URINE HAPTOGLOBIN AND HAPTOGLOBIN-RELATED PROTEIN PREDICT RESPONSE TO SPIRONOLACTONE IN RESISTANT HYPERTENSION PATIENTS

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ABSTRACT

Resistant Hypertension prevalence is progressively increasing and prolonged exposure to suboptimal blood pressure control results in higher cardiovascular risk and end-organ damage. Among various antihypertensive agents, spironolactone appears the most effective choice to treat resistant hypertension once triple therapy including a diuretic fails. However success in blood pressure control is not guaranteed, adverse effects are not negligible and no clinical tools are available to predict patient's response. Complementary to our previous study of resistant hypertension metabolism, here we investigated urinary proteome changes with potential capacity to predict response to spironolactone. Twenty-nine resistant hypertensives were included. A prospective study was conducted and basal urine was collected prior to spironolactone administration. Patients were classified in responders or non-responders in terms of blood pressure control. Proteins quantitation was carried out by liquid chromatography- mass spectrometry; ELISA and target mass spectrometry analysis were performed for confirmation. Among 3310 identified proteins, Haptoglobin and Haptoglobin-related protein showed the most significant variations, with increased levels in non-responders compared to responders prior to drug administration (variation rate 5.98 and 7.83, respectively). Protein coordinated responses were also evaluated by functional enrichment analysis, finding oxidative stress, chronic inflammatory response, blood coagulation, complement activation and regulation of focal adhesions as physiopathological mechanisms in resistant hypertension. In conclusion, protein changes able to predict patients' response to spironolactone in basal urine were here identified for the first time. These data, once further confirmed, will support clinical decisions on patients' management while contributing to optimize the rate of control of resistant hypertensives with spironolactone.

KEYWORDS: Resistant Hypertension, Blood Pressure, Spironolactone, Proteomics, Haptoglobin, Haptoglobin-related protein.

INTRODUCTION

Resistant hypertension (RH) is defined as blood pressure (BP) above target despite the concurrent use of three antihypertensive drugs, including a diuretic, a renin-angiotensin inhibitor and a calcium channel blocker, prescribed at optimal doses. The prevalence of RH, including pseudoresistant hypertension, is about 13% of hypertensive adults estimated with a threshold of 140/90 mmHg or higher[1]. The consequent increase in cardiovascular disease (CVD), end-stage renal disease (ESRD) and death is about 2- to 6-fold compared to non-resistant hypertensives[2]. These data highlight the importance of an appropriate management of RH. Spironolactone is an aldosterone antagonist to treat RH, being able to decrease BP in 22/10 mmHg (ASCOT study)[3] or 9.8/1.0 mmHg (ASPIRANT study)[4]. It has been strongly demonstrated as the most suitable fourth drug to be added to the commonly prescribed triple antihypertension therapy. The ASPIRANT and ASPIRANT EXTENSION[4, 5] trials first, and the PATHWAY-2 study[6] later, showed that spironolactone was more effective than placebo or other BP-lowering drugs and the best fourth drug option[7]. Non-pharmacological treatment have been also evaluated, mainly renal denervation (RD), concluding again on the superiority of spironolactone administration[8, 9]. However, success in BP control by spironolactone therapy is not guaranteed, adverse effects are not negligible (e.g. gynecomastia, hyperkalemia) and no clinical tools are available to predict which patients will show a positive response in terms of BP diminishment. Physiopathology behind RH development is greatly unknown and studies at molecular level are scarce. By evaluating patients' response to different pharmacological treatment, salt retention was recently proposed as the physiopathological condition behind RH which could explain spironolactone positive action through mineralocorticoid receptor blockade[10, 11]. Recently, we identified metabolic alterations subjacent to RH detectable in urine[12]. The

citric acid cycle (CAC) was revealed as the most significantly altered pathway, and a specific metabolic signature composed by oxaloacetate, malate, citrate and α-ketoglutarate showed increased levels in RH non-responder patients compared to responders. Interestingly, these four urinary metabolites showed altered levels in urine collected prior to spironolactone administration, thus demonstrating a predictive capacity in patient's response. Complementary to these previous data, here we investigate molecular changes also in basal urine (prior to drug administration) at the proteome level subjacent to RH with potential capacity to predict response to spironolactone.

METHODS

The authors declare that all supporting data are available within the article and its online supplementary files.

Patient selection

We enrolled a total of 29 hypertensive patients arriving at the Hypertension Unit Hospital 12 de Octubre. They were considered as RH patients if they fulfilled the following entry criteria: age ≥18 years and BP levels with mean values on a 24-hour ABPM >130/80 mmHg while receiving ≥3 drugs at adequate doses. A 24-hour ABPM was performed at baseline to confirm true RH. Patients with secondary hypertension were excluded. A prospective study was conducted, aimed to predict response to spironolactone at protein level. Urine samples were collected prior to spironolactone administration (basal samples). RH patients were then treated with 25/50 mg/day of spironolactone, added on top of former antihypertensive treatment. According to patients' response to spironolactone in terms of BP control and not based on modulation on particular protein alterations, patients were classified in responders (RHr) and non-responders (RHnr). Responders were those patients whose 24-hour ambulatory systolic

BP dropped ≥20 mmHg (or attaining systolic BP levels ≤130 mm Hg post-spironolactone). In Table 1, clinical characteristics of patients are shown. The study was conducted according to the recommendations of the Declaration of Helsinki, it was approved by the local ethic committee and all subjects gave informed consent. Urine samples were processed and stored as described before[13-15].

BP Measurements

BP was measured at the office with a validated semiautomatic oscillometric device, after 5-minute rest in a sitting position. BP values were estimated as the mean of three readings. Thereafter, 24-hour ABPM was performed using PaceLabs 90207 (SpaceLabs Inc.; Redmond, WA, USA) automated noninvasive oscillometric device, programmed to register BP at 20-minute intervals for the daytime period and at 30-minute intervals for the night-time period. The majority of measurements were performed on working days, and the patients were instructed to maintain their usual activities, return the following morning for device removal, and keep the arm extended and immobile at the time of each cuff inflation. Daytime and night-time periods were defined individually according to the patient self-reported data of going-to-bed and getting-up times.

Protein alterations in RH: TMT-LC-MS/MS quantitative analysis of the urine proteome

Protein digestion and peptide isobaric labelling

Massive protein identification and proteomics analysis of urine samples were performed by liquid chromatography on-line coupled to mass spectrometry (LC-MS/MS) using isobaric labeling. Tandem Mass Tag™ (TMT™, Thermo Fisher) kit contains different isobaric compounds with the same mass and chemical structure to label urinary peptide samples in parallel. For each sample, a unique reporter mass is used to measure relative protein expression levels during peptide fragmentation and tandem mass spectrometry.

In detail, urine proteins were first subjected to tryptic digestion using the FASP protocol as previously described[16] with minor modifications. Proteins were digested with modified trypsin (Promega) using a ratio 30:1 protein:trypsin (w/w). The resulting peptides were desalted onto C18 Oasis-HLB cartridges and dried-down for further analysis. For stable isobaric labelling, the peptides were dissolved in 100 mM Triethylammonium bicarbonate (TEAB) buffer, and the peptide concentration was determined by measuring amide bonds with the Direct Detect system (Millipore). Equal amounts of each peptide sample were labelled using the 10-plex TMT Reagents according to manufacturer's protocol. For increasing proteome coverage, TMT-labelled samples were fractionated by high-pH reverse phase chromatography (High pH Reversed-Phase Peptide Fractionation Kit, Pierce) before analysis.

Peptide analysis by liquid chromatography and mass spectrometry in tandem (LC-MS/MS)

Labelled peptides were analysed by LC-MS/MS using a C18 reversed phase nano-column (75 µm I.D. x 50 cm, 2 µm particle size, Acclaim PepMap RSLC, 100 C18; Thermo Fisher Scientific) in a continuous acetonitrile gradient on an Orbitrap Fusion mass spectrometer (Thermo Fisher). An enhanced FT-resolution spectrum (resolution=70,000) followed by the MS/MS spectra from the most intense parent ions were analysed along the chromatographic run. Dynamic exclusion was set at 40s.

Peptide identification

For peptide identification, all spectra were analysed with Proteome Discoverer (version 2.1.0.81, Thermo Fisher Scientific) using SEQUEST-HT (Thermo Fisher Scientific). For database searching, the Uniprot database containing all sequences from human and contaminants (May 14th, 2016; 70611 entries) was used. Peptide identification was performed using the probability ratio method and false discovery rate (FDR) was

calculated using inverted databases and the refined method[17] with an additional filtering for precursor mass tolerance of 15[18]. Identified peptides had a FDR equal or lower than 1% FDR.

Statistical analysis TMT experiment

Statistical analyses were performed using the WSPP statistical model previously described[19]. In this model protein log2-ratios are expressed as standardized variables, i.e., in units of standard deviation according to their estimated variances (Zq values). Proteins identified with < 3 peptides and those not showing homogeneous Zq values within each group were discarded. Protein abundance changes between groups were calculated by comparing their Zq mean values for each protein (RHr *versus* RHnr).

Confirmation of protein variations in RH: target analysis by ELISA and SRM

Individual urine samples from the 42 hypertensive patients were analysed by commercial ELISA assays and by Selected Reaction Monitoring (SRM-LC-MS/MS). For ELISA analysis, manufactures' protocols were followed and optimal conditions were established for urine samples analyses (see Supplementary Table S1). SRM-LC-MS/MS was performed as previously published[13-15]. Briefly, tryptic digest from 30µg total protein were analyzed in a 6460 Triple Quadrupole LC/MS/MS on-line connected to nanoLC in a Chip-format configuration (ChipCube interface, Agilent Technologies) and 1200 Series LC Modules (Agilent Technologies). Peptide separation was carried out onto a ProtID Zorbax 300B-C18-5 µm chip with 43 × 0.075-mm analytical column and 40nL enrichment column (Agilent Technologies). Two microliters of sample were injected at 3µL/min and separation took place at 0.4 µL/min as follow: 1) 0-3 min 5% B, 2) At 10 min 70% B, 3) At 12 min 95% B, 4) At 14 min 95% B, 5) 14.2-16 min 5% B (A= 0.1% formic acid in double-distilled water; B = 0.1% formic acid in acetonitrile). The fragmentor was set to 130 V, dwell time to 20 ms, delta EMV to 600 V, and

collision energy was optimized for each SRM transition (see Supplementary Table S2). The system was controlled by Mass Hunter LC-MS Acquisition Software (v4.01). Individual signals were normalized based on urinary creatinine and normalized peak areas were calculated for inter-group comparison in both cases, ELISA and SRM analysis.

Statistical analysis

Statistical analyses were performed by means of GraphPad Prism 6 (version 6.01) software. The ROUT method was applied to detect outliers based on the False Discovery Rate (FDR), setting Q value to 5%. Mann-Whitney non-parametric test (95% confidence level) was performed. For those proteins showing alteration between RHr and RHnr, a multivariate analysis (combined ROC curve) was performed to evaluate their predictor potential using Metaboanalyst webserver[20, 21]. The multivariate ROC curve was obtained using Random Forest algorithm as built-in method.

Functional enrichment analysis

We applied the functional enrichment method FatiGO[22], as implemented in the Babelomics platform v5[23], to the lists of up-regulated proteins (FDR<0.05 and FC>2) in each (RHr and RHnr) conditions. Gene Ontology was used for the protein annotation. GO terms with FDR<0.05 were taken as significantly over-represented. GO terms were represented with their ontological relationships using own scripts and the cytoscape tool[24].

RESULTS

Prediction of RH patients' response to spironolactone treatment

Spironolactone reduced systolic blood pressure in responders by -21.4 mm Hg (95% CI -26.6 to -16.1) compared with a reduction of -2.6 mm Hg in non-responders (95% CI -11.9 to 6.6). We investigated significant protein alterations able to identify those RH

patients who will respond to spironolactone. For that purpose, quantitative proteomics was accomplished in basal urine samples (i.e. those collected before treatment). Once known patient's clinical response, individuals were classified in RH responders or those remaining with uncontrolled BP (non-responders). Table 1 compiles patient's clinical characteristics. No significant variation between RHr and RHnr was observed in presence of diabetes, smoking habits, previous CVD, duration of hypertension, cholesterol or concomitant therapies, among others.

In a first discovery stage urine samples were submitted to protein digestion and resulting peptides were isobaric labelled by TMT methodology and analysed by LC-MS/MS. Among the 3310 identified proteins, the most significant variations were found for Haptoglobin-related protein (HPR) and Haptoglobin (HP) showing increased levels in basal urine of RHnr compared to RHr (variation rate of 7.83 for HPR, and 5.98 for HP). For confirmation, ELISA assays were performed for absolute protein quantitation (Figure 1 A-C) finding 163.6±146.3 µg HP/g creatinine and 8.5±6.6 µg HPR/g creatinine for RHr, and 430.4±387.7 µg HP/g creatinine and 34.5±35.5 µg HPR/g creatinine for RHnr (p value = 0.0046 for HP and p value = 0.0051 for HPR). Data confirmed increased values for both proteins in non-responders (Table 2). Sensitivity and specificity were evaluated by means of univariate and multivariate ROC curve, resulting in AUC = 0.74 for HP (Figure 1A) and 0.67 for HPR (Figure 1B). Increased performance was obtained when combined both potential predictors, resulting in AUC value of 0.81 (Figure 1C).

As alternative to conventional ELISA assays of great potential in the clinical setting in terms of cost-effectiveness, easy of automatization, and high-throughput, we developed a target mass spectrometry assay based on SRM methodology. SRM methodology allows significantly shortening the analysis time compared to non-mass spectrometry-

based approaches. The best-performing analysis conditions for each protein are detailed in Supplementary Table S2. SRM analysis (target approach) confirmed again the variation found in the discovery stage (untargeted) with p value = 0.024 for HP and p value = 0.0479 for HPR (Figure 1D-F). In this case, AUC values were 0.80 for HP (Figure 1D), 0.75 for HPR (Figure 1E) and 0.82 when combined HP and HPR (Figure 1F). Best performance was obtained when combined HP and HPR proteins, for both ELISA and SRM assays, and, individually, SRM performed better than ELISA for HP and for HPR. Thus, SRM-LC-MS/MS represents a real alternative in clinical routine, with reduced analysis time and superior performance than ELISA for HP and HPR. A positive correlation with ACR was found for both HP (r=0.3735, p=0.0017) and HPR (r=0.5684, p=0.0021). When adjusted the potential influence of baseline characteristics as DBP, pulse pressure, eGFR, age or aldosterone/renin ratio (ARR), the predictability of HP and HPR is still significant (see Supplementary Table S3).

Coordinated behaviour of proteins in RH

The 3310 proteins identified in the discovery stage are compiled in (see Supplementary Table S4). Coordinated responses of up-regulated proteins either in RHr or in RHnr were evaluated by functional enrichment analysis. Single Enrichment Method was applied considering altered proteins with ratio of change ≥ 2.0 and compared with the rest of the genome. Figures 2 and 3 show Gene Ontology (GO) terms over-represented in RHr (up-regulated genes in RHr) or RHnr (up-regulated genes in RHnr), respectively. Supplementary Tables S5 and S6 compile the GO terms identified (FDR adjusted p value <0.05) and proteins included in each GO term. As can be seen in Figure 2, biological processes significantly over-represented in RHr point to cellular response to oxidative stress, NADP/H metabolic process, chronic inflammatory response, glucose catabolic process and regulation of NF-KappaB transcription factor, among others.

Those changes involve alterations in the proteins: ANXA1, CAT, HBA1, HBB, MPO, PGD, PLA2G2A, PRDX2, S100A12, S100A7, S100A8, S100A9, TALDO1 and TXN. In RHnr, over-represented proteins are AHSG, ALB, C8G, C9, CFB, FTL, HP, HPX, HRG, KDR, MLLT4, MUC6, NAPSA, ORM1, ORM2, PIP, PLG, SERPINA1 and TF, and point to regulation of blood coagulation, complement activation, acute inflammatory response, immune response, VEGF signalling pathway, iron homeostasis and regulation of focal adhesions as main subjacent physiopathological mechanisms (Figure 3).

DISCUSSION

Haptoglobin and haptoglobin-related protein were the most significant changes identified in the protein pattern (with the highest rate of variation), being able to distinguish between responders and non-responders in basal urine, i.e. before spironolactone had been administered. HP and HPR have been mainly described in plasma and it is known that circulating HP is 10-fold higher than HPR[25, 26]. As shown here (Figure 1A, B), this ratio is conserved in urine. Previous studies proposed HP to creatinine ratio as predictor of nephropathy complications in Type 2 diabetic patients[27]. Here we show, for the first time, its added value in predicting spironolactone response of RH patients. Both proteins share approximately 91% of their sequences and have high affinity for free haemoglobin (Hb) leading to HP-Hb and HPR-Hb complexes. However, these complexes present a significant difference in binding preferences after formation. HP-Hb present high affinity for the scavenger receptor CD163 while HPR-Hb complex has higher affinity for HDL particles[26]. These affinities differently contribute to immune response. The link between HTN and immune response was

previously shown by our group specifically in patients developing albuminuria under RAS inhibition[14, 28]. Here we show its activation in RH.

Pathological release of haemoglobin (Hb) in the blood stream triggers pro-inflammatory and oxidative injury. Several studies demonstrated a positive correlation between Hb levels and hypertension, and even between Hb and BP rise in healthy individuals, both men and women[29, 30]. Haptoglobin (HP) is a circulating glycoprotein and the primary Hb scavenger by irreversible binding, thus eliminating free Hb and neutralizing the oxidative damage[31, 32]. Hypertensive and oxidative effects induced by free Hb were reduced by HP administration or endogenous synthesis stimulation[33]. As acute phase protein, plasma HP was higher in coronary artery disease[34] and it was also found increased in plasma from hypertensive patients developing albuminuria by our group[35]. In agreement, urine HP level was shown to predict type 2 diabetic patients at risk of renal function decline prior to macroalbuminuria development[27], which was further corroborated while demonstrating the HP improved predictive performance compared to albuminuria[36]. Here we demonstrate a predictive capacity for HP and HPR in terms of spironolactone response when administered to RH patients. Similarly to albumin, an increased glomerular permeability would result in the observed higher levels of HP in urine from patients who do not respond to spironolactone. In this sense, both HP and HPR positively correlate with ACR. The fact that HP and HPR correlate with albuminuria indicates that an early damage in renal function, namely increased permeability of glomerular barrier, is present in those who do not respond to spironolactone. This indicates that this form of early renal damage is related to the poor response of BP to spironolactone in RH. More advanced degrees of renal damage have been shown to prevent the response to anti-hypertensive therapy in RH[37]. On the other hand, some authors point to increased expression of HP in the kidney as a protective

mechanism against oxidative injury. In the same way, HPR is hypothesized to protect the circulatory system from oxidative properties of Hb by the binding of HPR-Hb with ApoA-I [26]. In RH, vascular dysfunction is even more aggravated than in controlled hypertension with impaired endothelial homeostasis and vascular stiffness. Increased HP and HPR levels found in basal urine of future non-responders may also reflect an increased renal expression in an attempt to compensate negative effects of RH at vascular level.

Functional enrichment analysis revealed regulation of blood coagulation and focal adhesion assembly, tissue homeostasis, complement activation, immune response, homeostasis and VEGF signaling as biological responses over-represented in RHnr up-regulated signature. These physiological mechanisms, and specific proteins as ANXA1, MPO, S100A8, S100A9, PRDX2, TXN, PLG, SERPINA1 and TF, have been previously identified by our group in hypertensive patients developing albuminuria despite of being chronically RAS suppressed, thus confirming their activation in situations aggravating cardiorenal prognosis, as RHnr patients and albuminuric patients without RH[13, 14, 28, 38, 39]. These findings are in alignment with the frequent development of chronic kidney disease in RH[1]. Our data indicate that in RH these processes are activated since the initial stages of cardiovascular and renal complications. Hypertensives with mildly decrease creatinine clearance have been described to have an activated coagulation system[40]; hypertensive emergencies have been associated with high levels of inflammatory, endothelial activation or coagulation[41] and we have previously described and impairment in focal adhesions at early stages of atherosclerosis[42]. Vascular endothelial growth factor (VEGF) signaling pathway is also significantly involved probably activated as a compensatory mechanism to facilitate vasodilation. In responders to spironolactone (RHr), cellular response to oxidative stress

was found significantly up-regulated, in alignment with NADP mediation in antioxidation and ROS generation[43], and positive regulation of NF-kB. Confirming our previous data showing deregulation of CAC cycle in RH[12], glucose catabolism was also found altered at the proteome level.

In conclusion, this is the first study to identify protein changes reflected in urine subjacent to RH. Proteomics provided an un-biased approach to identify most significant protein changes which can be quantified in urine as a reflection of RH physiopathology at the same time that coordinated behavior of altered proteins can be evaluated. Protein variations identified in a first discovery stage were confirmed by different technical approaches. One limitation of the study could be the open-label administration of spironolactone and lack of placebo or a comparator agent. However, it was not our purpose to compare the spironolactone efficiency with other drugs. We aimed to identify potential protein predictors in urine collected prior to spironolactone treatment in the same patients' cohort who showed metabolic alterations with such predictive capacity[12]. It cannot be ruled out the possibility that HP and HPR could also predict BP response to antihypertensive drugs other than spironolactone. This study was not designed as an RCT but represents daily clinical practice in resistant hypertension according to the most recent guidelines[44]. This is a pilot study and the main limitation could be the relatively low number of patients from a clinical point of view (and unbalance between groups). However, the study fulfills the requirements of an Omics study in terms of group size and technical workflow[45] and the strength of the data found in this trial enhances the possibility that the required larger multicenter cohorts to further confirm their potential use in RH management could simply confirm them.

PERSPECTIVES

This is the first study to identify protein signatures in urine subjacent to RH and able to predict response to spironolactone. The urinary proteome was explored and individual proteins were quantified by mass spectrometry, a technique that can be easily implemented in daily routine clinical practice. Among the 3310 proteins identified, HP and HPR were the two showing the most dramatic change in basal urine from responders compared to non-responders, showing capacity to anticipate spironolactone effect. These findings are of great importance in the control of RH patients and opens a new field of research in RH management.

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DISCLOSURES

R.M. Ruilope has served as advisor/speaker for Astra-Zeneca, Bayer, Daiichi-Sankyo, Medtronic, Novartis, Pfizer, Relypsa, Sanofi, and Takeda. The other authors report no conflicts.

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NOVELTY AND SIGNIFICANCE

What is new?

- Physiopathological alterations subjacent to resistant hypertension (RH) at the protein level, and involved biological processes are shown here for the first time.
- Haptoglobin and Haptoglobin-related protein are able to predict patients' response to spironolactone when measured in urine collected prior to drug administration.
- Immune system and oxidative stress response arise as key players in Resistant Hypertension.

What is relevant?

-This study was conducted in patients with resistant hypertension, in urine collected prior to spironolactone treatment.
-The identification of protein markers able to predict response to spironolactone will contribute to optimize RH patients' management and avoid unnecessary side effects in non-responders.
- An alternative technical approach to ELISA assay is here proposed for HP and HPR analysis in urine, allowing the analysis of larger cohorts in shorter periods of time.

Summary

Resistant hypertension physiopathology has reflection in urine at the proteome level. Analyzed in urine collected prior to spironolactone administration, HP and HPR proteins showed increased levels in non-responders compared to responders, thus demonstrating their predictive capacity. Immune system and oxidative stress response confirm their activation in situations aggravating cardiorenal prognosis, as they are resistant hypertension and albuminuria development in non-resistant hypertensives.

LEGENDS OF FIGURES

Figure 1. Haptoglobin (HP) and haptoglobin-related protein (HRP) in urine distinguish between RH patients who respond to spironolactone (RHr) and non-responders patients (RHnr). A) HP analysis by ELISA assay and ROC curve; B) HPR analysis by ELISA assay and ROC curve; C) combined ROC curve and predicted class probabilities graph with ELISA data, showing good separation between responders and non-responders; D) HP analysis by SRM-LC/MS/MS and ROC curve (transition m/z 720.3 → 881.4); E) HPR analysis by SRM-LC-MS/MS and ROC curve (transition m/z 916.4 → 1122.0); F) combined ROC curve and predicted class probabilities graph with SRM-LC-MS/MS data, showing good separation between responders and non-responders. All analyses were performed in basal urine samples prior to drug administration. * p value <0.05, ** p value <0.01.

Figure 2. Coordinated responses of proteins up-regulated in RH patients responders to spironolactone (RHr). Functional enrichment analysis was performed considering altered proteins in comparison with the rest of the genome. Over-represented GeneOntology terms (GO) in RHr are shown, together with individual proteins variation rate when compared responders *versus* non-responders (RHnr). Terms are grouped according to their ontological relationships. Non-connected GO terms do not a have a direct link in GO database.

Figure 3. Coordinated responses of proteins up-regulated in RH patients non-responders to spironolactone (RHnr). Functional enrichment analysis was performed considering altered proteins in comparison with the rest of the genome. Over-represented GeneOntology terms (GO) in RHnr are shown, together with individual proteins variation rate when compared responders *versus* non-responders (RHnr). Terms

are grouped according to their ontological relationships. Non-connected GO terms do not a have a direct link in GO database.

Table 1. Baseline Clinical data of RH patients included in the study. RHr: resistant hypertension, responders to spironolactone; RHnr: resistant hypertension, non-responders to spironolactone; CVD: cardiovascular disease; SBP: systolic blood pressure; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; MDRD: modification of diet in renal disease; ARR: aldosterone to renin ratio; BMI: body mass index; HDL: high-density lipoprotein; LDL: low-density lipoprotein; ACEi: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor.

	Resistant Hypertension		
	RHr (n=19)	RHnr (n=10)	*p*-value
Characteristics			
Age, y	61±12	68±11	0.161
Gender, % male	58	50	0.709
Current smoking, %	21	10	0.483
Diabetes, %	37	50	0.519
Previous CVD, %	11	10	1.000
Duration of Hypertension, y	14±25	22±13	0.597
SBP, mmHg			
Office	157.1±10	169.8±21	0.063
24Hours	143.7±9	147.7±7	0.251
Daytime	146.9±9	151±8	0.251
Nightime	134.8±12	137.8±10	0.370
Central	135.7±11	138±20	0.755
DBP, mmHg			
Office	93.11±10	85.4±11	0.056
24Hours	88.68±8	80.5±10	0.035
Daytime	91.21±9	82.8±10	0.046
Nightime	81.42±10	73.9±12	0.090
Central	91.83±10	84.3±8	0.061
Albumin/creatinine, mg/g	13.27 [5.67-33.54]	27.06 [7.94-330.6]	0.176
eGFR by MDRD, mL/min/1.73m^2	89.54±26	82.23±14	0.506
ARR	0.69 [0.47-0.87]	1.11 [0.61-1.18]	0.065
BMI, kg/m^2	31.12±5	30.37±4	0.598
Total cholesterol, mg/dL	183.2±30	192.2±34	0.598
HDL cholesterol, mg/dL	53.06±15	60.74±20	0.396
LDL cholesterol, mg/dL	102.2±24	103.9±32	0.630
Triglycerides, mg/dL	127.4±68	138.3±57	0.551
Antihypertensive drugs, in total	4	4	0.504

ACEi, %	32	20	0.534
ARB, %	74	80	0.734
Diuretic, %	84	100	0.208
Calcium channel blocker, %	89	80	0.514
β blocker agent, %	53	70	0.390
α blocker agent, %	21	20	0.974
Spironolactone, %	100	100	1
Concomitant therapies			
Anticoagulant, %	11	0	0.321
Statins, %	42	80	0.059
Insulin, %	5	0	0.514
Oral antidiabetic, %	21	40	0.300

Table 2. Protein variation rates found for haptoglobin (HP) and haptoglobin-related protein (HPR) in the discovery and confirmation stages. By TMT-LC-MS/MS, protein abundance was expressed in terms of Zq mean values. By ELISA, concentration values were calculated referred to creatinine. Zq values: log2-ratios expressed as standardized variables; RHr: resistant hypertension, responders to spironolactone; RHnr: resistant hypertension, non-responders to spironolactone;

	DISCOVERY			
	Zq individual average		*ΔZq*	*p-value*
	RHr	RHnr		
HP	-2.59	3.39	5.98	0.00279
HPR	-3.65	4.17	7.83	0.00009
	QUANTITATION			
	Concentration (µg/g creatinine)			*p-value*
	RHr	RHnr		
HP	163.6	430.4		0.0046
HPR	8.51	34.47		0.0051