PLASMA MOLECULAR SIGNATURES IN HYPERTENSIVE PATIENTS WITH RENIN ANGIOTENSIN SYSTEM SUPPRESSION: NEW PREDICTORS OF RENAL DAMAGE AND *DE NOVO* ALBUMINURIA INDICATORS

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Running title: *de novo* and sustained Albuminuria indicators

Total words of manuscript: 5510

Word count of abstract: 210

Total number of figures: 5

ABSTRACT

Albuminuria is a risk factor strongly associated with cardiovascular disease, the first cause of death in the general population. It is well established that Renin-Angiotensin-System suppressors prevent the development of new-onset albuminuria in naïf hypertensive patients and diminish its excretion, but we cannot forget the percentage of hypertensive patients who develop *de novo* albuminuria. Here, we applied multiple proteomic strategy with the purpose to elucidate specific molecular pathways involved in the pathogenesis and provide predictors and chronic organ damage indicators.

1143 patients were followed for a minimum period of 3 years. Hypertensive patients chronically RAS suppressed were recruited, classified in 3 different groups depending on their albuminuria levels (normoalbuminuria, *de novo* albuminuria and sustained albuminuria) and investigated by multiple proteomic strategy. Our strategy allowed us to perform one of the deepest plasma proteomic analysis so far which has shown two proteomic signatures: l) with predictive value of *de novo* albuminuria and 2) sustained albuminuria indicator proteins. These signatures are related to inflammation, immune as well as in the proteasome activation occurring in situations of endoplasmic reticulum stress. Furthermore, these results open the possibility of a future strategy based on anti-immune therapy to treat hypertension which could help to prevent the development of albuminuria and hence, the progression of kidney damage.

Keywords: RAS suppression**,** albuminuria, hypertension, organ damage, immune system

INTRODUCTION

Albuminuria is a risk factor strongly associated with cardiovascular and renal complications particularly when diabetes and hypertension are present. However, in the absence of these two processes albuminuria can be equally relevant as a predictor.[1] These facts reflect the enormous relevance of albuminuria to monitor cardiovascular disease (CVD), the first cause of death in the general population.[2] Different factors promote the development of albuminuria[3] and lead finally to an increased albumin leakage through the glomerular barrier.[4-7]

Renin-Angiotensin- System (RAS) suppression is considered the therapy of choice to prevent the development of new-onset albuminuria in naïf patients[8,9] and to diminish the amount excreted albuminuria.[10] The positive effect depends on a reduction in blood pressure (BP),[11] as well as a modification of intraglomerular hemodynamics and permeability.[12] Recent studies conducted by our group have shown that despite an apparently adequate RAS suppression, a relevant percentage of hypertensive patients develop *de novo* albuminuria pointing to a progression of CVD probably facilitated by an inappropriate therapeutic response to ACEi or ARB.[13]

Moreover, we have also found an increased systemic oxidative damage in hypertensive patients who develop albuminuria under chronic RAS suppression suggesting that oxidative stress is one of the mechanisms underlying the development of albuminuria[14] in this situation.

These considerations highlight the need to continue searching for potential mechanisms and indicators of the development and progression of albuminuria in hypertensive patients under RAS suppression, considering that it will contribute to better understand the pathogenesis of the progression of CV and renal disease under chronic treatment with either an ACEi or an ARB[15, 16].

Recently, substantial improvements in the entire MS-based proteomics pipeline, including sample preparation, liquid chromatography mass spectrometry hardware and data analysis, have been produced. In this study, we present the application of an –omics strategy to approach the unbiased, non-targeted, study of albuminuria in hypertensive patients with RAS blockade searching for predictors and chronic organ damage markers in blood plasma (Figure 1A).

Our data show the existence of 2 characteristic plasma protein signatures described for the first time consisting of: 1) protein candidates to be markers of *de novo* albuminuria, composed by 4 proteins and 2) sustained albuminuria indicator proteins, made up of 16 proteins, which may be considered markers of end organ damage. The proteins hereby associated with albuminuria development point to the activation of the immune system, as well as inflammation and endoplasmic reticulum stress (ERS), which might be related to the underlying of simultaneous cardio-renal damage.

METHODS

Patient recruitment

Patient selection and classification was previously described in an initial paper showing the development of albuminuria in patients during chronic RAS suppression.[13] Briefly; 1143 patients were followed for a minimum period of three years with visits to the Hypertension Unit, Hospital Universitario 12 de Octubre, Madrid, at least, every 6 months. After that, the patients continued with their annual revisions. One hundred twenty nine hypertensive patients chronically RAS suppressed with or without high albuminuria (48 diabetics and 81 non-diabetics, HTA RAS patients) were recruited between January 2011 and June 2013 in the unit, (Figure 1B).

Albuminuria is defined as albumin/creatinine ratio (ACR) in the urine greater than 20 mg/24 h and 30 mg/24 h in men and women, respectively.

For the discovery phase, 24 HTA RAS patients were classified based on ACR in urine and development time of albuminuria in three groups: a) normoalbuminuric patients (N); b) *de novo* albuminuria (dnA), patients who developed *de novo* albuminuria in the last ten years and c) patients with sustained albuminuria (SA) at baseline, which maintained elevated albuminuria levels during follow-up. Then, we confirmed these proteins in a cohort of patients with or without diabetes with the objective of verifying our findings in a general population. All the patients were matched by baseline characteristics and medications (Table S4).

In the validation phase, 105 patients were classified in the same groups as previously described. The clinical characteristics and medications of these hypertensive patients are shown in Table 2. A significant decrease of Estimated Glomerular Filtration Rate (eGFR) (ANOVA, $p = 0.004$) was observed for both albuminuric groups (dnA and SA), which implies impaired renal function associated with kidney damage.

The study was conducted according to the recommendations of the Declaration of Helsinki and was approved by the "Hospital 12 de Octubre" ethics committee. In all cases informed consent was requested from subjects indicating that their participation in the study was not prejudicial in any way to the treatment and possessed no risk.

Proteomics pipeline

The experimental strategy consisted in: 1) an immunodepletion of the 14 more abundant plasma proteins, 2) a discovery phase using two different, complementary and robust proteomics techniques, 2D-DIGE (GE-Healthcare) and iTRAQ labeling (AB Sciex) followed by LC-MS/MS according to previous publications from our group[17] and 3) a validation phase

in an independent cohort of patients by Selected Reaction Monitoring (SRM) analysis[18]. See details in supplementary material.

For 2D-DIGE and iTRAQ experiments, we employed 24 plasma samples (n = 8, per group), which were combined to generate four sample pools per group (N1-N4, dnA1-dnA4 and SA1-SA4) (Tables S2 and S3). In the SRM assay, we grouped the 105 plasma samples (N = 53, dnA = 26 and SA = 26) into 32 pools (N1-N15, dnA1-dnA9 and SA1-SA8). The pool samples were perfectly grouped taking into account their clinical characteristics and albuminuria levels.

Statistical methods

Values for patients' characteristics are expressed as means ± standard deviation (SD) or percentages. For all comparisons, one-way ANOVA was used to calculate statistically significant differences of the values between the different groups studied. Post-hoc analysis of significant ANOVA results was performed by means of Tukey analysis. In iTRAQ results, we have considered differentially expressed those proteins identified with at least two peptides and log2-ratios expressed in form of the standardized variables (Zq) ± 2 with p values ≤ 0.05, being Zq the mean for the 4 replicates *versus* the normoalbuminuria group. In relation to 2D-DIGE analysis, we selected significant protein spots with a 1.3-fold difference in abundance between groups. For SRM, we have considered significant those peptides showing significant differences in at least two of the three analyzed transitions. In all cases, we have represented the transition of the most significant peptide. Receiver operating characteristic (ROC) curves were generated using SPSS 15.0 for windows software (SPSS Inc.). Statistical significance was accepted at *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

RESULTS

In this work, we investigated molecular changes involved in the development of albuminuria in the plasma proteome of hypertensive patients with RAS suppression using different proteomics approaches (iTRAQ and 2D-DIGE). On the one hand, the use of an iTRAQ LC-MS/MS strategy has allowed us to perform one of the deepest plasma proteomic analyses so far, which led us to identify 4935 proteins, 36 differentially expressed (Table S1). On the other hand, the 2D-DIGE analysis allows us to identify 12 differentially expressed proteins (Table S2 and Figure S1), which did not significantly expressed by iTRAQ. The combination of these two different and complementary proteomics techniques, in the discovery phase, guarantees a more extensive coverage of quantified proteins. Our results allowed us to describe 5 differential proteins that had never been before detected in plasma/serum; NALP9, UBP47, DNJC9, ORNT1 and CHADL, according to *Uniprot* and *The human protein atlas databases*, and therefore low abundance proteins could be quantified by the hereby presented mass spectrometry-based strategy. In order to validate these alterations, 39 proteins were analyzed by SRM assay; the whole proteins found significantly expressed by 2D-DIGE and 27 proteins selected among the total varied in iTRAQ analysis, taking into account the fold change and their implication in molecular mechanisms related to the pathology. A total of 17 proteins were validated by SRM in an independent cohort of 105 subjects (Figure 2A and Table S3). Confirmed altered proteins were classified according to their biological functions (Table 1), as we said before. Besides, these proteins were analyzed employing ROC curves, a useful tool, which allow us to discriminate the different groups of study attending to their protein expression levels. We performed a principal component analysis (PCA) with the whole of differentially expressed proteins validated (Figure 2B), which showed a correct grouping of patients with sustained albuminuria respect to the other two groups (Figure 2C). These results revealed 2 different signatures: one of them are, candidates markers of *de novo*

albuminuria, which are formed by 4 out of the 17 proteins validates by SRM, and the second one, may be considered markers of end organ damage since their levels are altered in sustained albuminuric patients and made up of 16 proteins (3 already varied in dnA) (Figure 2C).

Plasma proteomic signature with predictive value of *de novo* albuminuria: SRM results have shown the up-regulation of 3 proteins involved in immune response: CFAB, IGKC, IGHA2 (Figure 4A) and a decrease in HEMO (Figure 3D). ROC curve for these 4 proteins showed a significant classification value ($p = 0.003$) of this panel for the separation of dnA patients with respect to N, with a considerably high AUC of 0.874 (Figure 4A).

Panel of protein markers of sustained albuminuria: SRM results have shown a total of 5 up-regulated plasma proteins involved in immune response (IGKC, LV302, IGHA2, IGHG2 and IGHG3) (Figure 3A), among which two of them (IGKC and IGHA2) were found previously altered in sustained albuminuria. In addition, 5 altered proteins related with inflammation (A2GL, PON1, SAMP, NALP9 and HEMO) (Figure 3B), 2 involved in endoplasmic reticulum stress (UBP47 and DNJC9) (Figure 3C) and other 4 proteins with different functions such as vitamin D transport, blood coagulation, urea cycle and chondrocyte differentiation (VTDB, ORNT1, F13B and CHADL, respectively) were found altered in sustained albuminuric patients (SA) (Figure 3D). Moreover, ROC curves have shown a good sensitivity and specificity (AUC =1) indicating that these panels of proteins could discriminate sustained albuminuric patients from normoalbuminurics with a significant classification value ($p=1.07 \bullet 10^{-5}$) (Figure 4B).

DISCUSSION

Over the past 20 years the proteomic evolution has included a better understanding of the need to consider cardiovascular proteomics that resolve, identify and elucidate cardiovascular disease process.[19] Regarding this, we performed 2 complementary proteomic approaches to analyze plasma in order to search for potential indicators for an adequate risk assessment in hypertensive patients under chronic RAS suppression. With this purpose, we analyzed 3 groups of patients: N, dnA and SA with a different response to RAS suppression, therefore exploring underlying mechanisms of albuminuria development associated to inefficient therapeutic response. We have found a total number of 48 differentially expressed proteins, of which 17 were subsequently confirmed by SRM in an independent cohort of 105 patients. The proteomic alterations observed has allowed us to molecularly characterize several processes involved in albuminuria development and hence associated with cardio-renal risk (Figure 5).

The great majority of these 17 proteins are involved in immune response, inflammation and ERS, pointing to the activation of these processes in albuminuric patients. Moreover ROC curves, of great utility for evaluating diagnostic tests, have shown good sensitivity and selectivity for these protein markers, which prove the potential value of these indicators to classify hypertensive patients according to albuminuria onset.

A panel of 4 proteins (CFAB, IGKC, IGHA2 and HEMO) has shown alterations in plasma levels from patients who developed *de novo* albuminuria, compared to those remaining normoalbuminurics. Therefore, these proteins might have a potential predictive value for the development of albuminuria, which is furthermore enhanced by the ROC curve comparing dnA vs. N (Figure 4A). CFAB, a component of innate response, is the only protein that exclusively appears in the group of *de novo* albuminuria and its basal levels are subsequently recovered in sustained albuminuric patients. Previous studies have reported that innate

immunity is activated in early stages of hypertension and end-organ damage.[20] Similar findings have been described in different types of renal disease.[21,22] Analogous mechanisms may account for the observed increase of CFAB in plasma from *de novo* albuminuric patients.

On the other hand, we have found a different proteomic signature made up of 16 proteins, indicators of sustained albuminuria, whose levels may be related to persistent chronic organ damage. It is important to note that 3 out of these 16 proteins were already found altered in dnA patients (IGKC, IGHA2 and HEMO). These data show the augment of 2 immunoglobulins at an earlier stage of the pathogenesis which remained altered in more advanced stages, indicating that their up-regulation persists after the development of albuminuria. In total, we found higher levels of 5 immunoglobulins in the albuminuric patients, which could reflect intraglomerular inflammatory conditions contributing to an increase in glomerular permeability allowing the passage of albumin into the urine in such (a significant decrease of eGFR in patients is shown in Table 2). Similar increases in permeability should be taking place simultaneously in systemic vasculature.[23] A greater activation of this immune response involving LV302, IGHG2 and IGHG3 is observed and probably participates in albuminuria persistence. In fact, previous studies with chronic kidney disease are lined with our results describing an activation of immune cells associated with impaired renal function in such patients.[24] In addition, serum free light chains (FLCs) such as IGKC and LV302 have been associated with renal failure due to its accumulation in plasma as a result of inefficient clearing by the kidneys leading to GFR decrease.[25] Furthermore, the immune system plays a critical role in modulating renal injury through activation of cytokines and mediators of inflammation.[26]

Even more, a subset of 5 proteins (A2GL, SAMP, NALP9, HEMO, and PON1) related with inflammation processes has also been found altered in patients with sustained albuminuria, which seems related to the presence of persistent renal damage inferred by the observed significant decrease of eGFR (Table 2). These findings may suggest that, while kidney damage progresses, an enhanced inflammatory process is triggered leading to an alteration of the levels of these proteins observed in plasma that simultaneously contribute to progression of systemic atherosclerosis.[27-30] In addition, HEMO plays an important antioxidant role[31,32] and a decrease of this protein could participate worsening the endothelial dysfunction present in these patients, therefore increasing cardiovascular risk and renal damage. In fact, our previous studies showing an increased oxidative damage in albuminuric patients support this finding.[14]

Another group consisting of two proteins (UBP47 and DNJC9) has been involved in the proteasome activation in situations of endoplasmic reticulum stress (ERS). In the progression of CKD, albumin overload leads to ERS in the kidney, which has been associated with apoptosis of proximal tubular cells.[33] A defective proximal reabsorption of albumin can be invoked and explained through this proximal tubular damage.[34] Altered levels of UBP47and DNJC9 in SA patients point to the activation of a defense mechanism to counteract ERS, responsible for the degradation of misfolded proteins by the proteasome. In fact, DNJC9, which appears up-regulated in SA, is involved in the ubiquitination tagging of misfolded proteins driving their degradation by the proteasome, while UBP47 is a deubiquitinase, therefore conducting the opposite process to that of DNJC9, whose decrease favors as well proteasome-mediated degradation. The activation of the ubiquitination-proteasome system (UPS) may not be able to compensate ERS, which has been shown to lead to glomerular injury triggered by the activation of apoptotic pathways in the kidney as a consequence of

stress conditions.[35] ROC curve for UBP47 and DNJC9 has shown a perfect classification of SA with an AUC of 1 (Figure 4 B3), and considering that these proteins had never been before detected in plasma, this result points to a great potential of these 2 novel plasma proteins for monitoring renal function in hypertensive patients.

The hereby reported results show that hypertensive subjects who develop albuminuria under chronic RAS suppression exhibit an early activation of the immune response reflected by the increase of 2 immunoglobulins (IGHA2 and IGKC).With the evolution to sustained albuminuria, a greater immune activation is observed, since elevated levels of these 2 immunoglobulins together with 3 more: IGHG2, IGHG3 and LV302 were detected, which might be involved in the progressive renal damage observed. In addition, ROC curve value for this subset of immunoglobulins showed a correct classification of sustained albuminuric patients with respect to normoalbuminurics.

In this sense, our results are in line with previous studies pointing to a determinant role of the immune response in the pathogenesis of hypertension[36] and also provide additional molecular data showing particular immunoglobulins altered with albuminuria. Thus, the up-regulation of proteins involved in the immune system in patients with albuminuria could elucidate specific molecular pathways involved in the pathogenesis of organ damage, as well as support a hypothesis with increasing approval in the scientific community pointing to the great potential of a future strategy based on anti-immune therapy to treat hypertension.[37] In fact, hypertensive rats treated with this therapy showed increased GFR, decreased urinary protein excretion,[38] as well as reduced blood pressure, inflammation and ROS[39] which supports its potential to prevent the development of albuminuria and hence the progression of kidney damage.

In this work we show the great potential of plasma proteomics to discover novel indicators of early albuminuria and end-organ damage in hypertensive patients under RAS suppression and provide new proteins present in plasma together with a subset of promising markers of albuminuria to the high AUC values observed. Some limitations of our study are the use of pools for the proteomic analysis and the necessity to increase the number of patients in order to confirm the potential value of the hereby described protein indicators. Moreover, a prospective study will be required to determine the clinical utility of these proteins as potential biomarkers which could allow distinguishing two stages in the evolution of albuminuric patients chronically RAS suppressed. Strategies to stimulate reverse translation from clinical observations to the bench and to better merge clinical and basic science projects to facilitate translation are also needed.[19]

PERSPECTIVES

Our goal was identified molecular signatures able to elucidate specific molecular pathways involved in the pathogenesis and may provide predictors and chronic organ damage indicators in albuminuric patients during chronic renin-angiotensin suppression. More importantly, proposed markers allow early identification of patients at cardiovascular risk when clinical predictors as albuminuria are still in the normal range and this makes possible to adapt, better and earlier, the pharmacological intervention for those individuals at higher risk. According to these considerations, the key point of our study is that we provide the discovery of 2 different protein profiles which could allow distinguishing two stages in the evolution of albuminuric patients. Furthermore, these results open the possibility of a future strategy based on anti-immune therapy to treat hypertension which could help to prevent the development of albuminuria and hence, the progression of kidney damage.

AKNOWLEDGEMENTS AND SOURCES OF FUNDING

This work was supported by grants from the Instituto de Salud Carlos III (FIS PI070537, IF08/3667-1, PI11-02239, PI14/01917, PI11/01401, PI11/02432, PI13/01873, PI13/01746, PI13/01581, PI14/01650, PI14/01841) by PT13/0001/0013, PIE13/00051, PIE13/00045 , CP09/00229, IDCSalud (3371/002) Fundación Conchita Rábago de Jiménez Díaz, Fundación Mutua Madrileña and Redes Temáticas de Investigación Cooperativa (FONDOS FEDER, RD06/0014/1015, RD12/0042/0071). The drawings have been obtained by "*100 viñetas sobre hypertension, 2013. Daiichi Sankyo*", which is an original idea of this company for hypertension publications. These results are lined up with the Spanish initiative on the Human Proteome Project (SpHPP).

DISCLOSURES

DrRuilope has served as advisor/ speaker for Astra-Zeneca, Bayer, Daiichi-Sankyo, Medtronic, Novartis, Pfizer, Relypsa, Sanofi, Takeda.

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Novelty and Significance:

1) **What Is New**

The combination of two complementary proteomic approaches to analyze plasma has identified new potential predictors of renal damage and *de novo* albuminuria indicators. These protein signatures could allow us to monitor albuminuria in hypertensive patients with suppression of renin angiotensin system.

2) **What Is Relevant?**

The main question is that evolution of hypertensive patients who are chronically treated to control blood pressure is unpredictable in terms of albuminuria development. Thus, this is a clinical question of extreme importance and there are no current studies on it apart from previous research from our group[14,15,16]. The proteomic analysis has allowed identifying two proteomic signatures involved in the development of *de novo* and sustained albuminuria, which are related to inflammation, immune response and endoplasmic reticulum stress. The relevance of our findings is that this strategy may contribute to adequate risk assessement and earlier intervention and better outcome of these patients.

Summary

High prevalence of albuminuria in patients chronically RAAS suppressed has been demonstrated, and currently there are no available markers able to predict evolution of these chronically treated patients. Our strategy allowed us to perform one of the deepest plasma proteomic analysis so far which has shown two proteomic signatures: l) with predictive value of *de novo* albuminuria and 2) sustained albuminuria indicator proteins.

FIGURES LEGENDS:

FIGURE 1:- **(A)** Experimental design based on –omics strategy to approach the unbiased, non-targeted study of albuminuria in hypertensive patients searching for predictors and chronic organ damage markers in blood plasma. **(B)** Schematic representation of the clinical monitoring of the 1433 patients during 3 years in the Hypertension Unit of Hospital 12 de Octubre, Madrid, Spain. The protocol includes a baseline study followed by 3 month period of stabilization. Patients were subsequently followed during 3 years with visits to the unit every 6 months. The proteomics analysis started at the end of this period.

FIGURE 2:- A) Schematic picture of differentially expressed proteins identified by 2D-DIGE and iTRAQ and those validated by SRM. B) Principal component analysis where different groups were separated based on the 17 differentially expressed proteins validated by SRM. C) Two different plasma signatures, one of *de novo* albuminuria and the second of sustained high albuminuria are shown. N: normoalbuminuria; dnA: *de novo* albuminuria; SA: sustained albuminuria.

FIGURE 3:- Validation of 17 proteins by Selected Reaction Monitoring (SRM). A) Panel of proteins related with immune response. B) Proteins involved in inflammation. C) Proteins related with endoplasmic reticulum stress. D) Proteins with other functions. In the bar charts, we have represented the transition of the most significant peptide. N: normoalbuminuria; dnA: *de novo* albuminuria; SA: sustained albuminuria. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, # $p = 0.051$.

FIGURE 4:- Receiver Operating Characteristic (ROC) curves for classification of patients with *de novo* and sustained albuminuria *vs* normoalbuminurics. A) Proteins with predictive value of *de novo* albuminuria. B) Protein markers of sustained albuminuria involved in the

immune system (B1), inflammation (B2), endoplasmic reticulum stress (B3) and proteins involved in other functions (B4). Area under the ROC curve (AUC) with 95% confidence intervals was calculated and *p*-value $\leq$ 0.05 was considered statistically significant. N: normoalbuminuria; dnA: *de novo* albuminuria; SA: sustained albuminuria.

FIGURE 5:- The activation immune system in hypertensive patients with high albuminuria under chronic RAS suppression. Mechanisms involved in the pathogenesis of renal damage and functional implication of observed protein alterations related with immune response, inflammation and apoptosis. The immune-suppression as future therapy could prevent the progression of kidney damage.

Table 1. Biological functions of 17 proteins validated by SRM

Protein name	Abbreviation	Function	P-value		
			dnA/N	SA/N	SA/dnA
Ig gamma-3 chain C	IGHG3	Immune system response		↑ 0.005	↑ 0.008
Ig gamma-2 chain C	IGHG2	Immune system response		↑ $9.29 \cdot 10^{-5}$	↑ 0.014
Ig alpha-2 chain C	IGHA2	Immune system response	↑ 0.028	↑ $3.14 \cdot 10^{-4}$	
Ig kappa chain C	IGKC	Immune system response	↑ 0.014	↑ 0.008	
Ubiquitin carboxyl-terminal hydrolase 47	UBP47	Apoptosis		↓ $6.17 \cdot 10^{-8}$	↓ $2.5 \cdot 10^{-5}$
NACHT. LRR and PYD domains-containing protein 9	NALP9	Inflammation		↑ $4.6 \cdot 10^{-4}$	↑ 0.002
Dna J homolog subfamily C member 9	DNJC9	Apoptosis		↑ $5.87 \cdot 10^{-6}$	↑ $4.24 \cdot 10^{-6}$
Chondroadherin-likeprotein	CHADL	Chondrocyte differentiation		↓ 0.003	↓ 0.002
Ig lambda chain V-III region LOI	LV302	Immune system response		↑ 0.020	
Mitochondrial ornithine transporter 1	ORNT1	Metabolic process			↑ 0.020
Vitamin D-binding protein	VTDB	Lipid binding		↑ $1.62 \cdot 10^{-6}$	↑ $1.09 \cdot 10^{-5}$
Complement factor B	CFAB	Immune system response	↑ 0.012		
Coagulation factor XIII B chain	F13B	Coagulation		↓ $6.2.19 \cdot 10^{-6}$	↓ 0.003
Hemopexin	HEMO	Inflammation	↓ 0.051	↓ 0.040	
Leucine-rich alpha-2-glycoprotein	A2GL	Inflammation		↑ 0.001	↑ $8.23 \cdot 10^{-5}$
Serum paraoxonase/arylesterase 1	PON1	Inflammation		↑ $4.31 \cdot 10^{-7}$	↑ $1.13 \cdot 10^{-5}$
Serum amyloid P-component	SAMP	Inflammation		↑ 0.002	↑ 0.001

N: normoalbuminuria; dnA: *de novo* albuminuria; SA: sustained albuminuria.

Table 2. Demographic data and clinical characteristics of the study population recruited for the validation phase

Variable	N (n=53)	dnA (n=26)	SA (n=26)	P-value
Age (years)	64 ± 11	69 ± 8	66 ± 11	0.264
Male sex (%)	40	70	70	0.005*
BMI (kg/m2)	30 ± 4	30 ± 5	30 ± 4	0.966
Current smoking (%)	9	15	11	0.743
Albumin/creatinine (mg/g)	7.61 ± 6,67	116 ± 126	371 ± 573	0.000006 †
Creatinine clearance rate (mg/mL)	165 ± 38	87 ± 48	80 ± 39	0.405
eGFR (ml/min/1.73m^2)	81 ± 17	68 ± 20	67 ± 26	0.004*
Total cholesterol (mg/dL)	186 ± 29	166 ± 26	168 ± 26	0.003*
Triglycerides (mg/dL)	123 ± 50	137 ± 36	131 ± 71	0.583
HDL cholesterol (mg/dL)	55 ± 12	50 ± 10	47 ± 13	0.018*
LDL cholesterol (mg/dL)	106 ± 27	89 ± 19	97 ± 18	0.010*
Glycaemia (mg/dL)	118 ± 40	127 ± 26	118 ± 33	0.583
Uricacid (mg/dL)	5 ± 2	6 ± 2	7 ± 2	0.000*
Systolicbloodpressure (mmHg)	139 ± 18	136 ± 20	141 ± 28	0.663
Diastolicbloodpressure (mmHg)	82 ± 11	80 ± 10	83 ± 14	0.761
Diabetes (%)	38	62	54	0.107
ACEi (%)	17	23	23	0,737
ARB (%)	77	73	70	0,73
Diuretic (%)	23	35	54	0.022*
Calciumchannelblocker (%)	47	46	61	0.428
Beta blockingagent (%)	26	35	27	0.733
Alphablockingagent (%)	17	35	19	0.191
Anticoagulantagent (%)	40	50	31	0.366
Lipidloweringagents (%)	74	69	81	0.627
Antidiabeticagent (%)	26	42	38	0.302

Values expressed as mean ± SD or percentages (%). BMI: body mass index; eGFR: estimated glomerular filtration rate; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; ACEi: angiotensin converting enzyme inhibitors; ARB: angiotensin receptor blockers. N: normoalbuminuria; dnA: *de novo* albuminuria; SA: sustained albuminuria. Statistical significance was accepted at *$p < 0.05$, †$p < 0.01$.

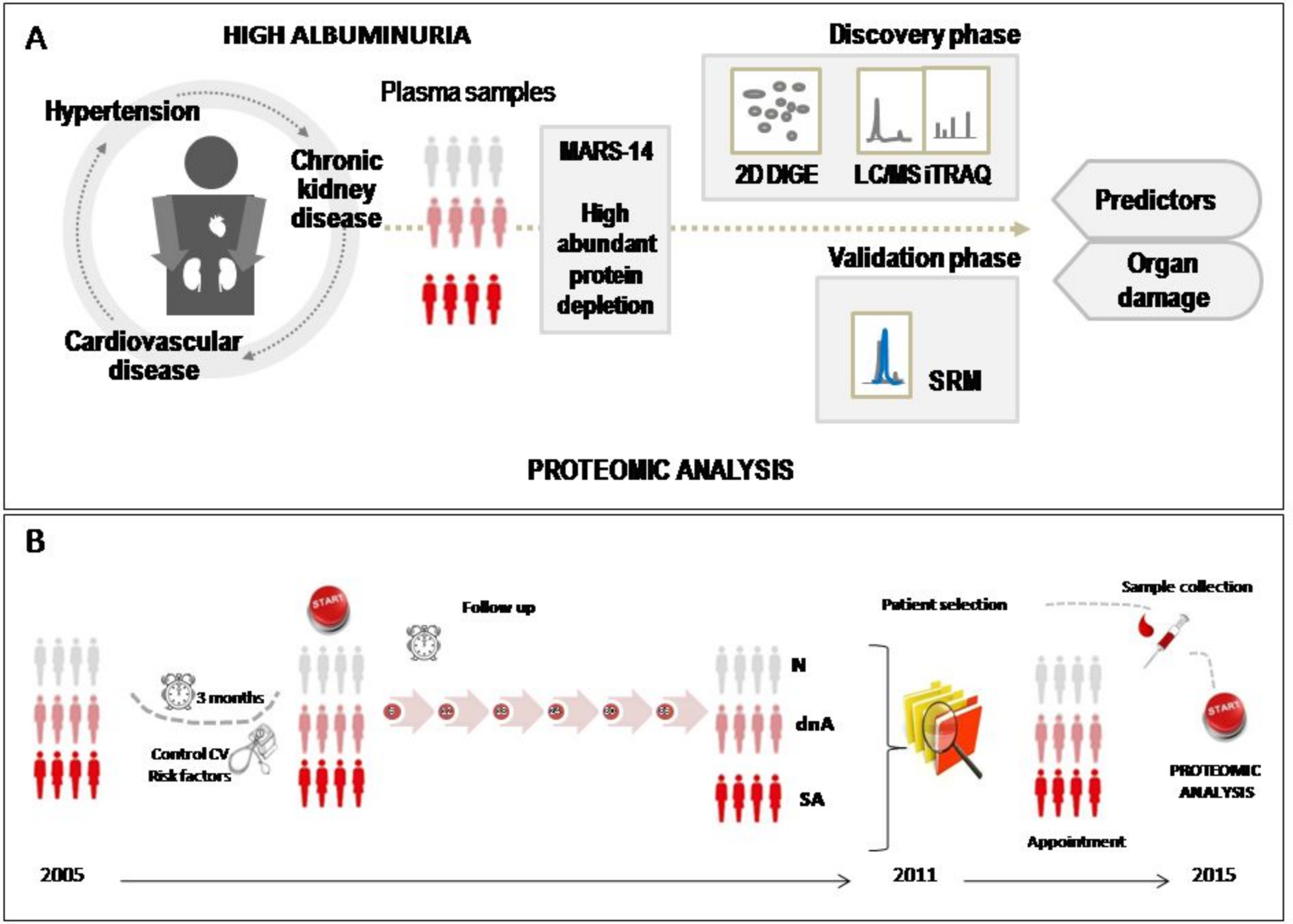

A
HIGH ALBUMINURIA
Hypertension
Chronic kidney disease
Cardiovascular disease
Plasma samples
MARS-14
High abundant protein depletion
Discovery phase
2D DIGE
LC/MS iTRAQ
Validation phase
SRM
Predictors
Organ damage
PROTEOMIC ANALYSIS
B
START
Follow up
3 months
Control CV Risk factors
N
dnA
SA
Patient selection
Sample collection
START
PROTEOMIC ANALYSIS
Appointment
2005
2011
2015

FIGURE 1

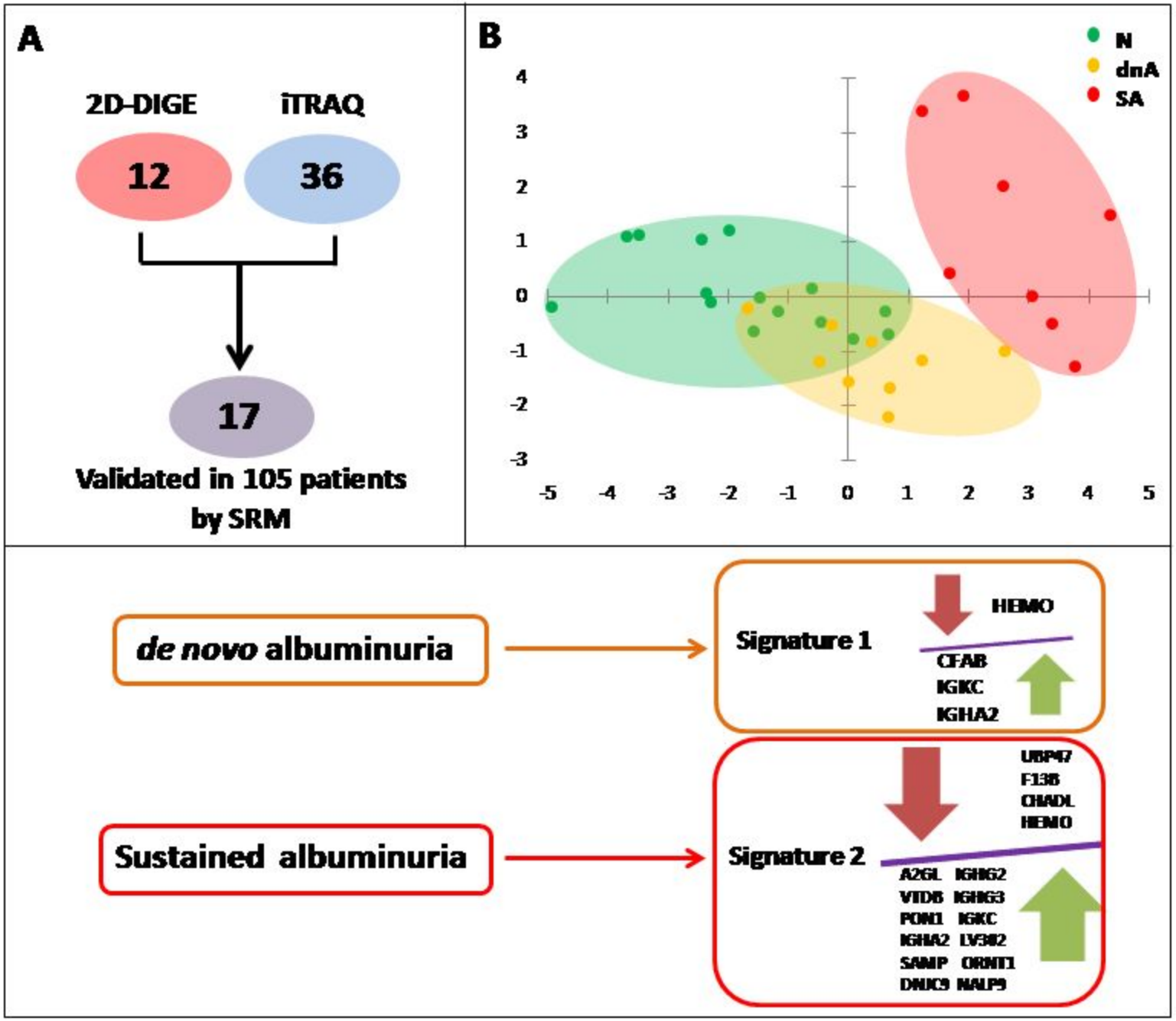

A
2D-DIGE
iTRAQ
12
36
17
Validated in 105 patients
by SRM
B
N
dnA
SA
4
3
2
1
0
-1
-2
-3
-5
-4
-3
-2
-1
0
1
2
3
4
5
de novo albuminuria
Signature 1
HEMO
CFAB
IGKC
IGHA2
Sustained albuminuria
Signature 2
UBP47
F136
CHADL
HEMO
A2GL IGHG2
VTDB IGHG3
PON1 IGKC
IGHA2 LV302
SAMP ORNT1
DNJC9 NALP9

FIGURE 2

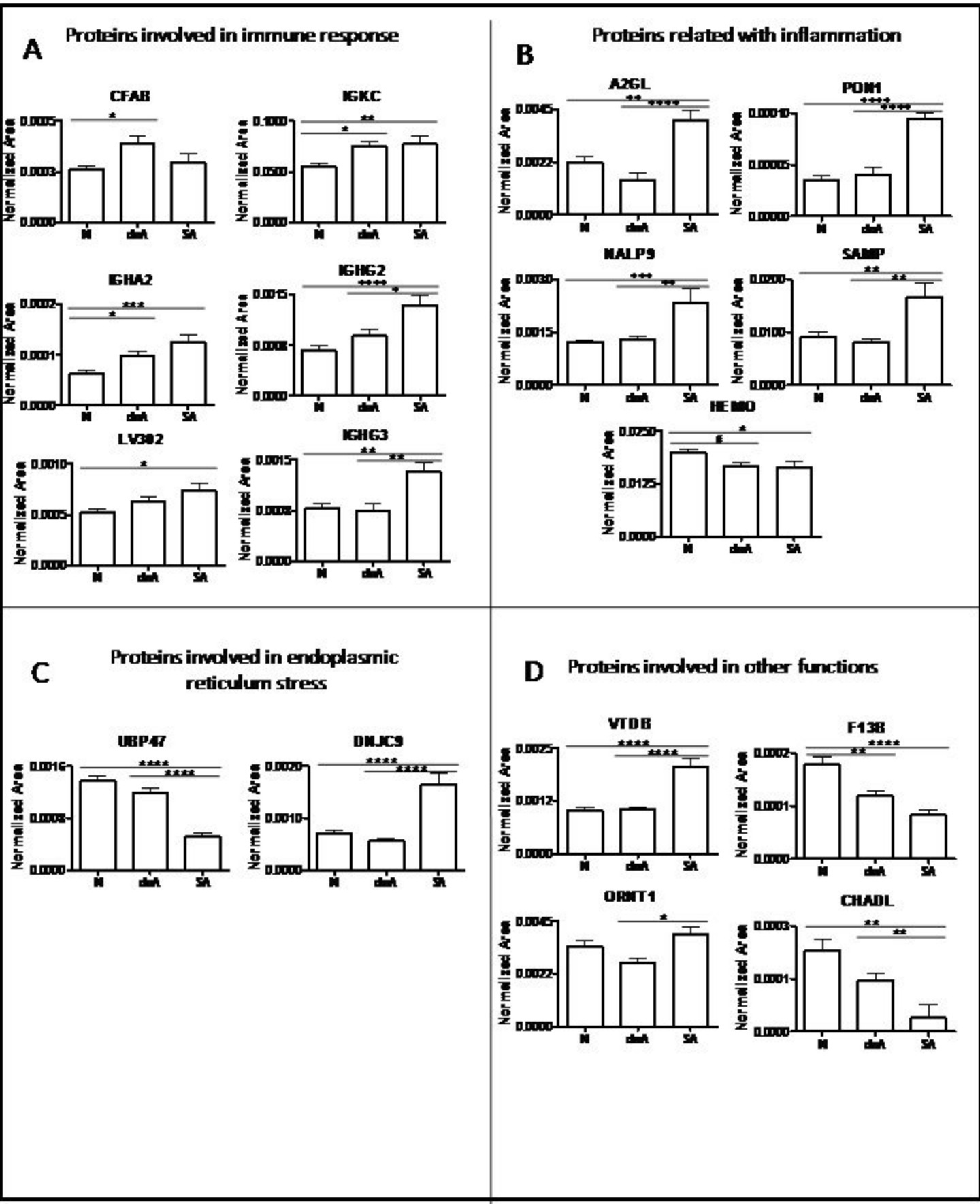
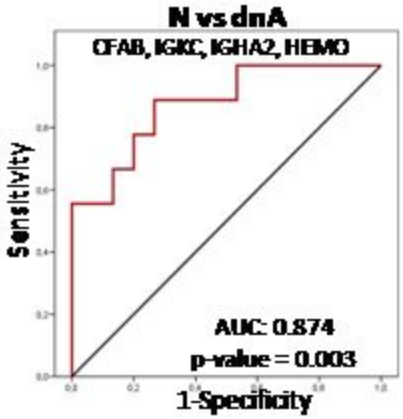
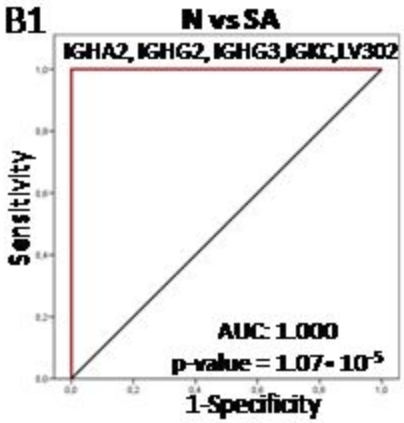

A
Proteins involved in immune response
CFAB
IGKC
IGHA2
IGHG2
LV302
IGHG3
B
Proteins related with inflammation
A2GL
PON1
NALP9
SAMP
HEMO
C
Proteins involved in endoplasmic reticulum stress
UBP47
DNJC9
D
Proteins involved in other functions
VTDB
F13B
ORNT1
CHADL
Normalized Area
N
daA
SA

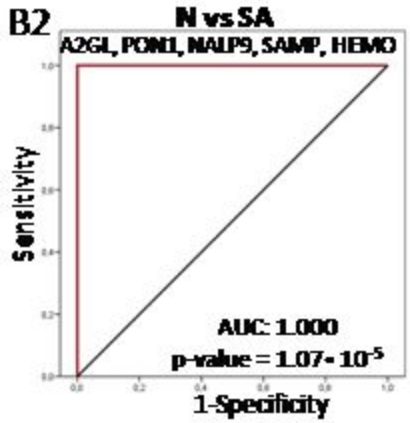
FIGURE 3

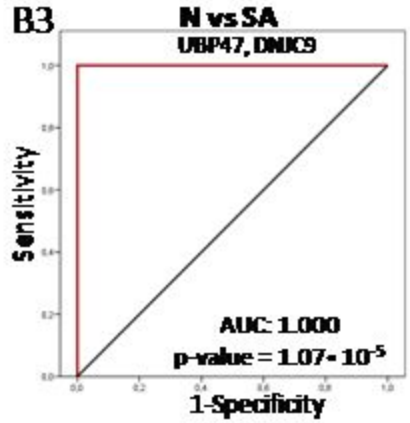
A Predictive value of *de novo* albuminuria



B Sustained albuminuria indicator proteins







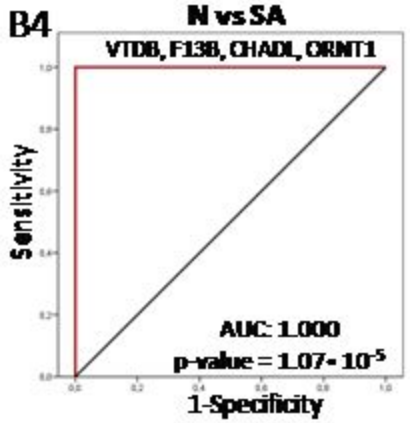


FIGURE 4

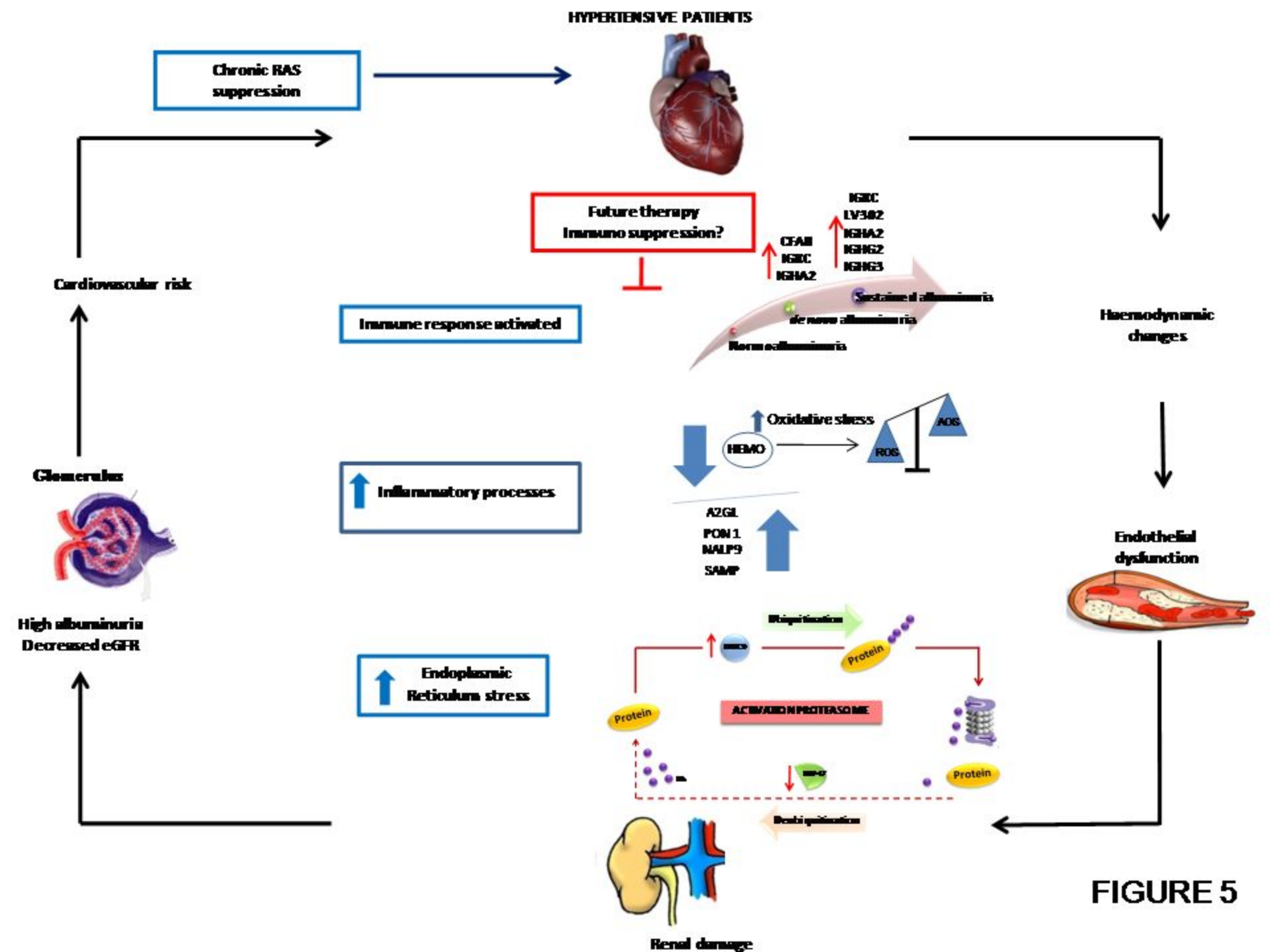

HYPERTENSIVE PATIENTS
Chronic RAS suppression
Future therapy Immuno suppression?
CFAH
IGEC
IGHA2
IGEC
LV302
IGHA2
IGHG2
IGHG3
Cardiovascular risk
Immune response activated
Sustained albuminuria
de novo albuminuria
Normoalbuminuria
Haemodynamic changes
Oxidative stress
HEMO
ROS
AOS
Glomerulus
Inflammatory processes
A2GL
PON 1
NALP9
SAMP
Endothelial dysfunction
High albuminuria
Decreased eGFR
Protein
ACTIVATION PROTEASOME
Protein
Endoplasmic Reticulum stress
Renal damage

FIGURE 5