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In a retrospective international study, circulating miR-148b and let-7b were found to be serum markers for detecting primary IgA nephropathy

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Immunoglobulin A nephropathy (IgAN) is a worldwide disease characterized by the presence of galactose-deficient IgA1 deposits in the glomerular mesangium. A kidney biopsy for diagnosis is required. Here, we measured two miRNAs (let-7b and miR-148b), previously identified as regulators of the *O*-glycosylation process of IgA1, in serum samples from patients with IgAN and healthy blood donors (controls) recruited in an international multicenter study. Two predictive models, based on these miRNAs, were developed and the diagnostic accuracy of the combined biomarkers was assessed by the area under the receiver operating characteristic (ROC) curve (AUC) carried out in three steps. In a training study, the combined miRNAs were able to discriminate between 100 patients with IgAN and 119 controls (AUC, 0.82). A validation study confirmed the model in an independent cohort of 145 patients with IgAN and 64 controls (AUC, 0.78). Finally, in a test study, the combined biomarkers were able to discriminate patients with IgAN from 105 patients affected by other forms of primary glomerulonephritis, supporting the specificity (AUC, 0.76). Using the same study design, we also performed two subgroup analyses (one for Caucasians and one for East Asians) and found that race-specific models were the best fit to distinguish IgAN patients from controls. Thus, serum levels of the combined miRNA biomarker, let-7b and miR-148b, appears to be a novel, reliable, and noninvasive test to predict the probability of having IgAN.

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KEYWORDS: biomarker; IgA nephropathy; microRNA; serum

Immunoglobulin A nephropathy (IgAN, MIM 161950) or Berger's disease is a worldwide renal disease with the highest prevalence in East Asian and Caucasian populations.[1] The annual incidence rate of biopsy-proven IgAN varies between 1.1 and 10.5 in 100,000 individuals.[2,3] Notably, this frequency is largely underestimated because in many cases, IgAN is asymptomatic. In fact, patients with persistent hematuria and/or mild proteinuria found during routine examination in children and adults are commonly encountered.[4,5] Hence, late referral for this disease to a physician or a nephrologist when there is a reduction in the renal function is often common. Currently, renal biopsy remains the standard invasive approach for diagnosing IgAN.[6]

Galactose-deficient IgA1 (Gd-IgA1) is the hallmark of IgAN, but, although their serum levels are frequently elevated in IgAN patients,[7–11] the low diagnostic performance of the test renders it unsuitable to replace renal biopsy.[11] Therefore, novel, reliable, and noninvasive diagnostic biomarkers are needed to support the diagnosis of IgAN.

We recently identified by microarray analysis six upregulated microRNAs (miRNAs) in peripheral blood mononuclear cells (PBMCs) of IgAN patients.[12,13] Interestingly, two of them, namely let-7b and miR-148b, are involved in the glycosylation process of IgA1 (Supplementary Figure S1 online). Let-7b

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regulates the enzyme *N*-acetylgalactosaminyltransferase 2 (GALNT2), and miR-148b regulates the enzyme core 1 β 1,3 galactosyltransferase 1 (C1GALT1).[12,13] We demonstrated that the overexpression of these two miRNAs in PBMCs of IgAN patients could explain the low activity of GALNT2 and C1GALT1, respectively, and the resulting decrease in GalNAc and Gal in IgA1 molecule observed in IgAN patients.[14]

In the last few years, several studies have revealed the opportunity to measure circulating miRNAs in serum samples of patients as biomarkers for different diseases[15,16] and neoplasias.[17,18] However, the specificity of biomarkers based on a single miRNA is relatively low because different genes and miRNAs may contribute to the pathophysiology of complex diseases.[19] Here, we tested, in individuals with different ethnic backgrounds enrolled in a multicenter study, the hypothesis that serum levels of let-7b and miR-148b could represent a robust and a noninvasive combined biomarker for IgAN.

RESULTS

International multicenter study

Mean age and sex distribution, reported in Table 1, were comparable between IgAN patients and controls in each group. At the time of serum sample collection, a proteinuria >500 mg per day and a mean value of estimated glomerular filtration rate were comparable between patients in the three groups (training, validation, and test). The study design is depicted in Figure 1.

Let-7b and miR-148b levels in serum samples

Starting from the assumption that circulating miRNAs seem to be largely released from the cellular compartment,[20] we initially investigated the presence of let-7b and miR-148b in the serum of IgAN patients and controls. We found that both miRNAs were expressed ($Ct \leqslant 32$) and accurately quantifiable.

Let-7b, upregulated in PBMCs of IgAN patients,[13] was also elevated in serum samples from a cohort of 100 IgAN patients (median 3.25, interquartile range (IQR) 1.58–6.44) compared with 119 healthy blood donors (HBDs) (median 1.27, IQR 0.74–2.64) from Caucasian (Italy, Greece) and East Asian (China, Japan) populations ($P<0.0001$; Figure 2a and b). Nevertheless, miR-148b serum levels measured in the same patients and controls were slightly lower in IgAN patients (median 0.25, IQR 0.18–0.43) compared with HBDs (median 0.32, IQR 0.23–0.47) ($P=0.005$; Figure 2c and d). However, this discrepancy may be due to the analysis of different sample types (PBMCs and serum). Other studies showed that changes in the serum levels of miRNA expression were the opposite of those observed in tissue samples.[21–23] However, the underlying mechanism and cause of these changes remain to be determined.

We found that there was no association between each miRNA and age and sex ($P>0.05$), but their expression levels changed significantly between Caucasians and East Asians irrespective of the disease status ($P<0.05$; Figure 2a–d).

To investigate the characteristics of these miRNAs as potential diagnostic biomarkers of IgAN, ROC curve analyses were performed. The ROC curve analyses showed that each miRNA individually displayed a modest diagnostic efficiency (Table 2).

Training study

Given the limitations of individual tests, we performed a logistic regression analysis using miR-148b and let-7b serum levels from 100 biopsy-proven IgAN patients and 119 HBDs

Table 1 | Demographic and clinical features of IgAN patients, non-IgAN patients, and HBDs included in the Training, Validation, and Test studies

	Training group		Validation group		Testing group
	IgAN (100)	HBDs (119)	IgAN (145)	HBDs (64)	non-IgAN patients (105)
Age (years)	42.9 ± 13.9	41.3 ± 12.3	44.5 ± 14.3	41.3 ± 12	48.1 ± 15.8
Sex (M/F)	62/38	86/33	70/74	32/32	62/43
Ethnicity					
Caucasians	49	61	52	29	41
East Asians	51	58	93	35	64
Proteinuria (>500 mg/24 h) (%)	39	nd	42	nd	48
Hypertension (%)	1	nd	54	nd	64
eGFR (ml/min per 1.73 m^2)	64.6 ± 34.2	nd	64.4 ± 36.1	nd	73.4 ± 30.3
CKD stage 1 (%)	25	nd	26	nd	33
CKD stage 2 (%)	26	nd	26	nd	33
CKD stage 3 (%)	29	nd	25	nd	24
CKD stage 4 (%)	11	nd	11	nd	4
CKD stage 5 (%)	9	nd	12	nd	6
miRNAs levels					
Serum let-7b level median (range)	3.25 (1.58–6.44)	1.27 (0.74–2.64)	2.30 (0.97–5.98)	1.15 (0.67–2.05)	1.04 (0.62–1.68)
Serum miR-148b level median (range)	0.25 (0.18–0.43)	0.32 (0.23–0.47)	0.23 (0.17–0.38)	0.31 (0.25–0.42)	0.23 (0.16–0.37)

Abbreviations: CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; F, female; HBDs, healthy blood donors; IgAN, IgA nephropathy patients; KDOQI, Kidney Disease Outcomes Quality Initiative; M, male; miRNA, microRNA; nd, not determined.
Values have been expressed as mean ± s.d. or median (IQR). eGFR has been calculated using the CKD-EPI Creatinine formula (ml/min per 1.73 m^2). CKD stages have been determined according to KDOQI guidelines. Ethnicity: Caucasians (Italy and Greece) and East Asians (China and Japan).

selected and matched from Caucasian (Italy, Greece) and East Asian (China, Japan) populations. The predictors used in the model included the two miRNAs expressed as log10 of their $2^{-\Delta Ct}$ values, as well as the ethnicity as covariate (Caucasians were used as reference).

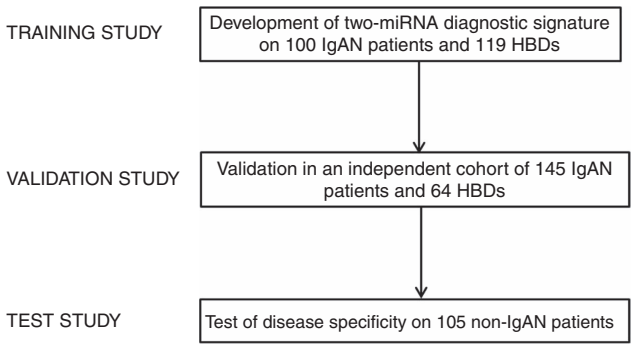

Figure 1 | Flow chart of the multicenter study. HBDs, healthy blood donors; IgAN, IgA nephropathy.

We obtained a potential classifier for detecting IgAN with the regression coefficients defining the following 'diagnostic signature':

$$-4.415 + 0.755 * \text{ethnicity} + 3.866 * \log_{10}(\text{let-7b}) - 5.115 * \log_{10}(\text{miR-148b}) (\textbf{Model 1}, \textbf{M1}).$$

The mean of the diagnostic signature was significantly different between biopsy-proven IgAN patients (median 0.41, IQR −0.18–1.94) and HBDs (median −1.09, IQR −1.81, −0.08) ($P<0.0001$, Figure 3a). The AUC was 0.82 (95% confidence interval (CI), 0.76–0.87; $P<0.0001$). A cutoff value of −0.19 showed the highest accuracy (sensitivity 76% (95% CI, 66–84); specificity 75% (95% CI, 66–82)) (Figure 3b). Interestingly, the signature was able to classify perfectly a good proportion of patients. In fact, approximately

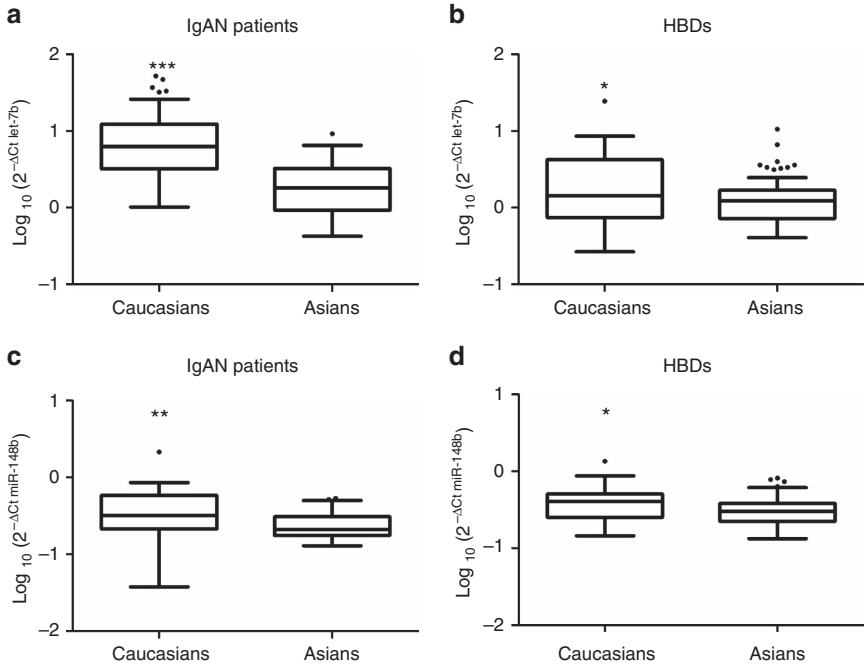

Figure 2 | Let-7b and miR-148b levels in serum samples. Serum levels of let-7b (**a**, **b**) and miR-148b (**c**, **d**) in a cohort of 100 IgAN patients and 119 HBDs considering Caucasian and East Asian populations independently. miRNA levels have been quantified by q-RT-PCR and expressed as $\log_{10}$-transformed of $2^{-\Delta ct}$. Let-7b and miR-148b were significantly higher in Caucasians than in East Asians (*$P<0.01$, **$P<0.001$, ***$P<0.0001$). HBDs, healthy blood donors; IgAN, IgA nephropathy; miRNA, microRNA; q-RT-PCR, quantitative real-time PCR.

Table 2 | Diagnostic performance of serum let-7b, miR-148b, miRNA combination (diagnostic signature), and miRNA combination with Gd-IgA1 in IgAN

	AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Positive LR	Negative LR	*P* value
let-7b	0.72 (0.65–0.79)	70	65	63	72	2.0	0.46	<0.0001
miR-148b	0.61 (0.53–0.69)	57	63	55	63	1.5	0.68	0.005
miRNA combination (training)	0.82 (0.76–0.87)	76	75	72	79	3.0	0.32	<0.0001
miRNA combination (validation)	0.78 (0.71–0.85)	64	74	84	47	2.6	0.42	<0.0001
miRNA combination (testing)	0.76 (0.71–0.81)	69	76	87	51	2.9	0.41	<0.0001
miRNA combination and Gd-IgA1	0.85 (0.80–0.90)	78	80	85	68	3.9	0.27	<0.0001

Abbreviations: AUC, area under curve; 95% CI, 95% confidence interval; IgAN, IgA nephropathy patients; LR, likelihood ratio; miRNA, microRNA; NPV, negative predictive value; PPV, positive predictive value.

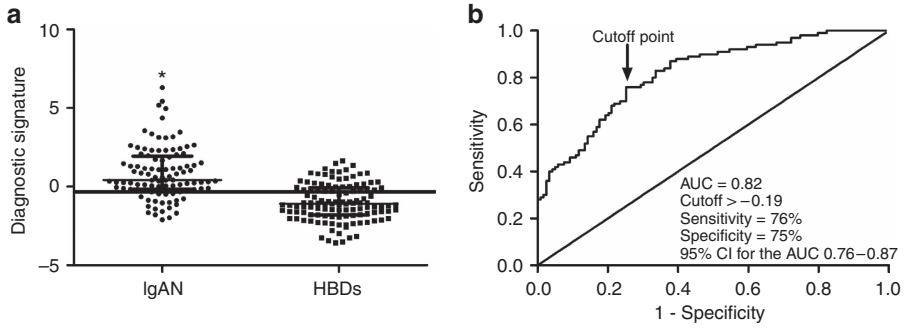

Figure 3 | Diagnostic performance of the combined miRNA biomarker (let-7b and miR-148b) in the Training group. The diagnostic signature was calculated by logistic regression: −4.415+0.755*ethnicity+3.866*$\log_{10}$(let-7b)−5.115*$\log_{10}$(miR-148b). Scatter plot (**a**) and ROC curve (**b**) were generated using the diagnostic signature derived by a logistic regression model applied to 100 biopsy-proven IgAN patients and 119 HBDs from Caucasian (Italy, Greece) and East Asian (China, Japan) populations. The signature was significantly higher in IgAN patients compared with HBDs ($P<0.0001$). The AUC was 0.82 (95% CI, 0.76–0.87). The median and IQR are shown on the scatter plots of individual subjects' diagnostic scores. The continuous horizontal line in the left panel represents the cutoff point at highest accuracy, −0.19. *$P<0.0001$. AUC, area under the ROC curve; CI, confidence interval; HBDs, healthy blood donors; IgAN, IgA nephropathy; IQR, interquartile range; miRNA, microRNA; ROC, receiver operating characteristics.

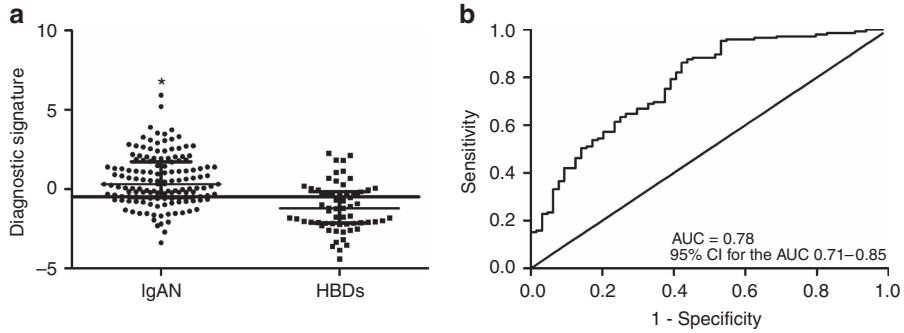

Figure 4 | Diagnostic performance of the combined miRNA biomarker (let-7b and miR-148b) in the Validation group. Scatter plot (**a**) and ROC curve (**b**) were generated applying the diagnostic signature, identified in the model developed in the Training cohort, to 145 biopsy-proven IgAN patients and 64 HBDs from two populations (Caucasian and East Asian). The signature was significantly higher in IgAN patients compared with HBDs ($P<0.0001$). The AUC was 0.78 (95% CI, 0.71–0.85) and was not significantly lower than the AUC of the training study ($P=0.57$). The median and IQR are shown on the scatter plots of individual subjects' diagnostic scores. The continuous horizontal line in the left panel represents the cutoff point at highest accuracy identified in the training group, −0.19. *$P<0.0001$. AUC, area under the ROC curve; CI, confidence interval; HBDs, healthy blood donors; IgAN, IgA nephropathy; IQR, interquartile range; miRNA, microRNA; ROC, receiver operating characteristics.

25% of the cases had diagnostic scores exceeding the highest value in controls, and approximately 20% of the controls had diagnostic scores below the smallest value in cases.

Thus, the combined miRNA biomarker differentiated IgAN patients from HBDs (Table 2).

Validation study

To confirm the relevance of the predictive model based on the two miRNAs, we validated the diagnostic signature found in the training study in an additional and independent large cohort of 145 IgAN patients and 64 HBDs comprising individuals from the two populations.

The diagnostic signature was significantly different between cases (median 0.31, IQR −0.54–1.73) and controls (median −1.21, IQR −2.15, −0.17) ($P<0.0001$; Figure 4a). The ROC curve of the diagnostic signature obtained in the validation study reached an AUC of 0.78 (95% CI, 0.71–0.85; $P<0.0001$; Figure 4b). This AUC was lower than the AUC for the training data set, but their difference was not significant ($P=0.57$). Using the cutoff value of −0.19 identified in the training cohort, IgAN status was predicted with a sensitivity of 64% (95% CI, 56–72) and a specificity of 74% (95% CI, 61–84).

In addition, we generated the ROC curve applying the same model to all IgAN patients ($n=245$) and all HBDs ($n=183$) after merging the training and validation set, and we found that the AUC of the two-miRNA signature was 0.80 (95% CI, 0.75–0.84; $P<0.0001$). At the cutoff value of −0.19, the sensitivity was 69% (95% CI, 63–75) and the specificity was 74% (95% CI, 67–80).

We next tested whether the diagnostic signature with the combined miRNA biomarker was independent of the renal function, and we found that there was no difference when stratifying IgAN patients by KDOQI stage (analysis of variance $P=0.36$; Supplementary Figure S2 online).

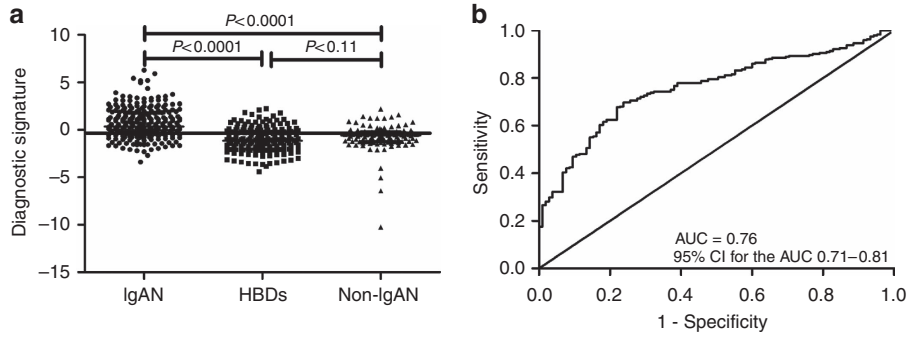

Figure 5 | Diagnostic signature of the combined miRNA biomarker (let-7b and miR-148b) in the Test group. Scatter plot (**a**) was generated applying the diagnostic signature to 105 biopsy-proven non-IgAN patients from two populations (Caucasian and East Asian). The signature was significantly different between IgAN patients and non-IgAN patients ($P<0.0001$), whereas there was no significant difference between non-IgAN patients and HBDs ($P=0.11$). ROC curve (**b**) was generated applying the diagnostic signature, identified in the model developed in the Training group, to all 145 IgAN patients and 105 non-IgAN patients as controls. The AUC was 0.76 (95% CI, 0.71–0.81). AUC, area under the ROC curve; CI, confidence interval; HBDs, healthy blood donors; IgAN, IgA nephropathy; IQR, interquartile range; miRNA, microRNA; ROC, receiver operating characteristics.

Test study

To study whether the diagnostic signature is specific and able to discriminate IgAN from other primary glomerulonephritides, we tested the model using a group of non-IgAN patients from the two populations. We selected patients with minimal change disease, focal segmental glomerulosclerosis, and membranoproliferative glomerulonephritis type I as disease controls.

Here, the diagnostic signature was significantly different between IgAN patients (median 0.35, IQR 0.46–1.77) and non-IgAN patients (median −0.59, IQR −1.25, −0.21) ($P<0.0001$), whereas there was no significant difference between non-IgAN patients and HBDs (median −1.16, IQR −2.03, −0.16) ($P=0.11$, Figure 5a). The ROC curve of the model, now discriminating between all IgAN patients and non-IgAN patients, yielded an AUC of 0.76 (95% CI, 0.71–0.81; $P<0.0001$; Figure 5b). At the cutoff value of −0.19, the sensitivity was 69% (95% CI, 63–75) and the specificity was 76% (95% CI, 67–84).

These results suggest that the diagnostic signature based on the two miRNAs could be considered a biomarker with good disease specificity.

Effect of corticosteroids

In order to assess the effect of corticosteroid therapy on the combined miRNA biomarker, we enrolled an independent cohort of 35 IgAN patients who were receiving prednisone at the time of blood sample collection. We found that the diagnostic signature was not influenced by corticosteroids ($P=0.07$; Supplementary Figure S3 online) and still remained significantly higher when compared with HBDs ($P<0.0001$).

Gd-IgA1 serum levels

We next measured serum Gd-IgA1 levels in a group of 150 randomly selected IgAN patients and 100 matched HBDs from the two populations. Elevated mean values of Gd-IgA1 were found in IgAN patients (median 0.25, IQR 0.21–0.28), and they were significantly different from HBDs ($P<0.0001$; Supplementary Figure S4A online). There was a significant positive correlation (Spearman $r=0.42$; $P<0.0001$, Supplementary Figure S4B online) between Gd-IgA1 serum levels and the diagnostic signature based on the two miRNAs, confirming a strong relationship between these two biomarkers.

Predictive model including Gd-IgA1 serum levels

In order to simultaneously associate the two miRNAs and Gd-IgA1 serum levels, we performed an additional logistic regression model including let-7b, miR-148b, and Gd-IgA1.

The identified diagnostic signature was the following: 2.008 +0.579*ethnicity+3.065*$\log_{10}$(let-7b)−2.699*$\log_{10}$(miR-148b) +7.019*$\log_{10}$(Gd-IgA1).

The ROC curve analysis of this model in the cohort of 150 IgAN patients and 100 HBDs yielded an AUC of 0.85 (95% CI, 0.80–0.90; $P<0.0001$). A cutoff value of 0.46 had the highest accuracy (sensitivity 78% (95% CI, 71–84); specificity 80% (95% CI, 71–87)) (Figure 6a and b).

Race influence

In order to ascertain the importance of the two miRNAs, specifically for the two different ethnic groups, we performed two additional logistic regression models (**Model 2, M2**) for Caucasians and East Asians separately, applying the same study design as in the main workflow.

The diagnostic signatures identified for each training group were as follows:

$$-4.038 + 4.102 * \log_{10}(\text{let-7b}) - 3.877 * \log_{10}(\text{miR-148b});$$

in the Caucasian cohort.

$$-4.569 + 3.440 * \log_{10}(\text{let-7b}) - 6.798 * \log_{10}(\text{miR-148b});$$

in the East Asian cohort.

The ROC curve analyses for these diagnostic signatures yielded an AUC of 0.86 (95% CI, 0.80–0.93; $P<0.0001$) in the Caucasian and 0.79 (95% CI, 0.70–0.87; $P<0.0001$) in the

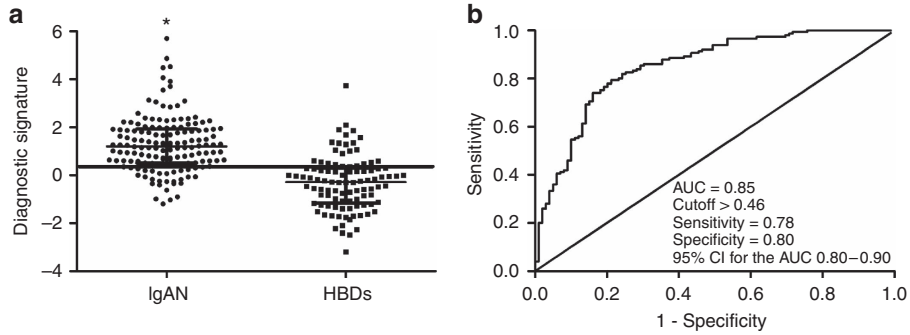

Figure 6 | Diagnostic performance of a model including miR-148b, let-7b, and Gd-IgA1 serum levels. The diagnostic signature was calculated by logistic regression: 1.037+0.579*ethnicity+3.065*$\log_{10}$(let-7b) − 2.699*$\log_{10}$(miR-148b)+7.019*$\log_{10}$(Gd-IgA1). Scatter plot (**a**) and ROC curve (**b**) were generated using the diagnostic signature derived by a logistic regression model applied to 150 biopsy-proven IgAN patients and 100 HBDs from Caucasian (Italy, Greece) and East Asian (China, Japan) populations. The diagnostic signature was significantly higher in IgAN patients compared with HBDs ($P<0.0001$). The AUC was 0.85 (95% CI, 0.80–0.90). The median and IQR are shown on the scatter plots of individual subjects' diagnostic scores. The marked lines represent the cutoff points at highest accuracy, 0.46. *$P<0.0001$. AUC, area under the ROC curve; CI, confidence interval; HBDs, healthy blood donors; IgAN, IgA nephropathy; IQR, interquartile range; miRNA, microRNA; ROC, receiver operating characteristics.

East Asian training group, respectively (Supplementary Table S1 online). These models were also confirmed using the validation and testing groups by comparing matched Caucasian and East Asian individuals independently (Supplementary Table S1 online). Finally, for each group in each cohort, we compared the AUC obtained applying M2 with the AUC obtained applying M1 (Supplementary Table S2 online). For Caucasians, M2 was significantly better than M1 in all groups (training, validation, and testing) ($P<0.05$), whereas for East Asians, M2 and M1 showed a similar diagnostic performance ($P>0.05$). However, according to the Akaike Information Criterion (AIC), both logistic regression models M2 showed a better fit than M1 and were preferable in discriminating IgAN patients from healthy subjects (M1 AIC = 230; M2 Caucasians AIC = 103; M2 East Asians AIC = 125; Supplementary Figure S5 online).

Altogether our results demonstrated that the two miRNAs are relevant clinical biomarkers, irrespective of the race.

DISCUSSION

Studies on miRNAs have remarkably improved the understanding of the pathogenesis of renal and non-renal diseases.[24–26] Recent publications have demonstrated that circulating miRNAs may serve as biomarkers for some diseases.[14–17] In this study, we present, for the first time, a combined miRNA biomarker that can predict the probability of being affected by IgAN.

A defective *O*-glycosylation of IgA1 is the key process in IgAN, and most patients have elevated serum levels of Gd-IgA1. In particular, two key enzymes catalyze the *O*-glycosylation process; specifically, GALNT2 regulates the first phase and C1GALT1 is involved in the attachment of galactose.[27–29] Our studies on miRNA expression patterns in PBMCs of IgAN patients demonstrated that there are strict interactions between let-7b and GALNT2 and miR-148b and C1GALT1, respectively.[12,13] Thus, these two miRNAs are the main regulators of the *O*-glycosylation process of IgA1, which has an important role in the pathogenesis of the disease. Nowadays, the measurement of Gd-IgA1 is considered a test of probability for diagnosis of IgAN, but the sensitivity and specificity of this laboratory measurement are insufficient for the test to replace kidney biopsy as the diagnostic standard.[11,30]

In this international multicenter study, we based our strategy to develop a noninvasive and a specific diagnostic test on three steps: (i) in the *training study*, we developed a model that combines let-7b and miR-148b serum levels to distinguish between IgAN patients and HBDs; (ii) the *validation study* was performed to confirm in an independent group of patients and controls in whom the combination of let-7b and miR-148b could be used to predict IgAN; and (iii) the *testing study* was carried out to check the disease specificity of the diagnostic signature.

Individually, the serum level of the two miRNAs displayed only a modest diagnostic efficiency (as indicated by AUC of 0.72 for let-7b and 0.61 for miR-148b). However, combined as a two-miRNA biomarker, in a diagnostic signature derived from a logistic regression model, the AUC increased to 0.82. The model (Model 1, M1) was validated in an independent cohort of patients and controls, and the signature was specific for IgAN when tested with other non-IgAN renal diseases. Moreover, it was independent of the renal function and stable to the effects of corticosteroids.

The model M1 defined the relevance of race as a risk factor in this context. An odds ratio of 2.13 was observed when we compared Caucasians with East Asians, in keeping with the known West-East gradient in disease risk. Thus, we performed two additional logistic regression models (Model 2, M2) for Caucasians and East Asians separately. These models showed a better diagnostic performance than M1 in discriminating IgAN patients from healthy subjects in all groups (training, validation, and testing).

Given these results, the test based on the combined biomarker may aid clinicians in the diagnosis of IgAN, particularly in those individuals with persistent urinary abnormalities who refuse renal biopsy and in relatives of IgAN patients with permanent microscopic hematuria and/or proteinuria.

Although it is noteworthy that the diagnostic model improved when we simultaneously associated let-7b, miR-148b, and Gd-IgA1 serum levels, it can be argued that the multistep ELISA assay used to accurately quantify small changes in Gd-IgA1 serum levels, as in this study, is hardly adapted by clinical laboratories. On the contrary, real-time PCR, a technology widely used in diagnostic practice, can accurately detect the change in the expression levels of the two miRNAs.

Intriguing questions are how let-7b and miR-148b exert their physiological role in blood vessels and why miR-148b is decreased in the serum of IgAN patients, but the answers remain unclear. Interestingly, we revealed a discrepant expression of miR-148b between PBMCs and serum of IgAN patients. In our previous work,[12] we demonstrated that miR-148b is an essential regulator of *O*-glycosylation process of IgA1 in IgAN. Many cells including B cells,[31] T cells,[32] dendritic cells,[33] mast cells,[34] epithelial cells,[35] and tumor cells[36] have the capacity to release exosomes. These exosomes contain both mRNAs and miRNAs, which can be delivered to another cell and function in a new location.[37] Thus, it might be possible that IgA1-producing cells specifically uptake miR-148b from the exosomes, and, as a result, miR-148b decreases in the serum. Another explanation could be that blood cells may specifically digest circulating miR-148b directly or indirectly. However, it is not yet clear why miR-148b is decreased in the serum, and future studies may demonstrate the mechanism of this miRNA in circulation. Despite the discrepancy in miR-148b expression between PBMCs and serum, we have demonstrated that the combination of let-7b and miR-148b could predict IgAN status.

Even though the combined miRNA signature showed a high diagnostic accuracy, this study has a few limitations. The main limitation is that both training and validation studies were carried out using retrospective samples. A prospective study is required to further confirm the clinical utility of both models in other centers and in particularly for early diagnosis of IgAN in asymptomatic individuals, thus reducing the increased risk of end-stage kidney disease for late referral. Second, we focused our attention to Caucasians and East Asians, in whom the frequency of the disease is relatively high, but additional studies in other races with low prevalence of IgAN are necessary. Finally, strength is that, in the absence of complete data unraveling the genetic determinants of the aberrant *O*-glycosylation process of IgA1, the combined biomarker could be used as a useful intermediate phenotype to identify asymptomatic individuals carrying IgAN susceptibility alleles not yet known.

In conclusion, the diagnostic signature based on serum levels of let-7b and miR-148b appears to be a novel, specific, and a noninvasive biomarker to test the probability of being affected by IgAN aiding clinicians in the diagnosis.

METHODS

Study design

A case–control international multicenter study was organized to test the clinical utility of let-7b and miR-148b in the diagnosis of IgAN. The study design is depicted in Figure 1. In total, 533 serum samples were collected at Renal Units from Italy (Polyclinic, Bari and Hospital of Verona), Greece (Hippokration Hospital and Hospital of Patras), Hong-Kong (Queen Mary Hospital), and Japan (Fujita Health University and Nagoya University). The diagnosis of biopsy-proven IgAN was based upon the demonstration, by immunofluorescence, of IgA as the dominant or the co-dominant immunoglobulin in mesangial deposits. Renal lesions were assessed according to the Oxford classification.[38,39] Individuals with secondary IgAN like lupus nephritis, Henoch-Schonlein purpura, and liver cirrhosis were excluded from the study.

The first cohort of 219 individuals (100 IgAN patients and 119 HBDs) from Caucasian and East Asian populations was enrolled in the training study, whereas an independent cohort of 209 individuals (145 IgAN patients and 64 HBDs) from the two populations was enrolled to validate our results.

Thereafter, 105 samples from non-IgAN patients were used for the test study. Thirty patients with minimal change disease, 30 focal segmental glomerulosclerosis, and 45 membranoproliferative glomerulonephritis type I were selected as disease controls.

Patients and controls in each group were first randomly sampled from the pool of available individuals and then matched using MatchIt.[40]

Next, we used an independent group of 35 IgAN patients, who were receiving prednisone at the time of blood sample collection, to study the effect of corticosteroid therapy. Clinical data including age, sex, blood pressure, serum creatinine, and 24-h urinary protein excretion at the time of blood sampling were collected. The estimated glomerular filtration rate (ml/min per 1.73 m^2) was calculated using the CKD-EPI Creatinine formula,[41] and the patients were classified as chronic kidney disease stages 1 to 5. Hypertension was defined as systolic blood pressure >140 mm Hg and/or diastolic blood pressure >90 mm Hg at rest or the use of antihypertensive medication.

The HBDs enrolled in the study were selected on the basis of their demographical characteristics and matched with the IgAN groups for age and sex. Urinalysis of HBDs was performed at the time of blood collection, and all HBDs included in this study had a negative urine test for blood and proteins.

Written informed consent was obtained from all study participants. The study was carried out according to the principles of the Declaration of Helsinki and was approved by the local Institutional Ethics Review Boards.

The main demographic and clinical features of our patients and controls included in the study are summarized in Table 1.

For the serum collection, blood sample from each participant (IgAN patients, HBDs, and non-IgAN patients) was collected in a tube with polymer gel and clot activator. After clotting at room temperature for 30 min, specimens were centrifuged at 3000 r.p.m. for 10 min at room temperature. Sera were stored at -80 °C until use.

RNA isolation

Total RNA, including miRNA, was extracted from 100 µl of serum using miRNeasy Serum/Plasma kit (Qiagen, Germany) according to the manufacturer's protocol. Finally, RNA was recovered in 50 µl of RNase-free water. The RNA concentration

was determined with NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Reverse transcription

The reverse transcription reaction was carried out with the miScript Reverse Transcription Kit (Qiagen) in 20 µl containing 200 ng of RNA extract, 4 µl of 5× miScript HiSpec Buffer, 2 µl of 10× miScript Nucleics Mix, and 2 µl of miScript Reverse Transcriptase Mix. For the synthesis of cDNA, the reaction mixtures were incubated at 37 °C for 60 min and at 95 °C for 5 min and then held at 4 °C. The cDNA specimens were stored at − 20 °C until PCR.

miRNA quantification by real-time quantitative PCR

The amounts of let-7b and miR-148b were quantified by quantitative PCR using the miScript SYBR Green PCR kit (Qiagen). One microliter of cDNA solution was amplified using 12.5 µl of 2× QuantiTect SYBR Green PCR Master Mix, 2.5 µl of 10× miScript Universal Primer, 2.5 µl of 10× miScript Primer Assay, and 6.5 µl of nuclease-free water in a final volume of 25 µl. Specific primers for let-7b, miR-148b, and miR-27a (Qiagen) were used. Quantitative PCR was run on iCycler (Bio-Rad Laboratories, Hercules, CA). The reaction mixtures were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 35 s. Normalization was performed using an internal control, the miR-27a, which has been found highly and equally expressed in our PBMC samples.[12] Comparative real-time PCR was performed in triplicate, including no-template controls. Let-7b and miR-148b relative expression was calculated using the $2^{-\Delta Ct}$ method where $\Delta Ct = Ct_{(\text{let-7b or miR-148b})} - Ct_{\text{miR-27a}}$.

Measurement of Serum IgA

IgA content in serum from each subject was measured in duplicate using enzyme-linked immunosorbent assay. Briefly, high adsorption polystyrene 96-microwell plates (Costar Corning, Corning, NY) were coated overnight with 5 µg/ml of $F(ab')_2$ fragment goat anti–human IgA antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), in phosphate-buffered saline (PBS) at 4 °C. After washes with PBS containing 0.05% Tween-20 (PBST), plates were blocked with 1% bovine serum albumin in PBS containing 0.05% Tween-20 (PBST) for 90 min at room temperature. Samples diluted in blocking buffer and standard human IgA (Calbiochem, San Diego, CA) were added to each well and then incubated for 90 min at room temperature. After washes, the captured IgA was then detected with biotin-labeled $F(ab')_2$ fragment of goat IgG anti–human IgA (Biosource, Camarillo, CA). Plates were washed, and the binding was measured after addition of avidin–horseradish peroxidase conjugate (ExtrAvidin; Sigma-Aldrich, Milan, Italy), and then the reaction was developed with the peroxidase chromogenic substrate o-phenylenediamine–H_2O_2 (Sigma-Aldrich). The color reaction was stopped with 2N H_2SO_4, and the optical density at 490 nm was determined in a microplate reader (GDV, Rome, Italy; model DV 990 B/V6).

Measurement of galactose-deficient IgA1 (Gd-IgA1)

The Gd-IgA1 was detected by binding of the lectin, Helix aspersa (Sigma-Aldrich), which is specific for terminal GalNAc residues, as previously reported.[9,42] High adsorption polystyrene 96-microwell plates (Costar Corning) were coated overnight with 3 µg/ml of $F(ab')_2$ fragment goat anti–human IgA antibody (Jackson ImmunoResearch Laboratories), in PBS at 4 °C. Plates were washed three times and blocked for 3 h at room temperature with 1% bovine serum albumin/PBST. Samples diluted in blocking buffer were added to each well and incubated overnight at 4 °C. The captured IgA was subsequently desialylated by treatment for 3 h at 37 °C with 20 mU/ml neuraminidase from *Vibrio cholerae* (Sigma-Aldrich) in 10 mM sodium acetate buffer, pH 5.3. After washes, samples were then incubated for 3 h at 37 °C with 2 µg/ml of GalNAc-specific biotinylated HAA lectin (Sigma-Aldrich) diluted in blocking buffer. Plates were washed, and the lectin binding was detected with avidin–horseradish peroxidase conjugate (ExtrAvidin, Sigma-Aldrich) diluted in blocking buffer, and the reaction was developed with the peroxidase chromogenic substrate o-phenylenediamine-H_2O_2 (Sigma-Aldrich). The color reaction was stopped with 2N H_2SO_4, and the optical density at 490 nm was determined in a microplate reader (GDV; model DV 990 B/V6).

The relative lectin binding per unit IgA1 was calculated as the optical density value of lectin over the optical density value of IgA concentration. The inter-assay and intra-assay coefficients of variation were 6.9 and 4.1, respectively.

Statistical analysis

All statistical analyses were performed using SPSS 20.0 software (SPSS, Chicago, IL) and R version 3.1.2 (http://www.R-project.org/[43]). Graphs were generated using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). For normally distributed variables, results were given as mean ± s.d., and they were compared between groups by Student's *t* test. Results for non-normally distributed variables were summarized as medians and IQR, and groups were compared by the Mann–Whitney U test. *P*-values <0.05 were considered to be statistically significant.

To test the diagnostic precision, we determined the intra-assay and the inter-assay variability by calculating the coefficient of variation, the ratio between the standard deviation, and the mean of replicate measurements. The intra-assay coefficient of variation was calculated for each sample performing real-time PCR in triplicate on the same plate. Then, to determine the inter-assay coefficient of variation, we randomly selected 10 samples and run them on three different days. The intra- and inter-assay coefficients of variation were 0.5% and 0.7% for let-7b and 0.9% and 0.5% for miR-148b, respectively.

Logistic regression analysis was used to develop combined miRNA models able to predict the probability of developing IgAN. Let-7b, miR-148b, and Gd-IgA1 levels were analyzed as a continuous trait (log-transformed).

The diagnostic accuracy of the models was assessed by the AUC.[44] The Younden index was used to determine the optimal cutoff value that maximizes the combined sensitivity and specificity.[45] ROC curves were compared using DeLong's test[46] for two ROC curves available in the R package 'pROC'. The AIC was used as a measure of goodness-of-fit. IgAN patients and controls were matched in each group using the genetic search algorithm implemented in MatchIt (http://www.jstatsoft.org/v42/i08/,[40]).

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

GS and FPS designed the experiments; GS, FS, GDP, SNC, and CC performed the experiments; GS and FP analyzed data; GS, FP, and FPS wrote the paper; FPS, FP, GZ, KL, JL, ST, AP, MS, DG, MG, KT, YY, SM, and EI collected blood samples and provided clinical and laboratory data.

SUPPLEMENTARY MATERIAL

Circulating miR-148b and let-7b in serum as markers for detecting primary IgA nephropathy: a retrospective international study.
Figure S1. The *O*-glycosylation process of the IgA1.
Figure S2. Diagnostic signature for the combined miRNA serum levels as a function of CKD stages in all IgAN patient group.
Figure S3. Effect of corticosteroids (CSs) on diagnostic signature derived from a logistic regression model comprising let-7b and miR-148b as predictors for IgAN.
Figure S4. Serum levels of Gal-deficient IgA1 in 150 IgAN patients and 100 healthy blood donors (HBD).
Figure S5. Logistic regression models with and without race as variable.
Table S1. Diagnostic performance of the two models (M2) developed in Caucasian and East Asian cohorts.
Table S2. Comparison of the diagnostic performance of different models in the three data set (training, validation, and testing).
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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