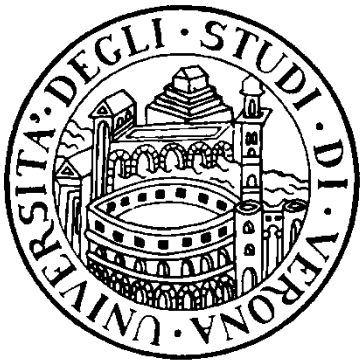

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Improving diagnosis and characterization of Primary Aldosteronism through different -omics approaches

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Improving diagnosis and characterization of Primary Aldosteronism through different -omics approaches

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Abstract

Primary aldosteronism (PA) is the most common form of secondary hypertension and it is characterized by a Renin Angiotensin System (RAAS) independent overproduction of aldosterone by the adrenal glands. The clinical features of primary aldosteronism resemble the ones of an essential hypertensive patient (EH) but PA patients have a higher risk of cardiovascular events and a worse prognosis.

Correctly diagnosed PA patients can benefit from specific treatments like the surgical removal of the aldosterone hyperproducing adrenal lesion or the administration of mineralocorticoid receptor antagonists (MRAs), depending on the features of the disease. Diagnosis and subtyping are highly challenging tasks and a complex diagnostic work-up was developed to be used in clinical practice. The identification of molecular markers of PA could provide new instruments that can help in the screening, diagnosis and characterization of this disease.

In our study, we carried out different experimental strategies to improve the identification and characterization of PA by identifying specific molecular signatures, also taking advantages of urinary extracellular vesicles (UEV) analysis to identify prospect biomarkers in a non-invasive way. We propose a protocol for the investigation of mRNA content in UEV that provided information on the pathophysiological regulation of the sodium-chloride cotransporter transcript (NCC) in our proof-of-principle study. We analyzed the protein content of UEV through an untargeted MS-based approach that identified several biomarker candidates separating EH and PA patients, and we also detected significant differences in key proteins involved in water reabsorption pathway.

Lastly, we carried out semi-targeted metabolomics analysis of serum samples from EH and PA patients, including a follow up of the metabolic changes in 7 aldosterone-producing adenoma (APA) patients after surgical therapy in a 2-year time window. Through this approach, we discovered differences in purine, lipid and energy related

metabolites in PA and EH patients, as well as in PA subtypes APA and bilateral hyperaldosteronism (BAH). In our follow-up study we detected marked variations in the level of metabolites related to endothelium homeostasis and lipid mediators of inflammation.

Through these strategies, we have identified several molecular markers that can improve the PA diagnostic work-up and we gained insight into the pathophysiological features of this disease, highlighting the usefulness of the -omics approaches in the investigation of complex diseases.

Sommario

L'iperaldosteronismo primario (PA) è la più comune forma di ipertensione secondaria ed è caratterizzata da una produzione e secrezione di aldosterone nella corteccia surrenalica, in completa o parziale indipendenza dal sistema renina-angiotensina. Le caratteristiche cliniche dell'iperaldosteronismo sono simili a quelle riscontrabili nei pazienti con ipertensione essenziale (EH) ma è noto che, rispetto ad essi, i pazienti con PA siano soggetti a un rischio maggiore di sviluppare eventi cardiovascolari. Una diagnosi corretta di PA permette a questi pazienti di poter ricevere un trattamento specifico, rappresentato dalla rimozione chirurgica della lesione surrenalica che causa la produzione incontrollata di aldosterone quando la malattia è monolaterale, o dalla somministrazione di inibitori del recettore dei mineralocorticoidi quando la malattia è bilaterale.

La diagnosi e la sottotipizzazione di PA sono processi difficoltosi che hanno richiesto lo sviluppo di un complesso e articolato algoritmo diagnostico utilizzato in clinica. L'identificazione di marcatori molecolari di PA potrebbe fornire nuovi strumenti per migliorare lo screening, la diagnosi e la caratterizzazione di questa malattia. Nel nostro studio abbiamo adottato differenti strategie sperimentali con l'obiettivo di migliorare il riconoscimento e la caratterizzazione del PA tramite l'identificazione di molecole peculiari, sfruttando anche le caratteristiche delle vescicole extracellulari urinarie (UEV) come fonte di potenziali biomarker.

In questo studio abbiamo proposto un protocollo per l'analisi del contenuto in mRNA delle UEV che abbiamo impiegato in un esperimento pilota per la quantificazione del trascritto del cotrasportatore sodio-cloro (NCC), una proteina chiave nel riassorbimento del sodio nel tubulo convoluto distale. Grazie a questo protocollo, abbiamo ottenuto informazioni sulla regolazione di NCC in condizioni patofisiologiche in pazienti PA ed EH. Abbiamo anche analizzato il contenuto di proteine nelle UEV tramite un approccio di tipo *untargeted* basato sulla spettrometria di massa, identificando diversi potenziali biomarker proteici capaci di

separare i pazienti EH dai PA ed evidenziando alcune differenze in proteine chiave nei meccanismi di riassorbimento dell'acqua a livello renale.

Infine, abbiamo condotto uno studio di metabolomica sui campioni di siero di PA ed EH, includendo anche un follow-up di pazienti con aldosterone-producing-adenoma (APA), i cui campioni sono stati prelevati nell'arco di due anni. Con questo approccio abbiamo osservato che fra i gruppi PA ed EH esistono differenze metaboliche che coinvolgono il metabolismo di purine, lipidi e carboidrati, mentre nello studio follow-up siamo riusciti a evidenziare delle notevoli variazioni in livelli di metaboliti coinvolti nell'omeostasi endoteliale e in lipidi mediatori dell'infiammazione.

Grazie a queste strategie, abbiamo illustrato alcuni meccanismi di regolazione di trascritti, proteine e metaboliti nel PA e abbiamo identificato diversi potenziali marcatori molecolari che potrebbero contribuire a migliorare l'iter diagnostico. Il nostro studio evidenzia anche l'utilità degli strumenti di -omica per l'indagine di malattie complesse e l'identificazione di biomarker.

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1. Introduction

1.1 Primary aldosteronism

Hypertension can pragmatically be defined as the level of blood pressure (BP) at which diagnosis and treatment are more beneficial than the associated risk without any intervention, and in clinic it is diagnosed when the levels of BP are above defined evidence-based cutoffs (*1*). Hypertension is a known major risk factor for cardiovascular disease, it is a major cause of death and it represents one of the most challenging public health issues in developed and developing countries (*2, 3*). Worldwide prevalence of hypertension has been estimated to be around 1.13 billion, with a global percentage ranging from 30 to 45% among adults (*4, 5*). Depending on its causes, hypertension can be classified as primary (essential) or secondary. While the causes of essential hypertension are not fully known being likely associated to interactions of several genetic, non-genetic and environmental risk factors, the secondary one is a consequence of clearly identifiable underlying causes. (*5*).

Primary aldosteronism (PA) is currently considered as the most common form of secondary hypertension; it is characterized by an overproduction and secretion of the mineralocorticoid hormone aldosterone by the adrenal glands. This overproduction is partially or completely independent of the renin-angiotensin-aldosterone system (RAAS) because it still occurs when renin is suppressed. High aldosterone causes an increased renal sodium and water retention with waste of potassium and ultimately leads to arterial hypertension and hypokalemia (*6*). As clinically important feature, the abnormal production of hormone may be due to bilateral hyperaldosteronism (BAH) or unilateral functioning adenoma.

1.2 Genetics and molecular mechanisms of PA

Several efforts have been made to understand the genetic basis of PA. In physiological conditions, aldosterone production responds to renin–angiotensin-

aldosterone system (RAAS) regulation. The protease renin processes angiotensinogen to produce angiotensin I. Angiotensin converting enzyme (ACE) converts then angiotensin I into the active peptide angiotensin II. This peptide is then bounded by specific surface receptors on the zona glomerulosa cells in the outer layer of the adrenal cortex where, in association to extracellular K+, it triggers membrane depolarization. Change in membrane potential stimulates the voltage-dependent L and T type Ca2+ channels and the increase in calcium influx stimulates the aldosterone synthase CYP11B2 (*7*).

Choi and colleagues were the first to demonstrate that one of the genetic causes of the unilateral form of PA aldosterone producing adenoma (APA) was a somatic mutation on KCNJ5 gene, encoding for the inward-rectifier potassium channel Kir3.4 (*8*). The mutations in KCNJ5 are located in the selectivity filter of this channel that becomes permeable to sodium ions, allowing them to contribute to membrane depolarization and consequent calcium entry. Their findings were then followed by the discovery of other mutations in several genes including the voltage-gated calcium channel Cav1.3 (CACNA1D), in Na+/K+ and Ca2+ ATPases (ATP1A1, ATP2B3) and beta-catenin (CTNNB1) (*9-13*). Despite some of the molecular mechanisms that characterize aldosterone over-production and secretion are now clear in APA, molecular bases of BAH, the bilateral form of PA, are still unknown (*14*).

Molecular mechanisms of aldosterone regulation on blood pressure involve the activity of several proteins. Aldosterone is a lipophilic molecule and can cross plasmatic membrane binding the cytoplasmatic mineralocorticoid receptor (MR) (*15, 16*). Once activated, MR works as a transcription factor regulating many downstream targets that promote sodium and water reabsorption. A complex network of transporters and channels is involved in sodium, chloride and potassium homeostasis in the kidney. A model of a distal convoluted tubule cell, the district that is critical for aldosterone-mediated sodium reabsorption, is shown in Figure 1.

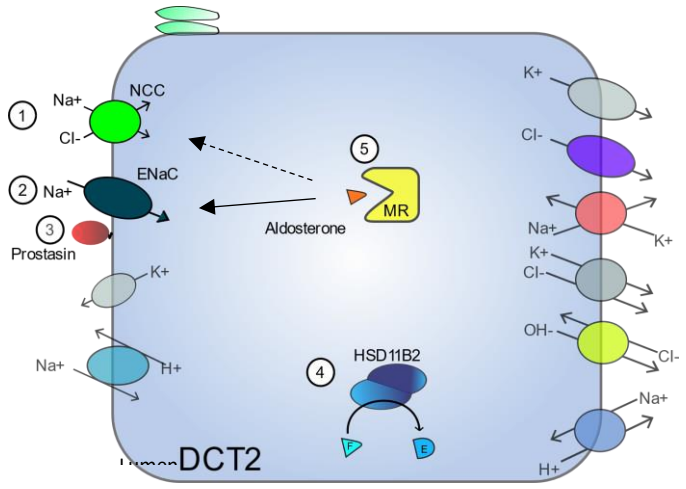

Figure 1: model of DCT cells. NCC and ENaC (1 and 2) are highlighted.

The epithelial sodium channel (ENaC) is the main and most characterized target of aldosterone-MR activity. This channel is also named after its amiloride-sensitivity and is composed by three homologous subunits (α, β, γ). This heteromultimeric membrane protein acts as a rate limiting step in the control of blood pressure and is regulated by mineralocorticoid signaling (*17, 18*). The GPI-anchored serine protease prostasin is one of the key regulators of ENaC. This protease cut an inhibitory peptide from the ENaC γ subunit, leading to an increased probability of the open conformation of the channel (*19, 20*). Prostasin was found to be secreted in urine and is considered to be a marker of ENaC activity in vivo (*21*) and our laboratory demonstrated that urinary excretion of prostasin is increased for PA patients (*22*).

Another key player in salt reabsorption tubule that is affected by aldosterone is the thiazide-sensitive sodium chloride cotransporter (NCC). This member of the SLC12 family of electroneutral cation chloride cotransporter is encoded by the SLC12A3 gene and is located in the apical plasma membrane of epithelial cells in the distal convoluted tubule (DCT). It has been shown that the abundance of NCC is modulated by aldosterone (*23, 24*) but several others hormonal and non-hormonal stimuli can contribute to regulate its activity (*25*). Together with ENaC, NCC is critical for regulating the arterial blood pressure and it has been found that NCC can be physically associated with the epithelial channel in the distal portion of the distal

convoluted tubule (DCT2), where the two proteins are co-expressed. This association is modulated by aldosterone and likely it has an impact on their salt reabsorption functionality (*26*).

1.3 Diagnosis and therapy

The clinical features of PA patient are largely similar to those observed in EH patients. This makes the right diagnosis not only a complex task but also a possible challenging issue for the National Health Systems in terms of cost/effects ratio considering the large prevalence of the primary hypertension In the general population (*27*). in addition, it is now acknowledged that PA patients are characterized by an increased risk in developing cardiovascular and renal events when compared to EH patients. In presence of a high-to-normal sodium intake, PA can convey several detrimental effects on the cardiovascular system, including an increased probability of atrial fibrillation and heart failure. It also induces tissue inflammation, myocardial remodeling and fibrosis and central sympathetic overactivation that can contribute to target organ damage in heart, blood vessels and kidney, and these effects are largely mediated by the interaction of aldosterone to the mineralocorticoid receptor (MR) (*15, 28-37*).

Initially, PA was considered to be rare, but its prevalence has recently been re-evaluated by several studies that reviewed the previous estimations of 2% among the hypertensive population to approximately 10%. The screening for PA is not systematically adopted in many Hypertension Centers but only when aldosterone is above center-defined thresholds or when the patient is hypokalemic. For these reasons, PA can be factually considered to be still underdiagnosed, especially for milder and normokalemic forms (*27, 38-40*).

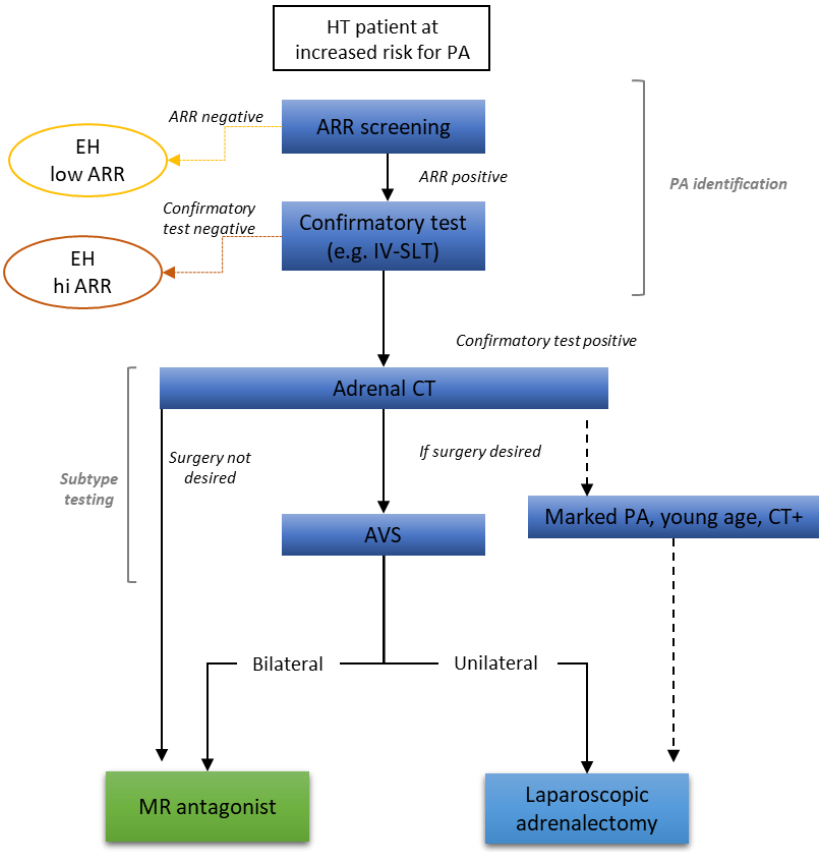

Figure 2: Diagnostic algorithm of PA. Modified from Funder, 2016.

The last guidelines for the diagnosis of PA (Figure 2) start from the calculation of aldosterone-to-renin ratio (ARR) as screening test for subject that are suspected of PA. In the laboratory practice, renin (measured as plasma activity or plasma concentration) and aldosterone are assessed using radio-immunometric assays, although tandem-MS methods and automated chemiluminescence-base methods that measure in parallel both renin and aldosterone have been proposed (*41-43*). One of the drawbacks of this screening test is that no validated protocol has been agreed upon yet, and ARR cutoffs vary between referring centers that use different assay protocols. (*44*). Since the aim of the ARR screening is detecting PA at high sensitivity minimizing the likelihood of missing true positive, this test is prone to

include false positives (*28, 45*). The suggested step after ARR screening is a confirmatory test (that works in fact as an exclusion test), aimed at demonstrating that the aldosterone secretion does not respond to RAAS regulation. The confirmatory tests include oral sodium loading test, saline infusion test, fludrocortisone suppression test or captopril challenge test. Performance of these confirmatory tests may vary from different clinical settings and no official gold standard or cutoff values have been definitively validated yet. Moreover, confirmatory tests are not always applicable to all patients, due to the possibility of complications such as hypertensive crisis or acute heart decompensation (*28, 46, 47*). However, the importance of a confirmatory test is that it can spare invasive procedures for PA subtyping to patients resulted falsely positive for ARR screening. It is suggested that the confirmatory test can be avoided in cases of spontaneous hypokalemia, when plasma renin is below detection level and plasma aldosterone concentration is above 20 ng dL^{-1} (or 550 pmol L^{-1}) (*28*). Computer Tomography (CT) and adrenal vein sampling (AVS) are aimed at the subtyping of PA, a fundamental step for the choice of the appropriate treatment, i.e. surgical or not. The use of CT to detect adrenal lesions has several limitations as lesions with size < 10mm may be omitted; actually, the large majority of adrenal functioning lesions (APAs) may be included in such range. In one study where the performance of CT was compared to AVS, it was found that the accuracy of the former was of 53%. In 22% of the cases, unilateral patients would have been excluded from receiving adrenalectomy while in 25% of the cases bilateral patients might have had unnecessary surgery (*28*). AVS is a procedure that measures directly the amount of cortisol-corrected aldosterone in both the adrenal glands by vein catheterization and overcomes most of the limitations of currently used imaging techniques. Despite being expensive, heavily reliant on the operator's skills and experience and inherently susceptible to complications like vein rupture (estimated in about 1% of the procedures), this technique remains a key component in the PA diagnostic work-up (*27, 48, 49*). Alternative method have been explored, including the promising

11C-metamidate PET, but due to its technical requirements it cannot be considered a suitable tool on large scale (*50*). The selection of the appropriate therapeutic treatment (surgical curable vs surgical uncurable PA) is crucial. In the first category, aldosterone producing adenomas (APA) account for about 30% of total PA cases, while bilateral hyperaldosteronism accounts for the remaining cases (*14, 27*).

Overall, the current work-up for the diagnosis of PA is cumbersome, time-consuming, relatively expensive, and requires specific technical skills or experience so that it cannot be easily applied routinely to the multitude of the hypertensive population. Nevertheless, considering the high prevalence of PA among hypertensive patients, the increased risk of PA subjects in developing cardiovascular events and the availability of specific treatments that can control (or cure) the disease, an effective diagnostic work-up is of paramount value. One of the most important challenges in PA research is to improve the sensitivity, specificity, cost and time effectiveness of the diagnostic work-up and replace the now necessary invasive procedures with viable alternatives.

A promising way to improve the identification and characterization of PA is finding specific molecular signatures (biomarkers) in matrixes collected from patients in non-invasive ways (e.g. urine) or less-invasive ways (e.g. blood).

1.4 Urine and urinary exosomes as source of biomarkers

Exosomes are extracellular membrane-bound vesicles with a diameter ranging from 30 to 120 nm and can be found in a wide variety of biological fluids, including blood and urine. They are selectively released from intracellular multivesicular bodies (MVB) of virtually any cell type (Figure 3) and they have a unique biogenesis and composition (*51*). Generation of vesicles, trafficking and cargo sorting in MVBs are strictly dependent on a group of proteins called Endosomal Sorting Complex Required for Transport (ESCRT) (*52, 53*).

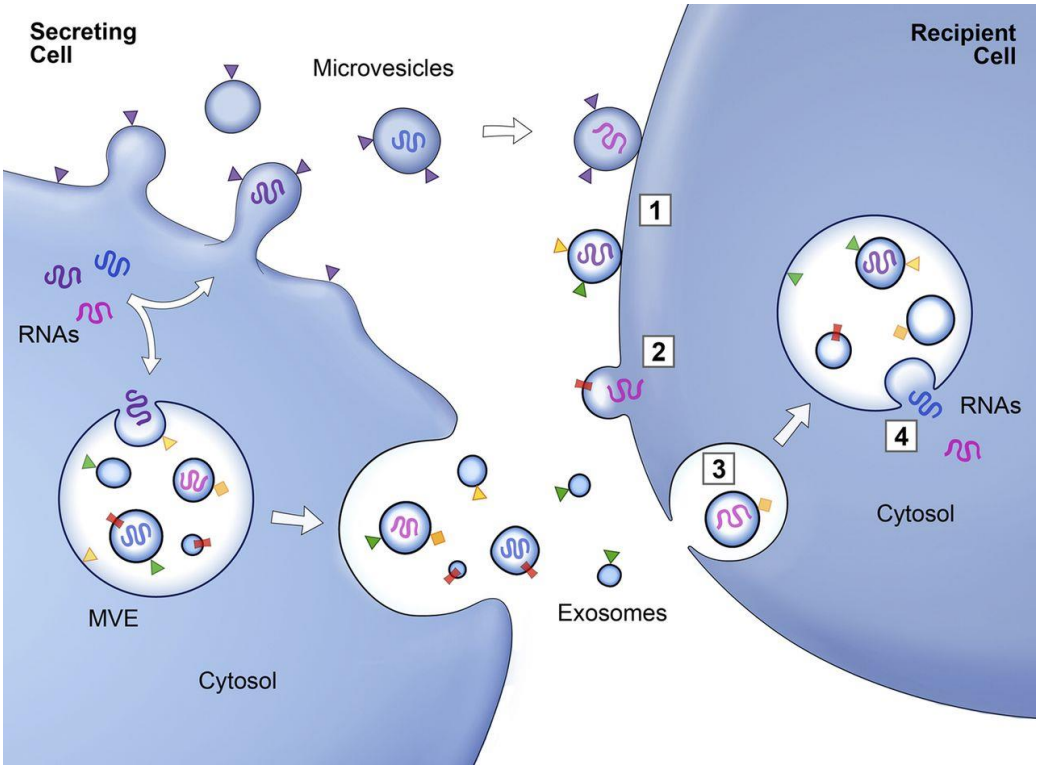

Figure 3: Exosomes (3) and microvesicles (1) biogenesis and internalization. From Raposo et al., 2013.

Exosomes differ from apoptotic bodies and other membrane bound-vesicles by size and by the presence of markers such as members of tetraspanins family (e.g. CD9, CD63, CD81), heat shock proteins (e.g. HSP70) or protein related to MVB biogenesis (e.g. ALIX, TSG101), although most of the components are shared also with extracellular vesicles, lysosomes, endosomes or alpha-granules and cannot be considered exosome-specific (*54*).

Exosomes play a significant role in cell-to-cell communication and carry selected components of the parent cell, such as proteins, metabolites, receptors and nucleic acids, including miRNAs and mRNAs. They are also rich in lipids and lipid-related proteins and phospholipases (*55*). Their biological functions have been related to many pathophysiological processes including cancer, immune-mediated diseases and cardiovascular diseases, and their content reflects the condition of the parent cell, making them an ideal source for potential biomarkers. The possibility of

isolating exosomes from body fluids appears therefore attractive as a potentially effective diagnostic tool (*56, 57*).

Exosomes that are isolated from urine (UE) are secreted from all the cells lining all the segments of the nephron and can provide information on kidney functionality, ion transport and tissue homeostasis. Their isolation is absolutely non-invasive and, since their cargo is protected by a lipid bilayer and theoretically reflect the pathophysiological condition of the cell-of-origin, low-abundant proteins and RNA that are present can be good putative biomarker candidates. (*58, 59*). For this reasons, several studies have explored the possibility to use UE as source of biomarker useful for the diagnosis of several diseases including kidney injury (CKD, AKI), glomerular injury, kidney fibrosis, cancer, diabetes and infections (*60-64*). Exosomes have already proved to be valuable also for PA and hypertension research: some studies have shown that UE cargo of patients suffering of mineralocorticoid-dependent arterial hypertension are enriched in proteins and miRNA related to salt reabsorption activity and regulation (*65-67*).

Over the last decade, the increasing interest in investigating this subcellular fraction has made available many isolation protocols that exploits different strategies for the isolation of exosomes. Each isolation protocol makes use of a different feature to separate exosomes from other similar vesicles, with the most common strategies exploiting the differences in size and density (size exclusion chromatography, differential ultracentrifugation, ultrafiltration, precipitation) or the presence of surface markers (immune-based precipitation). However, it has been reported that different methods may yield different results and the choice of the most appropriate isolation and characterization strategy may depend on the type of target (*60, 61*). Another challenging aspect of the experimental design is to find an appropriate normalization method to obtain a reliable quantification of the molecular content of exosomes (*64, 68*). The recent efforts in the standardization of definitions, isolation protocols and the creation of dedicated databases and tools for exosomes and

extracellular vesicles have considerably improved exosomes research, though many critical aspects still remain to be fully addressed. The exosomes research community is currently involved in an effort for reaching a consensus that will help in the translation from bench to bedside of exosomes as diagnostic and therapeutic tools (*69-72*).

1.5 Metabolomics in the study of hypertension and primary aldosteronism

Metabolic phenotyping is the study of low molecular weight molecules produced within a biologic system and represents a powerful tool for understanding disease complexity (*73*). Profiling of metabolites obtained with state-of-the-art mass spectrometry technologies and protocols can provide invaluable information on how a biological system responds to environmental conditions, changes in homeostasis and pathologies, as metabolites constitute the final product of the network of cellular activities. In hypertension research, metabolomics has already been successfully used as investigation tool both in human and animal models (*74-76*).

In the last three years, the research on PA has started to use metabolomics in the effort to improve the identification and characterization of the disease and to better understand the underlying pathophysiology. Our laboratory has started investigated the urinary metabolic signature in primary aldosteronism patients by using a non-invasive approach and we discovered that PA and hypertension are characterized by dysregulation in purine and sulfur metabolism, that BAH patients have a specific metabolic signature and that several metabolites were differentially regulated as function of sex variability (*77*). Arlt and colleagues have performed a profiling of steroid metabolome in patients with PA, discovering that glucocorticoid co-secretion is frequently found in primary aldosteronism and can contribute to associated metabolic risk (*78*). Murakami and colleagues have used the innovative approach of MSI (mass spectrometry imaging) to pinpoint metabolomics derangements in APA tissues, discovering that 18-oxocortisol was associated to a better clinical outcome after adrenalectomy (*79*). Together with state-of-the-art mass spectrometry

techniques, metabolomics could provide a significant amount of information relevant to the understanding of the underlying pathophysiology of PA but also useful to detect specific metabolic signatures that can be used as molecular markers in diagnosis and subtyping of the disease.

2. Description of the project and aims

The main aim of this project is to **improve the identification and characterization of PA** by identifying specific nucleic acids, proteins and metabolites that can be considered markers of this pathology. The ultimate goal is to provide information that can be useful in clinical practice to separate PA patients in a population of hypertensive subjects. To achieve this, we have analyzed different clinical samples using both targeted and untargeted approaches. By quantifying molecular markers of PA and investigating their networks and pathways, our research strategies enable us also to pursue an important side aim: to **gain insight on the pathophysiology of PA** itself and acquire information that can be relevant in clinical practice for improving its subtyping and treatment.

This project has been developed in three distinct parts:

1. Analysis of the mRNA content of urinary extracellular vesicles (UEV), targeting specifically the NCC mRNA
2. Analysis of the protein content of UEV of PA and EH patients (untargeted label-free MS quantification)
3. Metabolomics of PA and EH patients (semi-targeted MS relative quantification)

The first two parts are aimed at exploiting UEV as source of biomarkers for their capability of concentrating molecular information and for their non-invasive collection, while the last part is focused on serum metabolomics for the identification of specific metabolic markers of PA.

3. Materials and Methods

3.1 Selection of patients

We enrolled patients among hypertensives subjects referring to the Hypertension Unit of the Verona University Hospital (Verona, Italy). Patients were screened by mean of aldosterone-to-renin ratio (ARR). Screening samples were collected in the morning from fasting patients who remained out of bed for at least 2 h, after an adequate period of withdrawal of interfering drugs. As hypotensive treatment, only calcium-blockers or alpha-adrenergic blockers were allowed. PA diagnosis was confirmed in patients with elevated ARR value by intravenous salt loading test (IV-SLT). An ARR value of 32 was previously defined as the cutoff for the screening (*77*). Samples from IV-SLT were collected after intravenous saline loading (2 l of 9% NaCl infused in 4 h), to confirm an autonomous aldosterone secretion. The test was considered positive when post-test aldosterone concentration was higher than 50 pg ml-1 (138.7 pmol l-1). [28] In patients for whom plasma concentrations of aldosterone had an inadequate decrease after saline load, adrenal venous sampling was performed to define the disease subtype (BAH or monolateral APA) after a CT scan. Patients positive at both screening and confirmatory steps were diagnosed as PA and assigned to a disease subtype when possible (BAH or APA). Adrenal venous sampling (AVS) was performed under unstimulated conditions, and was considered successful for a selectivity index (which is the ratio between cortisol in the adrenal vein and cortisol in inferior vena cava) >2. For the diagnosis of monolateral or bilateral PA a lateralization index (calculated as the ratio between cortisol-corrected aldosterone levels in one adrenal compare to the contralateral) of 2 was adopted. Patients negative for the screening test (i.e. with an ARR lower than the threshold) were defined as EH. In NCC mRNA analysis and Metabolomics experiments these patients are referred to as EH low-ARR. Patients positive for the screening test but with a negative confirmatory test (i.e. IV-SLT) are indicated as EH hi-ARR in Metabolomics experiments.

Blood and urine samples were collected at the recruitment, after confirmatory test (IV-SLT) and after therapy or surgery (e.g. after adrenalectomy and after mineral corticoid antagonist administration). In the case of MRA treatment, the samples were collected one month after the first administration. For patients who underwent adrenalectomy, samples were collected 3 months, one year and two years after.

Routine laboratory test including measurement of biochemical and hormonal parameters were performed at the laboratory of the Clinical Chemistry Institute of the Verona University Hospital. Plasma aldosterone and renin levels were measured by using commercially available methods (Dia Sorin Diagnostics, Vercelli, Italy), as previously described (*80*).

The study was performed according to the principles of the Declaration of Helsinki. Each patient provided written informed consent. The Institutional Review Board Ethical Committee approved the protocol.

3.2 Biochemical and hormonal features of patients

Overview of the features of patients involved in the three separate studies are shown in the relative results section. Statistics in these tables was calculated through t-test and non-parametric tests were used when data were not normally distributed, or variance was not homogenous. Chi-square test was used for proportions.

3.3 Processing of clinical samples

Urine

Urine samples were collected and processed according to a previously reported protocol (*21*). Briefly, second morning urine samples were collected, chilled on ice and processed within one hour from collection. Urine pH was adjusted to 7.0 and a protease inhibitor cocktail was added (Complete Protease Inhibitor; Roche

Diagnostics, Basel, Switzerland) before centrifugation at 3500 rpm for 40 minutes at 4°C. Supernatant was subsequently filtered using 0.22 µm filters (Millipore, Billerica, Massachussets, USA). Cell-free urine (CFU) was then stored in aliquots at -20°C. Urine processed as illustrated here were then used for further analysis (e.g. exosomes isolation).

Blood

Clinic routine blood draw was collected in three different Vacutainer (BD, Franklin Lakes, New Jersey) collection tubes (separation gel, citrate and EDTA tubes) and immediately centrifuged at 1500 g for 15 minutes. Plasma and serum were collected and stored in aliquots at -20 °C. Buffy coat was collected and stored separately at -20°C. Remaining packed red blood cells were washed in saline solution and centrifugation step was repeated. Red blood cells were finally lysed with sterile water in 1:2 proportion and stored at -20 °C.

3.4 TEM characterization of vesicles

Exosomes pellet was thawed and resuspended in 100 µl of sterile PS and kept at +4°C. Aliquots of 6 µl of the suspension were absorbed for 1 minute on a ultra-thin carbon coated copper grid (CF200H-Cu-UL, Electon Microscopy Sciences) and excess of suspension was removed by gentle blotting.

Suspension adsorbed to grid was placed on 1 drop of UranyLess solution (Electon Microscopy Sciences) for 1 second. Operation was repeated and the second drop was left in place 30 seconds. Grid was then dried by gentle blotting and air. Sample was then visualized on a a Morgagni 268D (FEI Philips) transmission electron microscope, setting the voltage to 80kV.

A recent position paper on exosomes research suggest that the term “exosome” should describe only vesicles with a confirmed MVB origin and release. Where possible, in this thesis the term “urinary exosome” (UE) has been replaced by the

more generic term “urinary extracellular vesicles” (UEV) and the term “exosome” remained for referring to papers, databases and protocols that preceded this position paper (*72*).

3.5 NCC mRNA analysis

3.5.1 Isolation and characterization of urinary extracellular vesicles

Urine samples were thawed and extensively vortexed to increase the exosomes yield(*81*). Aliquots (5 ml) were mixed with an equal volume of a commercially available exosomes extraction reagent (Total Exosome Isolation Reagent from urine; ThermoFisher Scientific, Waltham, Massachusetts, USA) and incubated 1 hour at RT.

3.5.2 RNA extraction and analysis

Urine samples and the corresponding volume of the reagent were transferred into polypropylene centrifuge tubes and centrifuged 1 hour at 10.000 g and 4°C. Supernatant was carefully removed and UEV RNA was extracted with commercial kit (PureLink RNA Micro kit; Invitrogen, Carlsbad, California, USA) following the manufacturer’s instructions. RNA was eluted in a final volume of 15 µl and stored at -80 °C.

The cDNA synthesis was carried out using a commercial kit (iScript Advanced cDNA synthesis kit for RT-PCR; Biorad, Hercules, California, USA). To improve mRNA detection and measurement, a preamplification step was included (SsoAdvanced PreAmp Supermix; Biorad, Hercules, California, USA). Preamplification primer mix included the genes SLC12A3 and B2M. RealTime PCR was performed using SYBR green detection (SsoAdvanced Universal SYBR Green Supermix; Biorad, Hercules, California, USA) and all reactions were performed in 96-well plates in duplicate. All RealTime PCR runs included a negative control containing all the reaction reagents without cDNA. Specificity of the target was assessed by melt curve analysis. Intron-spanning primers (Biorad, Hercules, California, USA) were used for target and housekeeping genes. NCC primers amplify all the three isoforms of the gene (*82*).

B2M gene use as normalizer was based on primer efficiency and absence of PCR product detection after no reverse-transcriptase control experiments. Housekeeping gene was detectable in all samples investigated. When the NCC transcript was undetectable, a reference value of 40 Ct was used.

3.6 Proteomic analysis of urinary extracellular vesicles

3.6.1 Vesicles and proteins extraction

Ultracentrifugation protocol was adapted from Pisitkun et al. with the adjustments suggested by Livshits and colleagues (*83, 84*). Aliquots of urine (10 ml) were centrifuged at 17000 g for 15 minutes at 4°C to remove urinary sediment, including whole cells, large membrane fragments and other debris. Supernatant was then centrifuged at 50000 g for 120 minutes at 4°C to obtain a low-density membrane pellet. This pellet was resuspended either in 400 µl of ammonium bicarbonate 100 mM (AMBIC) or RIPA buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS with 1mM NaF and Complete Roche protease inhibitor cocktail).

3.6.2 Mass Spectrometry analysis

Sample processing for MS analysis and data collection were conducted at the Mass Spectrometry unit of the University of Piemonte Orientale (Novara, Italy). These analyses were performed in the frame of a collaborative project.

Proteins extracted from UEV were quantified using BCA assay (Pierce BCA protein assay kit; ThermoFisher Scientific). Samples were denaturated with TFE, reduced in DTT 200 mM and alkylated with IAM 200 mM before complete tryptic digestion with 2 µg of Trypsin/Lys-C (Promega, Madison, WI, USA). Digested peptides were desalted on the Discovery® DSC-18 solid phase extraction (SPE) 96-well Plate (25 mg/well) (Sigma-Aldrich Inc., St. Louis, MO, USA) and vacuum evaporated to be reconstituted with 20 µL of 0.05% formic acid in water.

For each sample, 4 µg of trypsin-digested proteins were analyzed with a micro-LC Eksigent Technologies (Eksigent Technologies, Dublin, CA, USA) system that included a micro LC200 Eksigent pump with flow module 5-50 µL, interfaced with a 5600+ TripleTOF system (Sciex, Concord, ON, Canada) equipped with DuoSpray Ion Source and CDS (Calibrant Delivery System). The stationary phase was a Halo C18 column (0.5 x 100 mm, 2.7 µm; Eksigent Technologies, Dublin, CA, USA). The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluting at a flowrate of 15.0 µL min−1 at an increasing concentration of solvent B from 2% to 40% in 30 min. For identification purposes the samples were subjected to a data dependent acquisition (DDA): the mass spectrometer analysis was performed using a mass range of 100–1500 Da (TOF scan with an accumulation time of 0.25 s), followed by a MS/MS product ion scan from 200 to 1250 Da (accumulation time of 5.0 ms) with the abundance threshold set at 30 cps (35 candidate ions can be monitored during every cycle).

For the label-free quantification the samples were subjected to cyclic data independent analysis (DIA) of the mass spectra, using a 25-Da window: the mass spectrometer was operated such that a 50-ms survey scan (TOF-MS) was performed and subsequent MS/MS experiments were performed on all precursors. These MS/MS experiments were performed in a cyclic manner using an accumulation time of 40 ms per 25-Da swath (36 swaths in total) for a total cycle time of 1.5408 s. The ions were fragmented for each MS/MS experiment in the collision cell using the rolling collision energy. The MS data were acquired with Analyst TF 1.7 (Sciex, Concord, ON, Canada). Two DDA and three DIA acquisitions were performed. The DDA files were searched using Protein Pilot software v. 4.2 (Sciex, Concord, ON, Canada) and Mascot v. 2.4 (Matrix Science Inc., Boston, MA, USA). The data normalization was carried out through total peak area method. The UniProt Swiss-Prot reviewed database containing human proteins was used and a target-decoy database search was performed. A minimum number of 2 peptides was used for the identification. False Discovery Rate was set at 1%.

3.7 Metabolomics

3.7.1 Sample processing and metabolite extraction

General metabolomics extraction: Serum samples were thawed on ice and vortexed. Aliquots of 20 ul of serum were mixed with 480 ul of ice-cold extraction buffer made of MeOH, ACN and H2O mixed in the proportion of 5:3:2. Samples were the n vortexed vigorously for 30 minutes at 4 C before being centrifuged for 10 minutes at maximum speed (the maximum speed of our benchtop centrifuge was 10,213 g). Aliquots of 100 ul of clear supernatant were transferred into labeled and cooled autosampler vials. As quality control, a pooled mixture of 10 ul aliquots of extracts was assembled. Blank was assembled from pure extraction buffer.

Oxylipins metabolomic extraction: procedure was similar to the one described for general metabolomics with some changes. 20 ul of serum were mixed with 180 ul of pure ice-cold MeOH. Samples were briefly vortexed and kept at -20 C for 30 minutes before the centrifuge step. Clear supernatant was mixed in proportion 1:2 with 10 mM ammonium acetate solution before transferring the extracts in autosampler vials.

3.7.2 Ultra-High-Pressure Liquid Chromatography-Mass Spectrometry metabolomics

Classic metabolomics was run on a 5 minutes gradient method. Column in use was a C18 (Kinetex® 1.7 µm C18 100 Å, LC Column 30 x 2.1 mm, Ea, Phenomenex, USA). (*85*)

For positive mode, mobile phases were A: H2O + 0.1% FA; B: CAN + 0.1% FA. Negative mode run used A: 5% ACN, 95% H2O + 1 mM ammonium acetate and B: 95% ACN, 5% H2O and 1 mM ammonium acetate.

Chromatography conditions were as follow: flow rate 0.25 mL/min; solvent composition 95% A, 5% B from time zero until 3 min; column temperature 25 C; sample compartment temperature 7 C.

For positive or negative mode, the MS settings were: 70,000 (Q Exactive) or 60,000 (Q Exactive HF), scan range 65-900 m/z, maximum injection time 200 ms, 2 microscans, automatic gain control (AGC) 3 x 106 ions, ESI source voltage 4.0 kV, capillary temperature 320 oC, and sheath gas 45, auxiliary gas 25, and sweep gas 0 (all nitrogen, measured in arbitrary units).

For oxylipins analysis, analytes were separated on a C18 column (Acquity HSS T3, 2.1 3 100 mm, 1.8 um, Waters). The mobile phase was composed of A: water/acetonitrile (75/25, vol/vol) with 5mM ammonium acetate and B: 2-propanol/acetonitrile/water (50/45/5, vol/vol/vol) with 5mM ammonium acetate. Chromatography method and conditions were as follow: column temperature 45 C; sample compartment 7 C. Solvent gradient: 0-1 min 25% B FR 0.3 mL/min; 1-2 min 25-5-% B; 2-8 min 50-90% B; 8-10 min 90-99% B; 10-14 hold B at 99%; 14-14.1 99-25B; 14.1-16.9 hold 25% B and FR 0.4 mL/min. 16.9-17 min hold 25% B and resume FR to 0.3 mL/min. (*86*)

MS settings: 4.0 kV source voltage, 320 ^{o}C capillary temperature, and 45 sheath gas, 15 aux gas, and 0 sweep gas (all nitrogen). Polarity was negative only.

3.7.3 Statistical Analyses

Multivariate analyses, including partial least square-discriminant analyses (PLS-DA) and hierarchical clustering analyses were performed with MetaboAnalyst 4.0.59. Other analyses and figures were prepared using R (v. 3.6.1).

Samples extraction and analysis were conducted in the Metabolomics Lab of the University of Colorado, Denver under the supervision of Prof. D'Alessandro (Department of Biochemistry and Genetics). These analyses were performed in the frame of a collaborative project.

4. Results and Discussion

4.1 NCC mRNA analysis in PA and EH patients

In this study, we analyzed the UEV transcript level of NCC, key player in salt reabsorption pathway in the distal nephron, across PA and EH patients. For this study, we have enrolled 13 EH patients and 12 PA patients (8 APA and 4 BAH). Their biochemical and hormonal features are reported in table 1. PA and EH groups were significantly different for plasma renin, aldosterone, ARR and serum potassium, as expected. Of note, all patients were studied without medications interfering with ARR determination.

Table 1: Biochemical and hormonal features of the patients enrolled in NCC mRNA study. Values are indicated as mean ± SD or median (CI) for non-normally distributed data.

	PA patients (12)	EH patients (13)	pval
Gender (m/f)	12/0	7/6	< 0.05
Age (ys)	51.8 ± 11.9	50.1 ± 12.2	NS
BMI	28.2 (22.6, 35.2)	27.6 (24.6, 31.0)	NS
Renin	2.2 (1.2, 5.7)	8.3 (4.5, 15.3)	< 0.01
Aldosterone (pg ml^{-1})	286.5 ± 113.2	202.5 ± 68.2	< 0.05
ARR	131.7 (45.1, 384.8)	22.9 (11.9, 43.7)	< 0.01
Serum K (mmol l^{-1})	3.5 ± 0.5	3.9 ± 0.3	< 0.05
Creatinine (mg dl^{-1})	1.0 ± 0.1	0.9 ± 0.3	NS
Glucose (mmol l^{-1})	5.0 (4.4, 5.7)	4.7 (4.4, 5.1)	NS
Cholesterol (mg dl^{-1})	176,4 ± 55.6	204.5 ± 43.8	NS

Triglycerides (mg dl^{-1})	84.2 (57.8, 124.3)	95.0 (68.1, 132.5)	NS

4.1.1 UEV TEM characterization

Our isolated UEV were characterized by TEM (Figure 4). The average size of our vesicles was around 90 nm, compatible with the described size for exosomes.

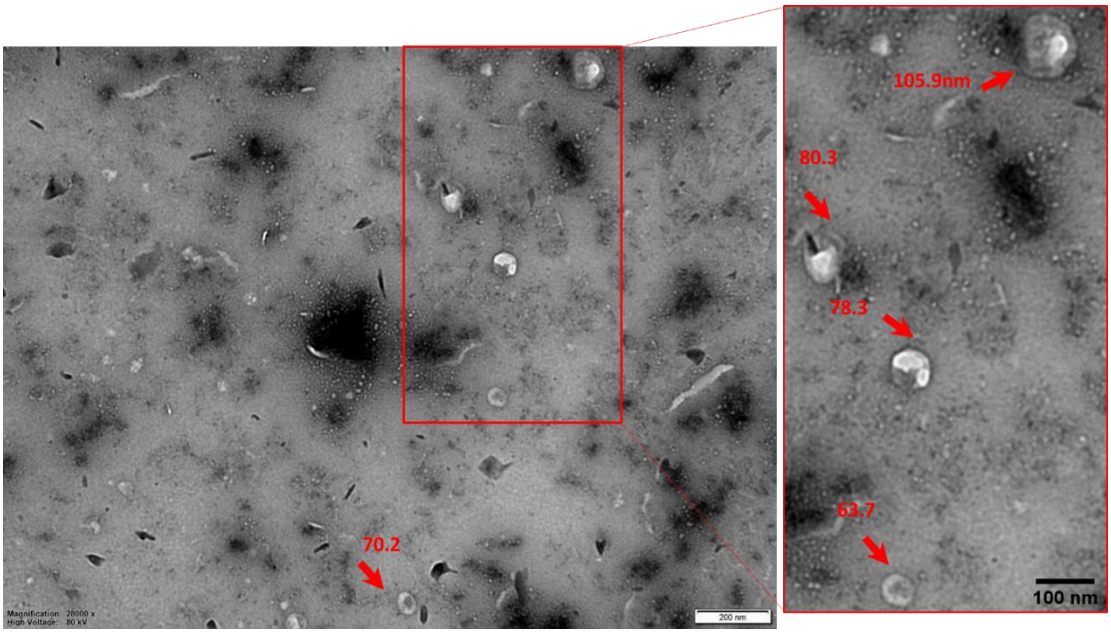

Figure 4: TEM analysis of UEV obtained using the chemical precipitation.

4.1.2 NCC mRNA analysis

Urinary extracellular vesicles (UEV) were collected and analyzed for NCC mRNA content in 25 hypertensive subjects. After RNA extraction and cDNA synthesis a step of preamplification was introduced to improve the detection, as the quantity of exosomal mRNA was very low (around 20 pg/ul in most cases). UEV NCC mRNA relative abundance was expressed as dCt (calculated as $C_{t\ TARGET} - C_{t\ HOUSEKEEPING}$) reversed from positive to negative for a more immediate interpretation. Higher values of dCt correspond to a more abundant transcript, and vice-versa.

Firstly, we assessed UEV NCC mRNA in patients at their enrollment. NCC mRNA levels' distribution for PA and EH subject is indicated in Figure 5 (panel a). PA patients were also subdivided in APA and BAH subtypes (panel b). In both cases, transcript relative abundance between groups was not significantly different.

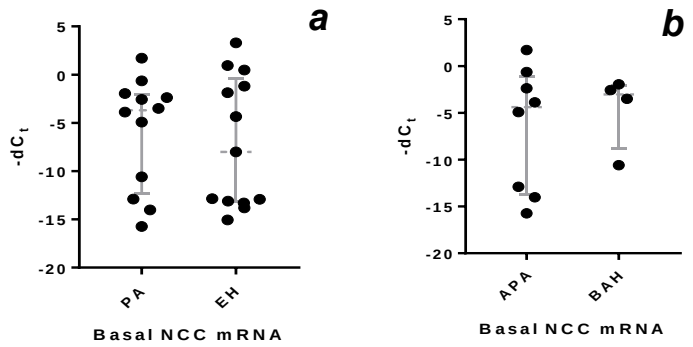

*Figure 5: (**a**) basal level of NCC mRNA in UEs of PA and EH patients. (**b**) basal NCC mRNA of PA patients separated for diagnosis subtype.*

Previous data have suggested that UEV of PA patients are characterized by an increased level of NCC protein (and its phosphorylated and active form: pNCC) when compared to exosomes from EH patients(*66*). According to this view, differences in protein abundance would be reflected by the level of the corresponding transcript. Thus, an intriguing hypothesis was that NCC mRNA could have been employed as a more stable and reliable marker of PA than the corresponding protein. Our results did not confirm such hypothesis and no statistically significant difference in NCC mRNA expression was observed in UEV from PA or EH patients (Fig 5a). Similarly, no difference was observed between the PA subtypes, i.e. patients with monolateral (APA) or bilateral (BAH) adrenal lesions (Fig. 5b).

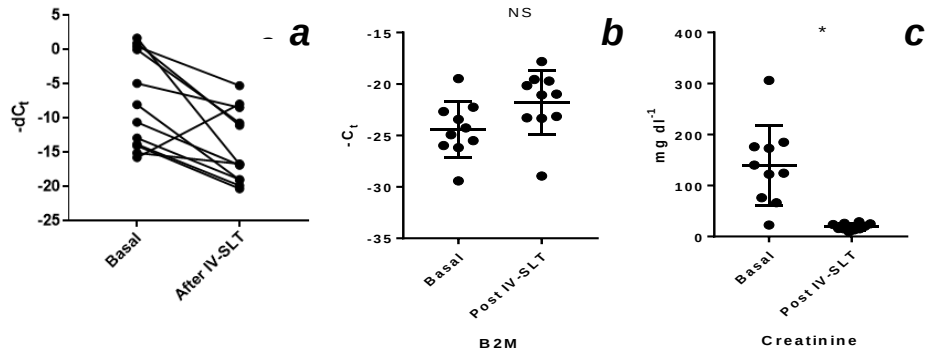

*Figure 6: (**a**) Scatterplot of UEV NCC mRNA relative abundance before and after IV-SLT (pairwise T test, p<0.05). (**b**) Relative abundance of the housekeeping transcript B2M. (c) Creatinine concentration of the urine samples.*

To investigate factors that can influence the NCC mRNA abundance, we measured the transcript in UEV samples from patients who resulted positive to the ARR screening and subsequently underwent confirmatory test (IV-SLT). In all EH patients and in 5 PA patients out of 6, the saline infusion led to a marked decrease of the level of NCC mRNA (Figure 6a). The PA patient characterized by an increase in NCC mRNA level was also an outlier for an extremely elevated ARR, due to the lowest level of plasma renin and the highest level of PAC measured among the subjects in our study (1 pg/ml and 532 pg/ml respectively). Unlike the creatinine values, we observed that B2M normalizer was not reduced after IV-SLT (Figure 6b and 6c).

Creatinine is a spontaneous breakdown product of creatine, which is secreted by muscle cells at a constant rate throughout the day. This small molecule passes through the glomerulus filtration and is not reabsorbed in the renal tubule. This suggests that the NCC mRNA reduction is not an artifact due to changes in urine concentration after IV-SLT and that the accumulation of exosomes in urine could be not linearly compared to glomerular filtration capability and time. One of the assumptions of creatinine normalization for urinary vesicles is that the rate of release, uptake and clearance of vesicles in urine is constant. To present time, there are no data that support this claim. Measurement of urinary creatinine provides a readout of the filtration capability of the kidney and, for this reason, creatinine level has also been used in different studies for the normalization of urinary and

exosomal proteins. In UEV research, the quantification of known vesicles markers (e.g. CD9) represents a viable alternative to urinary creatinine normalization, though it has been reported that the two normalization strategies are not necessarily correlated and CD9 can also be affected in immune response (*62, 87*). Taken together, these observations prompt to a careful evaluation of urinary creatinine as normalization factor for urinary exosomes, especially in experimental settings where spot urine samples are used and where short-term effects that can impact urine concentration and volume are analyzed. Nevertheless, in the context of exosomal transcripts evaluation, creatinine has shown several limitations dependent on the experimental settings (*68, 88*).

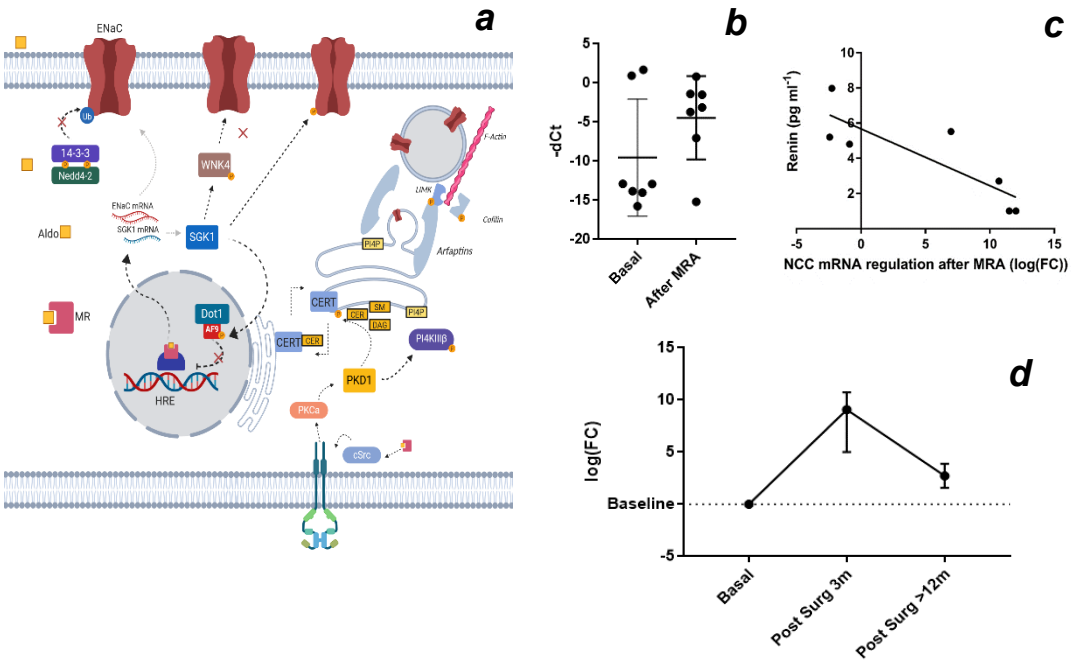

*Figure 7: (**a**) MR and aldosterone regulate ENaC abundance and activities in genomic and non-genomic ways: MR+aldosterone complex can enhance the transcription of ENaC mRNA and SGK1 which inhibits the chromatin repression complex Dot1 and AF9. SGK1 can also block ubiquitination of ENaC and promote the channel activation by phosphorylation. MR acts also on PKD1 which contributes to increase he membrane trafficking of ENaC in multiple ways. Modified from Valinsky, 2019 and Quinn, 2013. (**b**) NCC mRNA level before and after administration of MRA. (**c**) Scatterplot of plasma renin and NCC mRNA level of patients after MRA treatment (Spearman R=-0.81; p=0.04). NCC mRNA variation is expressed as the log2 of the fold-change. (**d**) Trends of NCC mRNA after adrenalectomy performed on 4 APA patients (median and range are shown).*

Spironolactone (SL) is a specific pharmacological inhibitor of the mineralocorticoid receptor (MR). MR is a key transcription factor in the salt reabsorption pathway that acts on different downstream targets, being ENaC one of the most relevant ones (Figure 7a). The binding of SL on the MR blocks its signaling activities, preventing an excessive sodium reabsorption and potassium exchange at the tubule level, so it is categorized as a potassium-sparing diuretic (*89*).

SL it is the most acknowledged therapeutic choice for the control of blood pressure in PA and in resistant hypertension, and constitutes a first-line treatment for bilateral forms of PA. It is also indicated for treating unilateral forms of PA (including APA), when adrenalectomy is not feasible or desired, and prior to the surgery (*28*). Seven of the patients involved in this study were taking 25 mg/day of SL and urine samples were collected at baseline and after one month of treatment. UEV NCC mRNA level was measured and compared before and after MRA treatment (Figure 7b).

In 4 patients, the level of UEV NCC mRNA was found to be increased by the treatment. By analyzing biochemical and hormonal features of patients, we observed that plasma renin concentration was in correlation with NCC mRNA variation in abundance and such correlation was inversely proportional (Figure 7c). MR acts on different targets including ENaC and NCC. In particular, MR has been demonstrated to regulate ENaC abundance, transcription, trafficking and activity through a complex network of interactions (Figure 7a) (*16, 90-98*). On the other hand, it has been demonstrated that aldosterone stimulates the phosphorylation of NCC in a WNK-SPAK dependent manner but without affecting the total abundance or surface expression of the cotransporter (*23*). In a MR random-deletion experiment in mice it has been shown that, while alpha-ENaC was undetectable in MR^{ko} tubule cells, there was no difference in NCC abundance (*18*). Several other regulators can impact NCC functionality in an aldosterone independent way, including vasopressin and upstream players in the RAAS like angiotensin II (*99-104*).

The treatment with MRA has the ultimate effect of reducing sodium reabsorption and, consequently, blood volume. It is supposed that changes in the abundance of NCC mRNA are an indirect consequence of the reduced functionality of ENaC in the sodium reabsorption pathway and that such effects are not directly mediated by MR activity. In this view is interesting to consider that in never-treated hypertensive patients, ARR and low renin activity may predict the response to spironolactone (*105*). We are collecting more samples in order to confirm this hypothesis.

We measured the NCC mRNA in UEV of 4 APA patients who underwent adrenalectomy, all achieving complete or partial clinical success according to the PASO criteria (*106*). We observed the changes in the transcript abundance 3 months and one year after surgery (Figure 7d). After surgery, NCC mRNA increased in all patients, suggesting that the monolateral adrenalectomy and the related hormonal adjustment both deplete volume and stimulate the mRNA expression of NCC. Interestingly, after a longer period of follow-up (12 months), a clear trend was recorded toward pre-surgery levels, consistently with a complete reversal of the disease and full recovery of a normal blood volume, as generally observed in the clinical history of these patients.

Taken together, our data indicate that the transcriptional regulation of NCC is closely regulated by Na-driven volume, with compensatory increases in case of hypovolemia following drug or surgical treatment or- in opposite direction- in case of Na loading. We also hypothesize different regulatory layers for NCC mRNA and protein in the distal convoluted tubule, and together with the short-term regulation that acts at post-translational level by modifying apical trafficking of NCC and its activation, we can hypothesize the existence of a long-term adaption active at transcriptional level.

While there are no indications that NCC mRNA can be considered as a putative biomarker of PA, we showed that the analysis of mRNA content of UEV can provide information on the physiology and pathophysiology of the renal tubule, and with

this preliminary study we are proposing a new protocol for a non-invasive investigation of the transcriptional status in the kidney.

4.2 Proteomic analysis of urinary extracellular vesicles

In this study we have analyze the proteomic content of UEV of PA and EH patients using an untargeted approach. Our isolated UEV were characterized by TEM and their average diameter was estimated to be around 80 nm, compatible to what is reported in literature for exosomes (Figure 8). Features of patients are reported in Table 2.

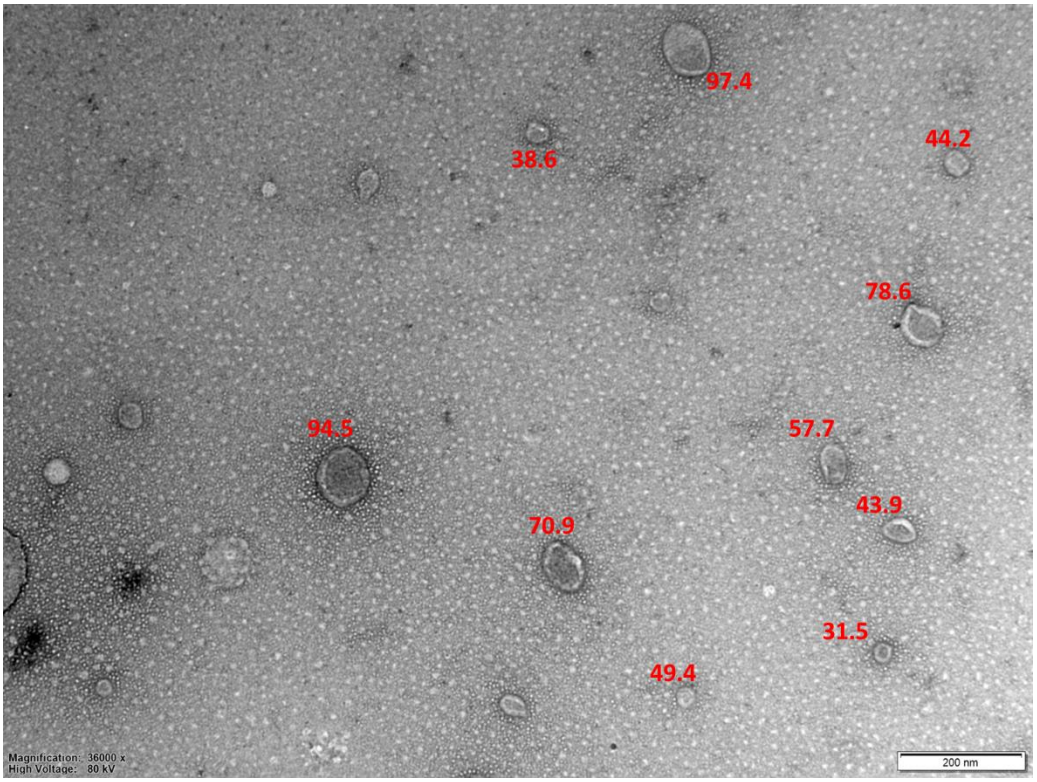

Figure 8: representative picture of TEM characterization of UEV isolated with utltracentrifugation

Table 2: biochemical and hormonal features of patients enrolled in UEV proteomics study. Values are indicated as mean ± SD.

	PA patients – 24 (13 APA, 11 BAH)	EH patients - 13	pval
Gender (m/f)	15/9	8/4	NS
Age (ys)	53.6 ±1.43	42.1 ± 4.8	<0.05
BMI	27.9 ± 0.9	27.3 ± 0.8	NS
Renin (pg/ml)	3.25 ± 0.8	18.7 ± 2.7	<0.01
Aldosterone (pg/ml)	372.4 ± 31.3	174.4 ± 25.8	<0.01
ARR	163.7 ± 47.3	11.33 ± 2.39	<0.01
Serum K (mmol l^{-1})	3.29 ± 0.12	4.0 ± 0.10	<0.05
Creatinine (mg dl^{-1})	0.88 ± 0.04	1.03 ± 0.26	<0.05
Glucose (mmol l^{-1})	4.9 ± 0.17	4.9 ± 0.23	NS
Cholesterol (mg dl^{-1})	192.3 ± 7.2	188.7 ± 11.1	NS
Triglycerides (mg dl^{-1})	99.29 ± 13.2	91.67 ± 20.2	NS

4.2.1 PA vs EH

In the early diagnosis stages of PA, it is important to separate PA patients from EH subjects. To identify putative biomarkers, we compared the UEV proteome of 12 EH patients and 23 PA patients. In our proteomics analysis, we identified a total of 301 proteins. T-test and fold-change analysis were used to identify prospects biomarker and subsequent network analysis was carried out to gain insight on the pathways involved in the definition of the pathology.

We have identified a total of 26 proteins differentially regulated between the two conditions, 13 proteins were up-regulated in PA (Figure 9 a, b). We compared our positive hits with proteins previously found in proteomics study on urinary exosomes in the database Vesiclepedia (VP) using FunRich 3, a free and standalone tool for functional enrichment (*70*). 8 of our differentially regulated proteins were not reported in VP.

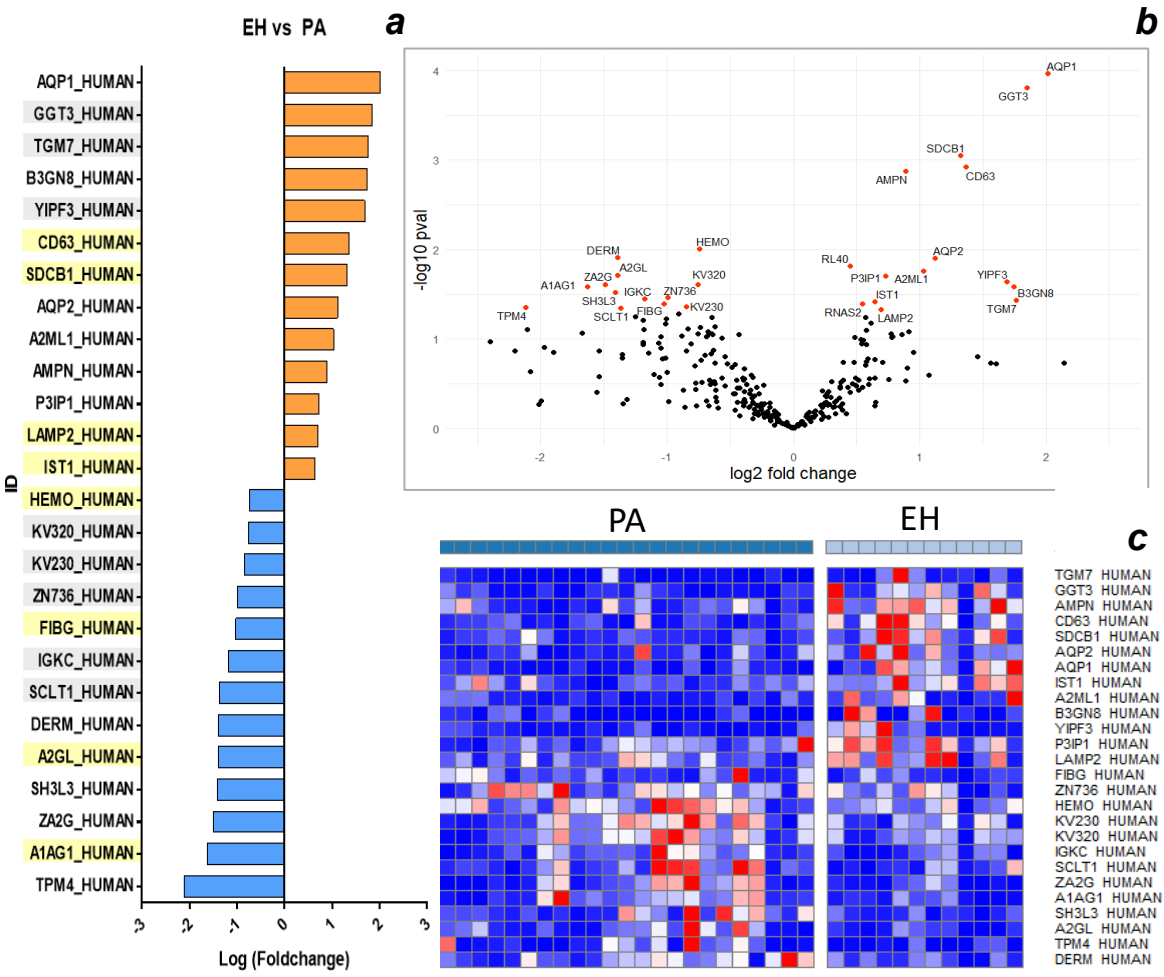

*Figure 9:(**a**) Barplot of Log(fc) for proteins with pval < 0.05. Fold change cutoff was set to 1.5. Grey highlighted names indicate protein not previously reported in the Vesiclepedia database (http://microvesicles.org/ , filter: urinary exosomes experiments). Yellow highlighted names indicate proteins belonging to clusters identified with STRING (https://string-db.org/). (**b**) Volcano plot and distribution of pval and fold change of the positive hits. (**c**) Heatmap of the top 26 proteins resulting from t-test analysis.*

In previous studies, we observed that there are significant sex-dependent differences in proteins and metabolites in urine (*77, 107*). Thus, we verified through t-test if the distribution of regulated proteins was influenced by sex. No significant differences were found analyzing the distribution of proteins in all the patients. However, analyzing the distribution within the diagnosis groups (PA and EH) we found significant differences for AQP1, YIPF3, AMPN in PA and B3GN8 in EH.

To assess the performance of the putative biomarkers, we performed Receiver-operating characteristic analysis (ROC) on the significant proteins. Setting the threshold of the area-under-curve (AUC) to 0.7, we identified 9 proteins increased in EH patients and 2 in PA patients as biomarker candidates (Figure 10 and Table 3).

Table 3: AUC and CI values for selected proteins

Protein	AUC	CI_inf	CI_sup	Up-regulated
GGT3	0.85	0.72	0.99	EH
CD63	0.83	0.65	1.00	EH
AMPN	0.83	0.66	1.00	EH
AQP1	0.83	0.64	1.00	EH
IST1	0.81	0.65	0.97	EH
AQP2	0.81	0.66	0.95	EH
SDCB1	0.79	0.61	0.96	EH
TGM7	0.77	0.60	0.94	EH
P3IP1	0.76	0.58	0.94	EH
A1AG1	0.74	0.57	0.91	PA
TPM4	0.73	0.55	0.92	PA

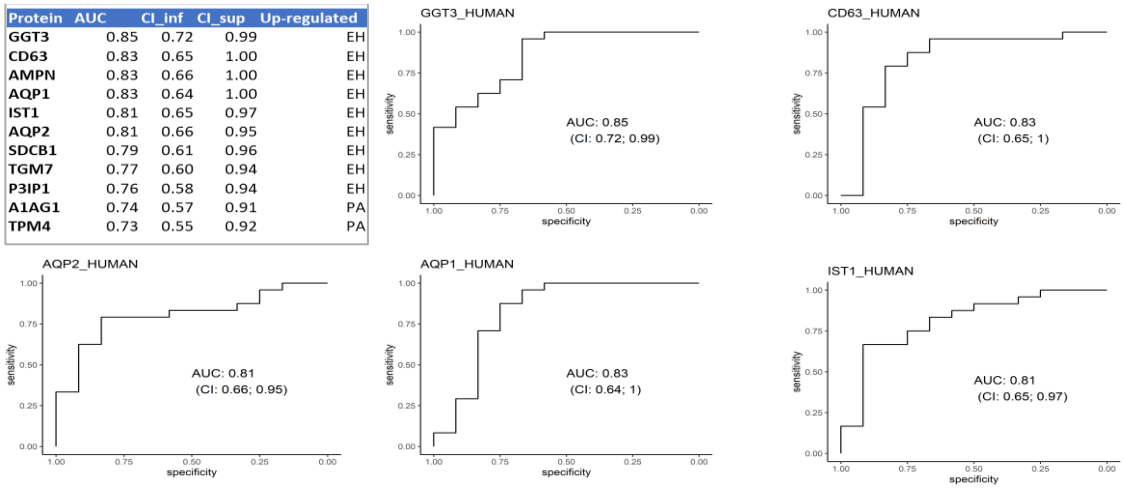

Figure 10: ROC curves of some of the candidate biomarkers.

Due to the significant sex-dependent difference in the distribution of AQP1 in PA patients, we calculated ROC curve separately for men and women. AUC remained unchanged (Female AUC= 0.81; Male AUC=0.82) but the lower limit of CI dropped to 0.42 for women (F CI=0.42, 1; M CI=0.61, 1).

Interestingly, the best performing protein GGT3 is a putative glutathione hydrolase, considered to be a non-functional product of a pseudogene (*108*). According to the database Genecards (www.genecards.org), kidney and thyroid tissues are the ones with the highest abundance of GGT3 mRNA. The role of this protein in these two organs is unknown.

To identify pathway involved in the different regulations of UEV proteins, we carried out network analysis using Gene Ontology and the database STRING. In PA patients, Gene Ontology reported an enrichment for proteins involved in *positive regulation of immune response* and *receptor-mediated endocytosis*. In EH patients, proteins were found to be involved in *renal water transport* and *cell activation involved in immune response.*

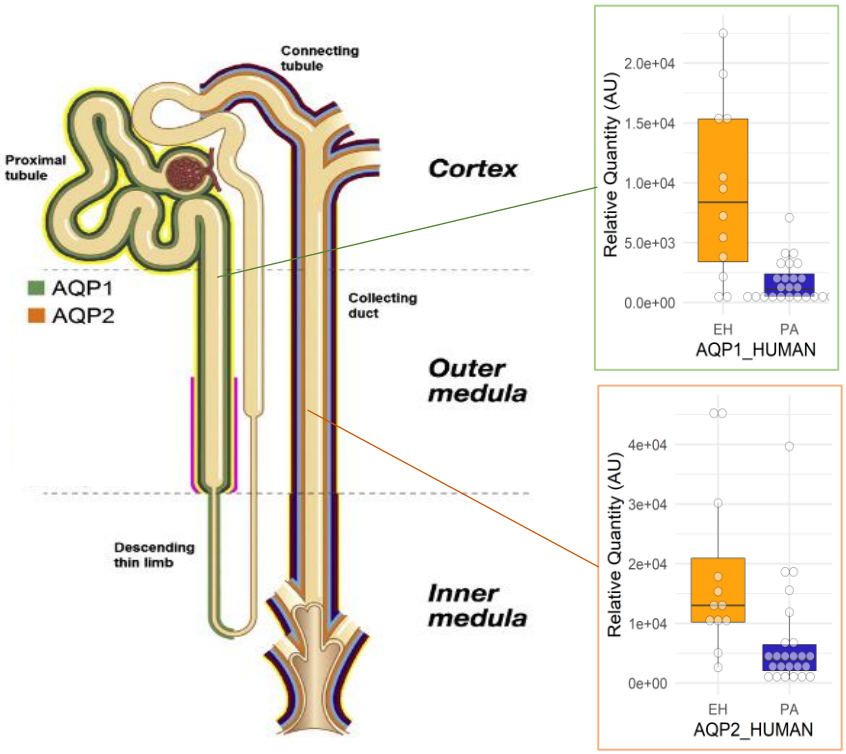

Figure 11: Boxplots and relative position in the nephron of AQP1, AQP2 and the target o. Adapted from Kortenoeven, 2014

AQP1 and AQP2 proteins, belonging to the renal water transport class, were found to be up-regulated in subjects of the EH group (Figure 11). Aquaporins are a family of transmembrane proteins that form pores in the membrane facilitating the transport of water using osmolarity as the driving force. Among the 13 identified aquaporins (AQP0-12), eight have been found in renal epithelial cells (AQP1-4, 6-8, 11) and they are critical for the maintenance of the body water balance (*109*).

AQP1 is localized in the apical and basolateral membrane of the epithelial cell in the proximal tubule, where a large part of the water filtered by the glomeruli (around 65%) is reabsorbed through an isosmotic mechanism. AQP1 is stimulated by ANGII, that effects directly the expression of AQP1 mRNA (*110*). Thus, the increased expression of AQP1 (5.5-fold in EH over PA) may be a consequence of the status of the RAAS in PA patients, where plasma aldosterone is high and renin is suppressed.

AQP2 is located in the apical plasma membrane in the collecting duct (CD) and it is the primary target for AVP regulation of CD permeability through trafficking of AQP2-loaded vesicles to the apical region or through an increase in AQP2 mRNA expression (*111*). By measuring the plasma concentration of copeptin (C-terminal portion of provasopressin) in PA and EH, it has been shown that cleavage of provasopressin was higher in PA patients, suggesting a higher and chronic activation of AVP signaling (*112*). Moreover, in an experiment aimed at the identification of aldosterone effects on AQP2, it has been demonstrated that, even though aldosterone causes a short-term decrease of AQP2 mRNA in $mpkCCD_{C14}$ cells, it increases long-term (48 h) protein turnover and reduces the channel degradation in the lysosome, despite a persisting low level of AQP2 mRNA transcription (*113*). This is in contrasts with our observed 2.6-fold increase of AQP2 protein in UEV of EH patients. However it is possible to hypothesize that a longer and chronic exposure to high plasmatic aldosterone could negatively affect the abundance of AQP2 by acting on the abundance of mRNA or by altering the trafficking of intracellular vesicles and apical localization (*114*).

Notably, the level of AQP1 and AQP2 protein in exosomes correlates (with some exceptions) with their renal protein level, and it has been observed that the urinary fraction of AQP2 is mainly located in exosomes with preserved transport activity (*115, 116*). This protein (AQP2), in fact, it is commonly utilized as a marker of exosomal origin for urinary vesicles characterization.

To identify protein-protein interactions and functional networks, we queried the database STRING (https://string-db.org/) with all the significant proteins together or separated for experimental groups. Two nodes differentially regulated were identified and the GO analysis for biological process reported an involvement of the "*secretion* pathways", while the most significant GO term for cellular component was *extracellular exosome* (Figure 12). Proteins up-regulated in PA patients were found to be involved in *regulated exocytosis* and, more specifically, they were classified under *alpha granule of platelets* according to Reactome

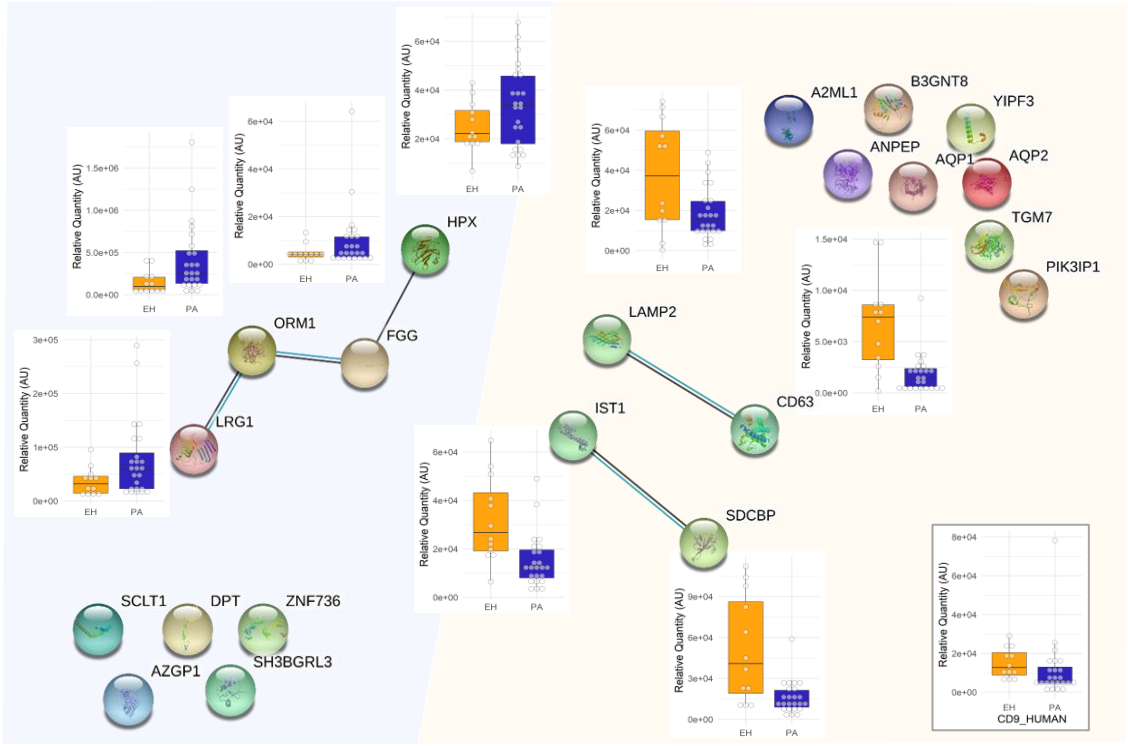

Figure 12: STRING analysis of regulated proteins. Text mining interactions were excluded. Nodes-relative boxplots are shown. CD63 resulted to be significantly higher in EH patients. No significant difference was found for CD9.

(https://reactome.org) classification. Alpha-granules are the most abundant secretory granules in platelets. Their content includes several growth factors and they are involved in many different functions, including wound healing and angiogenesis. Their size ranges from 200 to 500 nm and their maturation and release is strictly dependent on mature MVB, where also exosomes are assembled and sorted. Both alpha-granules and exosomes carry several surface markers in common, including CD9 and CD63 (*117*). Despite the number of common features, the alpha-granules are indeed characterized by a larger size when compared to exosomes. The TEM size assessment of the vesicles we isolated revealed an average size around 80 nm and the same proteins identified in the alpha-granules are listed in several exosomes experiments on the database Vesiclepedia, including experiments where exosomes were extracted from cell culture (FGG in VP ID and cell line: 348 (LIM1836) ; 591 (HT-29); 401 (THP-1); 738 (JEG-3) or urine (VP ID: 63; 179; 382; 508; 498). These observations suggest the possibility that the proteins included in the up-regulated node in PA (e.g. gamma fibrinogen or the acute-phase glycoprotein ORM1) are not exclusive of alpha-granules but can be also carried by other smaller vesicles originated in the MVB, as the exosomes. The role of these proteins in the kidney is not yet understood and needs to be investigated.

All the proteins found to be up-regulated in EH patients in the STRING node analysis showed association to exosomes biogenesis. Interestingly, one of the up-regulated proteins was CD63, a member of the tetraspanins family, commonly used as marker of exosomes and extracellular vesicles (albeit not exclusive of this subcellular fraction) and quantified for an indirect readout of the abundance of exosomes in a sample (*64, 69, 87, 118*). CD63 was found in association with LAMP2 (lysosomal associated membrane protein-2), glycoprotein frequently found in exosomes and late endosomes and significant correlations were found with other proteins up-regulated in EH and involved in exosomes biogenesis (SDCB1) and members of the ESCRTIII complex (IST1) (CD63-SDCB1: R=0.72, p=8.1e-07; CD63-IST1: R= 0.45, p=0.005).

Extracellular vesicles and exosomes are populations characterized by a high level of heterogeneity and it has been demonstrated that differences in the markers exposed on the surface of exosomes can reflect differences in their cargo (*119*). As a relevant example of this, it has been found that the PA marker NCC is associated with CD9+ but not CD63+ urinary vesicles.(*87*). While the CD63+ fraction of UEV is more abundant in EH patients, we detected no significant differences in the abundance of CD9 marker (Figure 12).

Taken together, our results indicate a strong variability in the composition of vesicles isolated from the urine of PA and EH patients. It is possible to hypothesize that this may depend on a different pathophysiological condition of the releasing tissue or a different tissue-of-origin of the vesicles. In addition, we found that in our settings CD63 and CD9 UEV markers do not correlate and their separate evaluation for normalization purposes may introduce biases when vesicle populations are enriched in different surface markers. For proteomics investigations on UEV, we hypothesize that normalization strategies independent of the vesicle composition (i.e. protein quantification or NTA count) could provide more reliable results.

AQP1 and 2 values were found to be different between EH and PA patients and it has been shown that their level in urinary vesicles mirrors the level and functionality in the tissue. We can speculate that the reduced water absorption activity of AQP1 and 2 in PA patients is a sort of long-term response to the expanded-volume hypertension.

A subset of proteins significantly altered between the two groups have been already proposed as urinary biomarkers in other settings or have been observed to be altered under specific conditions (table 4).

Table 4: details on the significant protein hits in PA vs EH comparison, including literature references when used as urinary biomarkers in other settings.

Protein	Name	Description	Up-reg in	Previously proposed as urinary biomarker?	REF
TPM4	Tropomyosin alpha-4 chain	Actin filament binding, Calcium ion binding	PA	Up-regulated in TAL of Henle's loop cells under osmotic stress	(*120*)
A1AG1	Orosomucoid 1	acute-phase response	PA	Proposed as urinary marker of chronic heart failure, bladder cancer and active lupus nephritis in children. Is protective against renal ischemia-reperfusion injury (anti-inflammatory effect); Has been observed to ameliorate nephrotic syndrome in a mouse model.	(*121-125*)
ZA2G	Zinc-alpha-2-glycoprotein	transmembrane transporter	PA	Proposed as prognostic marker in prostate cancer. Exerts Antifibrotic Effects in Kidney	(*126, 127*)
SH3L3	SH3 Domain Binding Glutamate Rich Protein Like 3	electron transfer activity; cell redox homeostasis	PA	Proposed as potential prognostic marker for urothelial carcinoma (binds EGFR)	(*128*)
A2GL	Leucine-rich alpha-2-glycoprotein	TGFb binding; regulation of endothelial cells proliferation	PA	Increased in severe IgA nephropathy	(*129*)
DERM	Dermatopontin	cell adhesion	PA	-	
SCLT1	Sodium channel and clathrin linker 1	chlatrin binding	PA	Interacts with the voltage-gated Na channel Na(v)1.8 (SCN10A) and chlatrin. SCN10A is expressed also at kidney level.	(*130, 131*)
IGKC	Immunoglobulin Kappa Constant	antigen binding, ig receptor binding	PA	Increased in urine of lung adenocarcinoma patients	(*132*)
FIBG	Fibrinogen gamma chain	blood coagulation, cell adhesion molecule inding, positive regulator	PA	Proposed as early diagnostic biomarker for AKI	(*133*)

		of exocitosis			
ZN736	Zinc Finger Protein 736	nucleic acid binding	PA	-	
KV230	Immunoglobulin kappa variable 2-30	antigen binding	PA	-	
KV320	Immunoglobulin kappa variable 3-20	antigen binding	PA	-	
HEMO	Hemopexin	heme transporter activity, cellular ion homeostasis	PA	Proposed for diagnosis of chronic predisposition to AKI, urinary exosomal biomarker of acute T-cell mediated rejection in kidney transplant	(*134, 135*)
IST1	IST1 homolog	cadherin binding, MVB assembly	EH	-	
LAMP2	Lysosome-associated membrane glycoprotein 2	enzyme binding, lysosomal protein cataboli process	EH	Increased in Cholangiocarcinoma patients	(*136*)
P3IP1	Phosphoinositide-3-kinase-interacting protein 1	negative regulation of PI3K activity	EH	-	
AMPN	Aminopeptidase N	aminopeptidase activity (ANGIII, IV are targets)	EH	Activity of aminopeptidases has been proposed as biomarker for bladder cancer.	(*137*)
A2ML1	Alpha-2-macroglobulin-like protein 1	peptidase inhibitor activity	EH	Proposed as diagnostic and prognostic marker for necrotizing enterocholitis in infants	(*138*)
AQP2	Aquaporin-2	water transport	EH	Proposed as diagnostic biomarker of diabetic nephropathy	(*139*)
SDCB1	Syntenin-1	cytoskeletal adaptor activity (with PDCD6IP regulates exosome	EH	-	

		biogenesis)			
CD63	CD63 antigen	cell-matrix adhesion	EH	-	
YIPF3	Protein YIPF3	cell differentiation	EH	-	
B3GN8	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 8	acetylglucosaminyltransferase activity, protein glycosylation	EH	-	
TGM7	Protein-glutamine gamma-glutamyltransferase Z	protein-glutamine gamma-glutamyltransferase activity, peptide crosslinking	EH	-	
GGT3	Putative glutathione hydrolase 3 proenzyme	Pseudogene. Transcript is abundant in the kidney	EH	-	(*108*)
AQP1	Aquaporin-1	water transport	EH	AQP1-containing exosomes were proposed as biomarker of dialysis efficiency. AQP1 in urine has been proposed as screening marker for renal cell carcinoma	(*140*)

4.2.2 APA vs BAH

We performed a similar analysis to identify proteins that can separate the two main PA subgroups and help in the characterization of the disease. We compare the UEV proteome of 13 APA patients against 11 BAH patients. In this comparison we found a total of 25 differentially regulated proteins, 6 of which were not reported in VP database (as shown in Figure 13 a). Nine proteins were up-regulated in APA while 16 were up-regulated in BAH patients (Figure 13).

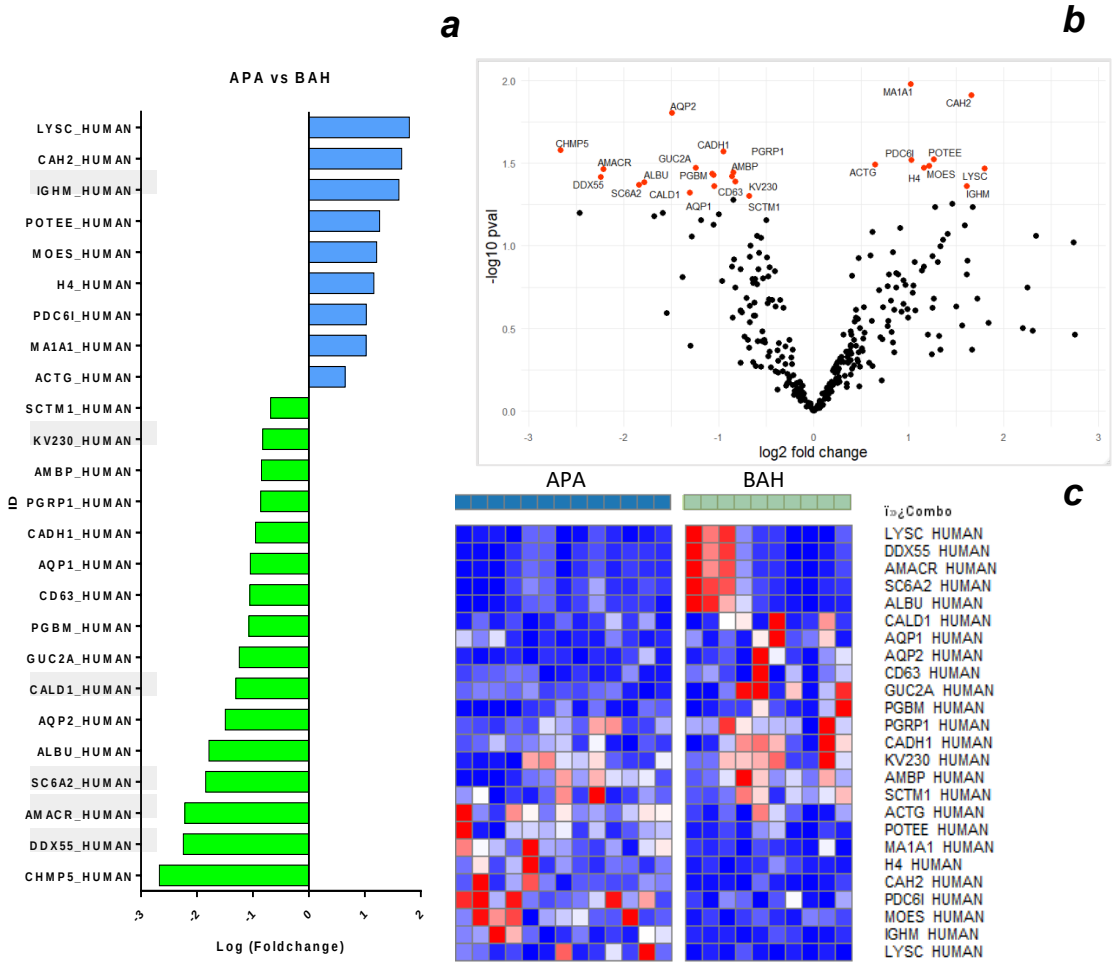

*Figure 13: (**a**) Barplot of Log(fc) for proteins with pval < 0.05 and FC cutoff of 1.5. Grey highlighted names indicate protein not previously reported in the Vesciclepedia database. (**b**) Volcano plot and distribution of pval and fold change of the positive hits. (**c**) Heatmap of the top 25 proteins resulting from t-test analysis.*

ROC curve analysis setting AUC threshold to 0.7 and lower limit of CI to >0.5 gave 8 prospect biomarkers, all up-regulated in APA (Table 5 and Figure 14). Among the up-regulated proteins in BAH patients we found CD63, AQP1 and AQP2 that were suggested as candidate biomarkers for differentiating PA vs EH patients in the previous comparison, even though none of them were above the threshold we set for AUC and CI in the shortlisting of the best candidates.

As we showed that sex variable may affect the distribution of proteins, we performed Mann-Whitney test between the two PA subtypes. With the exception of CAH2, the proteins listed in the table 2 resulted all significantly different for the variable sex. This is probably due to the uneven distribution of the variable sex in our sample set. To improve the reliability of the selection of prospect biomarkers, we performed sex-separated ROC analysis. H4, LYSC, CAH2 and M1A1 maintained an AUC > 0.8 in all cases and a CI lower limit >0.5.

Table 5: AUC and CI values for proteins

Protein	AUC	CI_inf	CI_sup	Up-reg
H4	0.88	0.74	1.00	APA
MA1A1	0.87	0.71	1.00	APA
PDC6I	0.83	0.67	1.00	APA
LYSC	0.84	0.66	1.00	APA
POTEE	0.81	0.64	0.99	APA
CAH2	0.82	0.63	1.00	APA
ACTG	0.81	0.62	1.00	APA
MOES	0.80	0.62	0.99	APA

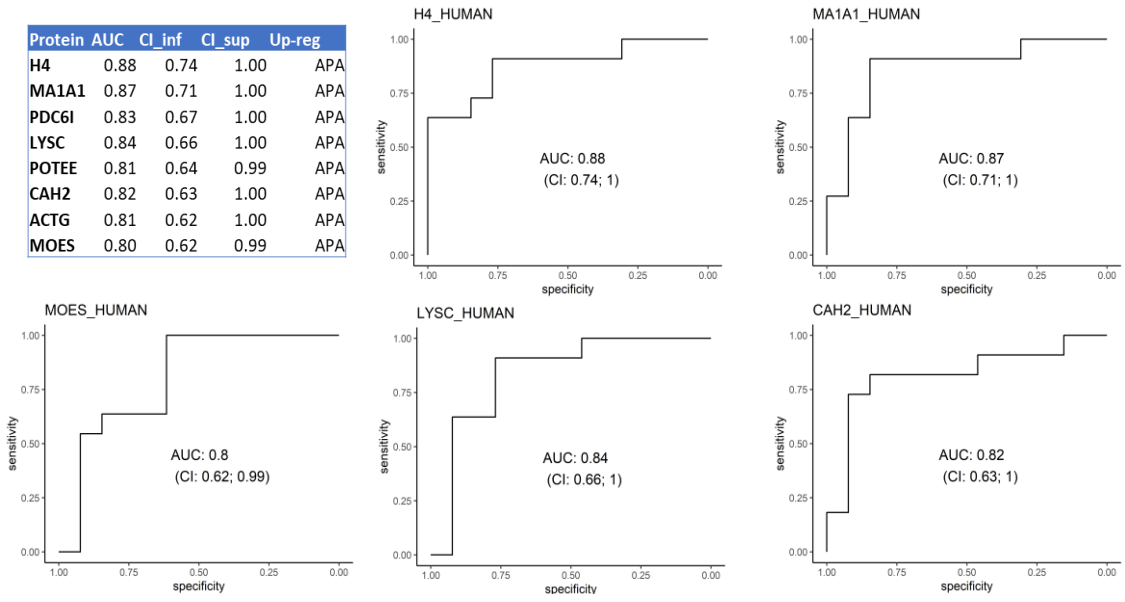

Figure 14: ROC curves of selected candidates.

One of the best performing protein from the ROC analysis resulted to be histone H4. Histones were previously considered to be an indication of apoptotic bodies

contamination in a vesicle isolation experiment (*141*). However, an increasing number of reports is showing that histones are frequently included in exosomes, where they can participate in the inclusion of DNA in the vesicular cargo, and some members of this family have been already included in panels of putative biomarkers (*142-144*). In addition, in our experimental setting we did not find correlations between H4 and other nuclear proteins, event that could point to a possible contamination due to large apoptotic bodies. For these reasons, histone H4 was included among the candidate biomarkers, though its canonical nuclear localization should be taken in consideration in the future validation experiments for this protein, as sources other than exosomes can interfere with its abundance. Another well performing candidate was the carbonic anhydrase II (CAH2). CAH2 is an enzyme that increases the speed of the reversible reaction that convert carbon dioxide and water to carbonic acid, that becomes bicarbonate ion in water with a loss of a proton. This enzyme is expressed along the nephron and interacts with a large number of transporters and ion exchangers: it has been shown that can stimulate the epithelial sodium-proton exchanger NHE3 in the proximal tubule, increases the activity of the electrogenic Na+/HCO3- cotransporter NBCe1, and regulates the activity of Pendrin (*145-148*). CAH2 is able also to physically interact with the cytosolic C-terminal of AQP1, stimulating water reabsorption. In CAH2-deficient mice it has been observed that AQP1 expression was increased while the levels of AQP2 and NKCC2 remained equal to *wt* (*149*). Although AQP1 is more abundant in BAH patients that have also significantly less CAH2 compared to APA and EH patients, we could not find a correlation and we cannot support the view of a direct regulation of AQP1 by CAH2. However, given the amount of interactions involving CAH2 and that AQP1 and 2 were statistically different even between APA and BAH, we can hypothesize that the two subtypes of PA may be different in their water reabsorption pathways mechanisms.

Network analysis was carried out to detect functional protein associations. GO analysis for biological process identified 14 proteins involved in transport (*vesicle-*

mediated transport, *renal water transport*, *bicarbonate*) (Figure 15). Proteins involved in water transport and vesicle-mediated transport were up-regulated in BAH patients while structural proteins (ACT1, MSN) were up-regulated in APA. Although STRING identified several protein-protein interactions, no relevant nodes were found, since interacting proteins were differentially regulated between the two experimental groups.

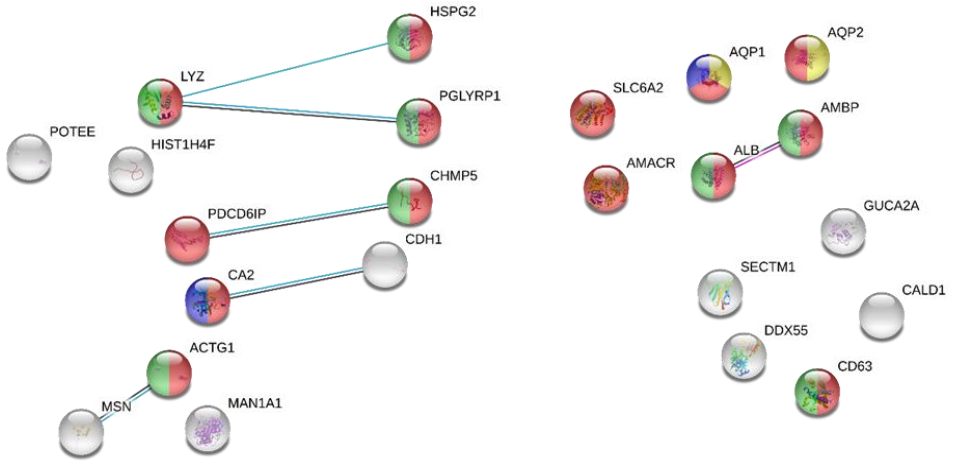

Figure 15: String analysis of protein differentially regulated between APA and BAH patients. Text mining interactions were excluded. Function of these protein is color-coded: red=transport; yellow=renal water transport; green= vesicle-mediated transport; blue= bicarbonate transport.

In previous studies, we demonstrated striking metabolic differences in urine samples of the two PA subtypes, and in our UEV proteomic study we detected differences in water reabsorption proteins and key regulators of acid-base equilibrium and ion transporters (*77*). Our exploratory study has pioneered the possibility to exploit these differences in UEV proteins for improving in, a non-invasive way, the identification and characterization of PA subjects. However, some inherent limitations need to be taken into consideration. The main limitation is the limited number of patients included in our analysis. This limitation required the use of more stringent criteria to short-list our biomarker candidates, although is still possible that the separation performances have been overestimated due to overfitting. To confirm our findings, future studies should enroll a larger cohort of

patients for the verification step, that would be conducted in parallel with different analysis or detection protocols (e.g. MRM, Elisa). Due to the sample size limitations, we also limited the stratification of patients and did not explore the possibility of identifying sex-specific biomarker candidates or testing multiple candidates in a panel to increase specificity. Both possibilities will however become viable in a larger sample size study. Once validated, UEV biomarkers able to discriminate between PA and EH could be used in clinic to help in the diagnostic workflow of primary aldosteronism, limiting the use of confirmatory test that can give rise to complications for patients. Biomarker able to characterize the PA subtype may improve the current diagnostic work-up providing an alternative to AVS and allowing a time-effective choice of the appropriate treatment.

5. Metabolomics

We have performed serum metabolomics in PA and EH patients adopting a semi-targeted approach MS strategy. Table 6 shows the biochemical and hormonal features of the patients in this study. As expected, significant differences are detected only for renin, aldosterone, ARR and serum potassium.

Table 6: the biochemical and hormonal features of the patients in metabolomics study. Values are reported as mean ± SD.

	PA patients – 17 (13 APA, 4 BAH)	**EH patients - 37 (27 low-ARR, 10 hi-ARR)**	**pval**
Gender (m/f)	23/12	13/6	NS
Age (ys)	48.56 ± 2.5	44.32 ± 2.26	NS
BMI	27.18 ± 1	28.37 ± 0.83	NS
Renin (pg mL^{-1})	4.178 ± 0.93	11.24 ± 1.43	<0.01
Aldosterone (pg mL^{-1})	367.6 ± 48.25	158.5 ± 9.49	<0.01
ARR	123.2 ± 34.13	33.25 ± 6.9	<0.05
Serum K (mmol l^{-1})	3.45 ± 0.16	3.86 ±0.05	<0.05
Creatinine (mg dl^{-1})	0.92 ± 0.08	0.87 ± 0.03	NS
Glucose (mmol l^{-1})	4.89 ± 0.12	5.18 ±0.23	NS
Cholesterol (mg dl^{-1})	202.9 ± 9.6	201.2± 7.53	NS
Triglycerides (mg dl^{-1})	114.1 ± 15.85	100.8 ± 7.77	NS

5.1 Diagnosis and subgroups comparison

We have analyzed 56 serum samples through a UHPLC-MS semi targeted approach, obtaining the relative quantitation of 164 metabolites. We started our analysis by comparing the different metabolites in all the experimental subgroups (characterized by a different diagnosis) that are involved in the diagnosis work-up of PA. In this setting, we included the group of hypertensive patients who resulted positive in the ARR screening (labeled as HI ARR), but negative to the IV-SLT, to have an insight on the metabolic status of hypertensive patients with a suppressible high level of aldosterone. We identified 19 metabolites significantly different within the groups of patients (ANOVA, adj. p<0.05). These metabolites were related to energy,

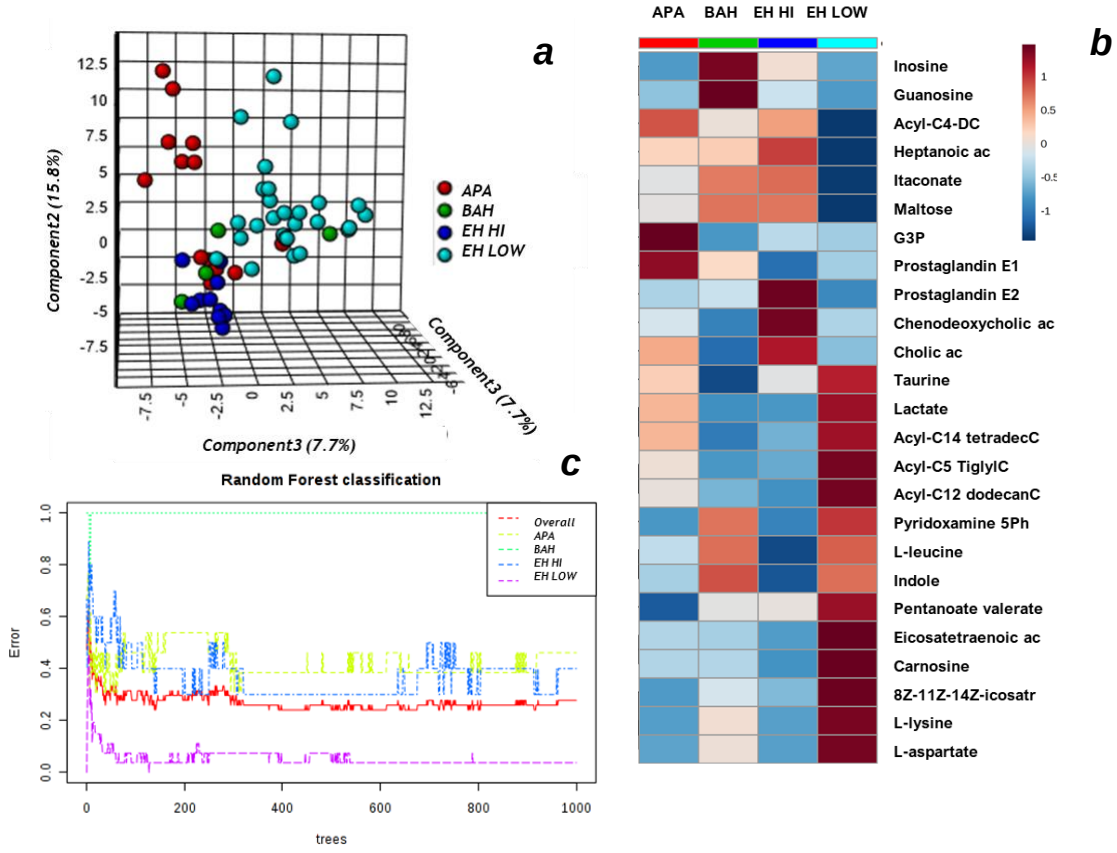

*Figure 16: (**a**) PLS-DA analysis clustering. (**b**) Clustering analysis of best 25 metabolites obtained with ANOVA analysis. PLS-DA VIP score and clustering analysis have identified the same classes of metabolites. (**c**) Random forest analysis confirmed the group clustering and the clear separation between low-ARR and hi-ARR EH patients.*

nucleotides metabolism, fatty acid metabolism and lipid mediators of inflammation (Figure 16).

PLS-DA was conducted to identify the metabolite with the highest contribution to group separation. We found specific metabolic signatures for PA subtypes and, interestingly, we found that there was a clear separation between EH patients who result positive for ARR screening (hi-ARR) compared to patients that are tested negative. Results obtained with PLS-DA were validated by random forest analysis and clustering analysis, both confirming the separation of hi- and low-ARR clusters and the metabolic heterogeneity in APA and BAH groups.

5.2 PA and EH comparison

We then analyzed serum metabolome of the two main groups (PA and EH) including hi-ARR patients, to identify metabolic changes reflecting a constant and non-suppressible high level of plasma aldosterone (Figure 17).

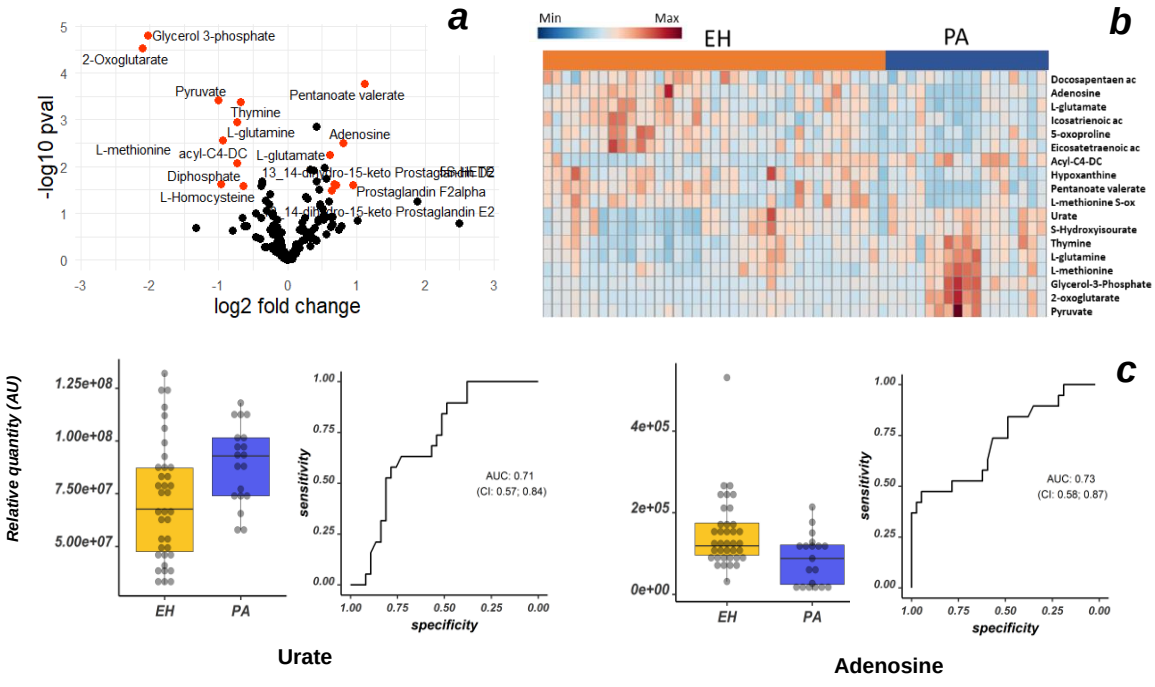

*Figure 17: (**a**) Volcano-plot showing metabolites with FC cutoff set at 1.5 and p<0.05. (**b**) Heatmap showing significant metabolites. APA subgroup was characterized by a high level of heterogeneity in energy-related metabolism. (**c**) Box and dotplots of some of the most significant hits and their relative ROC curve.*

We found that purine metabolism was altered, with a decrease in adenosine and hypoxanthine plasma concentration for PA patients, that had also an increased plasmatic level of urate. Altered purine metabolism was also observed in our previous analysis on urine metabolome of PA and EH patients (*77*).

Several reports have described associations between serum uric acid and hypertension or cardiovascular disease. Hyperuricemia has been found to be predictive of the development of hypertension and it has been reported a strong correlation with blood pressure in the majority of adolescents with EH of recent onset (*150*). In a rat model, mild hyperuricemia was induced by inhibiting uricase, resulting in hypertension that recapitulates the hemodynamic and histologic features observed in clinic and activation of RAAS, showing another link between uric acid and hypertension (*151*). Despite its physiological role as an antioxidant, high level of uric acid in serum have been independently correlated with cardiovascular events and it has been shown that this metabolite can perturbate blood pressure regulation and induce endothelial tissue damage through different mechanisms, including altered NO production and promotion of inflammation (*150, 152-155*). Multiple cross-sectional studies and meta-analysis have reported that PA patients are exposed to a higher risk of cardiovascular events compared to hypertensive patients and, in this view, urate could be a promising marker for the evaluation of incidence of such effects (*29, 30*). Another interesting finding of this analysis is that adenosine is significantly lower in PA patients. Adenosine binds specific receptors (A2A) on vascular smooth muscle cells and induces muscle relaxation and vasodilation (*156*). In addition, it exerts many other activities that are protective on the blood pressure and endothelium homeostasis, including the modulation of cardiac remodeling after ischemia-reperfusion and the regulation of inflammatory response (*157, 158*). Consistently with our observations, van den Berg and colleagues have shown that adenosine plasma concentration is lower in PA patients and hypothesized that this can contribute to their higher susceptibility to cardiovascular events (*159*).

Energy metabolic pathways were also found to be different between PA and EH patients, showing an increased level of glycolysis intermediates as glycerol-3-phosphate and pyruvate for PA patients, levels that were higher in the APA subgroup. Notably, PA patients were characterized also by a higher level of alpha-ketoglutarate and glutamine, while the level of glutamate and oxoproline resulted to be lower. This may be indicative of a different regulation of glutamate shunting in the TCA, with possible downstream effects on the homeostasis of glutathione. No differences between PA and EH patients were found in arginine or citrulline metabolism, key metabolites in the NO pathway.

Differences in metabolism of fatty acids were mainly found in the class of polyunsaturated fatty acids, including arachidonic, icosatrienoic and docosapentaenoic acids, found to be higher in EH patients. Differences were also detected in acyl-carnitines profile, where we detected an increased level of short chain carnitines (acyl-C4) for PA and hi-ARR patients, while medium and long chain carnitines were higher in low ARR patients.

Sex-dependent differences were detected in the distribution of several long-chain fatty acids, proline, branched-chain amino acids, intermediates of tryptophan metabolism and prostaglandin E1, supporting the previous observations on sex-specific differences in metabolic profiles.

Our results indicate that PA and EH patients have specific metabolic signatures and that a non-suppressible high plasma level of aldosterone can have impact on purine and fatty acids metabolism. Interestingly, several metabolites of PA signature have been correlated with higher incidence of cardiovascular effects and, as a future perspective, these metabolites and the pathways involved should be investigated to identify promising therapeutic targets or markers that can provide prognostic information in clinical practice.

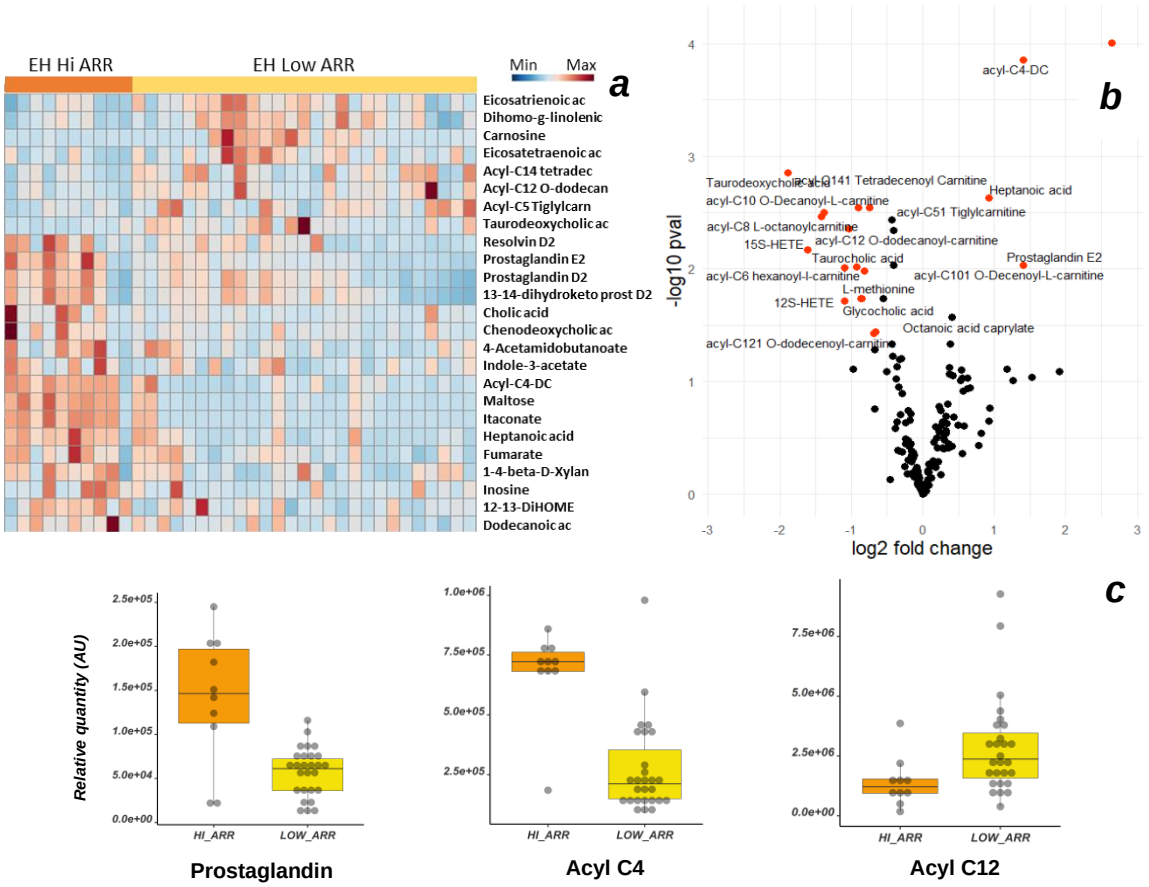

*Figure 18: (**a**) Heatmap of the metabolic comparison of low-ARR and hi-ARR EH patients and (**b**) volcano-plot showing metabolites with FC cutoff set at 1.5 and p<0.05. Both representations show a clear difference in acyl-carnitines profile and oxylipids. (**c**) Box and dotplots of some of the most significant hits.*

In our study we also found striking differences in the clustering of metabolic profile of hi-ARR and low-ARR hypertensive patients (Figure 16b). Patients positive for the ARR screening but negative for the confirmatory test are considered to be not affected by PA and treated as essential hypertensive patients (*28*). Our data indicates that EH patients who result positive for ARR screening have a different acyl-carnitines, eicosanoids and prostaglandin profiles when compare to ARR negative EH patients (Figure 18). Kisaka and colleagues used ARR screening to classify EH patients and investigated differences in cardiovascular risk in these two subpopulations, finding that high ARR can be an independent predictive marker of CV events (*160*). On the basis of this observation, we speculate that these metabolic

differences may have clinical and therapeutic implications and should be investigated further. Should the clinical relevance of these difference be confirmed, it could be advisable to routinely perform ARR screening also to assess the status of the RAAS in hypertensive patients, to specifically address their increased risk in developing adverse cardiovascular effects.

5.3 APA and BAH comparison

PA subtyping is essential to determine the best therapeutic strategy for the treatment of the disease. In our previous study on urine metabolomics, we have found a strong metabolic discrimination between BAH and APA. We have investigated differences in serum metabolites of these two subtypes, as they are representative of the majority of PA cases.

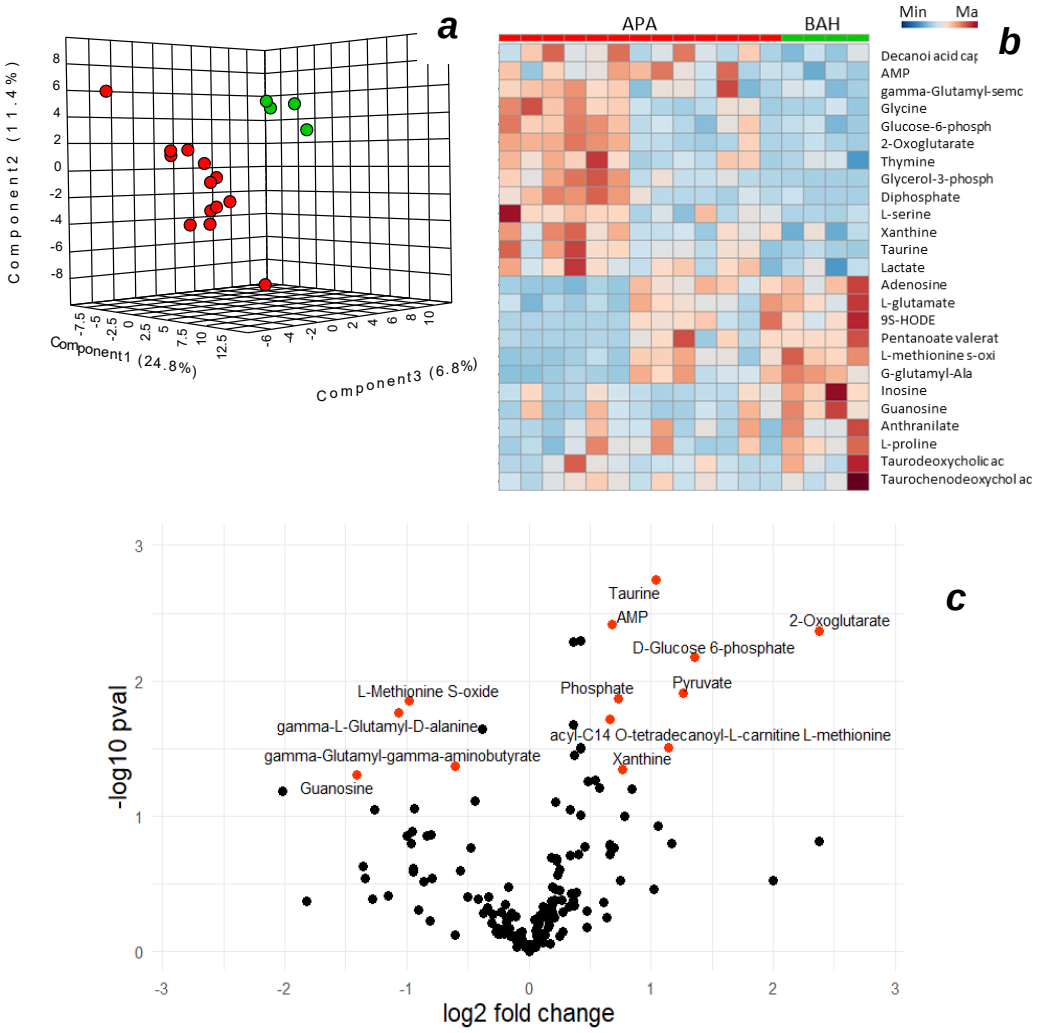

*Figure 19: (**a**) PLS-DA clustering of APA (in red) and BAH (green) samples. (**b**) Heatmap of the top-25 metabolites identified with t-test analysis. VIP score and clustering analysis concordantly identified the same metabolic pathways as differentially regulated between the two groups of PA patients. (**c**) Volcano plot, statistically significant metabolites in t-test are highlighted.*

PLS-DA analysis identified differences in metabolites mainly involved in energy and purine metabolism and sulfur-containing metabolites. Data were confirmed through clustering analysis (Figure 19). Top 25 metabolites resulting from PLS-DA analysis are included in table 7.

Table 7: contributors to PLS-DA separation. Not all metabolites reached statistical significance in t-test comparison.

Metabolite	Pathway	pval	FoldChange
Inosine	Purine metabolism	<0.01	4.05 UP BAH
Guanosine	Purine metabolism	0.01	2.65 UP BAH
9S-HODE	Eicosanoids	NS	2.57 UP BAH
Taurodeoxycholic acid	Bile acids	NS	2.54 UP BAH
Taurochenodeoxycholic acid	Bile acids	NS	2.43 UP BAH
Pentanoate valerate	Saturated Fatty acids	NS	2.4 UP BAH
gamma-L-Glutamyl-D-alanine	Gamma-glutamyls	0.02	2.09 UP BAH
L-Methionine S-oxide	Sulfur metabolism	<0.01	1.97 UP BAH
Adenosine	Purine metabolism	0.03	1.92 UP BAH
L-glutamate	Aminoacids	0.04	1.74 UP BAH
Anthranilate	Indole and Tryptophan	NS	1.39 UP BAH
L-proline	Aminoacids	NS	1.36 UP BAH
Decanoic acid caprate	Saturated Fatty acids	NS	1.29 UP APA
L-serine	Aminoacids	NS	1.29 UP APA
gamma-Glutamyl-Se-methylselenocysteine	Gamma-glutamyls	NS	1.34 UP APA
glycine	Aminoacids	NS	1.34 UP APA
Lactate	Glycolysis	NS	1.5 UP APA
AMP	Nucleotides, purine metabolism	0.02	1.6 UP APA
Xanthine	Purine metabolism	0.03	1.7 UP APA
Thymine	Nucleotides	NS	1.79 UP APA
Taurine	Sulfur metabolism	0.05	2.05 UP APA
D-Glucose 6-phosphate	Glycolysis	0.04	2.56 UP APA
2-Oxoglutarate	TCA cycle	NS	5.2 UP APA
Glycerol 3-phosphate	Glycerophospholipid biosynthesis	0.03	7.72 UP APA
Diphosphate	Phosphates	NS	9.75 UP APA

APA patients were characterized to have a higher level of glycolysis and TCA cycle intermediates. In agreement to what our laboratory has previously found in the urine metabolomics experiment, we have also found significant differences in purine

metabolism intermediates (although no differences were detected for uric acid levels) and sulfur-containing metabolites.

Dysregulation in carbohydrate metabolism, lipid profiles and circulating free fatty acids have been linked with hypertension (*74*). Altered purine metabolism has been shown strictly related with hypertension in different settings, although the focus of the majority of the studies was the end product uric acid (*161, 162*). Adenosine, a vasodilation effector involved in the "basal" arterial tone decreasing the overall vascular resistances, was altered in PA vs EH. Evidence in favor of a blunted vasodilation in response to acetylcholine in PA as compared with EH patients (in spite of similar BP values) is already reported in literature (*163*). It may be interesting to further investigate this aspect, that could be specifically ascribed to the mineralocorticoid activity on muscle vessels rather than the BP values.

APA and BAH are both characterized by a hypertension caused by non-suppressible aldosterone secretion and their clinical distinction is paramount for identifying the appropriate therapeutic strategy. Our metabolomics analysis has shown that the two diagnosis subgroups are also characterized by a distinct metabolic signature in pathways related to hypertension, and those differences were detected in two different matrixes (serum and urine). This suggests that different causes of primary aldosteronism may impact differently the metabolic pathways related to hypertension. Little is known about the pathophysiological differences between APA and BAH subtypes, and genetic mechanisms of unilateral and bilateral primary aldosteronism are yet to be understood (*14*). The reason on why APA and BAH patients have a specific metabolic signature, despite being characterized by the qualitatively same expanded-volume aldosterone-dependent mechanism, is still unknown, but we can speculate that the extent of metabolic and pathophysiologic alterations of adrenal tissue may dysregulate corticosteroids homeostasis and the downstream metabolic pathways (*78, 164*).

The different metabolic signature between APA and BAH patients opens the possibility of integrating metabolic profiling in the diagnostic work-up, to improve the PA characterization step.

5.4 APA patients and post-adrenalectomy follow up

To identify metabolic changes associated with therapy in PA, we have followed up 7 APA patients who underwent adrenalectomy 3 months, one year and two years after surgery.

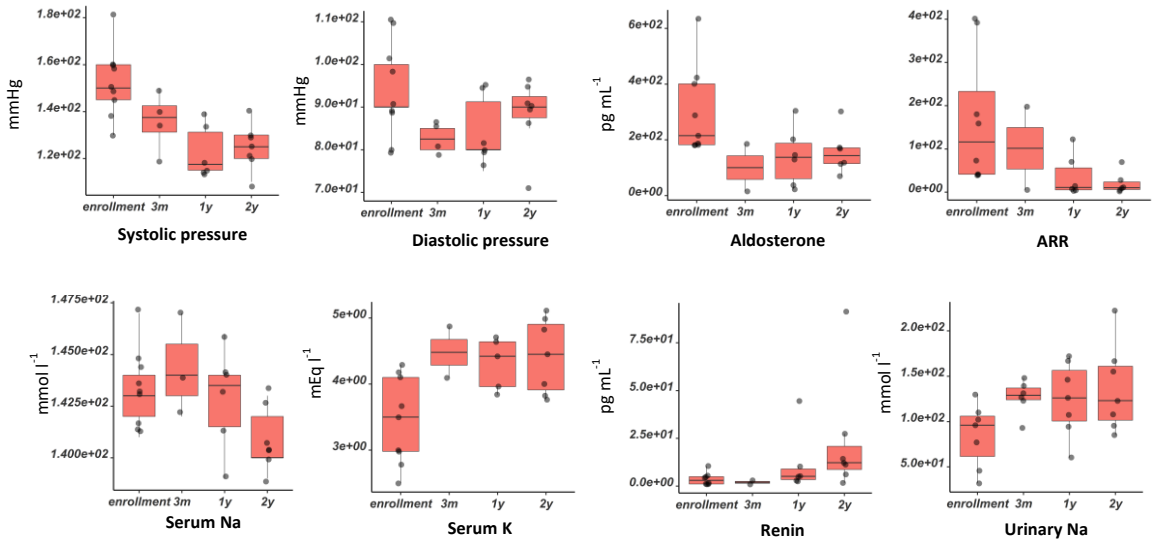

Figure 20: trends in biochemical and hormonal features of APA patients in the adrenalectomy follow-up.

After surgery, systolic and diastolic blood pressure returned to normal ranges in all patients. Restoring of RAAS regulation was evident by a significant increase in renin plasma level and decrease of plasma aldosterone. Excretion of sodium in urine was found to be increased as well as potassium levels in the blood. In most cases, changes in biochemical and hormonal parameters were evident from the first follow up point (3 months after surgery) (Figure 20).

Data were analyzed using the *Multivariate Empirical Bayes Analysis of Variance (MEBA) for Time Series* plugin in Metaboanalyst. Most of the metabolic changes were detected within the first 3 months after surgery and were maintained across the 2 years of follow up timespan.

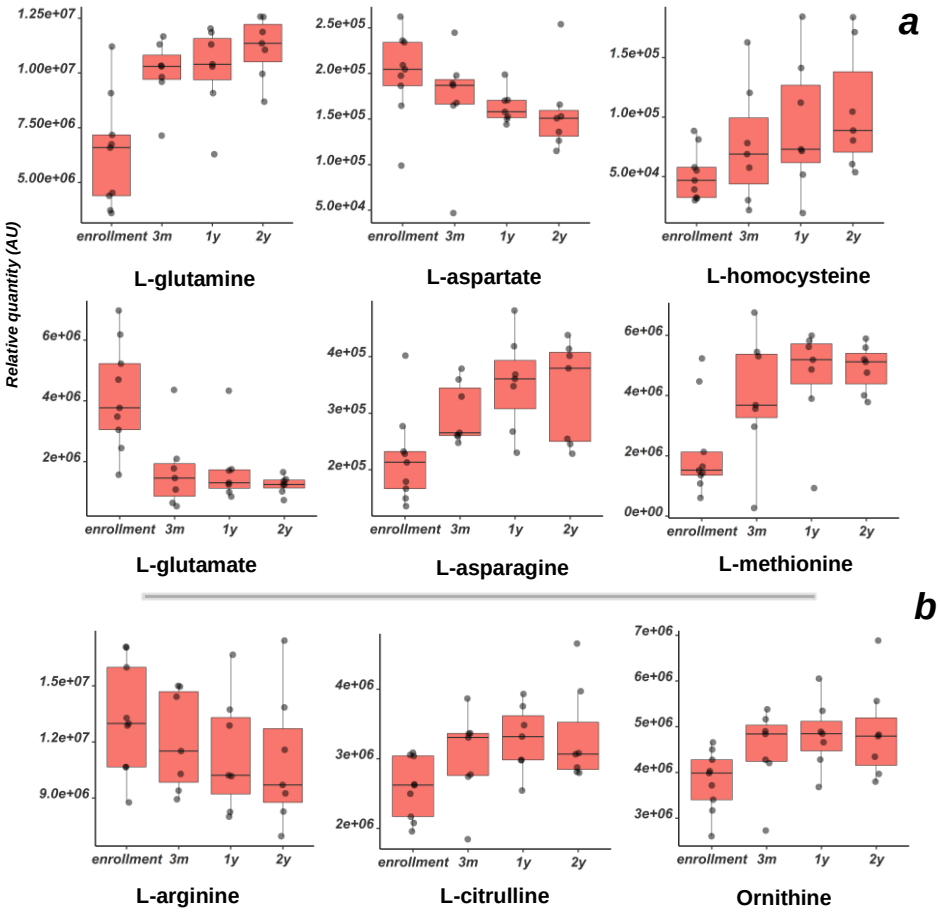

*Figure 21: (**a**) overview on the regulation of some amino acids and related products. (**b**) key metabolites in NO synthesis pathway*

We have measured an increase in free levels of the amino acids asparagine and glutamine and a correspondent decrease in aspartic and glutamic acids (figure 21a). Sulfur-containing compounds (methionine, homocysteine, taurine) were also found to be increased, suggesting a different regulation in sulfur exchange and a higher availability of compounds with ROS scavenging capabilities. Glutamine plays an important role in nitrogen exchange reactions and it has been reported to exert protective effects on cardiovascular physiology through different mechanisms including fueling NO generation, reducing blood glucose level and contributing in the maintenance of endothelial cells homeostasis (*165*). Key metabolites in NO pathway

(figure 21b) were found to be differentially regulated after adrenalectomy. Citrulline and ornithine were found to be increased 3 months after surgery; and higher serum level of these metabolites were maintained throughout the follow up. Conversely, arginine was not found to be significantly changed.

Purine metabolism was found to be heavily influenced by aldosterone-producing adenoma removal (Figure 22). Specifically, adenosine, AMP and guanosine were decreased over the course of follow up, with adenosine level dramatically reduced within 3 months after surgery.

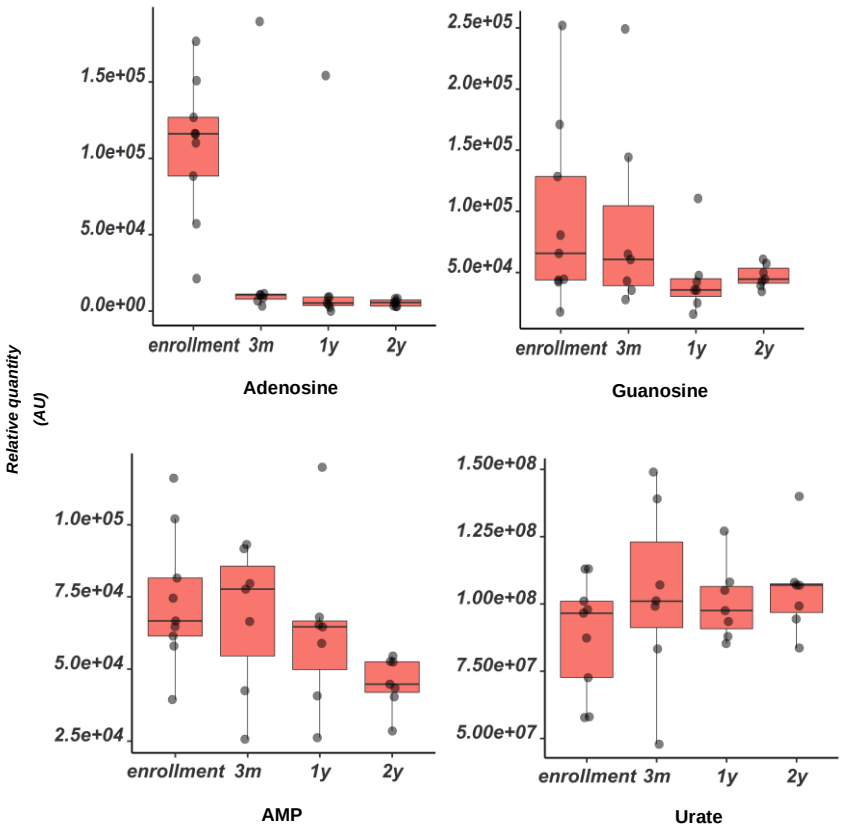

Figure 22: Overview of purine metabolism.

Despite alterations to purine metabolites, we have not detected significant variations in urate levels. In the previous analysis, we have found that adenosine is lower in PA patients when compared to EH patients. A possible explanation for the drop in adenosine within the first 3 months after adrenalectomy is that circulating

adenosine is tightly regulated by blood pressure, and with its normalization, the need for its vasodilation function is reduced altogether.

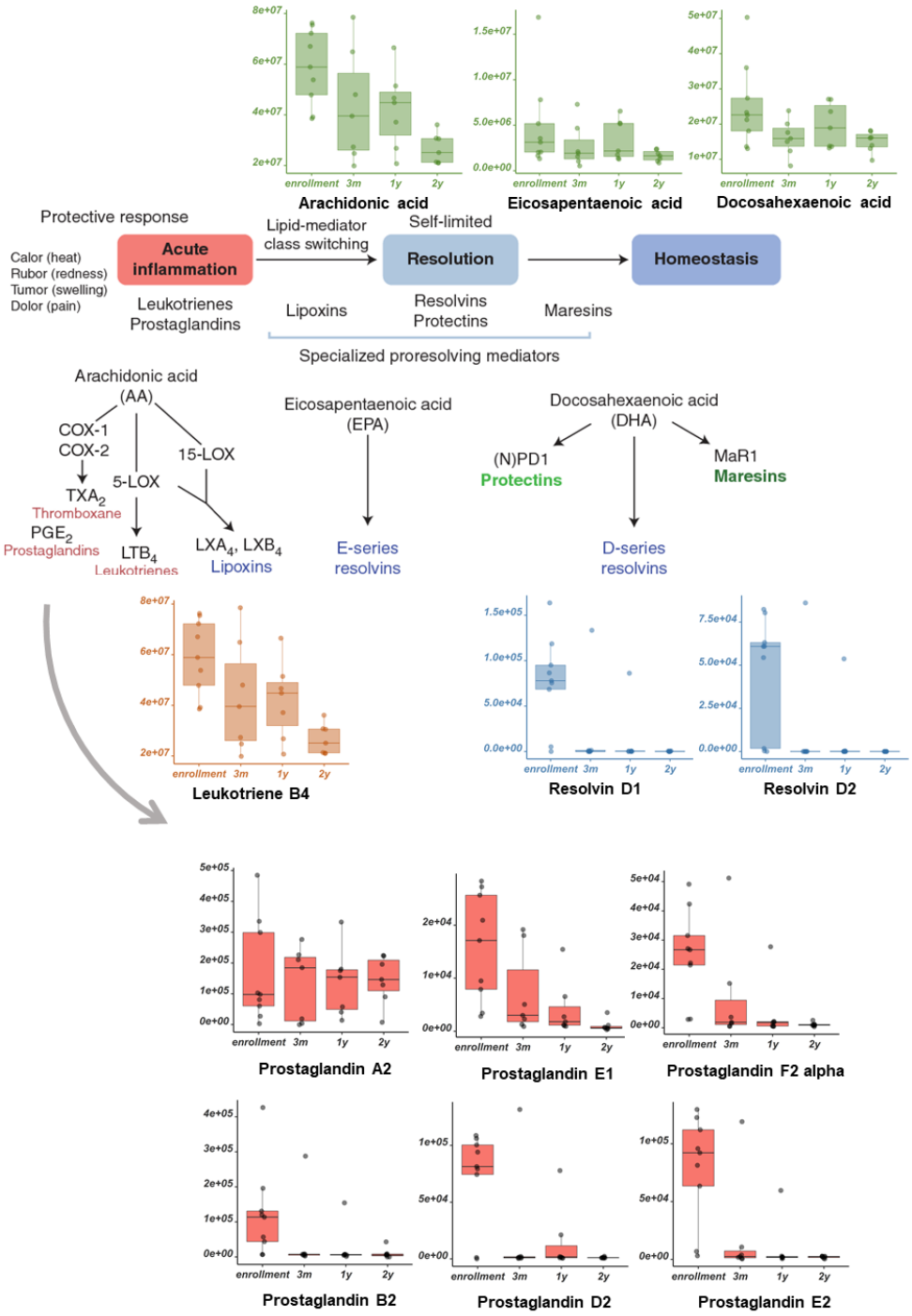

Figure 23: Boxplot of lipids involved in inflammation signaling. Diagram adapded from Serhan et al. 2014

Future quantitative metabolomics study could provide more information about adenosine role in hypertension by investigating variations in serum adenosine concentration in a cohort of hypertensive patients (PA and EH) compared to normotensive subjects in basal conditions or exposed to volume changes.

Lipidic mediators of inflammations class of compounds was found to be significantly changed after adrenalectomy (Figure 23). With the exception of prostaglandin A2, the other inflammatory lipids we have detected and measured are generally described by a decrease within the first 3 months after surgery. This was evident for pro-inflammatory lipids (prostaglandins, leukotrienes) but also for lipid associated with resolution of inflammation (resolvin D1 and 2). Arachidonic acid, precursor in the synthesis of prostaglandins, was found to be significantly decreased two years after surgery, while no significant changes were detected for eicosapentaenoic acid and docosahexaenoic acid (precursors of resolvins). Observations in human studies and animal models have established the existence of a link between hypertension and inflammation, even though cause-effect relation between the two factors is still a matter of debate (*166*). Several studies have reported that aldosterone can promote inflammation through various mechanisms including the stimulation of the expression of ICAM-1, MCP-1 and COX-2, responsible of the synthesis of prostaglandins. These effects are reduced in eplerenone treatments, suggesting that they are largely dependent on MR signaling (*34-36, 167-169*). Our data suggest that APA patients are characterized by a degree of basal (and persistent) activation of pro-inflammatory signals that is counterbalanced by pro-resolutions mediators. Adenosine, guanosine and lipid mediators of inflammation were found similarly reduced after 3 months after surgery, and their level was maintained similar up to 2 years after surgery. One patient out of 7 was an exception to this trend (outlier evidenced in boxplots) and level reduction for these metabolites was reached only 2 years after the adrenalectomy. Biochemical and hormonal parameters of this patient including plasma aldosterone, renin, systolic BP, diastolic BP were no different from those of the other patients (Figure 20).

In our follow-up studies we have indications that APA patients are characterized by alterations of vasodilating metabolites and an inflammatory burden that is resolved after surgery. With the exception of prostaglandin E2 (higher in hi-ARR hypertensive patients), we could not find differences in inflammatory mediators between PA and EH or APA and BAH, suggesting that this inflammatory burden is common in all the hypertensive conditions. As a limitation of the study, a cohort of normotensive subjects was not included to confirm the hypothesis that inflammatory mediators characterize the hypertensive status.

Two hypotheses can be formulated to explain the decrease (or "normalization") of these systems: the first one is that removal of aldosterone-producing adenoma lowers the plasma level of aldosterone and the downstream cascade of pro-inflammatory events that depend on MR signaling. The second one is that hypertension itself promotes and sustains inflammation, and the normalization of the blood pressure itself is sufficient to damp lipids pro-inflammatory mediation. In some cases, PA patients refuse to undergo surgery and they are successfully treated with MR antagonists. A prospective metabolomics study on this specific cohort of patients could help in understanding the contributions to inflammation in PA, as their plasma aldosterone level is still above the normal ranges but MR signaling is inhibited. One of the patients in our follow-up study was characterized by a slower reduction in adenosine or inflammation mediators, despite a rapid normalization of biochemical and hormonal parameters, suggesting that factors other than blood pressure, aldosterone, renin and serum potassium could contribute to reduction of inflammation and, possibly, the restoring of endothelium homeostasis after surgery.

Taken together, our data on APA follow up indicate that APA removal not only improves blood pressure and restores the RAAS functionality but also impacts considerably the level of inflammation mediators, purine metabolism and key compounds in NO pathway.

6. Conclusions

Identification and classification of primary aldosteronism requires a complex diagnostic work-up for the clinical similarity of symptoms with essential hypertension. In the effort to identify molecules able to separate the two classes of patients and to get new insights into the molecular features of PA, our lab has approached the problem with multiple strategies. These strategies included the analysis of UEV content to find potential biomarkers and up-to-date proteomics and metabolomics MS-based approaches, proven to be valuable for clinical oriented research in different settings.

First, we developed a protocol for the analysis of mRNA content in UEV. To address the technical problem of scarcity of mRNA in UEV, we added a pre-amplification step for stabilizing target detection, and we decided to use normalization on housekeeping genes not prone to introduce DNA contamination. Notably, a similar protocol was developed in parallel by Bazzell and colleagues, who addressed the problem of the scarcity of mRNA content by adding a pre-amplification step as well, but suggesting the use of not normalized delta-CT for measuring the transcript level (*88*). We specifically focused on the mRNA of the NCC, a nucleic acid contained in UEV. Previous data have suggested that urinary exosomes of PA patients are characterized by an increased level of NCC and pNCC proteins when compared to exosomes from EH patients (*66*). Our hypothesis was that differences in protein abundance could be reflected by the level of the corresponding ribonucleic transcript and we investigated if it was possible use of the urinary NCC mRNA as a more stable and reliable marker of PA than the corresponding protein. The results did not support the use of NCC mRNA as marker of PA or to separate APA and BAH subtype, and there were indications that NCC protein abundance and mRNA regulation follow separated dynamics. Nevertheless, a secondary aim was to understand how the NCC mRNA is regulated in response to salt infusion and therapy (MRA and adrenalectomy). Our findings indicate that NCC mRNA expression is

down-regulated in response to Na-driven volume and that is up-regulated as compensatory mechanisms in case of hypovolemia following drug or surgical treatment. In our proof-of-concept study we showed that our UEV analysis protocol can provide information on the status of the renal tubule and help in the characterization of disease that impact on nephron homeostasis. We also provided evidence that, in the frame of the normalization debate for UEV, the use of creatinine should be taken with caution. The introduction of pre-amplification step in our protocol increased the sensitivity of the qPCR assay, thus allowing to perform the analysis with a limited amount of sample. However, it has been observed that this step may introduce bias and lower the reliability of qPCR results (*170*). To address this limitation, we selected a small panel of genes and used primers with efficiency close to 100%. The recent technological developments of droplet-based digital PCR platforms (ddPCR), coupled with new protocols for the detection of rare transcripts, could open the possibility of avoiding the pre-amplification step, thus improving further our protocol for UEV mRNA analysis in the near future (*171*).

In the second part of our project, we analyzed the UEV content using an untargeted label-free MS approach for biomarker-discovery purpose. Our findings showed that groups of protein associated to UEV composition are different between PA and EH, suggesting that UEV of different composition are released in the urine in the two conditions, thus UEV themselves could be considered as prospect biomarkers. Moreover, proteins related to water reabsorption and ion homeostasis were found among the most significant hits (AQP1, AQP2 for PA vs EH and CAH2 for APA vs BAH) indicating a different physiological equilibrium in renal transport between groups of patients and identifying possible targetable pathways that can be investigated further. The use of state-of-the-art proteomic techniques allowed us to obtain a list of promising candidates that could help in the diagnosis and characterization of PA. The use of network analysis for the identification of protein-protein interactions can extend the information obtained on the single proteins and lead to the identification of dysregulated pathways or cellular process that can be of clinical interest. As an

immediate future perspective of this study, we plan to investigate the clinical relevance of our candidate biomarkers for the identification and subtyping of primary aldosteronism by enrolling a larger cohort of patients and performing a parallel two-protocols validation with MS MRM and ELISA (*172*).

To perform this proteomics analysis, we attempted at first to repurpose and adapt the same EV isolation protocol we used for the mRNA analysis. Preliminary data obtained comparing three different isolation methods (e.g. precipitating reagent, ultracentrifugation, ultrafiltration) indicated that the chemical precipitating reagent was interfering with the peptide detection and the ultracentrifugation resulted to be the best isolation method for proteomics, in terms of number of identified peptides. For this reason, we adopted the ultracentrifugation protocol that, while requiring a higher volume of sample, was shown to be better compatible with the downstream MS analysis. We believe there is potential for integrating the proteomics data with the transcriptomics information obtainable from the UEV, however the impact that may derive from the use of different isolation techniques on this kind of analysis is to be assessed.

In our study we characterized the dimension of our isolated UEV through TEM. Their dimensions were found compatible with those of exosomes, although the clear demonstration of an exosomal nature of the isolated UEV was not accomplished as it would have required techniques that were not available at our site. Nevertheless, we acknowledge that a better characterization of the isolated UEV may have helped to evaluate qualitative differences between the proteomics and transcriptomics experiments. Further developments of this study will include a quantification step through Nanoparticle Tracking Analysis (NTA) and a surface marker profiling step through flow cytometry, as per indication of the ISEV position paper (*72*).

Lastly, our metabolomics investigation has provided a considerable amount of information on the metabolic status of PA patients in comparison to EH patients. We have found that lipid, energy and purine metabolism are differentially regulated

between the two groups and that key metabolites in those pathways have been already associated to the risk of developing cardiovascular events. We showed that metabolism is different among hypertensive patients that are separated by the ARR scoring system. Though ARR was specifically designed to detect PA cases at high sensitivity and is not optimized for the classification of EH patients, it is clear that the RAAS status has a considerable impact on several metabolic pathways. The previous observation made on hi-ARR patients and their higher propensity of developing cardiovascular events points out that there are potential clinical benefits in scoring EH patients for their RAAS functionality. ARR score itself is subject to improvements and more sensitive alternatives are being investigated, opening to the opportunity to understand if the stratification of EH patients for their RAAS status is feasible and if it can provide benefits for their management (*173*).

Interestingly, in the last part of our metabolomics study we have shown that adrenalectomy has mid-term effects on purine metabolism (with possible implications on endothelium homeostasis) and it leads to a decrease in inflammation-related signaling. Though it is still unclear if these effects are mediated by MR signaling and/or are a secondary effect of the normalization of blood pressure, this result strengthens the concept that adrenalectomy is beneficial for APA patients, considering the reported associations between inflammation and cardiovascular disease. Most of the metabolic changes occur in the first 3 months after surgery and, in the same time window, we have found an increase in NCC transcript in UEV. It will be of interest to integrate the metabolomics data by investigating, from a transcriptomics and proteomics perspective, how the profile of ion channels and transporters changes in the DCT district after adrenalectomy or MRA treatment.

Mutations in at least 5 genes have been observed to be associated with APA, suggesting different possible biochemical origins for this phenotype. Indeed, despite a rapid normalization of blood pressure and plasma potassium occurred in all APA

patients after the adrenalectomy, we have observed some differences in the associated metabolic changes. It is possible that the differences in the underlying causes of APA may be reflected in differences in clinically relevant features. To better characterize this subtype, one current developments of this study is the metabolomic analysis of pathological and non-pathological adrenal tissue from the same APA patients here described. Gene sequencing is also being carried out with the aim of understanding the role of the known mutations in the biochemical and metabolic features of APA.

Several serum metabolites and protein markers associated to UEV seem to have a potential clinical relevance in the diagnosis and characterization of this disease and for monitoring its treatment. As a future perspective, we plan to enroll a larger cohort of hypertensive patients for the verification step to confirm the efficacy of these prospect biomarkers. However, for the subsequent validation step there are some key factors to take into consideration. First, it is important to consider that the PA diagnosis algorithm is still codified by guidelines that do not identify “gold standards” techniques or cut-offs for screening, confirmation and subtyping. Starting from the same population of hypertensive subjects, the differences in the available techniques, know-how, instruments and adopted cut-off values across referral centers will probably lead to differences in the identification and sub-classification of the PA population. As consequence, if the validation of these prospect markers is run separately in different settings, there may be discrepancies in the biomarker panel that is finally suggested to be adopted in clinic. Second, the selection of patients in this study was made by following stringent criteria, to limit the effects of possible confounding factors. It is known that the diagnosis of PA of recent onset or mild cases of PA is challenging and indeed we have observed that there are metabolic similarities between PA and hi-ARR hypertensive patients. We expect that the specificity of some biomarkers identified in a clearly distinguishable population of PA patients will likely decrease in a real-world application. To better understand which biomarkers have a solid possibility to be used in clinic, we believe it is strongly

advisable that validation and verification steps enroll a large-number heterogeneous population and that the validation step is carried out in a multi-center setting.

Taken together, our data highlight the importance of adopting a multiple -omics strategy coupled with multivariate statistics in biomarker discovery and, in general, to approach complex diseases. The use of UEV as a sort of “liquid biopsy” is a valuable and non-invasive method for the understanding of the pathophysiological mechanisms involved in PA. In the light of our findings and proposed protocols, we also foresee an opportunity for future studies pursuing a multi-omics approach that is integrated by design.

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