLA4 -H (n=9)	*P* value
DBP, mmHg	78.33 ± 8.86	78.11 ± 10.03	0.961
24h UTP, g/d	0.44 [0.26, 0.92]	0.74 [0.31, 1.22]	0.566
Scr, μmol/L	103.41 ± 42.27	113.14 ± 50.41	0.663
eGFR, mL/min per 1.73m^2	82.26 ± 22.88	72.10 ± 34.81	0.475
Gd-IgA1, ug/mL	8660.86 [7478.60, 10,650.71]	5358.78 [4635.99, 11,282.03]	0.895
C3, g/L	101.85 ± 16.54	108.50 ± 23.99	0.549
C4, g/L	22.76 ± 7.95	24.68 ± 5.28	0.619
IgA, g/L	388.12 ± 123.33	551.33 ± 173.84	0.061
IgA/C3	3.86 ± 1.28	5.29 ± 1.97	0.126
IgE, g/L	53.20 ± 62.17	56.56 ± 85.13	0.933
IgG, g/L	988.25 ± 558.48	967.50 ± 434.97	0.941
VLA-4, ng/mL	3002.78 ± 435.29	4708.67 ± 579.38	<0.001
VEGFD, ng/mL	120.81 ± 15.35	183.41 ± 15.77	<0.001
Drug-used, n (%)			
ARB	6 (66.7)	8 (88.9)	0.571
Hydroxychloroquine	5 (55.6)	5 (55.6)	1.000

Abbreviations: VLA4 –L, group with low concentration of VLA-4; VLA4 –H, group with high concentration of VLA-4; BMI, Body-mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; 24h UTP, 24-hour urinary total protein; Scr, Serum creatinine; eGFR, estimated glomerular filtration rate; ARB, Angiotensin receptor blocker.

Table 4 Clinical and Immunological Features of Participants Across Different Groups in Validation Model

	NC (n=6)	LN (n=6)	MN (n=6)	IgAN (n=6)	*P* value
Male sex, n (%)	4(66.7)	3(50.0)	3 (50.0)	4(66.7)	1.000
Age, year	35.17 ± 4.29	47.50 ± 5.93	47.17 ± 6.30	38.33 ± 3.65	0.254
BMI, kg/m^2	23.98 ± 0.86	22.18 ± 0.68	25.44 ± 1.15	24.40 ± 1.04	0.143
SBP, mmHg	126.53 ± 3.78	121.86 ± 2.22	118.65 ± 2.98	121.63 ± 1.58	0.278
DBP, mmHg	82.56 ± 6.63	73.16 ± 5.35	71.30 ± 2.82	74.45 ± 5.27	0.454
Smoking, n (%)	1(16.7)	0(0)	2(33.3)	2(33.3)	0.695
Alcohol use, n (%)	3(50.0)	2(33.3)	4(66.7)	3(50.0)	0.941
Hypertension, n (%)	0 (0)	2 (33.3)	4 (50.0)	3 (44.4)	0.154
Diabetes, n (%)	0 (0)	2 (11.1)	1 (16.7)	0 (0)	0.573
24h UTP, g/d	NA	1.61 [0.52, 3.02]	3.01 [0.15, 6.49]	0.67 [0.45, 1.85]	0.003
Scr, μmol/L	72.37 ± 2.22	94.41 ± 8.14	80.56 ± 13.57	87.35 ± 10.22	0.419
eGFR, mL/min per 1.73m^2	115.40 ± 5.04	79.23 ± 9.16	97.57 ± 11.84	96.72 ± 8.45	0.072

Abbreviations: NC, normal control; LN, lupus nephritis; MN, membranous nephropathy; IgAN, IgA nephropathy; BMI, Body-mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; 24h UTP, 24-hour urinary total protein; Scr, Serum creatinine; eGFR, estimated glomerular filtration rate.

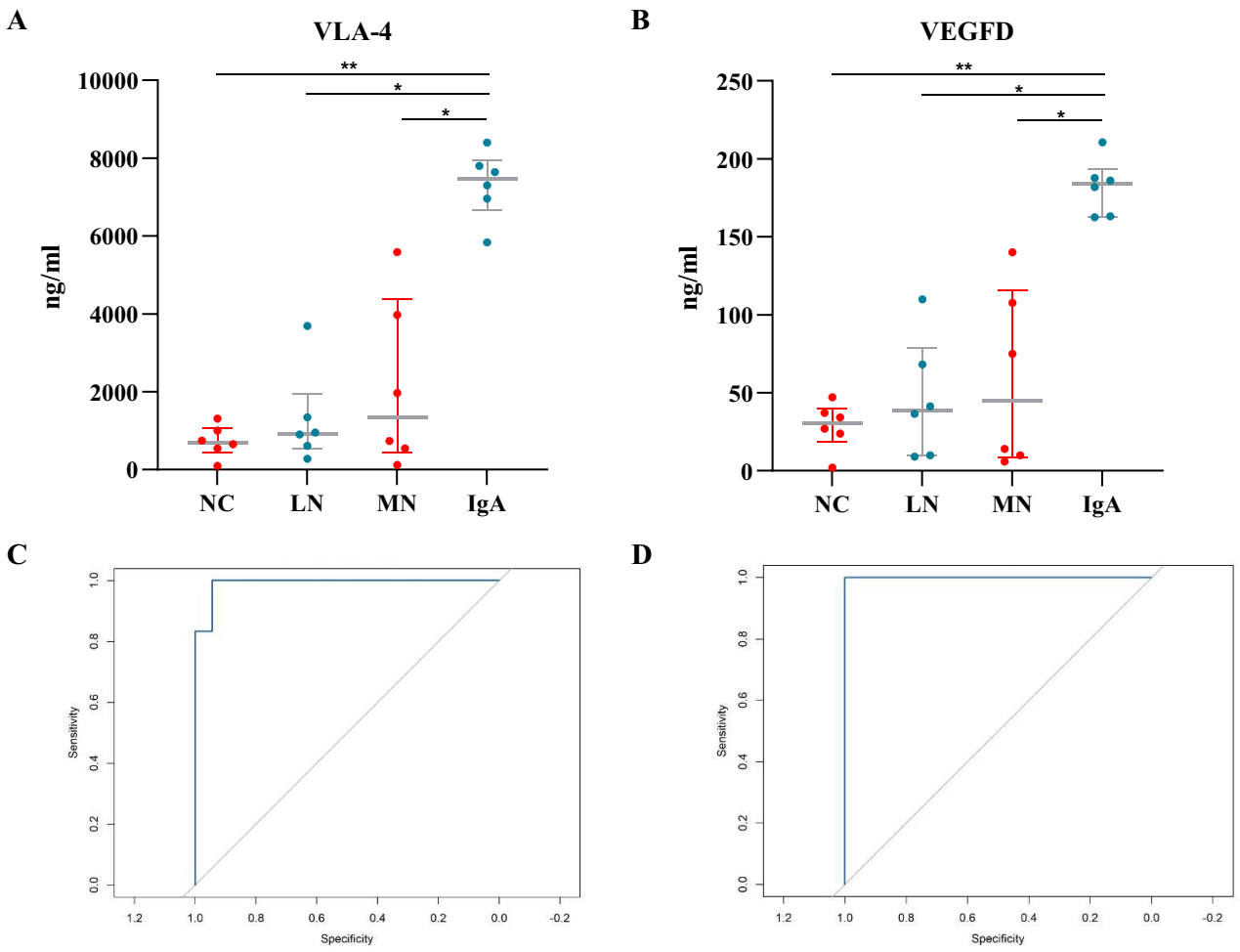

Figure 3 Validation the diagnostic effectiveness of VLA-4 and VEGFD in validation set. Circulating levels of VLA-4 (**A**) and VEGFD (**B**) were significantly higher in IgAN patients compared to the other three groups (**, $P<0.01$; *, $P<0.05$). The diagnostic effectiveness of VLA-4 (**C**) and VEGFD (**D**) were assessed with ROC curves using the LOOCV method.

Abbreviations: NC, normal control; LN, lupus nephritis; MN, membranous nephropathy; IgAN, IgA nephropathy.

modulating cell-matrix interactions, a mechanism that may be operative in IgAN.[19] Additionally, research had suggested that integrins could serve as potential receptors for IgA1.[20] The central pathological mechanism of IgAN involves complement activation and inflammatory responses triggered by the deposition of aberrant IgA1, with the upregulation of VLA-4 potentially serving as a direct indicator of the activity of this pathological process. Although no correlation was found between Gd-IgA1 and VLA-4 within the IgAN subgroup, patients exhibiting elevated VLA-4 levels had higher serum IgA concentrations and IgA/C3 ratio, implying that VLA-4 may play a pivotal role in mucosal immunity mechanisms, thereby influencing disease progression.[21] Based on these observations, we can speculate that the abnormal glycosylation of IgA1 may exacerbate local inflammation and tissue damage through the activation of VLA-4 expressed on endothelial or immune cells.

The VEGF family includes VEGFA, VEGFB, VEGFC, VEGFD, and placental growth factor, which is responsible for regulating the development and growth of vascular endothelial cells.[22] Among these members, VEGFD stands out due to its potent lymphangiogenic and angiogenic abilities,[23] and had been reported as a diagnostic biomarker for lymphangioleiomyomatosis.[24] Previous study had suggested that VEGFD might participate in Th17-dominant autoimmune ocular disease in part through its induction of lymphangiogenesis.[25] It was suggested that chronic inflammation is required to initiate neolymphangiogenesis with formation of lymphatic vessels under the influence of mononuclear cell-derived growth factors, predominantly of the VEGF isoforms including VEGFD.[26] Neolymphangiogenesis has been demonstrated in transplanted kidneys, chronic interstitial nephritis and IgA nephropathy.[27,28] The role of VEGFD in

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IgAN can be linked to its ability to promote lymphangiogenesis and angiogenesis. The formation of new lymphatic vessels in the kidney may contribute to the accumulation of immune cells and the progression of inflammation.[29] The study demonstrating the diagnostic value of VEGFD for IgAN patients suggests that VEGFD could serve as a biomarker for both diagnosis and prognosis evaluation. This finding highlights the potential of VEGFD as a therapeutic target for IgAN, as modulating its activity might help reduce inflammation and slow down the progression of the disease.

According to preliminary research, IgAN patients exhibit aberrant plasma vWF, a particular indicator of endothelial cell injury, which is marked by elevated levels or molecular defects.[12,30] Additionally, thrombotic microangiopathy (TMA) was found to be common (up to 53%) in renal tissue with IgAN.[13] TMA is characterized by the formation of thrombi in small blood vessels, leading to endothelial injury and tissue damage.[31] Although it have been reported instances of IgAN patients complicated by TMA, the relationship between VEGF and TMA in IgAN remains unclear.[32–34] VEGF is a key regulator of vascular homeostasis, and its dysregulation is closely associated with the pathological processes of kidney diseases.[35] Previous studies have indicated that the occurrence of TMA is strongly associated with reduced VEGF levels, such as those induced by VEGF inhibitors.[36,37] However, hour findings revealed a significant elevation of VEGF in patients with IgAN. This seemingly contradictory phenomenon actually highlights the micro-environment-dependent regulatory mechanisms of VEGF in different renal pathologies, underscoring its complex and context-specific roles in kidney disease progression.

Gd-IgA1 refers to IgA1 with abnormal glycosylation, which plays a significant role in the pathogenesis of IgA nephropathy.[38] Gd-IgA1 is more prone to deposit in the mesangial area of the glomerulus, activate the complement system, thereby leading to inflammatory responses and glomerular damage.[39] The insights into understanding regarding the crosstalk between Gd-IgA1 and endothelial injury are quite scarce. It has been reported that co-culturing immune complexes containing Gd-IgA1 with human renal glomerular endothelial cells (HRGECs) leads to increased expression of endothelial adhesion molecules and pro-inflammatory mediators,[6] suggesting that Gd-IgA1 immune complexes may directly promote glomerular endothelial injury. In this study, we initially sought to explore the potential associations between Gd-IgA1, a well-established biomarker in IgAN pathogenesis, and two emerging endothelial-related biomarkers, VLA-4 and VEGFD. Levels of plasma Gd-IgA1 showed a significantly correlation with VLA-4 and VEGFD, indicating that high levels of Gd-IgA1 may affect endothelial cells via VLA-4 and VEGFD systematically, not just those confined to glomerular endothelial cells. However, our analysis did not reveal any significant correlations between Gd-IgA1 and either VLA-4 or VEGFD among the 18 IgAN patients. Despite the absence of such correlations, our stratified analysis uncovered clinically meaningful differences in serum IgA and the IgA/C3 ratio across varying concentrations of VLA-4 (and VEGFD). This findings highlight the potential diagnostic and prognostic value of VLA-4 and VEGFD as biomarkers in IgAN. The observed variations in serum IgA and the IgA/C3 ratio may reflect distinct pathophysiological pathways associated with varying expression levels of VLA-4 and VEGFD. The incorporation of VLA-4 and VEGFD as complementary biomarkers could enhance the comprehensive understanding of IgAN, facilitating more personalized therapeutic strategies and potentially improving patient outcomes.

The relationship between immunosuppressive therapies and endothelial biomarkers remains complex and context-dependent. Immunosuppressive agents can influence endothelial function through various mechanisms, including the regulation of adhesion molecules, cytokine release, and leukocyte migration. For instance, corticosteroids and other immunosuppressive drugs have been shown to reduce the expression of adhesion molecules such as ICAM-1 and VCAM-1.[40] Drugs like RTX could indirectly affect T-cell function which may consequently influence the expression of VLA-4.[41] Immunosuppressive therapies including corticosteroids and cyclophosphamide, have been shown to modulate angiogenic processes and may impact VEGFD levels.[42] To minimize potential confounding effects of immunosuppressive agents on endothelial function markers, we excluded patients with LN and MN who had received corticosteroid or immunosuppressive therapy from subsequent analyses. The between-group comparison was subsequently repeated, demonstrating consistent results with our initial analysis (Supplemental Table 3). These findings suggest that the study conclusions maintain statistical robustness and are not substantially influenced by pharmacological interventions. This stability suggests that these biomarkers may reflect intrinsic endothelial pathology rather than treatment-induced modifications, potentially offering more reliable diagnostic parameters.

In both the overall cohort analysis and the subgroup analysis of IgAN patients, no significant correlation was observed between VLA-4 or VEGFD levels and proteinuria, despite previous studies demonstrating associations between endothelial biomarkers and proteinuria.[43–45] This discrepancy may be attributed to the predominant involvement of VLA-4 and VEGFD in immune regulatory functions rather than direct endothelial dysfunction. Furthermore, the potential loss of VLA-4 and VEGFD through increased urinary protein excretion may counterbalance the elevation of these biomarkers induced by immune-mediated injury, thereby attenuating their correlation with proteinuria.

This study certainly has several limitations. Firstly, this is a cross-sectional study, so the generalizability of the findings needs to be examined in prospective studies. Secondly, further clarification is needed regarding the underlying mechanisms of changes in endothelial-associated biomarkers in IgAN patients. Finally, due to the small number of enrolled patients, the impact of the drug on these biomarkers was not ascertainable.

Conclusions

In conclusion, we have identified two endothelial-associated biomarkers that are valuable for diagnosing IgAN patients and are correlated with renal outcomes. These biomarkers could be utilized in the development of diagnostic tests and as therapeutic targets. Additionally, beyond the well-established role of endothelial injury in IgAN, the potential role of neolymphangiogenesis warrants further investigation.

Abbreviations

IgAN, Immunoglobulin A nephropathy; LN, lupus nephritis; MN, membranous nephropathy; BMI, body mass index; Gd-IgA1, galactose-deficient immunoglobulin A1; vWF, von Willebrand Factor; VEGF, vascular endothelial growth factor; sFlt-1, soluble fms-like tyrosine kinase-1; VCAM-1, vascular cell adhesion molecule-1; EnMPs, endothelial-derived microparticles; 24h UTP, 24-hour urinary total protein; eGFR, estimated glomerular filtration rate; VIM, variable Importance Measures; CKD, chronic kidney disease; FABP4, fatty acid-binding protein 4; TNF-α, tumor necrosis factor α; TMA, thrombotic microangiopathy; HRGECs, human renal glomerular endothelial cells; LOOCV, leave-one-out cross-validation; AUC, area under curve; ARB, angiotensin receptor blocker.

Ethical Approval and Informed Consent

This study was approved by the Institutional Ethics Committee of First Medical Center of Chinese PLA General Hospital in accordance with the Declaration of Helsinki (S2023-111-01). All participants provided informed consent.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no competing interests in this work.

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