Excess aldosterone as a mechanism of resistant salt-sensitive arterial hypertension

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To my father LT, a 'founding figure' in my life. I miss you dad.
FT

Contents

Chapter 1

General introduction and outline

GENERAL INTRODUCTION

Arterial Hypertension (HT) is the most common condition seen in primary care, currently affecting approximately one billion people worldwide, with an estimated raise to 1.5 billion in 2025[1].

In most cases, HT is an asymptomatic condition. However, it may ultimately result in cardiovascular diseases such as myocardial infarction, stroke, heart failure, renal failure and peripheral artery disease. Moreover, HT accounts for 18% of cardiovascular disease deaths (9.4 million deaths each year) and is considered the leading risk factor for global disease burden, disability and premature death from cardiovascular disease[2]. Accordingly, HT diagnosis and a prompt treatment should be a priority in every health care system.

HT is defined as "essential", or "primary", when the exact etiology of hypertension is unknown[3,4], and "secondary" when it derives from a specific underlying disease. The prevalence of secondary HT is lower (less than 10%) than that of primary hypertension, but it is likely that it has been underestimated in most epidemiological studies because appropriate tests were not generally performed at the general population level[5]. In fact, in Specialized Centers for HT the prevalence of secondary forms of HT is about 30%.

The identification of a secondary cause of HT and the underlying pathophysiology is crucial since it allows achieving cure of the HT, especially in younger patients, or, when this is not feasible, a better control of blood pressure (BP) and a better prevention of specific target-organ damage and cardiovascular events by a more targeted pharmacological treatment.

The European Society of Cardiology and the European Society of Hypertension (ESC/ESH) guidelines on the diagnosis and management of HT, published in 2018, recommended the systematic search of secondary HT, especially in presence of clinical characteristics that should raise the suspicion of secondary HT[6]. Clinical clues that should raise suspicion for a secondary cause of hypertension include snoring/daytime sleepiness, abrupt onset of hypertension, hypertension onset < 30 years of age, malignant hypertension, abrupt loss of BP control in a patient with prior BP control, use of BP raising substances such as NSAIDs/amphetamines/immunosuppressive agents, resistant (taking 3 or 4 antihypertensive drugs, including a diuretic and BP above goal or taking ≥4 drugs, including a diuretic and BP below goal) or refractory hypertension (taking ≥5 drugs, including a diuretic, and BP above goal), unprovoked (not taking a diuretic) or excessive hypokalemia, and/or the onset of diastolic hypertension in older patients (≥65 years). Common and uncommon causes of secondary HT are listed in **Table 1**.

Table 1. Causes of Secondary Hypertension with clinical indications (adapted from 2017 American College of Cardiology/American Heart Association (ACC/AHA) Guidelines[7])

Common causes	Prevalence	Clinical clues
Renal parenchymal disease	1%–2%	Urinary tract infections; obstruction, hematuria; urinary frequency and nocturia; analgesic abuse; family history of polycystic kidney disease; elevated serum creatinine; abnormal urinalysis
Renovascular disease	5%–34%	Resistant hypertension; hypertension of abrupt onset or worsening or increasingly difficult to control; flash pulmonary edema (atherosclerotic); early-onset hypertension, especially in women (fibromuscular hyperplasia)
Primary aldosteronism	8%–20%	Resistant hypertension; hypertension with hypokalemia (spontaneous or diuretic induced); hypertension and muscle cramps or weakness; hypertension and incidentally discovered adrenal mass; hypertension and obstructive sleep apnea; hypertension and family history of early-onset hypertension or stroke
Obstructive sleep apnea	25%–50%	Resistant hypertension; snoring; fitful sleep;

		breathing pauses during sleep; daytime sleepiness
Drug or alcohol induced	2%–4%	Sodium-containing antacids; caffeine; nicotine (smoking); alcohol; NSAIDs; oral contraceptives; cyclosporine or tacrolimus; sympathomimetics (decongestants, anorectics); cocaine, amphetamines and other illicit drugs; neuropsychiatric agents; erythropoiesis-stimulating agents; clonidine withdrawal; herbal agents
Uncommon causes	**Prevalence**	**Clinical clues**
Pheochromocytoma/ paraganglioma	0.1%–0.6%	Resistant hypertension; paroxysmal hypertension or crisis superimposed on sustained hypertension; "spells," BP lability, headache, sweating, palpitations, pallor; positive family history of pheochromocytoma/paraganglioma; adrenal incidentaloma
Cushing's syndrome	<0.1%	Rapid weight gain, especially with central distribution; proximal muscle weakness; depression; hyperglycemia
Hypothyroidism	<1%	Dry skin; cold intolerance;

		constipation; hoarseness; weight gain
Hyperthyroidism	<1%	Warm, moist skin; heat intolerance; nervousness; tremulousness; insomnia; weight loss; diarrhea; proximal muscle weakness
Aortic coarctation (undiagnosed or repaired)	0.1%	Young patient with hypertension (<30 y of age)
Primary hyperparathyroidism	Rare	Hypercalcemia
Congenital adrenal hyperplasia	Rare	Hypertension and hypokalemia; virilization (11-beta-hydroxylase deficiency [11-beta-OH]); incomplete masculinization in males and primary amenorrhea in females (17-alpha-hydroxylase deficiency [17-alpha-OH])
Mineralocorticoid excess syndromes other than primary aldosteronism	Rare	Early-onset hypertension; resistant hypertension; hypokalemia or hyperkalemia
Acromegaly	Rare	Acral features, enlarging shoe, glove, or hat size; headache, visual

		disturbances; diabetes mellitus

Primary aldosteronism

First described by Jerome Conn over 60 years ago, primary aldosteronism (PA) is now held to be the most common group of familial and sporadic form of secondary HT. It is caused by inappropriately high aldosterone production, relatively autonomous of renin-angiotensin system and non-suppressible by sodium loading[8]. Such inappropriate production of aldosterone causes HT, cardiovascular damage, sodium retention, suppression of plasma renin, and increased potassium excretion that (if prolonged and severe) may lead to hypokalemia. Familial Hyperaldosteronism (FH) is relatively uncommon and to date are held to exist in four forms. Sporadic PA is by far the most common form and is generally caused by adrenocortical aldosterone-producing adenoma (APA), unilateral (UAH), or bilateral adrenal hyperplasia (BAH).

Previously considered a rare disease, recent prevalence studies demonstrate that PA is actually a very common and vastly underdiagnosed etiology of HT[8,9]. The reasons accounting for the underdiagnoses of PA are the lack of systematically screening by general practitioners, the lack of hypokalemia in the majority of cases and the within normal range levels of aldosterone with suppressed renin, a condition often mislabeled as low-renin essential hypertension.

The diagnosis of PA and its targeted treatment is of crucial important since aldosterone excess, independently from the degree of BP, induces fibrosis, generation of reactive oxygen species, and inflammatory changes; patients with PA have higher cardiovascular morbidity and mortality than age- and sex-matched patients with essential hypertension and the same degree of BP elevation. Thus, a timely diagnosis allows better chances of prevention of hypertension-mediated organ damage (HMOD) in PA patients (**Table 2**)[10,11].

Table 2. Assessment of hypertension-mediated organ damage in PA patients.

Comorbidity	Basic clinical screening	Instrumental and biochemical evaluation
Obstructive Sleep Apnea	History of snoring, excessive daytime sleepines, nocturnal cough, headach, etc.	Validated questionnaires (i.e. STOP-Bang and NoSAS); home sleep apnea testing (HSAT); attended polysomnography (PSG)
Atrial fibrillation	History of palpitations, arrhythmias, dyspnea.	12-lead ECG, Holter monitoring, Echocardiography
Cardiac remodeling, left ventricular hypertrophy	Hystory of chest pain, shortness of breath, oedema, myocardial infarction, coronary revascularization, syncope, heart failure.	12-lead ECG, Echocardiography
Metabolic disease	Hystory of non type 1 diabetes, insuline-resistance	Fasting blood glucose and glycated HbA1c
Renal function and Chronic Kidney Disease	History of polyuria, nocturia, haematuria, urinary tract infections.	Urinary albumin excretion (UAE), estimated glomerular filtration rate (eGFR)

The 2016 Endocrine Society Clinical Practice Guidelines recommend the screening for PA in patients with sustained BP above 150/100mmHg on each of three measurements obtained on different days, with hypertension (BP>140/90mmHg) resistant to three conventional antihypertensive drugs (including a diuretic), or controlled BP (>140/90 mm Hg) on four or more antihypertensive drugs; HT and spontaneous or diuretic-induced hypokalemia; hypertension and adrenal incidentaloma; HT and sleep apnea; HT and a family history of early onset hypertension or cerebrovascular accident at a young age (<40 years); and all HT first-degree relatives of patients with PA[9].

The diagnosis of PA requires demonstration of low or undetectable renin levels and plasma aldosterone concentrations (PAC) that are inappropriately high for salt and volume status. This is commonly done with concomitant measurements of plasma aldosterone levels and renin activity or concentration and calculation of the aldosterone-to-renin ratio (ARR), the more sensitive and accurate biochemical test in identification of PA. The plasma potassium

measurement is both insensitive and not specific for the screening of PA, as the prevalence of hypokalemia among patients with PA is low (30%)[12]; thus, while hypokalemia, when present and not attributable to obvious causes, is a strong clue of PA, normokalemia does not allow to exclude it. Moreover, it has to be taken into account the potential confounding factors that may interfere with ARR measurements, such as medications, leading to positives and false negatives results (**Table 3**)[9].

Confirmatory tests, such as captopril challenge test and saline infusion test, are a controversial issue since their accuracy was not supported by studies following the STARD recommendations[13]. Although they are still used in some centers to confirm the diagnosis of PA, these tests function as exclusionary rather than confirmatory tests because their negative predictive value largely exceeds their positive predictive value, as clearly shown in the PAPY study post hoc analysis[14].

The most common forms of PA are APAs and UAH, which can be treated with unilateral adrenalectomy, and BHA, which requires lifelong mineralocorticoid receptor antagonists. The distinction between APA and BHA is crucial to identify the appropriate treatment and to allow cure in those patients with unilateral aldosterone secretion[15,16]. Subtyping of PA is, therefore, a key step in the diagnostic-therapeutic process.

The distinction between unilateral and bilateral form of secretion in PA is optimally made by bilateral adrenal vein sampling (AVS), a procedure in which a catheter is introduced via the femoral vein, and blood from the left and (more difficult) right adrenal veins, plus the inferior vena cava, is sampled. The procedure should be performed by an experienced interventional radiologist at third level referral centers that can perform and interpret AVS. A major difference in aldosterone levels (measured as aldosterone/cortisol ratio) between the 2 sides is evidence for a unilateral source of hyperaldosteronism and thus curable by laparoscopic unilateral adrenalectomy[17]. As a preliminary test for adrenalectomy, AVS should be reserved for patients who are seeking long-term cure of PA with surgery and are reasonable candidates for general anesthesia and adrenalectomy. The Endocrine Society guidelines suggest that unilateral adrenalectomy should not be performed without prior demonstration of lateralized aldosterone production by AVS. An exception to this rule may be young patients (age <35 years) with a florid PA phenotype, comprising spontaneous hypokalemia, marked aldosterone excess, suppressed plasma renin, a unilateral adrenal lesion with radiologic features consistent with a cortical adenoma, and a contralateral normal gland on CT[9].

Table 3. Drugs and conditions affecting plasma aldosterone concentration, renin, and the ARR.

Factor	PAC	Renin	ARR	FP rate	FN rate
Potassium status					
Hypokalemia	↓	→↑	↓	↓	↑
Potassium loading	↑	→↓	↑	↑	↓
Sodium status					
Sodium depletion	↑	↑↑	↓	↓	↑
Sodium loading	↓	↓↓	↑	↑	↓
Medications					
ß- blockers	↓	↓↓	↑	↑↑	↓
Long-acting CCB	→↓	↑	→	→↓	→↓
ACE inhibitors	↓	↑↑	↓	↓	↑
ARBs	↓	↑↑	↓	↓	↑
K^+-sparing diuretic agents	↑	↑↑	↓	↓	↑
K^+-losing diuretic agents	↑	↑↑	↓	↓	↑
Central α-2 agonist	↓	↓↓	↑	↑	↓
NSAIDs	↓	↓↓	↑	↑	↓
Others conditions					
Aging	↓	↓	↑	↑	
Renal impairment	→	↓	↑	↑	↓
Pregnancy	↑	↑↑	↓	↓	↓
Renovascular HT	↑	↑↑	↓	↓	↑
Malignant HT	↑	↑↑	↓	↓	↑

PAC, plasma aldosterone concentration; ARR, aldosterone to renin ratio; ACE, angiotensin-converting enzyme; ARBs, angiotensin II type 1 receptor blockers; NSAIDs, non-steroidal anti-inflammatory drugs; HT, hypertension; FP, false positive; FN, false negative.
Adapted from J. W. Funder et al[9]

Thus, even if expensive and usually available only in major centers, AVS is the most accurate method to lateralize hyperaldosteronism, since the lateralization by adrenal imaging, usually by computerized tomography (CT), is less successful. Several studies have strongly demonstrated that adrenal imaging (CT or MRI) alone is insufficient for PA lateralization, since very small APAs (<10 mm) or adrenal zona glomerulosa hyperplasia cannot be visualized. In 2004, a Mayo Clinic study that examined the diagnostic accuracy of a CT based strategy for the subtyping of PA using cosyntropin-stimulated AVS, as reference test, reported between-test concordant results in only 53% of the cases, unilateral disease at AVS in 22% of CT-negative cases, and unilateral mass at CT with bilateral or

contralateral disease at AVS in 25%of the cases[18]. In 2009 Kempers et al. retrospectively analyzed 38 studies comprising a total of 950 patients studied for PA, concluding that discordant results between AVS and CT/MRI were found in 38% of the patients, inappropriate adrenalectomy or inappropriate exclusion from adrenalectomy would have occurred in 15% and in 19% of cases, respectively, if only imaging had been used[19].

However, according to Endocrine Society Guidelines, adrenal imaging should be performed in patients affected by PA, for two main reasons: it is useful to detect large lesions suspected for adrenocortical carcinoma or to identify the adrenal venous drainage for the ensuing interventional radiological and surgical procedures[9].

After the demonstration of a unilateral aldosterone hypersecretion, unilateral adrenalectomy should be offered since it allows the cure of PA in almost all patients and the normalization or considerably reduction of BP in a substantial proportion of the patients. AVS-guided transperitoneal or retroperitoneal laparoscopic adrenalectomy has become the standard surgical method in term of safety and feasibility for unilateral PA, since it offers many advantages over conventional open surgery, such as reduced postoperative pain, less postoperative ileus, shorter hospitalization durations after surgery, lower wound-related complication rates, less operative blood loss, and lower postoperative complication rates[20].

A complete biochemical success in terms of correction of hypokalemia and hyperaldosteronism after unilateral adrenalectomy occurs in more than 98% of cases[15,16,21,22]. As regards the blood pressure outcome, it was differently stratified in the different studies as cure, marked improvement, mild improvement or no improvement in the AVIS-2 Study or as complete, partial, and absent success, in the PASO Study[16,22]. Identified predictors of a good clinical response were age, sex, short duration of hypertension, and high number of antihypertensive medications[23,24].

In PA patients with bilateral disease and in those with AVS-diagnosed unilateral PA who are not willing or cannot be candidate for adrenalectomy, mineralocorticoid-receptor antagonists (MRAs), alone or in combination with other antihypertensive agents, are recommended in order to normalize BP and obtain normokalemia[25].

Traditionally, unilateral disease has been synonymous with a pathological diagnosis of an APA, while bilateral disease is most commonly associated with idiopathic adrenal hyperplasia. However, in recent years with the availability of monoclonal antibodies for human CYP11B2, other pathological forms of unilateral PA have been described, including unilateral adrenal hyperplasia and multiple unilateral adrenal adenomas[26–28]. Therefore, the

conclusive diagnosis of the PA subtype should include the demonstration of a CYP11B2-positive adenoma at pathology, which in lateralized forms of PA can be an APA or unilateral multinodular adrenocortical hyperplasia, as reported in the "five corners" criteria[29] (**Table 4**).

Table 4. The "five corners" criteria for the diagnosis of APA (Adapted from[29]).

1. Biochemical evidence of PA (e.g., an inappropriately high aldosterone/renin ratio)
2. Lateralized aldosterone secretion by AVS
3. Detection of a nodule by imaging (CT or MRI) and an adenoma at pathology
4. Biochemical correction of PA after adrenalectomy
5. Detection of a CYP11B2-positive adenoma in the resected adrenal cortex at immunohistochemistry with a monoclonal antibody for human CYP11B2

Resistant hypertension and primary aldosteronism

HT is defined as "resistant" to treatment when BP remains above goal (SBP >140 and/or DBP >90mmHg, confirmed by ABPM or HBPM) despite appropriate lifestyle measures and the concurrent adherence to therapy with optimal or best-tolerated doses of three or more drugs that should include a diuretic[6].

Patients with RH are characterized by distinct demographics, comorbidities, and metabolic abnormalities, such as nondipping or reverse dipping BP and sympathetic nervous system overactivity, visceral obesity, and aldosterone excess (**Table 5**).

The majority of studies indicate that PA is a common cause of RH. Observational studies from different countries have demonstrated a prevalence rate of PA of ≈20% in patients with RH[6].

Therefore, the Endocrine Society Guidelines recommend the screening for PA in all such patients. However, these patients are, by definition, on complex therapeutic regimen entailing drugs, as ACE-inhibitors, angiotensin-type 1 receptors blockers, diuretics, and beta-blockers, which deeply affect the renin-angiotensin-aldosterone system and, therefore, can preclude identification of primary aldosteronism (PA), the most common secondary form of HT. In this respect, it is also important to recognize that antihypertensive medications other than spironolactone, eplerenone, and amiloride can alter screening test results for PA. For example, β-adrenergic receptor blockers, central α2-receptor agonists, and renin inhibitors suppress PRA, and ACE inhibitors, ARBs, non–potassium-sparing diuretics, and dihydropyridine CCBs increase PRA, thus altering plasma ARR values. If

the initial screening test results are not convincing, these medications can be selectively withdrawn for at least 2 weeks while BP is controlled with other agents that do not influence the renin-angiotensin-aldosterone system such as slow-release verapamil, hydralazine, or an α1-adrenergic receptor antagonist (prazosin, doxazosin, or terazosin)[6,30].

Table 5. Resistant hypertension characteristics, secondary causes and contributing factors. CKD, chronic kidney disease; HMOD, hypertension-mediated organ damage; LVH, left ventricular hypertension (*Adapted from ESC/ESH Guidelines*[6]).

Characteristics of patients with resistant hypertension	Causes of secondary resistant hypertension	Drugs and substances that may cause raise of blood pressure
Demographics • Older age (>75 years) • Obesity • Black people • Excess dietary sodium intake • High baseline BP and chronicity of uncontrolled HT	**More common causes** • Primary aldosteronism • Atherosclerotic renovascular disease • Sleep apnoea • CKD	**Prescribed drugs** • Oral contraceptives • Sympathomimetic agents • Non-steroidal anti-inflammatory drugs • Cyclosporine • Erythropoietin • Steroids • Some cancer therapies
Concomitant disease • HMOD: LVH and/or CKD • Diabetes • Atherosclerotic vascular disease • Aortic stiffening and isolated systolic HT	**Uncommon causes** • Pheochromocytoma • Fibromuscular dysplasia • Aortic coarctation • Cushing's disease • Hyperparathyroidism	**Non-prescription drugs** • Recreational drugs (e.g amphetamines, cocaine) • Excess liquorice ingestion • Herbal remedies

Sodium intake, blood pressure and cardiovascular risk

Historically, the use of salt goes back only several thousand years, with the advent of agriculture and civilization. Nowadays, the average dietary salt intake greatly exceeds the advice of the World Health Organization (WHO) that limit daily salt intake to 5 grams, which is equal to 2 grams of sodium[31–34], due to the increased consumption of processed foods.

The notion to limit sodium consumption originates from the strong correlation between sodium intake and blood pressure (BP) that has been demonstrated by many studies. The International Study of Salt and Blood Pressure (INTERSALT) was one of the first large epidemiological studies, including over 10.000 subjects, that demonstrated a positive association between BP and dietary sodium intake. A 2 gram higher daily sodium intake

was associated with a systolic and diastolic BP increase of 6 and 3 mmHg, respectively [4]. Moreover, the Intersalt Study showed that the BP increase normally seen with increasing age was not observed in populations consuming low sodium diets, indicating that high sodium intake plays a role in the development of hypertension. These results have been subsequently confirmed by many randomized controlled trials that have shown that BP decreases in response to a reduction in dietary sodium intake[35,36].

However, the role of salt in the pathogenesis of HT is not well understood and is much debated[36,37]. According to the classical physiology, the central mechanism linking sodium and BP balance has been described by Guyton and Borst, who both demonstrated that long-term control of BP is closely related with body fluid homeostasis, placing the kidney at the very center of long-term BP regulation[38,39]. Being the principal cation in the extracellular volume, sodium is responsible for preservation and regulation of the effective circulating and extracellular volume. In this concept, the kidney is responsible for matching sodium excretion with sodium intake. The kidney has therefore been thought to be the most important regulator of total body sodium, and thereby extracellular volume. Renal responses to changes in sodium intake are not instant since the kidney needs about 3 to 5 days to adapt to a new level of sodium intake[40]. To control extracellular osmolality in response to an acute increase in body osmoles following sodium intake or infusion, water will shift from the intracellular to the extracellular compartment (**Figure 1**)[41]. In addition, within hours to days, the elevated plasma osmolality will stimulate water intake and antidiuretic hormone (ADH) production, which results in water retention and an increase in extracellular volume and BP. When a new steady state of sodium homeostasis is achieved, in which the kidney is able to match sodium intake and excretion again, this will be at the expense of an increase of extracellular volume and BP. The increase in BP is the result of an increased cardiac output and total peripheral resistance, of which the latter is caused by autoregulatory vasoconstriction.

Therefore, one would expect a strong relationship between dietary sodium intake and BP, and between sodium intake and cardiovascular risk. However, the effect of sodium intake on BP varies considerably among individuals, discriminating sodium sensitive individuals, characterized by a BP increase after an increase in sodium intake, from sodium resistant individuals, who do not develop a BP increase. Sodium sensitive individuals have an impaired renal capacity for sodium excretion and need a higher BP to excrete sodium, as the kidney is considered to be the key organ for regulation of total body sodium content. Many risk factors for sodium sensitivity have been described such as aging, hypertension,

kidney disease, African-American ancestry and a low nephrons number[42–44]. Moreover, sodium sensitive individuals show higher risk of cardiovascular morbidity and mortality and, even when normotensive, had a similar incidence of mortality as hypertensive individuals[45].

The exact mechanism underlying sodium and water homeostasis and the different BP responses to sodium intake has not yet been resolved. Recently, Titze et al introduced a new paradigm about sodium homeostasis, inspired by previous investigations that have challenged the accepted concept of the two-compartment model[46]. Already Cannon suggested a role for the connective tissue in storage of salt and water in situations of "intolerable excess" designated as inundation and that the skin could serve as a reservoir for sodium chloride that could be stored in an osmotically inactive form[47]. Cannon made reference to work of Wahlgren, who in 1909 observed that one-third of the body's chloride (he could not measure sodium) was stored in the skin[48]. Moreover, Guyton et al suggested that strongly negatively charged mucopolysaccharides (later called glycosaminoglycans [GAGs]) could attract, and thus create, a higher density of cations such as Na+ and that "tissue fluids, pressures and gel" could, as proposed in his extensive model, influence overall regulation of circulation[49].

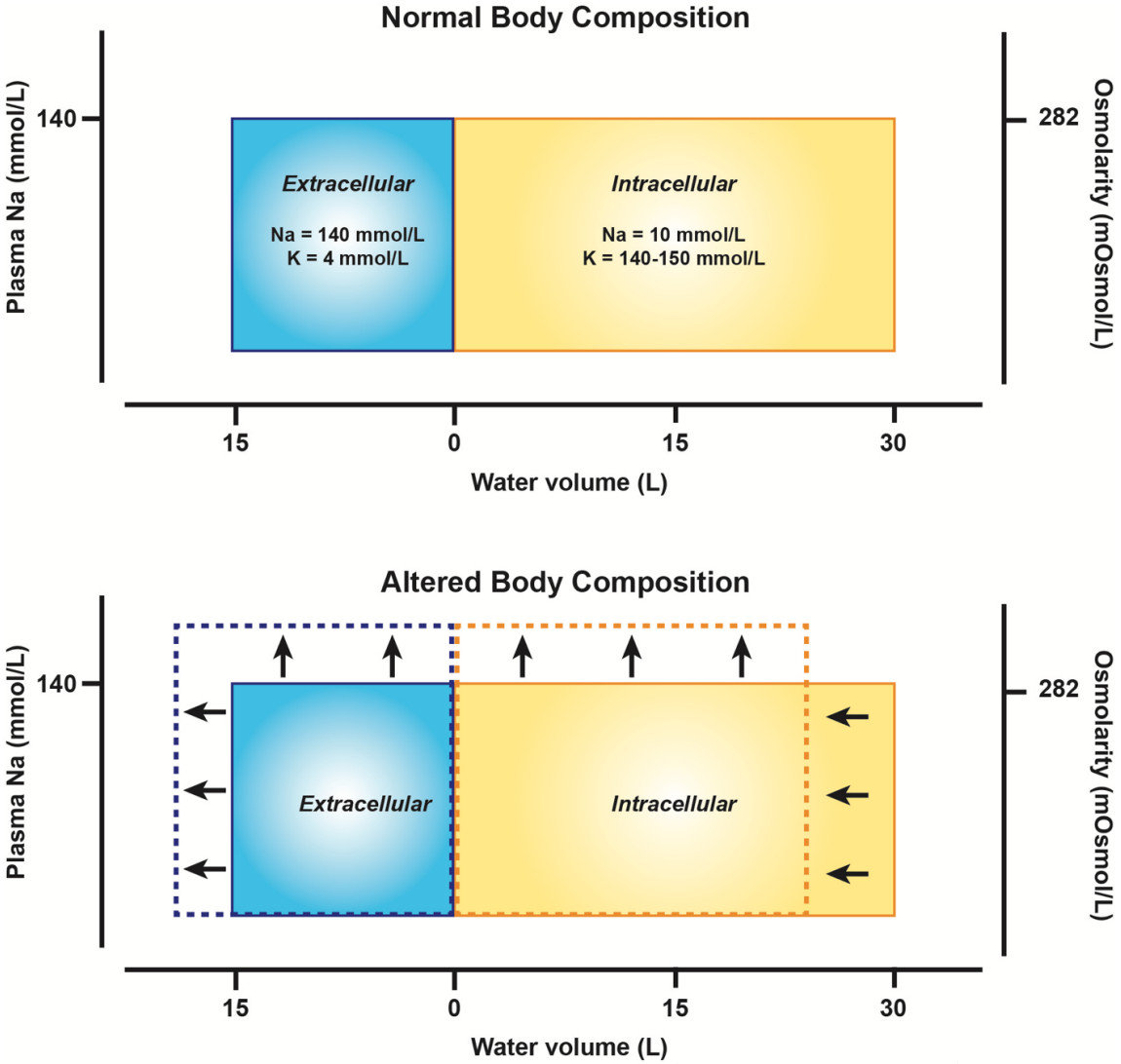
Recent long-term sodium balance studies by Titze and coauthors have shown that sodium homeostasis is more complicated than the classical two-compartment model. These studies, which accurately measured sodium intake and excretion during 200 consecutive days in an enclosed habitat, demonstrated that 24-hour sodium intake and 24-hour sodium excretion may differ up to 100 mmol during constant sodium intake[50]. This view runs against the assumption of the two compartment model that sodium intake and excretion is perfectly matched by the kidney during stable sodium intake. As a result of these differences between sodium intake and sodium excretion total body sodium varied up to 400 mmol. Surprisingly, these large changes in total body sodium did not result in any change of extracellular volume, body weight or BP. This is not in line with the current physiological concept that changes in total body sodium are always accompanied by changes in extracellular volume and body weight.

These data advocate the presence of a buffer where large amounts of sodium can be stored non-osmotically (e.g without water retention). Experimental studies have identified such a buffer in the skin interstitium and muscle where negatively-charged polysaccharides, called GAGs, are able to bind and osmotically inactivate sodium. As a result of sodium binding by GAGs, sodium retention is not accompanied by water retention. Experiments in rats and

mice have showed that that the hypertonic interstitial fluid compartment in the skin activates, through the transcription of the tonicity-responsive enhancer-binding protein (TonEBP, also known as NFAT5), mononuclear phagocyte system (MPS) cells, mainly macrophages, to initiate expression and secretion of VEGFC. VEGF-C is the primary lymphatic vessel growth factor, and its release results in hyperplasia of the lymph capillary network that facilitates sodium and chloride clearance from the tissue. The deletion of TonEBP in mouse MPS cells prevents the VEGF response to a high-salt diet and increases blood pressure. Additionally, an antibody that blocks the lymph-endothelial VEGFC receptor, VEGFR3, selectively inhibited MPS-driven increases in cutaneous lymphatic capillary density, inducing salt-sensitive hypertension. These experiments suggested an homeostatic immune function of skin MPS cells driven by Ton-EBP-VEGF-C signaling. As a part of the interstitial matrix, the lymphatic vasculature forms a vessel network in the interstitium of most tissues that has a role in BP control.

To summarize, even if the role of the kidney in salt homeostasis and hypertension is undoubtful, there are other extrarenal, tissue-specific regulatory mechanisms that regulate the release and storage of Na+ from a kidney-independent reservoir. The elucidation of how salt in the interstitium influences fluid homeostasis and blood pressure should be further investigated and will provide new treatment modalities for salt-sensitive hypertension.

Figure 1. The classic view of sodium homeostasis.



(A) Body water is divided over the intracellular ($2/3^{rd}$) and extracellular ($1/3^{rd}$). Because cell membranes are permeable for water, the osmolality is equal in both compartments. Within the extracellular compartment, sodium is the principal cation. The principal cation in the intracellular compartment is potassium. (**B**) External sodium will be added to the extracellular volume. To control body water osmolality, water will shift from the intracellular compartment to the extracellular compartment resulting in a slight rise of body water osmolality and plasma sodium concentration. Adapted from[41]

OUTLINE OF THE THESIS

The present thesis consists of a collection of the main research studies about primary aldosteronism conducted during the PhD Course.

In **CHAPTER 2** we hypothesized that adrenal vein sampling (AVS), the key test recommended by the current guidelines to demonstrate a unilateral surgically curable form of PA, is feasible even in RH patients, who by definition are on multiple drugs. Moreover, we wondered whether if AVS-guided unilateral laparoscopic adrenalectomy can represent an effective strategy to identify, among RH patients, who have a concealed form of PA and can benefit from surgery.

In **CHAPTER 3** we assessed the impact of unilateral adrenalectomy on health related quality of life (QoL), both in Mental and Physical components, and depression status of patients suffering from PA and we compared the results with a control group of patients with non-secreting adrenal tumor who also underwent adrenalectomy.

Based on previous studies in rodents, in **CHAPTER 4** we have investigated skin electrolytes and water accumulation in PA, the main curable cause of human salt-sensitive endocrine hypertension and a suitable model to investigate the changes in skin-Na+ content in relation to aldosteronism and its surgical correction. We hypothesized that extracellular Na+ storage in tissues and the ensuing tissue immune cell response are mechanistically related to the development of HT in PA and impact on organ damage. By proving this hypothesis, we expect to provide instrumental insight for developing novel treatment modalities for salt-sensitive hypertension.

In **CHAPTER 5** we developed a protocol study titled “Fibromuscular Dysplasia: can skin-Na^+ and water homeostasis influence the Blood Pressure Variability?”, that has been submitted to the Ethic Committee of Maastricht University Medical Center. We hypothesized that renal FMD patients may have higher blood pressure variability markers compared to primary hypertensive subjects and that this finding might be correlate to higher skin-Na^+ accumulation.

In **CHAPTER 6** I discussed the crucial role of screening for secondary causes of HT and in particular for PA in RH patients. Moreover, I discussed the impact of unilateral adrenalectomy on quality of life in patients affected by PA. Finally, I discussed the potential impact of nonosmotic Na^+ storage in PA and its significant reduction after unilateral adrenalectomy.

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Chapter 2

Resolution of Drug-Resistant Hypertension by Adrenal Vein Sampling-guided Adrenalectomy

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Abstract

Context. Drug-resistant hypertension (RH) is a very high cardiovascular risk condition that involves many hypertensive patients, in whom primary aldosteronism (PA) can be commonly overlooked.

Objectives. Our study aimed to investigate if adrenal vein sampling (AVS) can identify PA in patients with RH. Moreover, we sought for determining if AVS-guided adrenalectomy can be useful to resolve blood pressure resistance to treatment in these patients.

Design and setting. We searched for RH and additional clues of PA in 1016 consecutive patients referred for "difficult-to-treat" hypertension to an ESH Center of Excellence. Seventy-seven patients with these features were identified. As they wished to pursue surgical cure, they underwent AVS, which showed unilateral PA in 25 patients (17 men and 8 women), who were submitted to AVS-guided laparoscopic unilateral adrenalectomy.

Results. Their blood pressure fell after surgery from 161/99±26/14 mmHg at baseline, to 133/84±14/9 mmHg at 6 months ($p<10^{-4}$ and $p=10^{-2}$, for systolic and diastolic, respectively), notwithstanding the fall of antihypertensive drugs required to achieve blood pressure control (from 3.6±0.2 agents at baseline to 1.2±0.2 at 6 months; and from 4.8±2.1 to 1.2±1.4 defined daily doses; $p<10^{-4}$ for both). RH was resolved in all and 20% of the patients were clinically cured in that they no longer needed antihypertensive treatment; moreover, 96% were biochemically cured and cardiac and renal organ damage regressed.

Conclusion. In conclusion, AVS allowed identification of unilateral PA in patients presenting with RH. More importantly, AVS-guided adrenalectomy effectively resolved RH in these patients.

Prècis. AVS allows detection of unilateral PA in patients with RH. AVS-guided unilateral adrenalectomy allowed to achieve resolution of RH and biochemical cure of PA.

Abbreviations:

HT, hypertension;

BP, blood pressure;

RH, drug-resistant hypertension;

PA, primary aldosteronism;

AVS, adrenal vein sampling;

ESC/ESH, European Society of Cardiology/European Society of Hypertension;

PAC, plasma aldosterone concentration;

PCC, plasma cortisol concentration;

DRC, renin concentration;

ARR, aldosterone-renin ratio;

DDD, defined daily dose;

PRA, plasma renin activity;

HMOD, hypertensive-mediated target organ damage;

LV, left ventricular;

LVF, left ventricular hypertrophy;

LVM, left ventricular mass;

eGFR, estimated glomerular filtration rate;

SD, standard deviation;

IQR, interquartile range;

PASO, Primary Aldosteronism Surgical Outcomes;

MRA, mineralocorticoid receptor antagonist;

BMI, body mass index;

UAE, urinary albumin excretion.

Introduction

More than one hundred medications are available, as single agent or in combination, for the treatment of arterial hypertension (HT); yet, a substantial proportion of the patients do not reach the optimal blood pressure (BP) values for their cardiovascular risk profile [46]. These are high-risk patients, not just because of their uncontrolled BP, but also because of the common concurrence of overt signs of hypertension-mediated organ damage. For these reasons the European Society of Cardiology/European Society of Hypertension (ESC/ESH) and the American Heart Association have introduced the term of "drug-resistant hypertension" (RH) [47–49], which, with minor differences, define as RH patients those with high BP, who are not at target BP level, despite a therapy with multiple drugs, including a diuretic and adherence to life style changes [50], and even patients who are at target but require four or more drugs to achieve this goal [49,51].

These patients are by definition on multiple drugs, including diuretics, angiotensin-converting enzyme inhibitors (ACEI), angiotensin-type 1 receptors blockers (ARBs), and beta-blockers, that deeply affect the renin-angiotensin-aldosterone system and, therefore, can preclude identification of secondary forms of HT, of which primary aldosteronism (PA) is the most common.

Adrenal vein sampling (AVS) is the key test recommended by current guidelines for subtyping of PA [8,52]. Whether it is feasible and clinically useful in patients on multiple drugs remains unexplored. If proven, these hypotheses could be of paramount importance for the clinical management of the highly challenging cohort of patients with RH. Hence, in a proof-of-concept study we investigated if AVS can allow to identify unilateral PA as the underlying cause of RH [8,53]. We next tested the hypothesis that AVS-guided unilateral laparoscopic adrenalectomy could be an effective strategy to resolve RH in these patients.

Methods

Selection of the patients

We determined beforehand to select from a large population of consecutive patients referred for difficult-to-control HT to the University of Padua Center of Excellence of the European Society of Hypertension a cohort that met the ESC/ESH 2013 definition [50] for RH (Figure 1), because RH is listed among the features that should prompt the search for PA in the Endocrine Society Clinical Practice Guidelines [8]. We next planned to consider for further work-up those who had one or more additional clues suggesting PA, including

spontaneous or diuretic-induced hypokalemia; adrenal nodules on imaging; obstructive sleep apnea; a family history of early onset hypertension or cerebrovascular accident at a young age (< 40 years).

Moreover, the finding of low plasma active renin concentrations (DRC) ≤2 mIU/L in spite of administration of drugs (as ACEI, ARBs, diuretics) that should raise renin, and/or of elevated plasma aldosterone (PAC) on treatments (with ACEI and ARBs) expected to lower aldosterone secretion, were regarded as further clues to PA [8]. However, in the patients on beta-blockers, which can lower DRC and thus factitiously raise the ARR, raised PAC values were considered a condition sine qua non to suspect PA.

All patients fulfilling these criteria, who were reasonable candidate for general anesthesia and surgery and sought for pursuing surgical cure, were offered AVS following the Endocrine Society Clinical Practice Guidelines [8]. Consenting patients underwent the test while on multiple antihypertensive drugs, following the protocol described in detail elsewhere [54–56]. They also underwent adrenal imaging by 3 mm-thick slices computed tomography (CT) with contrast medium to rule out adrenocortical aldosterone-secreting carcinoma [57], and identify adrenal vein anatomy [8].

Study design

Considering the clinical challenges posed by the patients with "difficult-to-treat" HT, we set up a protocol for their evaluation which was used routinely with clinical success and constituted the basis of this prospective study. The study was approved by the Institutional Review Board and followed the Helsinki recommendations. For each patient, at baseline, and at one- and six-month follow-up after adrenalectomy, we determined the anti-hypertensive drugs burden by both number and defined daily dose (DDD) of antihypertensive drugs. The total DDD of each patient was calculated as sum of the DDD of the different drugs administered (Table 1).

Adrenal vein sampling

Bilaterally simultaneous AVS was performed by highly experienced radiologists, using catheters shaped for each adrenal vein. Blood was collected by gravity from the right and left adrenal veins and from the infrarenal inferior vena cava for the measurements of PAC, plasma cortisol concentration (PCC) and androstenedione [56], under unstimulated conditions. Cosyntropin was not used, because while facilitating the ascertainment of catheterization success lowered the lateralization index, as confirmed

in the largest available series of PA patients who underwent AVS both under unstimulated conditions and during cosyntropin-stimulation in the AVIS-2 Study [22].

The choice of the cut-offs used for AVS interpretation were those recommended in an expert consensus document [52]. Briefly, AVS was used to make the diagnosis only if bilaterally successful, as defined by a selectivity index > 2.0. Unilateral PA was diagnosed and used to refer the patients for laparoscopic adrenalectomy if the lateralization index exceeded 2.0. These cutoffs were validated in multiple studies that used an unambiguous diagnosis of aldosterone producing adenoma (APA) as reference following the STARD recommendations (10,15,16), and are supported by the AVIS-2 Study results [22].

Biochemical and hemodynamic evaluation and diagnostic criteria

All patients underwent a full biochemical and clinical assessment at baseline, which included measurement of serum sodium and potassium, 24-hour urine sodium and potassium excretion, direct measurement of DRC, PAC (LIAISON Direct Renin™ and Aldosterone Kits™, both from Diasorin, Saluggia, Italy), and calculation of the aldosterone-renin ratio (ARR), and again at one- and six-months follow-up post-adrenalectomy. Biochemical follow-up included measurements of the same variables, if possible without potentially confounding antihypertensive medications, i.e. when the patients received only a long-acting calcium channels blockers and/or doxazosin.

For the analysis of BP outcome, attended BP values were measured with automated devices in the office considering the average of at least three measurements taken three minutes apart. Each patient provided a written consent to participate in the study and allowed use of his/her de-identified data.

The diagnosis of APA was established unambiguously by the “five-corner-criteria”, which besides a biochemical diagnosis of PA, require biochemical cure, e.g. normalization of plasma renin activity (PRA) and PAC after adrenalectomy [64], and immunochemical demonstration of an aldosterone synthase positive adenoma at immunostaining with a monoclonal antibody for human CYP11B2 [29].

Hypertension-mediated-organ-damage evaluation

Hypertension-mediated-organ-damage in the heart was determined by Doppler echocardiography. Briefly, left ventricular (LV) hypertrophy (LVH), geometry and LV end systolic and end diastolic volume, LV stroke work, LV work, Doppler-flow velocity

indexes of early (E wave) and late (A wave) LV filling, the E/A wave ratio, the tissue-Doppler E/e', and the mitral E wave deceleration time (mDecT) were measured by the same expert cardiologist (M.C.), following the American Society of Echocardiography guidelines as reported [65]. LVH was defined according to the ESC/ESH cut-offs, i.e. left ventricular mass (LVM)/height ≥50 g/m$^{2.7}$ for men and ≥47 g/m$^{2.7}$ for women (5). Because of its dependence on the duration of diastole the mDecT was normalized for heart rate.

Hypertension-mediated-organ-damage was also assessed in the kidney by 24-hour urinary excretion of albumin normalized per gram of excreted creatinine, and estimated glomerular filtration rate (eGFR with the CKD-EPI equation) and categorized in chronic kidney disease (CKD)-classes.

Statistical analysis

One-way ANOVA followed by Scheffe's post-hoc test, paired t test, or the non-parametric Wilcoxon test were used to compare quantitative variables between groups. The distribution of categorical variables was compared by chi-square analysis. Results were expressed as absolute numbers, ratio, percentage, mean (± standard deviation, SD) or median (range, IQR). Significance was set at two-tailed $p < 0.05$. For the analysis we used SPSS (version 25 for Mac; IBM Italy Spa, Rome, Italy) and GraphPad Prism (version 8.2 for Mac; GraphPad Software, La Jolla, CA) software.

Results

From September 2011 to September 2018, 1016 patients were evaluated at our specialized Center for "difficult-to-control" arterial hypertension. In about half of the cases, they were either referred by their physicians. In the rest they directly presented after their own web-based search for a specialized ESH hypertension center. Of the 1016 patients, 71% did not fulfill the RH definition either because they were on < 3 medications, or were not on optimal medical treatment (18%), or were judged to be not fully adherent to prescribed treatment and, therefore, they were excluded from this study (Figure 1). The remaining 110 patients fulfilled the ESC/ESH definition of RH [49,50] in that they exhibited BP values (both systolic and diastolic) above 140/90 mmHg in spite of an average of 3.6 of drugs at baseline, which corresponded to 4.8 ± 2.1 Defined Daily Doses (DDD) (Table 1 and Figure 2).

Seventy-seven of these patients had at least one of the aforementioned clues of PA, mostly spontaneous or diuretic-induced hypokalemia [8]. Owing to their high-risk, their wish to pursue surgical cure, and the lack of contraindications to general anesthesia and surgery, they were offered AVS, which was performed after verification that their plasma renin levels were not overtly elevated [52], but because of their high BP values and the associated high risk, without withdrawal of interfering drugs.

The procedure was performed with no intra- and post-procedural complications and was bilaterally selective in 82% of the patients. Unambiguous evidence of a unilateral form of aldosteronism by the aforementioned criteria was achieved in 25 of patients who, therefore, underwent AVS-guided laparoscopic adrenalectomy. Surgery was followed by pathology and immunohistochemistry analysis, and biochemical and clinical follow-up to confirm the diagnosis of APA (Figure 1).

Follow-up studies

Examination at one-month post-adrenalectomy entailed a comprehensive clinical and biochemical evaluation, as per protocol at our institution. It showed that BP markedly fell by 31 mmHg systolic and 17 mmHg diastolic, from 161/99 ± 26/14 mmHg at baseline to 130/82 ± 9/7 mmHg ($p<10^{-4}$ for both systolic and diastolic), notwithstanding the tapering (from 3.6 ± 0.2 at baseline to 1.0 ± 0.2, $p<10^{-4}$) of the number of drugs required to achieve BP control.

Six months after surgery the BP fall was well maintained (133/84 ± 14/9 mmHg ($p<10^{-4}$ for systolic and $p=10^{-2}$ for diastolic) despite the persistently reduced drug therapy to 1.2 ± 0.2 ($p<10^{-4}$ vs. baseline) (Table 1 and Figure 2).

By the Primary Aldosteronism Surgical Outcomes (PASO) criteria, all patients showed a clinical benefit: 20% of the patients were completely cured, i.e. were normotensive on no antihypertensive drugs; 80% showed a partial clinical success, but importantly, none showed persistence of RH (Figure 3) [64]. Accordingly, the number of drugs needed to achieve BP control showed a clear-cut highly significant shift to the low values as compared to baseline (Table 1 and 2 and Figure 2).

These changes were paralleled by a significant increase of serum potassium and DRC levels, and by a decrease of PAC (Table 1). Thus, 96% showed complete biochemical cure and only 4% partial success, with no differences between sexes (Figure 3) [64].

At variance, in the group assigned to antihypertensive treatment based on a mineralocorticoid receptor antagonist because of either bilateral PA on AVS, or lack of

bilateral success on AVS, none were biochemically or hemodynamically cured ($p< 10^{-3}$ for cure rate between adrenalectomy and medical treatment).

Hypertension-mediated organ damage

In spite of the relatively short follow-up, LV mass index decreased (from 60 ± 14 g/m$^{2.7}$ to 52 ± 13 g/m$^{2.7}$, $p<10^{-4}$) at the last follow-up, mainly because of a decrease of both LV end diastolic volume and posterior wall thickness; the relative wall thickness remained unchanged (Table 3). The left atrium size decreased; the bulbar aorta did not; therefore, the change of the ratio did not attain statistical significance. The mDecT and the mDecT normalized for heart rate increased significantly from baseline (Table 4); however, the E/A ratio and the E/e' ratio values, which were within the normal range at baseline, showed no significant changes. The LV stroke volume and LV stroke work fell significantly as a result of decreased LV mass and volume (Table 4). While arterial compliance exhibited a 21%, non-significant trend to increase, systemic resistance index did not show any significant changes (Table 4).

As regard renal function, we observed a significant fall of urinary albumin excretion and a slight decrease of eGFR after adrenalectomy (Table 1).

Discussion

Previous studies have documented biochemical cure, and cure or improvement of hypertension with adrenalectomy and target medical treatment in PA [66–69]. However, to the best of our knowledge, none was focused on patients with RH. Thus, this is the first proof-of-concept study to reveal that identification of a unilateral aldosterone excess by means of AVS is feasible in high-risk patients with RH. It is also the first to demonstrate, albeit in a highly selected cohort, that AVS-guided unilateral laparoscopic adrenalectomy allows total biochemical cure of PA in 96% of the patients, and also, more importantly, resolution of RH in all (Table 1). Not only the BP values were brought down to target levels after surgery, but a highly significant decrease of antihypertensive medications burden, in terms of both number and DDD needed to control HT, was also seen (Table 1 and Figure 2). These impressive changes were accompanied by regression of cardiovascular damage (Tables 3 and 4), indicating that the correction of hyperaldosteronism and the ensuing favorable hemodynamic changes were instrumental in lowering the overall cardiovascular risk of the patients.

We would like to underline that these results were made possible by unambiguous identification of a unilateral APA by means of AVS. All adrenalectomized RH patients showed an adenoma (average diameter 16.3 ± 5.6 mm) at pathology examination that was conclusively identified as aldosterone-producing at immunohistochemistry with a monoclonal antibody specific for CYP11B2 (18). Thus, in at least one third of our patients, RH was due to a surgically curable tumor, whose removal led to resolution of RH in all of our patients, and could be identified by AVS in RH patients despite their being, by definition, on multiple potentially interfering drugs.

It is worth mentioning that the biochemical and hemodynamic improvement was accompanied by impressive changes in hypertension-mediated organ damage. We found a reduction of LV mass index, which occurred early, i.e. within six months after adrenalectomy and involved an inward remodeling, i.e. rearrangement of the LV around a smaller cavity (Table 3), a finding consistent with previous observations [66], and with the view that PA involves a slight expansion of blood volume [70], that is corrected when biochemical cure is accomplished.

This decrease of LV mass and volume has profound implications from the functional standpoint: it implies a highly significant decrease of LV stroke work and, therefore, of LV myocardial O_2 consumption, with favorable consequences in terms of myocardial susceptibility to ischemia.

Notwithstanding the changes in LV mass and volumes, the indexes of LV filling, which were already normal at baseline, did not decrease further. However, the left atrium size also decreased significantly, suggesting a decrease in left atrial pressure and an improvement of LV filling, a contention supported by the significant increase of the mitral valve deceleration time, even after correction for the duration of diastole (Table 4). Collectively these findings indicate an enhancement of LV emptying, in keeping with what observed in a larger series of PA patients without RH [71].

Somewhat unexpectedly, given the prominent fall of systolic and diastolic BP and the trend toward an increase of arterial compliance (Table 4), total systemic vascular resistance did not change significantly, a finding that deserves further scruting in a large cohort of RH patients.

As regards renal damage, microalbuminuria decreased significantly after surgery, likely because of the decrease of BP and the correction of hypertension- and hyperaldosteronism-induced hyperfiltration [72]. Due to the same mechanism, estimated GFR also showed a trend toward a decrease, thus confirming in RH patients what

previously observed in larger series of PA patients not selected because of RH [73]. Thus, on the whole, these findings indicate that the removal of the source of excess aldosterone secretion not only furnished resolution of RH, but also regression of cardiac and renal damage, when present.

Both the latest European and the American Guidelines on hypertension (4), considering the results of studies with mineralocorticoid receptor antagonist (MRA) agents in RH patients [74–79], recommend prescription of an MRA in RH patients. The present findings provide further indirect but compelling evidence in support of a crucial role of hyperaldosteronism in the pathogenesis of RH.

There are few limitations to be acknowledged in this as in all studies: the observational design; the lack of systematic use of 24-hour ambulatory BP measurement to confirm RH and exclude white-coat HT and the absence of a control group to rule out a Hawthorne effect (23). We believe that these potential limitations are offset by the careful AVS – centered diagnostic work-up of PA, the availability of APA as gold reference, the painstaking assessment of hypertension-mediated organ damage, that allowed to rule out pseudo-RH, and the availability of follow-up data which allowed to conclusively diagnose APA.

In summary, this study provided three novel observations that are critically important for the clinical management of the patients with RH: i) the demonstration that AVS is feasible and allows identification of unilateral PA in RH patients, a challenging PA phenotype owing to the need of multiple antihypertensive drugs potentially confounding AVS; ii) the finding that AVS-guided adrenalectomy allowed resolution of RH in those with underlying PA; iii) the prominent clinical benefit in spite of severity of arterial hypertension, presence of hypertension-mediated organ damage, and documented resistance to treatment. The high rate of biochemical cure, the 20% rate of complete cure of arterial hypertension and the marked improvement in the rest (Figure 3) allowed a highly significant decrease of the drug burden, as assessed by both number of antihypertensive agents and total DDD in the patients who still required to control BP after adrenalectomy (Figure 2).

Accordingly, these results suggest that patients with RH should be referred to centers that can successfully perform and interpret AVS and thus can identify the patients who markedly benefit from unilateral laparoscopic adrenalectomy.

Table 1. Clinical and biochemical characteristics of the recruited patients at baseline and after unilateral adrenalectomy.

Variables	Baseline	Post-surgery (6 months)	*p-Value*
BMI (Kg/m^2)	26.8 ± 3.3	26.4 ± 4.1	=0.35
Office SBP (mmHg)	161 ± 26	133 ± 14	$<10^{-4}$
Office DBP (mmHg)	99 ± 14	84 ± 9	=0.01
Heart rate (bpm)	73 ± 10	72 ± 9	=0.81
Drugs (number)	3.6 ± 0.2	1.2 ± 0.2	$<10^{-4}$
Defined Daily Dose	4.8 ± 2.1	1.2 ± 1.4	$<10^{-4}$
Adrenal adenoma size	16.3 ± 5.6	-	-
PRA (ng/mL/h)	0.7 (0.2-3.4)	4.5 (0.2-40.9)	=0.01
DRC (mIU/L)	4.9 (2.0-27.9)	36.8 (2.0-334.6)	=0.01
PAC (ng/dL)	31 (14 - 152)	7 (2 -13)	=0.01
ARR	168 (30 - 624)	10 (0 - 40)	=0.01
serum Na^+ (mmol/L)	142 ± 2.6	140 ± 2	=0.03
serum K^+ (mmol/L)	3.6 ± 0.8	4.5 ± 0.6	$<10^{-4}$
24h urinary Na^+	178 ± 64	163 ± 47	=0.28
24h urinary K^+ excretion	77 ± 28	64 ± 22	=0.01
UAE (mg/g creatinine)	330 (5-1770)	138 (5-3820)	=0.01
eGFR (mL/min/1.73 m^2)	78 ± 4	71 ± 4	=0.03

Data reported as mean (±SD) or median (IQR) as appropriate.

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; PRA, plasma renin activity; PAC, plasma aldosterone concentration; ARR, aldosterone-renin-ratio; UAE, urinary albumin excretion; eGFR, estimated glomerular filtration rate.

Table 2. Antihypertensive drugs at the initial screening and after adrenalectomy.

Antihypertensive drugs	At the screening		Post-adrenalectomy (6 months)	
	DDD	Patients (%)	DDD	Patients (%)
Calcium-channel Blockers:				
• Dihydropyridine	2.0 (1.0-2.0)	20.0	1.0 (1.0-2.0)	48.0
• Non-dihydropyridine	0.7 (0.7-0.8)	32.0	0.9 (0.7-1.0)	16.0
β-blockers	0.5 (0.5-0.5)	4.0	0.5 (0.5-0.7)	12.0
Diuretics:				
• Thiazide	0.2 (0.2-0.2)	20.0	1.0 (1.0-1.0)	4.0
• Loop	1.0 (1.0-1.0)	4.0	1.0 (1.0-1.0)	4.0
• Potassium sparing	0.5 (0.5-0.5)	12.0	-	-
Mineralocorticoid Receptor Antagonists	0.7 (0.7-0.7)	4.0	-	-
α1-blockers	1.0 (0.5-1.0)	52.0	0.5 (0.5-0.6)	16.0
Angiotensin-type I Receptors Blockers	2.0 (2.0-2.0)	28.0	2.0 (2.0-2.0)	4.0

Data reported by median (IQR).
Please note that no ACE-inhibitors and/or central blockers were administrated to control blood pressure at the initial screening.

Table 3. Cardiac remodeling indexes of the patients at baseline and after unilateral laparoscopic adrenalectomy.

Variables	Baseline	Post-surgery (6 months)	*p-Value*
Cardiac remodelling			
LVED diameter (mm)	50 ± 4	48 ± 4	$<10^{-4}$
LVES diameter (mm)	30 ± 4	29 ± 4	NS
IVSd (mm)	1.32 ± 0.18	1.27 ± 0.17	=0.006
PWd (mm)	1.30 ± 0.17	1.21 ± 0.13	=0.004
LVED Volume (ml)	122 ± 24	107 ± 20	<0.05
LVES Volume (ml)	37 ± 14	35 ± 10	NS
LVMI ($g/m^{2.7}$)	60 ± 14	52 ± 13	$<10^{-4}$
RWT	0.52 ± 0.05	0.55 ± 0.08	NS
Left atrial size (mm)	38.8 ± 0.5	36.9 ± 0.5	$=10^{-4}$
Aorta (mm)	36.2 ± 0.3	35.7 ± 0.3	NS
Aorta/Left atrial size	1.07 ± 0.14	1.04 ± 0.14	NS

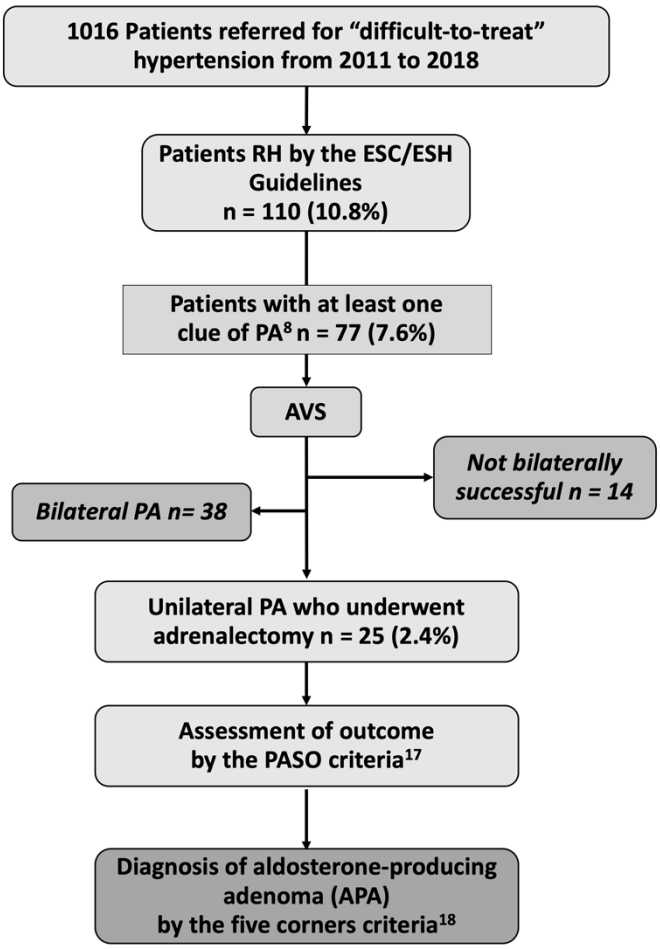
Data reported as mean ± SD.
IVSd, interventricular septum thickness at diastole; LVED, left ventricular end-diastolic; LVES, left ventricular end-systolic; LVM, left ventricular mass; PWd, posterior wall thickness in diastole; RWT, relative wall thickness

Table 4. Hemodynamic parameters of the patients at baseline and after unilateral laparoscopic adrenalectomy.

Variables	Baseline	Post-surgery (6 months)	*p-Value*
Hemodynamic parameters			
Stroke Volume (ml/beat)	85 ± 14	72 ± 14	$<10^{-4}$
Stroke Work (g/m)	192 ± 43	142 ± 29	$=3 \cdot 10^{-3}$
Cardiac Index (L/min/m^2)	2.87 ± 5.02	2.56 ± 7.30	=0.05
Mitral Deceleration Time (msec)	239 ± 64	282 ± 80	=0.02
Mitral Deceleration Time normalized for HR (msec/beat)	3.5 ± 1.2	4.2 ± 1.1	=0.03
Systemic resistance index (din s/cm^{5}m^2)	3083 (2576-3590)	3075 (2568-3582)	NS
Arterial compliance (ml/mmHg)	1.32 ± 0.41	1.60 ± 0.38	NS

Data reported as mean ± SD, or median (IQR) as appropriate. HR, heart rate.

Figure 1: Flow-chart of the study for selection, recruitment and analysis of the patients.



[1]Refer to Figure 3 for the outcome according to the PASO criteria.

ESH, European Society of Hypertension; AVS, adrenal vein sampling; PA, primary aldosteronism; RH, resistant hypertension.

Figure 2: Changes of systolic and diastolic blood pressure values, the number and the defined daily dose of drugs in resistant hypertensive patients before adrenalectomy and at 1-month and 6-months follow-up.

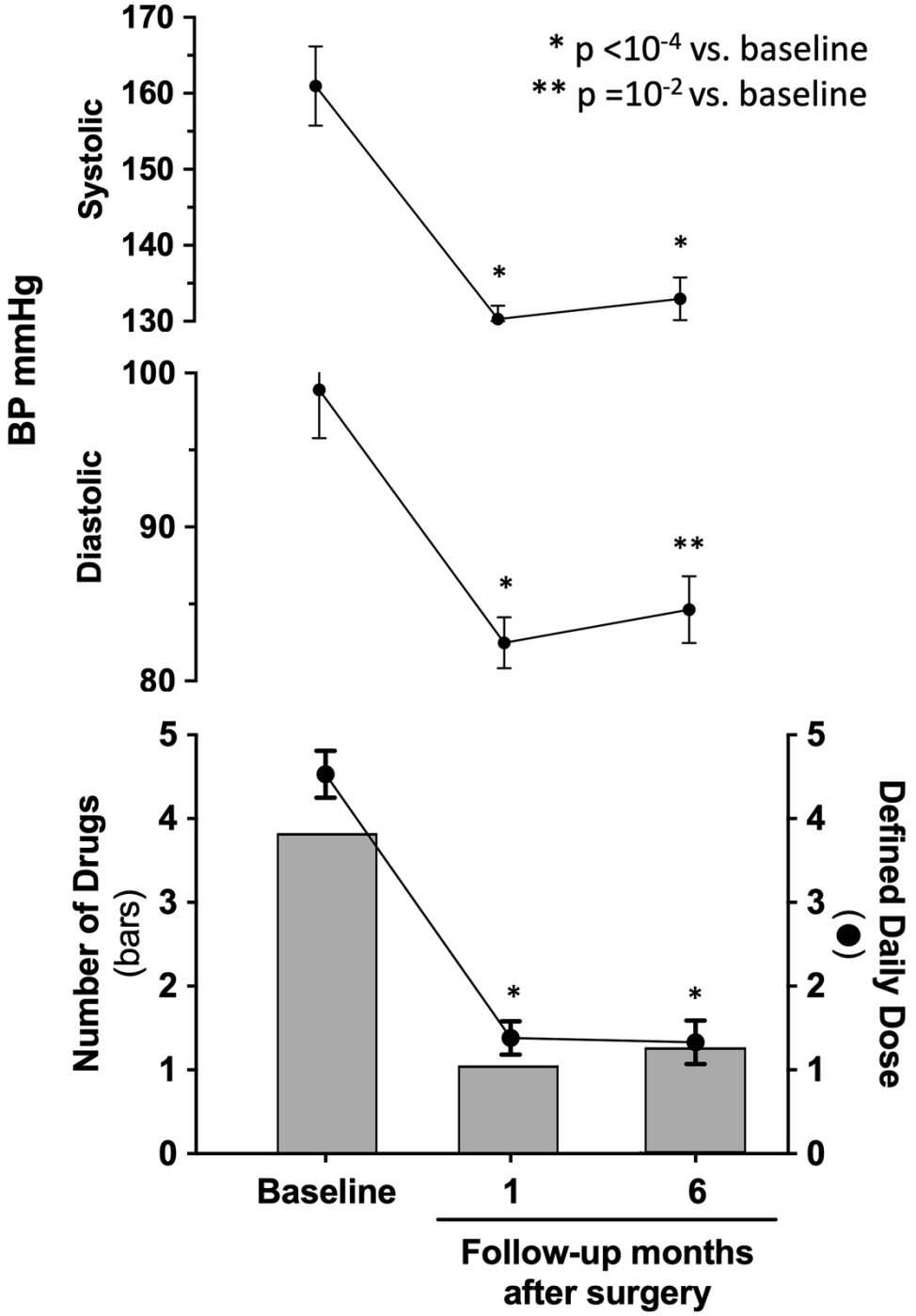
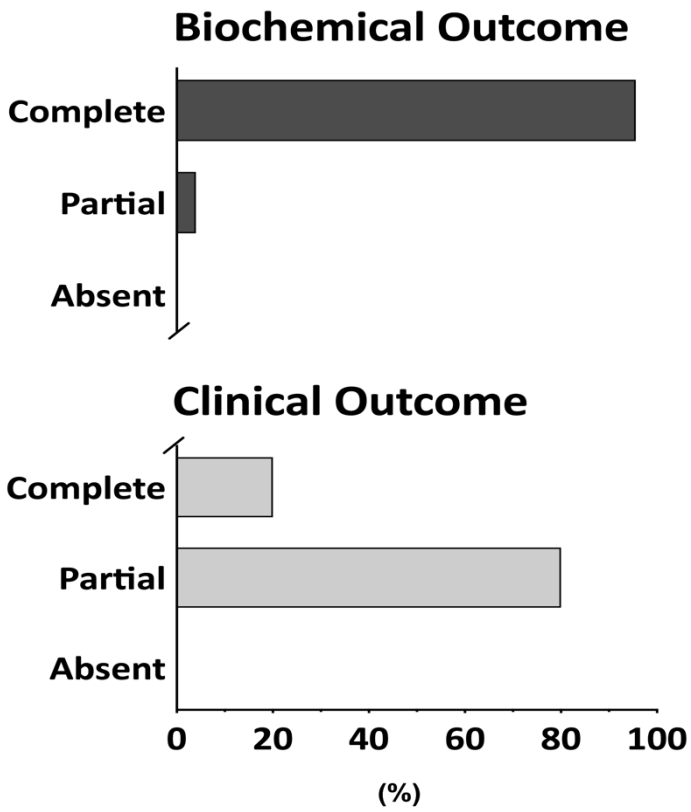


Figure 3: Clinical and biochemical outcomes after AVS-guided unilateral laparoscopic adrenalectomy in 25 patients with resistant hypertension by the PASO criteria as defined below.



Absent clinical success: unchanged or increased blood pressure (BP) [unchanged difference (pre- vs post-surgery) in systolic/diastolic BP of < 20/10 mmHg or increased in systolic/diastolic BP ≥ 20/10 mmHg respectively] with either the same amount or an increase in antihypertensive medication [unchanged difference is n defined as less than 0.5 of the defined daily dose (DDD) or an increase of 0.5 or more times the DDD between pre- and post-surgery];

Partial clinical success: the same BP as before surgery [difference (pre- vs post-surgery) in systolic/diastolic BP of < 20/10 mmHg respectively] with less antihypertensive medication [defined as less than 0.5 times the DDD between pre- and post-surgery] or reduction in BP [defined as a difference in systolic and/or diastolic BP of ≥ 20/10 mmHg respectively] with either the same amount or less antihypertensive medication [unchanged antihypertensive medication is defined as a change (decrease or increase) of less than 0.5 times the DDD; less antihypertensives defined as a decrease of 0.5 or more times the DDD between pre- and post-surgery];

Complete clinical success: defined as office systolic/diastolic BP < 140/90 mmHg respectively without the aid of antihypertensive medication.

Absent biochemical success: persistent hypokalaemia [defined as K^+ < 3.6 mmol/L after surgery] and/or persistent raised aldosterone-to-renin ratio (ARR) [defined as ARR ≥ 25 (ng/dL)*(ng/mL/h)$^{-1}$ after surgery];

Partial biochemical success: correction of hypokalaemia [defined as K^+ ≥ 3.6 mmol/L after surgery] and a raised ARR with ≥ 50% decrease in baseline plasmatic aldosterone concentration [defined as ARR ≥ 25 (ng/dL)*(ng/mL/h)$^{-1}$ and plasmatic aldosterone concentration ≥ 15 ng/dL after surgery];

Complete biochemical success: correction of hypokalaemia [defined as K^+ ≥ 3.6 mmol/L after surgery] and normalization of ARR [defined as ARR < 25 (ng/dL)*(ng/mL/h)$^{-1}$].

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Chapter 3

Effect of unilateral adrenalectomy on the quality of life of patients with lateralized primary aldosteronism

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Abstract

Background: Primary aldosteronism (PA) is associated with an increased prevalence of anxiety and depression. Subnormal quality of life (QoL) scores in PA patients may be improved after surgical treatment. The aim of the study was to assess the impact of surgery on health-related QoL and depression status of patients suffering from PA, comparing the results with a control group of patients undergoing surgery for non-secreting adrenal tumors.

Methods: Data on QoL and depression status were prospectively collected, from January 2014 to January 2017, before, early after surgery (at 1 month) and at late follow up (at least 6 months) in patients with unilateral PA and in a control group with non-secreting adrenal tumors submitted to unilateral laparoscopic adrenalectomy. QoL was assessed using the Short Form 36 (SF-36) Health Survey for Physical (PCS) and Mental Component (MCS); the depression status by a 20-item depression scale (DS) questionnaire.

Results: Twenty-six PA patients and 15 controls were recruited. Biochemical cure of the disease was achieved following surgery in all PA patients; hypertension was cured in 31% of cases and improved in the remaining 69% of cases. No morbidity occurred in both groups. There were no significant differences between PA patients and controls concerning demographics, preoperative PCS, MCS and DS values. In patients with PA, MCS values improved at early (42.72±13.68 vs 51.56±9.03, $p=0.0005$) and late follow up (42.72±13.68 vs 51.81±7.04, $p<0.0001$); also DS values improved at early (15.92±11.98 vs 8.3±8.8, $p=0.0002$) and late follow up (15.92±11.98 vs 4.57± 6.11, $p<0.0001$). In PA patients PCS values significantly improved at late follow up (51.02±8.04 vs 55.85±5.1, $p=0.013$). Also in controls an improvement of MCS and DS scores was found at early and late follow up compared to preoperative values, while no significant differences in PCS were found.

Conclusions: Both PA and non-secreting adrenal tumors affect health-related QoL, worsening MCS and DS scores. Adrenalectomy is effective in curing PA, and improving MCS and DS scores at early and late follow-up, in patients with PA and non-secreting adrenal tumors. In PA patient surgery also significantly improves PCS at late follow up.

Key words

Primary aldosteronism, quality of life, depression status, adrenalectomy

Background

Primary aldosteronism (PA) due to unilateral or bilateral overproduction of aldosterone, is the most common cause of endocrine hypertension, and it has been reported in more than 11 % of referred hypertensive patients [1-2].

The lateralization of aldosterone hypersecretion is crucial, because only patients with unilateral PA are held to be surgically curable [3]. Unilateral laparoscopic total adrenalectomy is currently the preferred strategy in patients with lateralized excess [4-7].

Besides leading to increased cardiovascular morbidity and mortality [1], PA has been claimed to be associated to higher prevalence of anxiety and depression, with potential impact on health-related quality of life (QoL), through mechanisms and pathways that remain to be clarified [8-9].

Undoubtedly, both surgical and medical treatment of PA can control hypertension and severe cardiovascular damages in the long term [3]. However, whether these two strategies have the same beneficial effects on QoL and depression remains uncertain. In fact, they show substantial differences, as surgery eliminates the source of aldosterone excess, while mineralocorticoid receptor antagonists simply control it [10-11].

Measurement of QoL investigates the functional status of the individual and the patient's appraisal of health, allowing assessment of the impact of a disease and/or treatment from the patient's perspective.

Therefore, only few studies investigated health-related QoL has been in patients suffering from PA [12-13], reporting subnormal scores compared to normal population. A recent systematic review, not focusing on this issue, [14] highlighted that, in patients with PA, health-related QoL as well as scores for depression and anxiety [11] ameliorated after surgery with respect to medical treatment.

However, it could not be excluded that non-specific psychological effects secondary to surgery could be at least partly responsible for the improvement in QoL [10].

The aim of the present study was, therefore, to assess the impact of surgery on health related QoL (both in Mental and Physical components) and depression status of patients suffering from PA and to compare them with a control group of patients with non-secreting adrenal tumor who also underwent adrenalectomy.

Methods

Data were prospectively collected from January 2014 to January 2017 at Endocrine Surgery Unit of Padua University Hospital, Italy.

The present prospective non-randomized study included patients with unilateral PA and a control group of patients with non-secreting adrenal tumor submitted to laparoscopic transperitoneal

adrenalectomy by flank approach performed by the same surgeon (M.I.). The institutional ethics committee approved the study and informed consent was obtained from all patients.

PA was diagnosed based on a plasma aldosterone concentration greater than 15 ng/dL and an aldosterone/renin ratio greater than 40 ng/dL:ng/mL/h, measured after washout of interfering drugs or after changes of the drug treatment as previously detailed [15]. The diagnosis was confirmed by saline infusion and/or the captopril test [15].

Arterial hypertension was defined by a systolic blood pressure (BP) of 140 mm Hg or greater, diastolic BP of 90 mm Hg or greater, or both and/or the presence of antihypertensive medical treatment.

Surgery was performed in patients with lateralized PA, according to the results of preoperative lateralizing techniques. Lateralizing techniques included adrenal venous sampling (AVS), CT scan and/or MR as previously described [16].

AVS was performed with bilateral simultaneous catheterization, by using one catheter for each adrenal vein. Successful selective catheterization was usually confirmed when the ratio between cortisol concentration in each adrenal vein and the inferior vena cava was greater than 1.1; unilateral aldosterone hypersecretion was usually confirmed when the ratio of adrenal vein aldosterone concentration to the homolateral cortisol concentration on the side with the higher ratio over the contralateral aldosterone to cortisol ratio (AVS ratio) was greater than 2.

The control group was composed by patients suffering from non-secreting adrenal tumor, defined as asymptomatic adrenal mass, incidentally detected on imaging not performed for suspected adrenal disease, in patients without glucocorticoid, catecholamine or mineralcorticoid hypersecretion (assessed by plasmatic ACTH, 24-hour urinary free cortisol levels and 1 mg overnight dexamethasone suppression test, 24-hour urinary catecholamine or metanephrine levels and plasma renin and aldosterone levels, respectively). In these patients, surgery was indicated by the presence of adrenal mass with suspicious radiological findings (even without evidence of local invasion or distant metastases), evidence of significant tumor growth during follow-up imaging and/or patient preference. Patients with adrenal or extra-adrenal malignancies or any psychiatric disorders were not included in the study.

The surgical procedure was performed with the patient in lateral decubitus flank position. Pneumoperitoneum (at 12-14 mmHg by CO_2) was made by Hasson cannula inserted by open technique. A subcostal port was placed for the laparoscope, and two/three other 5/10 mm ports sited. For right adrenalectomy the liver was mobilized and retracted and the right medium adrenal vein was identified by following the lateral edge of the vena cava. The main right adrenal vein was identified and divided early. Then the adrenal branches from the inferior phrenic artery, aorta, and the renal

artery were divided. On the left side the colonic flexure was mobilized and the splenorenal ligament dissected, allowing the fall of the spleen medially and the identification of the tail of the pancreas. The avascular plane separating adrenal gland from the tail of the pancreas was opened, allowing the view of the inferior adrenal vein, going into the left renal vein. The inferior adrenal vein was divided and then the gland was dissected and removed. Small arterial vessel from superior, medium and inferior pedicle were coagulated. In all cases, the adrenal gland was removed via a retrieval bag [7, 17].

Health-related QoL and depression status, were assessed preoperatively (at the time of hospital admission) and postoperatively at 1-month outpatient control and at long term (at least 6 months after surgery).

QoL was evaluated using the Italian version of Short Form 36 (SF-36) Health Survey for a Physical (PCS) and a Mental Component (MCS). The SF-36 is a 36 item self-administered questionnaire measuring QoL across eight domains obtaining eight scaled scores, which are the weighted sums of the questions in their section. These domains are physical functioning, social functioning, role limitations due to physical problems, role limitations due to emotional problems, vitality, bodily pain, general health perceptions, and general mental health. PCS and MCS are a summary of physical and emotional QoL respectively. Scores may range from 0 (poorest health status) to 100 (best health status). The depression status was evaluated using a 20-item depression scale (DS) questionnaire; the score may range from 0 (best status) to 60 (poorest status) [18-19]. The results were compared with published normative values for the Italian population [20].

Records of the patients were reviewed to gather relevant demographics, body mass index (BMI, defined as body weight (kg)/height (m^2), normal values 20 to 24.9), hormonal parameters (including glucocorticoid, mineralcorticoid and catecolamine assays), BP values, number of antihypertensive drugs, side and size of adrenal masses, intra and postoperative morbidity and definitive pathology.

Postoperative follow-up data (including hormonal, BP parameters, number of antihypertensive drugs), were assessed 1 month after surgery and at long-term.

Results were expressed as absolute numbers, ratio, percentage, mean (± standard deviation) or median (range).

Statistical analysis was performed using Fisher's exact test for categorical variables, Student's paired *t* test, Wilcoxon matched-paired test, Mann–Whitney *U* test, as appropriate. $P < .05$ was considered statistically significant.

Results

Twenty-six PA patients and 15 patients with non-secreting adrenal tumor undergoing laparoscopic transperitoneal adrenalectomy were recruited.

No significant differences were found between PA patients and controls concerning demographics, BMI, side and dimension at preoperative imaging of the mass (**Table 1**). The size of the adrenal mass at preoperative imaging was significantly higher in controls than in PA patients ($p<0.0001$) (**Table 1**).

Hypertension was present in all patients with PA and in five patients with non-secreting adrenal tumor ($p<0.0001$); hence, the mean systolic and diastolic BP were significantly higher in PA patients than in controls (154±19 vs 125±9 mmHg, $p<0.0001$ and 91±12 vs 75±8 mmHg, $p=0.0002$, respectively); likewise, the number of antihypertensive drugs was significantly higher in PA patients than in controls (3.42±1.47 vs 0.60±0.99, $p<0.0001$).

Preoperative MCS and DS scores were impaired in PA patients compared to normal Italian reference population; similar findings were found also in the control patients.

No significant differences were found between PA patients and controls concerning preoperative PCS (51.02±8.04 vs 51.16±9.63, $p=0.75$), MCS (42.72±13.68 vs 39.39±12.81, $p=0.39$), and DS values (15.92±11.98 vs16.26±12.56, $p=0.91$).

All patients underwent uneventful laparoscopic surgery; no conversion to open approach was performed, no blood transfusion was required and no intra- or post-operative morbidity occurred in both groups.

Pathological specimens revealed benign adrenal tumors in all cases in both groups.

Early follow-up

At one month postoperative follow up, PA was biochemically cured in all patients, according to the normalization of the aldosterone/renin ratio and serum potassium levels; hypertension was cured in 10 cases (38%), and improved in the remaining 16 cases (62%). The systolic and diastolic BP were significantly reduced (from 154±19 to 130 ± 15 mmHg, $p<0.0001$ and from 91±12 to 78±9 mmHg, $p<0.0001$); also the mean number of antihypertensive drugs was significantly reduced (from 3.42±1.47 to 1.15±1.1, $p<0.0001$).

In patients with PA, MCS values significantly improved (42.72±13.68 vs 51.56±9.03, $p=0.0005$) (**Figure 1**), mainly due to an amelioration in the "mental health" (42.7±15.7 vs 53.17±7.33, $p=0.001$) and "emotional role" (45.5±11.83 vs 51.23±9.84, $p=0.001$) scores. Also DS values significantly improved (15.92±11.98 vs 8.3±8.8, $p=0.0002$). Conversely, no quite significant differences were found regarding PCS scores (51.02±8.04 vs 48.01±6.85, $p=0.07$) (**Figure 1**).

In controls, no significant changes in BP levels and number of antihypertensive drugs was found; also in the five hypertensive patients no changes in BP values and number of antihypertensive drugs were detected after adrenalectomy.

Also in controls MCS (39.39±12.81 vs 50.62±8.68, p=0.0005) and DS (16.26±12.56 vs 7.86±7.19, p=0.001) values improved after surgery, mainly due to a significant amelioration in the "mental health" (46.69±12.01 vs 53.79±6.36, p=0.006) and "emotional role" (33.19±13.69 vs 46.93±9.26, p=0.001) scores (**Figure 1**). No significant differences were found regarding PCS values (51.16±9.63 vs 48.60±6.99 p=0.26).

No significant differences were found between PA and control patients concerning postoperative MCS and PCS and DS (p= 0.69, p=0.81 and p= 0.93, respectively).

Long term follow up

At long term follow up (median 8 months, range 6-13), all patients that underwent surgery for PA were still biochemically cured, but two patients that at one month had achieved the cure of hypertension, restarted antihypertensive therapy; thus, the hypertension cure was achieved in 8 patients (31%).

PA patients had systolic and diastolic BP values significantly reduced (from 154±19 to 129± 13 mmHg, p<0.0001 and from 91±12 to 79±8 mmHg, p<0.0001), compared with preoperative values; also the mean number of antihypertensive drugs was significantly reduced (from 3.42±1.47 to 1.28±1.2, p<0.0001).

These patients had no significant differences in BP values and number of antihypertensive drugs one month after surgery and at long term follow up.

In patients with PA, a significant improvement in PCS (51.02±8.04 vs 55.85±5.1, p=0.013), MCS (42.72±13.68 vs 51.81±7.04, p<0.0001) and DS (15.92±11.98 vs 4.57± 6.11, p<0.0001) values was described, compared with preoperative period; PCS values improved significantly, compared with values recorded one month after surgery (p=0.0002) (**Figure 1**).

At late follow up, also in controls MCS (39.39±12.81 vs 49.30±10.46, p=0.001) and DS values (16.26±12.56 vs 6.80±5.64, p=0.002) improved after surgery, mainly due to a significant amelioration in the "mental health" (46.69±12.01 vs 54.64±7.87, p=0.007) and "emotional role" (33.19±13.69 vs 51.62±9.87, p<0.0001) scores (**Figure 1**). No significant differences were found regarding PCS values (51.16±9.63 vs 53.91±4.30 p=0.25).

No significant differences were found between PA and control patients concerning MCS and PCS and DS (p= 0.58, p=0.16 and p= 0.18, respectively).

Discussion

PA is a common, albeit often overlooked, cause of hypertension which is often severe and/or drug resistant and, therefore, associated with cardiovascular damage and a worse prognosis [21-22]. Moreover, it implies clear cut alterations of the renin-angiotensin-aldosterone system, which suggests that it entails multiple reasons to imply a worsened QoL, given that the derangements of this important system can affect QoL [8-9].

Hypertension is an important factor for reduced QoL: an impairment of QoL has been reported in patients with essential hypertension compared to normal controls in the somatization and psychological distress [23].

Along with this hypothesis, at the best of our knowledge, Health-related QoL in patients suffering from PA has been previously investigated only by 3 studies [12-13, 24].

In 2010, Sukor et al [24] examined health-related QoL in 22 patients with unilateral PA before and after unilateral adrenalectomy at 3 and 6 months, using SF-36 questionnaire. They found a significant improvement in the QoL of these patients both in physical and mental condition. However, they did not examine depression and anxiety.

In 2011, the same authors [12] compared the results of the previous study with those from 21 patients with bilateral PA, before or after commencing medical treatment, and with those of the normal Australian population. They confirmed that PA patients had subnormal QoL scores compared to normal population. Moreover, they described that QoL improved in all patients with bilateral PA undergoing medical treatment, but more slowly and to a lesser degree than in patients undergoing surgery for unilateral PA.

In 2012, Kunzel et al [13] published the results of a cross sectional study on the data from German Conn Registry examining health-related QoL using SF12 questionnaire, in which they investigated acute impairment of QoL and long-term treatment effects in patients with PA. The study included 132 patients with PA, stratified according to the treatment status: 27 newly diagnosed, untreated; 52 in chronic medical treatment, 49 patients treated with adrenalectomy. The study confirmed that PA patients had a worse physical and mental condition than the normal German reference population; untreated and medically treated patients reported the lowest scores.

Our study differed substantially from these previous studies, since we enrolled as controls patients undergoing the same surgical procedure for non-functioning adrenal mass.

This allowed adjustment for the potentially confounding effect of being harboring an adrenal tumor and of being submitted to adrenalectomy. Moreover, our study was aimed to clarify the impact of surgery on health-related QoL (both in Mental and Physical components) and for the first time on depression status, in patients suffering from PA.

In agreement with previous studies, we confirmed that patients with PA have an impaired QoL and depression status compared with normal population, as assessed by worse MCS and DS scores.

In PA patients, MCS (especially in the mental health and emotional role dimensions) and DS values significantly improved 1 month after surgery and at long term follow-up, confirming the beneficial effect of adrenalectomy previously reported [12]. However, we failed to find significant differences with the controls, since also these patients (without aldosterone excess) showed an impaired preoperative MCS and DS scores, and a significant amelioration one month and at long term after surgery. Interestingly, also in controls MCS improvement was mainly due to mental health and emotional role amelioration. However, the amelioration was more evident in PA patients.

Even if controls and PA patients were similar according to demographics and type of surgical procedure, the former had lower BP levels, larger adrenal masses and underwent surgery mainly because of a suspicion of malignancy, while there was not suspicion for the latter.

Thus, it remains unclear if it may be related to the reduction of BP levels or antihypertensive drug treatment (with a possible reduction of drug-related size effects), to the aldosterone/renin system normalization or a non-specific psychological effect of surgery.

We may argue that in control population the thought of impending surgery and the uncertain nature of the non-secreting adrenal mass might have affected the mental component and increased the depression status before surgery. Obviously, both factors might have been solved after surgery, since in all cases definitive pathology described a benign adrenal tumor; however, other psychologic effects of adrenalectomy may not be excluded, as previously reported [10].

In the present study, no significant amelioration of PCS scores was detected in PA patients one month after surgery; this finding might be related to the sequelae of recent surgery. In fact, in PA patients the improvement in PCS values become evident at long term follow-up. This is in agreement with previous studies that demonstrated at 3 months a significant increase of physical condition, after disease cure and BP normalization or amelioration [24].

In the control group, no significant variations were detected in PCS values at one month and at long term, compared with preoperative values.

However, some limitations to the present pilot study that might have biased the results should be underlined, including the limited number of cases and length of follow up. Moreover, the mental component of health–related QoL is difficult to explore; the SF-36 and DS questionnaires are not disease specific. Furthermore, the administration of the baseline questionnaire during hospitalization for surgery, when factors such as optimism or anxiety and fear of surgery could have some effects, might have affected the results in terms of worsening QoL and depression as compared to the normal population. However, if any, the effect was likely similar in both PA and controls.

Conclusions

PA affects the health-related QoL, worsening the mental component and the depression status. Adrenalectomy is effective in curing PA, and improves the mental component of health-related QoL and depression status at 1 month and at long term. At long term, surgery determines an improvement also in the physical component of health-related QoL of PA patients.

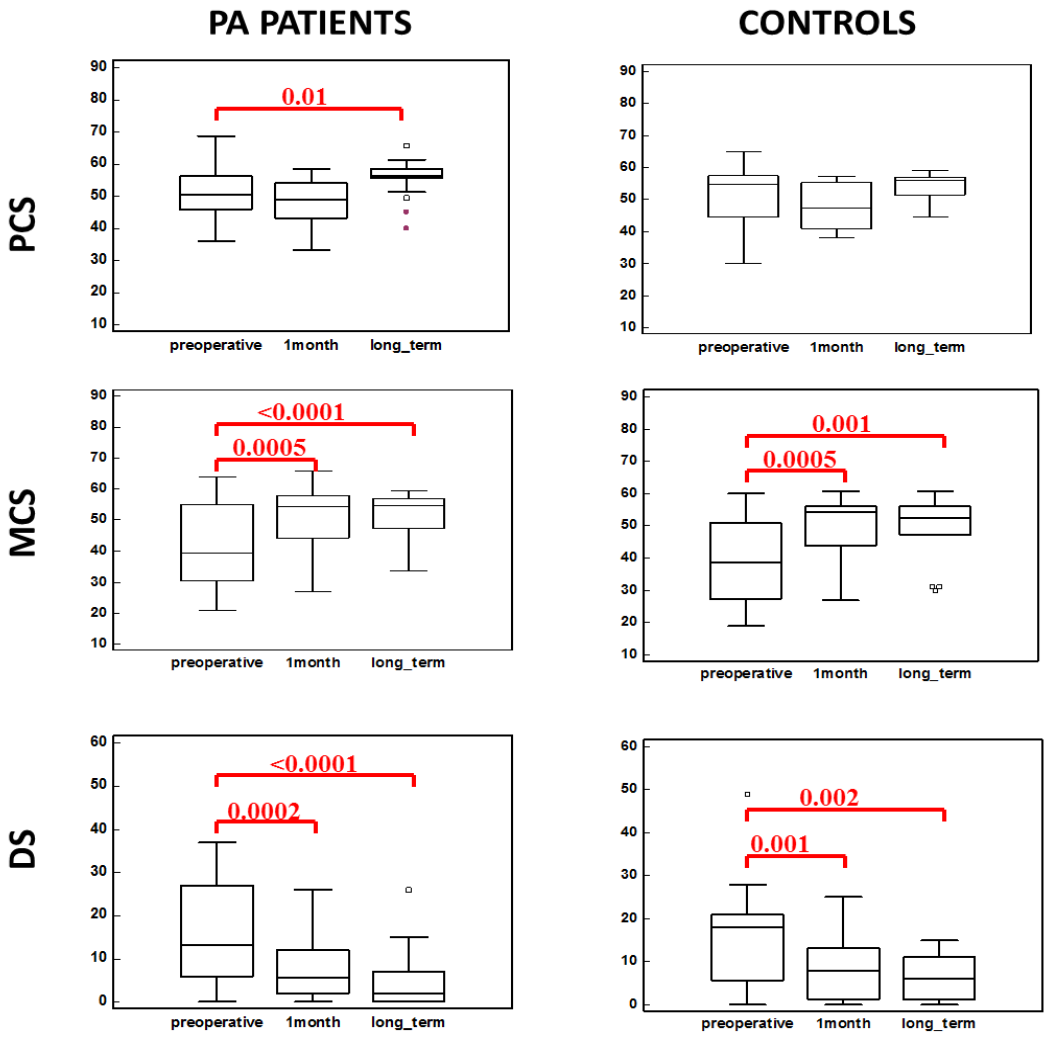
The role of hormonal cure of PA and the possible weight of the psychological effects of surgery itself in affecting QoL need to be further explored, since some relevant results may be observed also in patients undergoing surgery for non-secreting adrenal masses. Further studies are needed to confirm these results at a longer follow up and with a larger population.

Table 1. Demographics, and general features in (PA patients) patients with Primary Aldosteronism and Controls undergoing laparoscopic adrenalectomy.

	PA PATIENTS (n=26)	**CONTROLS (n=15)**	**p-Value**
Sex (female/male)	10/16	6/9	0.92
Age (years)	54±11	56±9	0.54
Body Mass Index (kg/m^2)	26.6±4	27.4±6	0.95
Side of the mass (left/right)	12/14	9/6	0.59
Size of the mass (mm) median (range)	15 (6-30)	44 (25-60)	<0.0001

PA: Primary aldosteronism

Figure 1. PCS, MCS and DS values in PA patients and controls, preoperatively, 1 month after surgery and at long term. *PA: primary aldosteronism; PCS: Physical Component Score; MCS: Mental Component Score; DS: depression scale.*



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Chapter 4

The skin-sodium compartment in human hypertension caused by aldosteronism: role of skin macrophages and lymphatic vessels

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Overall Summary

Hypertension (HT) accounts for 18% of the cardiovascular deaths worldwide and therefore is the major risk factor for stroke, myocardial infarction, and renal failure. In the setting of inappropriately high aldosterone secretion, excess sodium (Na^+) intake with ensuing body fluid volume expansion plays a key role in causing many forms of HT. In fact, according to classical physiology, pressure natriuresis and renal control of Na^+ homeostasis are central for blood pressure (BP) regulation and for the pathogenesis of HT. However, the precise molecular mechanisms whereby Na^+ retention raises BP remained poorly defined until recently, when accumulating evidences pointed to a role of extrarenal regulatory mechanisms involving salt-skin storage as a reservoir of free extracellular Na^+, and macrophages-mediated modulation of lymphatic vessels drainage of water and electrolytes from interstitium. Experimental studies in animal models, demonstrating that skin and muscle tissues can store Na^+, support this hypothesis. More specifically HT has been linked with excess skin-Na^+ content, because of dysregulation of skin lymphatic expansion during high Na^+ intake.

In this study we used primary aldosteronism (PA), a paradigm of salt-dependent low-renin HT, the most common cause of secondary HT, as a model for investigating the changes in skin-Na^+ content.

Abbreviations:

ARR, aldosterone-renin ratio;
AVS, adrenal vein sampling;
BMI, body mass index;
BP, blood pressure;
DBP, diastolic blood pressure;
DRC, renin concentration;
DW, dry weight;
eGFR, estimated glomerular filtration rate;
EH, essential hypertension;
HMOD, hypertensive-mediated target organ damage;
HT, hypertension;
IQR, interquartile range;
LI, lateralization index;
Ln, logarithm;
MRA, mineralocorticoid receptor antagonist;
MRI, magnetic resonance imaging;
PA, primary aldosteronism;
PAC, plasma aldosterone concentration;
Post-ADX, after adrenalectomy
PRA, plasma renin activity;
Pre-ADX, before adrenalectomy;
SBP, systolic blood pressure;
SD, standard deviation;
TonEBP, tonicity-enhancer binding protein;
VEGF-C, vascular endothelial growth factor-C;
WW, wet weight.

Introduction

Arterial hypertension (HT) is a major cause of mortality and disability worldwide. Na^+ is the most important electrolyte for maintaining effective circulating volume and for causing HT. According to classical physiology, Na^+ is held to be present almost exclusively in the extracellular space where it osmotically equilibrates with water. The kidney is believed to be mainly responsible for matching Na^+ excretion with Na^+ intake, resulting in an almost perfect equilibrium during constant Na^+ intake. Hence, it is considered to be the primary regulator of Na^+ and BP[1]. Recent well-controlled Na^+ balance studies, in which total body Na^+ varied by as much as 200 mmol during fixed Na^+ intake[2], have however shown that Na^+ can accumulate in the human body without concurrent water retention[2,3]. Surprisingly, the observed variation in total body Na^+ did not induce any changes in body weight or BP[2], suggesting that there is an additional compartment able to store excessive Na^+ without volume effects. In the last decade, the skin has emerged as a new player in the control of BP through local homeostasis of Na^+, but neither the mechanisms underlying excess extracellular interstitium skin-Na^+ deposition nor those by which it may affect the activity and signaling of target cells are currently known[4–6].

Recent animal experiments suggest that large amounts of Na^+ are non-osmotically stored, bound to negatively charged glycosaminoglycans (GAGs), which are abundantly present in the skin[7]. Moreover, following a high salt diet, the mononuclear phagocyte immune cells, accumulating as macrophages in the skin, have been implicated in the regulation of skin salt homeostasis and BP[8]. Elegant experiments in mice showed that the local hypertonicity induced by Na^+-storage in the skin leads to immune cell-driven induction of local tissue electrolyte clearance, via modulation of cutaneous lymph capillary density. This response initiates with the activation of the tonicity-enhancer binding protein (TonEBP), a transcription factor activated by osmotic stress[9]. In macrophages entering Na^+-skin storage sites, TonEBP binds to the promoter of vascular endothelial growth factor-C (VEGF-C), resulting in increased VEGF-C secretion into the skin interstitium. As a result, lymph capillary density increases, and skin electrolyte clearance improves. Moreover, increased Na^+ levels induce expression of pro-inflammatory genes while inhibit that of anti-inflammatory genes in macrophages [8]. Recent evidences suggest that high-Na^+ TonEBP-dependent activation occurs via intracellular cytosolic Ca^{2+} elevation-mediated MAPK pathway activation, pointing to a role of the intracellular Ca^{2+} second messenger in this biological phenomenon[10].

To date only scant studies in humans where the Na^+-skin content has been quantified indirectly by ^{23}Na-magnetic resonance imaging (MRI) exist. They suggested that Na^+ accumulation in skin and muscle is associated with ageing and HT[11,12], particularly in patients with poorly controlled BP or primary aldosteronism (PA), where skin-Na^+ accumulation was reversed after treatment[13]. However,

these findings were obtained: i) with a tiny amount of PA cases (n=5) that were not phenotypically well-characterized; ii) by ^{23}Na-MRI which, despite being validated against tissue chemical analysis, lacks its sensitivity and precision in the quantification of water and electrolytes. More robust evidences toward a potential mechanistic role for Na^+ accumulation in cardiovascular diseases were recently offered by Schneider et al., who showed that skin-Na^+ content is an even stronger predictor of left ventricular mass than systolic BP or whole body hydration status in patients with chronic kidney disease[14]. On the whole, these findings support the idea that skin-Na^+ control by lymph capillaries is relevant for BP control, suggesting the importance of local clearance mechanisms for electrolyte homeostasis.

Based on these premises, this study aimed to investigate if skin electrolytes and water accumulation occurs in human PA, the main curable cause of human salt-sensitive endocrine hypertension, which can be a suitable model to investigate the changes in skin-Na^+ content in relation to aldosteronism and its surgical correction. Most relevant to this study is the compelling evidence that skin-Na^+ storage and tissue immune cell response could play an essential role in pathogenesis of BP and subsequently in HT-related target organ damage.

Aim of the study

The general hypothesis was that extracellular Na^+-storage in tissues is mechanistically related to the development of HT in PA, a model of human HT where Na^+ retention and blood volume expansion play a key role. By proving this hypothesis and elucidating how salt in the interstitium is connected to the vasculature and impact on organ damage, we expect to provide instrumental insight for developing novel treatment modalities for salt-sensitive HT. We elected to use the skin, rather than other tissues, as experimental system because is the simplest model to study the extracellular electrolytes content.

In summary the following hypotheses were assessed in this study:

1) The skin-Na^+ content is higher in PA patients than in comparable groups of essential hypertensive patients and normotensive subjects.

2) Adrenalectomy in PA and ensuing cure of PA correct the skin-Na^+ accumulation.

3) Exploration of the molecular mechanism thought which Na^+ is stored in the skin due to aldosterone hyperproduction, in PA patients in comparison with a group of essential hypertensive patients and a control group of normotensive patients.

Specific aim 1: to confirm that skin-Na^+ is higher in PA patients (PA Group) compared to essential hypertensive patients (EH Group) and normotensive patients (Control Group), a skin biopsy was

performed immediately before adrenalectomy in consecutive patients with confirmed and lateralized PA, who according to current guidelines have the indication to adrenalectomy; a skin biopsy was also obtained in essential hypertensive and normotensive patients undergoing surgery for benign diseases.
Specific aim 2: to verify if adrenalectomy improves skin-Na^+ clearance in PA Group patients, a skin biopsy immediately before adrenalectomy and one month after adrenalectomy was performed. Moreover, to compare skin-Na^+ between bilateral versus unilateral form of PA, a skin biopsy was also obtained during adrenal vein sampling (AVS) without interfering drugs.
Specific aim 3: To investigate the molecular mechanisms driven by skin-Na^+ undergoing the crosstalk between macrophages and lymphatic vessels in the interstitium in PA patients, in normotensive patients and in patients with essential hypertensive undergoing surgery for benign diseases. The expression level of TonEBP and the vascular endothelium grow factor C VEGF-C, which are two fundamental players in the regulation of the interstitial electrolyte homeostasis, will be evaluated in primary skin fibroblasts obtained from patients PA patients, normotensive subjects and essential hypertensive patients. In detail, we will directly evaluate TonEBP/VEGF-C expression level both by real-time quantitative PCR and by Western blot (WB) analysis. We will also perform biochemical analysis of intracellular pathways, focusing on the activation of MAPK cascade, which are at the upstream level in the activation of TonEBP. We also checked the expression level of the ion channels/pumps of the plasma membrane (PM), with a particular interest in the PM Na+/Ca2+ exchanger (NCX), by real-time quantitative PCR, WB analysis and immunofluorescence. We will monitor the intracellular Ca2+ signaling using the ratiometric fluorescent dye Fura-2 and genetically encoded probe (Cytosolic-targetted Aequorin), to determine how high skin-Na^+ content can alter the ion balance of the plasma membrane.

Methods

Study design

The study protocol was submitted to the local Institution review Board and received a first approval. Written informed consent was obtained from all the participants.
The research process is represented as flowchart in **Figure 1.**

Patient recruitment

1. PA patients (**PA Group**) submitted to surgery, selected at the local PI's Institution (Hypertension Unit, an ESH Center of Excellence and a tertiary referral Center for secondary HT and, particularly, for PA).

2. Essential HT patients (**EH Group**), selected from those where a secondary HT was excluded by

biochemical/radiological /clinical findings and need surgery for benign diseases.

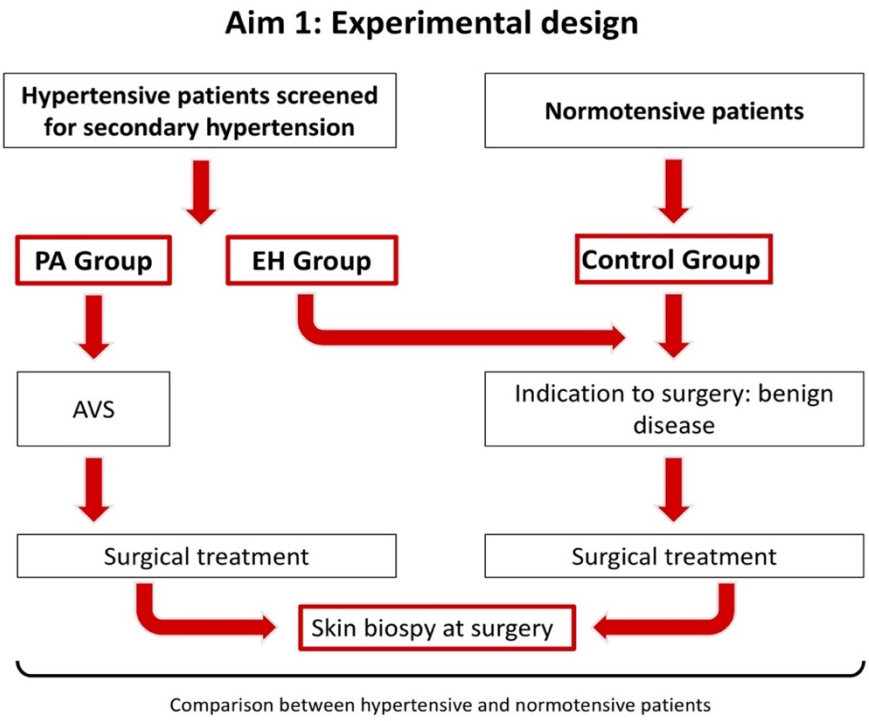
3. Normotensive patients (**Control Group**), selected from patients that need surgery for benign diseases. Written informed consent was obtained from all participants.

Inclusion criteria

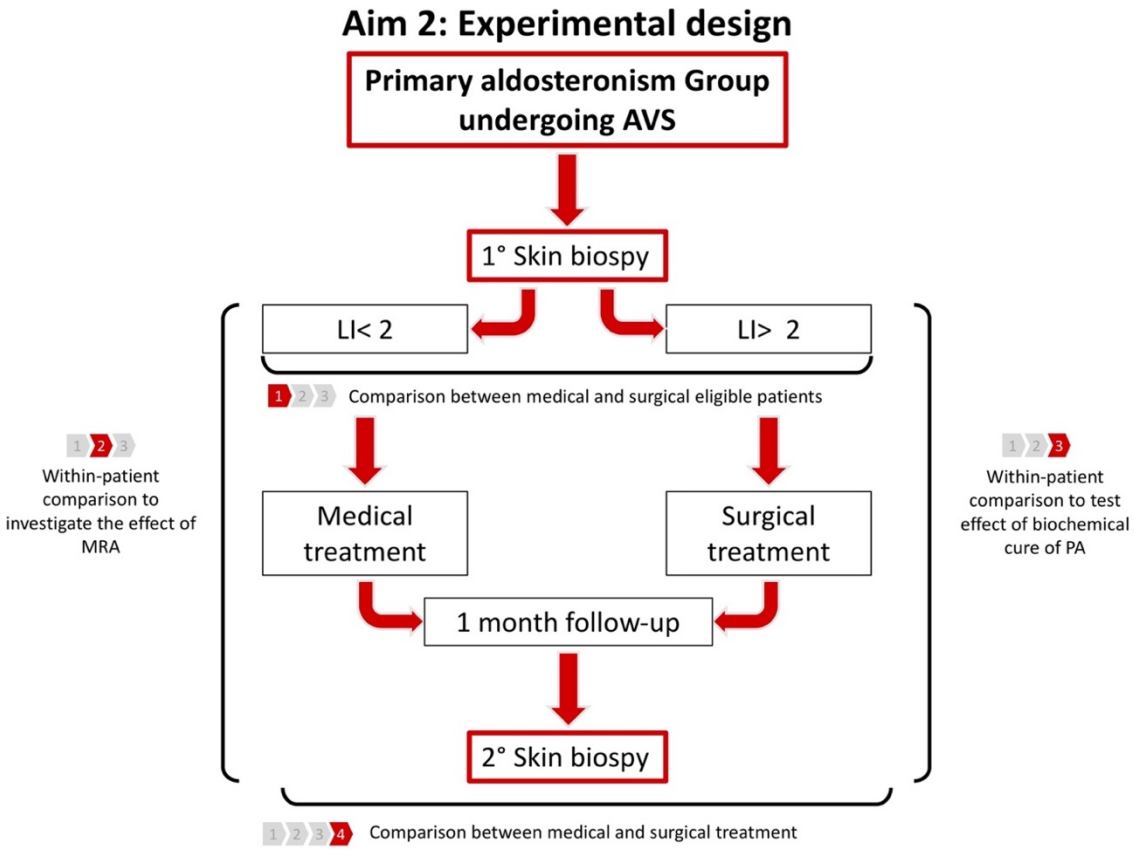
PA Group

- Age: 18 to 75 years old;
- Signed informed consent form;
- Diagnosis of PA defined as:
  - ✓ Plasma aldosterone concentration greater than 15 ng/dL and aldosterone/renin ratio greater than 2.06 ng/dL:mIU/L, measured after washout of interfering drugs or after changes of the drug treatment as previously detailed.

Figure 1. Research process flowchart (Aims 1 and 2).



***PA**=primary aldosteronism; **EH**=essential hypertension; **Control**=normotensive control group; **AVS**=adrenal vein sampling.*



***PA**=primary aldosteronism; **EH**=essential hypertension; **Control**=normotensive control group; **LI**=lateralization index; **AVS**=adrenal vein sampling; **MRA**=mineralocorticoid receptor antagonists.*

EH Group

- Age: 18 to 75 years old;
- Signed and dated informed consent form;
- A diagnosis of essential hypertension defined as:
  - ✓ Use of antihypertensive drug (s)
  - ✓ Arterial hypertension: in untreated patients confirmed by daytime ambulatory blood pressure monitoring (ABPM), or home blood pressure monitoring, with blood pressure higher or equal to 140 mmHg for systolic blood pressure and/or higher or equal to 90 mmHg for diastolic blood pressure.
  - ✓ Exclusion of secondary hypertension by hormonal biochemical screening (aldosterone, renin, ARR<2.06 ng/dL:mIU/L, ACTH, 24h urine cortisol, morning plasma cortisol level, 24h urine metanephrines).

Control Group

- Age: 18 to 75 years old;
- Signed informed consent form;

- Normal arterial blood pressure defined either as:
  - ✓ None antihypertensive drug (s)
  - ✓ Normal arterial blood pressure confirmed by daytime ambulatory blood pressure monitoring (ABPM), or home blood pressure monitoring, with blood pressure lower or equal to 135 mmHg for systolic blood pressure and/or lower or equal to 85 mmHg for diastolic blood pressure.
  - ✓ Exclusion of hormonal hypersecretion by biochemical screening (aldosterone, renin, ACTH, 24h urine cortisol, morning plasma cortisol level, 24h urine metanephrines and catecholamines).

Exclusion criteria

- history of allergy/intolerance to local anesthesia;
- personal history of malignant neoplasm;
- refusal of the patient to undergo dynamic testing;
- refusal of the patient to undergo AVS and/or contraindications to the general anesthesia that is required for laparoscopic adrenalectomy in PA Group;
- cortisol–aldosterone co-secreting adenoma or pheochromocytoma.

Skin biopsy collection

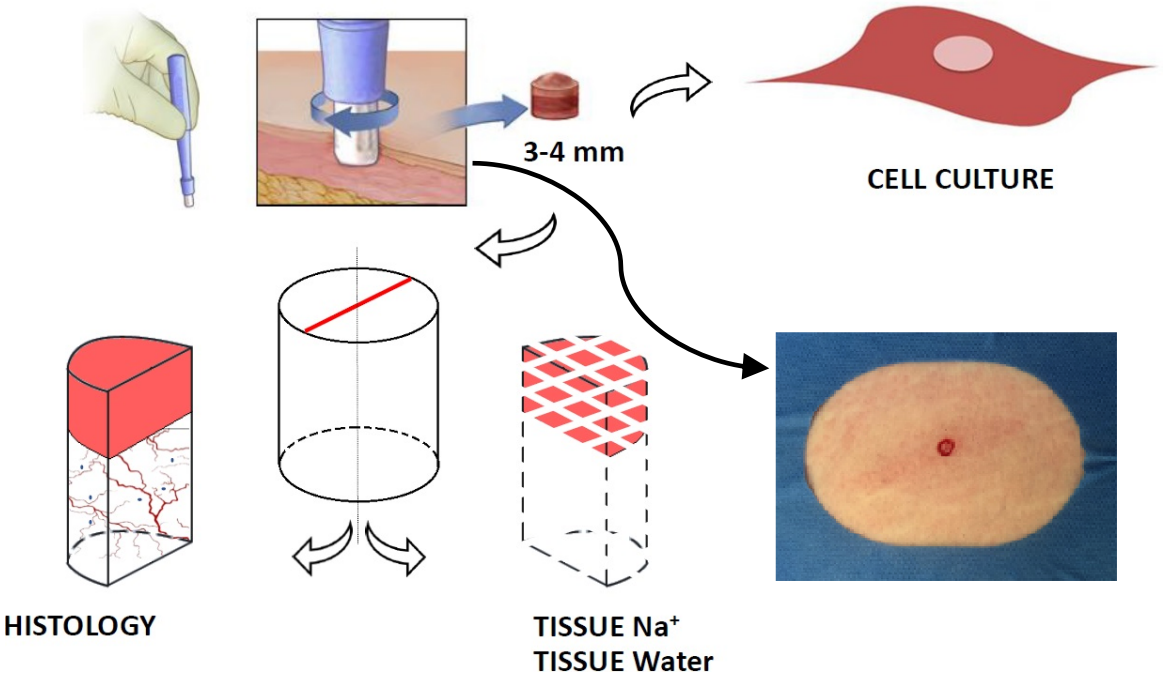
Aim 1. In informed consenting patients undergoing AVS-guided adrenalectomy for lateralized PA (PA Group), a skin biopsy was performed at the site of the surgical wound after adequate preparation to insure sterile conditions. The same procedure has been performed in EH Group and Control Group patients during surgery for benign diseases, as elective colecistectomy, hernia, abdominal subcutaneous lipoma. The surgical site was prepared, re-cleaned with alcohol swabs and draped. The epidermis and superficial dermis have been pierced by a sterile punch, the punch removed, and hemostasis obtained with sterile gauze. The biopsy was placed in a sample pot for bench preparation and immediate frozen in liquid nitrogen (**Figure 2**). The biopsy site was closed with steri-strips. Wound care information sheet and materials were provided to the participant.

Aim 2. In informed consenting PA patients, a skin biopsy was collected during AVS and one month after adrenalectomy with the same procedure described above. The skin biopsy obtained during adrenalectomy was within-patient compared to that collected one-month after. The gluteus was the preferred site in all recruited patients. The area of skin where the biopsy has been performed was anesthetized by a Lidocaine cream without Na^+ as excipient (LMX4 cream, Lidocain 4%). The biopsy was performed with the participant lying prone or in the lateral position.

Skin sample analysis for water and electrolytes content

All frozen skin samples were weighted to determine total (WET) weight and then desiccated at 90°C for 72 hours to determine the DRY weight. We found in preliminary experiments that after this period the weights remained unchanged with further drying. The difference between total WET and DRY weight estimated the tissue water content. The measurement of Na^+ and K^+ in the skin was carried out as follows. Homogenates of the dry skin were suspended overnight in 1N nitric acid to displace free Na^+ and K^+. The supernatant was then diluted as needed to get in the working range of concentrations of the atomic adsorption spectrophotometer (SpectrAA 50/55 Varian, Agilent) and read using the lamp and wavelength appropriate for each element.

Figure 2. Skin punch biopsy. Representative image of a gluteal skin punch biopsy performed in a patient one-month after AVS guided adrenalectomy for PA.



Immunohistochemical analysis of the skin tissue.

Human skin biopsies obtained from patients with dermopunch were immediately frozen in liquid nitrogen. Successively, freshly cryosections, 14µm thick, were fixed in PFA 4% for 20 min in the dark. Then, cryosections were blocked in PBS containing 2% goat serum and 5% bovine serum albumin (BSA) for two hours at room temperature and incubated in the same solution with primary antibodies in a overnight at 4°C. After three PBS washes, sections were incubated with

AlexaFluor488- or AlexaFluor555-conjugated secondary antibodies (Life Technologies) for 1 hour at room temperature. Hoechst (Sigma Aldrich) was used to stain nuclei. Images were acquired with Leica SP5 confocal microscope.

Statistical analysis

Results were expressed as mean ± SD, or median and interquartile range, as appropriate. Continuous variables were tested for normal distribution with Kolmogorov-Smirnov test. Parametric and non-parametric statistics were used for log-transformed data and variable with a skewed distribution, respectively. Within-patient comparison of paired t-samples test was used for PA patients before and after surgery; Pearson's χ^2 test was used for categorical variables.

Significance was set at $p < 0.05$. Since several comparisons was undertaken for Aim 1, the post-hoc Scheffè test was used to warrant against false positive findings.

Specific licensed softwares, including GraphPad (vers 8.1.2) and SPSS (for Mac vers 25) were used for these tasks.

Sample size calculation. For Aim 1 using a specific software (R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.), we have calculated that a sample size of 6 patients in each group had 98% power to detect a difference in means of 7 nmol/L of Na^+ (the difference between a PA Group mean, μ1, of 26 nmol/L and a Control Group mean, μ2, of 19 nmol/L) assuming that the common standard deviation is 3 nmol/L using a two group t-test with a 0.05 two-sided significance level. With 300 consecutive hypertensive patients we expect to have 42 cases of PA (14% prevalence) and given the rate of surgically curable cases identified at our center, we expect to have at least about 15 cases of PA.

For Aim 2 and 3, a sample size of 6 had more than 99.84% power to detect a difference in means of 6 nmol/L of Na^+ (the difference between PA preoperative skin Na^+ content mean, μ1, of 26 nmol/L and PA postoperative skin Na^+ content mean, μ2, of 20 nmol/L) assuming that the common standard deviation is 3 nmol/L using a paired two group t-test at a 0.05 two-sided significance level.

Results

1. Characteristics of the study population

1.1 Demographics, clinical and biochemical features of the study cohort.

The clinical and biochemical characteristics of the patients are summarized in **Table 1**. Thus far, 21 patients affected by PA, 8 controls normotensive and 6 EH patients were recruited for the study. Of

the 21 PA patients, 19 had unilateral aldosterone excess and were treated by AVS-guided unilateral adrenalectomy; the remaining two patients had a bilateral aldosterone hypersecretion.

The three groups of patients were well-matched for sex, age and BMI ($p>0.05$). Systolic and diastolic blood pressure was significantly higher in PA patients compared to EH patients and controls ($p<0.05$). No significant differences were observed between groups regarding renal function, preoperative serum Na^+ and K^+ levels, plasma renin activity (PRA) and direct renin concentration (DRC). Conversely, aldosterone to renin ratio was significantly inappropriately higher in patients with PA compared to EH patients and controls ($p=10^{-4}$).

1.2 Skin sodium, potassium and water content

Figure 3 shows the raw and Log-transformed distribution of skin Na^+ **(a)**, water **(b)** and K^+ **(c)** content.

In an exploratory analysis, we investigated whether PA patients had salt and fluid accumulation in the skin compared to normotensive (Controls) or essential hypertensive (EH) subjects (**Figure 4** and **5**).

Skin water content assessed by drying until the weight stabilized was not different between the three groups (**Figure 4**; $p>0.05$ for all groups). There was, however, a significant increase in Na^+ content relative to dry weight (**Figure 5a**) as well as Na^+ content relative to water (**Figure 5c**) in PA patients compared to normotensive control patients ($p=0.04$ and $p=0.02$, respectively), in line with previous experiments in rodents fed with high salt diet or DOCA-treated[4–7]. No significant differences in Na^+ content relative to dry weight or water content were observed between PA patients and EH patients or between EH patients and controls ($p>0.05$).

Regarding skin K^+, no significant differences have been observed between the three groups (**Figure 5b** and **5d**; $p>0.05$).

Table 1. Clinical and biochemical characteristics of the recruited patients.

***PA**= primary aldosteronism; **EH**=essential hypertensive group; **Controls**=normotensive group; **NA**=not available. Data reported as percentage, mean (±SD) or median (IQR) as appropriate. Statistical analysis between groups was performed by ANOVA followed by Sheffe's post-hoc test or Kruskal-Wallis non-parametric test, and Pearson X^2 for qualitative variables.*

Variables	**PA (n=21)**	**p-Value (PA vs EH)**	**EH (n=6)**	**p-Value (EH vs controls)**	**Controls (n=8)**	**p-Value (PA vs controls)**
Age (years)	54 ± 8	>0.05	57 ± 9	>0.05	46 ± 12	>0.05
Sex (Male, %)	57%	>0.05	83.3%	>0.05	33.3%	>0.05
BMI (Kg/m^2)	26 (9)	>0.05	25.5 (6)	>0.05	25.5 (5)	>0.05
Office SBP (mmHg)	161±18	**=3x10^{-3}**	135 ± 10	>0.05	115 ±6	**<10^{-4}**
Office DBP (mmHg)	99 ± 18	=0.05	82 ± 8	>0.05	70 ± 8	**<10^{-4}**
Heart rate (bpm)	76 ± 10	>0.05	71 ± 8	0.64	66 ± 11	0.62
PRA (ng/mL/h)	0.25 (0.44)	>0.05	1.16 (1.21)	>0.05	0.64 (-)	>0.05
DRC (mIU/L)	2.1 (3)	>0.05	9.5 (9.8)	>0.05	2 (-)	>0.05
PAC (ng/dL)	296 (283)	**=2x10^{-2}**	45 (35)	>0.05	52 (-)	**=3x10^{-3}**
ARR ([ng/dL]*[(ng/mL/h)$^{-1}$])	115 (182)	**=3x10^{-3}**	5 (25)	**=3x10^{-3}**	20 (-)	**=3x10^{-3}**
serum Na$^+$ (mmol/L) at screening	141 ± 2	-	NA	-	NA	-
serum K$^+$ (mmol/L) at screening	3.2 ± 0.68	-	NA	-	NA	-
serum Na$^+$ (mmol/L) preoperatively	142 ± 2.6	>0.05	141 ± 1.7	>0.05	141 ± 2.2	>0.05
serum K$^+$ (mmol/L) preoperatively	4.1 ± 0.5	>0.05	4.3 ± 0.4	>0.05	4.1 ± 0.2	>0.05
24h urinary Na$^+$ excretion (mmol/24h)	140 ± 63	-	NA	-	NA	-
24h urinary K$^+$ excretion (mmol/24h)	86 ± 42	-	NA	-	NA	-
eGFR (mL/min/1.73 m^2)	82 ± 18	>0.05	87 ± 9	>0.05	97 ± 12	>0.05

Figure 3. Raw and Log-transformed distribution of skin sodium (a), water (b) and potassium content (c).

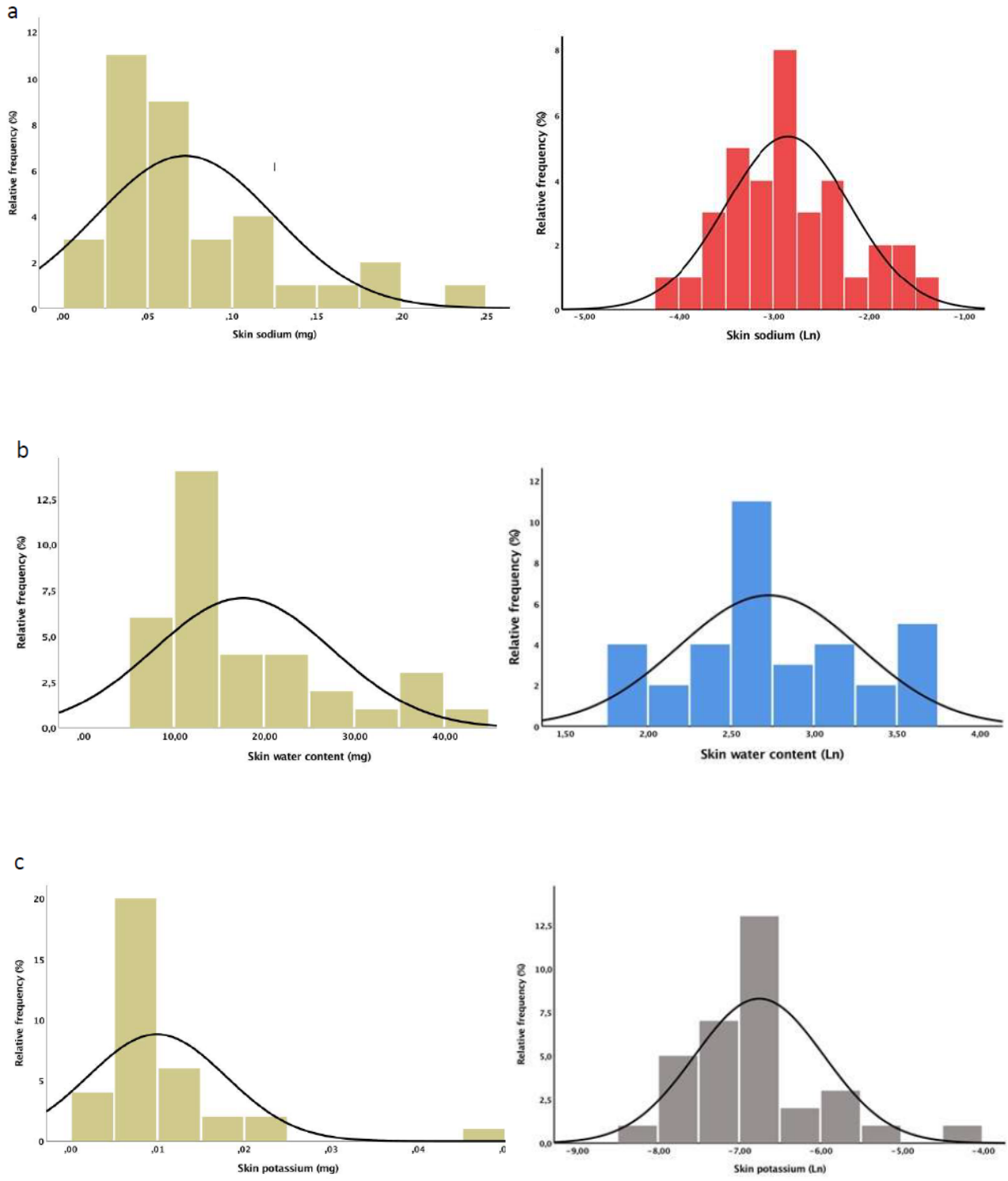
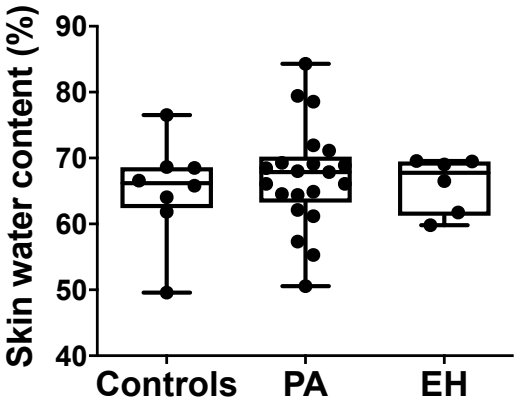
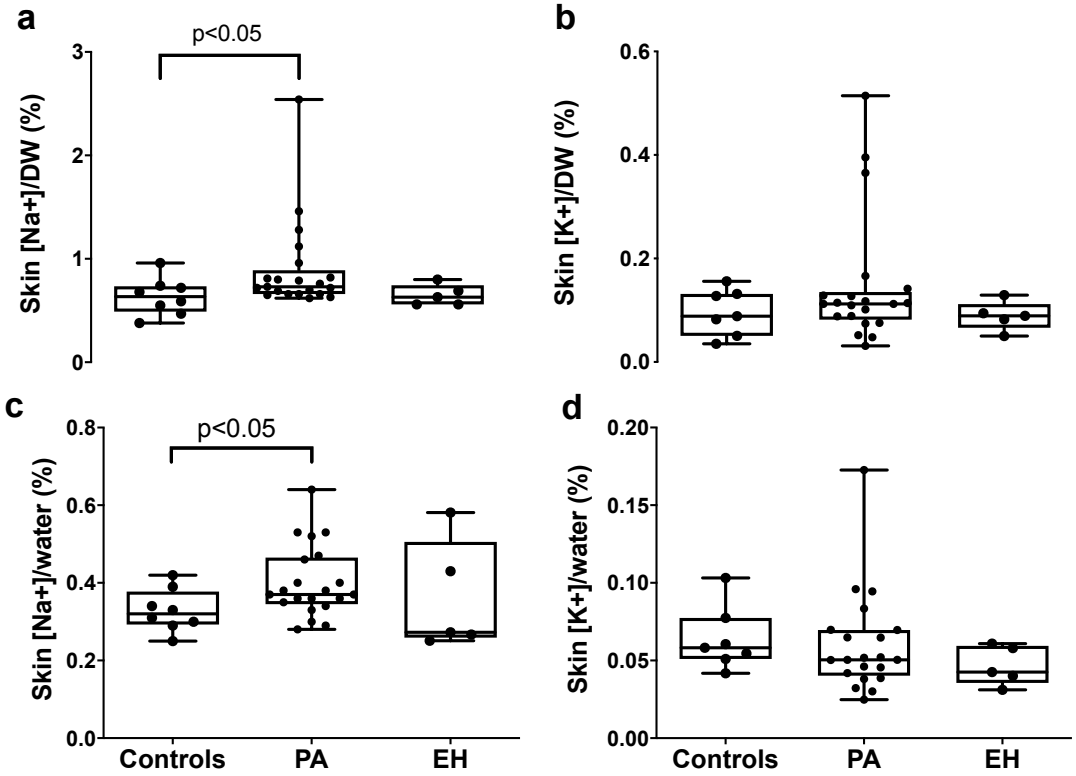


Figure 4. Skin water content in primary aldosteronism patients (n=21), essential hypertensive patients (n=6), and normotensive patients (n=8).



Individual values of skin water content expressed as percentage relative to wet weight.
***Controls**=normotensive control patients; **PA**=primary aldosteronism patients; **EH**=essential hypertensive patients.*
Statistical analysis was performed on log-transformed data by unpaired t-test.

Figure 5. Skin-Na^+ and K^+ content in primary aldosteronism patients (n=21), essential hypertensive patients (n=6), and normotensive patients (n=8).



a,b *Individual values of Skin-Na^+ and K^+ content expressed as percentage relative to dry weight (DW)* **c,d** *Individual values of skin Na^+ and K^+ content relative to skin water.*
Statistical analysis was performed on log-transformed data by unpaired t-test.
***Controls**=normotensive control patients; **PA**=primary aldosteronism patients; **EH**=essential hypertensive patients.*

2. Characteristics of the PA group

2.1 Clinical and biochemical characteristics of the recruited unilateral PA

Nineteen PA patients had a unilateral aldosterone hypersecretion and therefore underwent AVS-guided unilateral adrenalectomy. All nineteen PA patients undergoing unilateral adrenalectomy fulfilled the "five corners" criteria[15], as shown in **Table 2**.

Table 2. Clinical and biochemical characteristics of the recruited PA patients undergoing surgery, at baseline and after unilateral adrenalectomy.

Variables	At baseline	After adrenalectomy	*p-Value*
Office SBP (mmHg)	159 ±17	140 ± 13	$=8x10^{-3}$
Office DBP (mmHg)	98 ± 22	89 ± 10	0.25
Heart rate (bpm)	76 ± 9	73 ± 13	0.56
Adrenal adenoma size (mm)	18.6 ± 7.4	-	
AVS LI (lateralization index)	38 ± 43	-	
PRA (ng/mL/h)	0.25 (0.44)	0.55 (1.68)	$15x10^{-3}$
DRC (mIU/L)	2.1 (4.80)	4.5 (11.20)	$23x10^{-3}$
PAC (ng/dL)	296 (283)	33 (43)	$=10^{-3}$
ARR ([ng/dL]*[(ng/mL/h)$^{-1}$])	115 (182.5)	10 (10)	$=10^{-3}$
serum Na^+ (mmol/L) at screening	141 ± 2	140 ± 2	0.27
serum K^+ (mmol/L) at screening	3.3 ± 0.6	4.5 ± 0.4	$<10^{-4}$
serum Na^+ (mmol/L) pre vs post surgery	142 ± 1.8	140 ± 2	$2x10^{-2}$
serum K^+ (mmol/L) pre vs post surgery	4.1 ± 0.6	4.5 ± 0.4	$6x10^{-2}$
24h urinary Na^+ excretion (mmol/24h)	147 ± 61	155 ± 53	$=10^{-3}$
24h urinary K^+ excretion (mmol/24h)	89 ± 50	70 ± 34	0.89
eGFR (mL/min/1.73 m^2)	82 ± 21	67 ± 18	$7x10^{-3}$

Data reported as percentage, mean (±SD) or median (IQR) as appropriate. P refers to within-patient comparison (paired-t test or Wilcoxon sign-rank test, as appropriate).

As expected, BP markedly fell from 159/98 ± 17/22 mmHg at baseline to 140/89 ± 13/10 mmHg one-month after adrenalectomy ($p=8x10^{-3}$ for systolic blood pressure), as well as PRA, DRC, PAC and ARR ($p<0.05$). Serum potassium significantly improved after adrenalectomy from 3.3 ± 0.6 mmol/L to 4.5 ± 0.4 mmol/L ($p<10^{-4}$). 24h-urinary sodium excretion significantly increased after adrenalectomy ($p=10^{-3}$) while 24-h urinary potassium excretion did not differ significantly after adrenalectomy (p=0.89). Glomerular

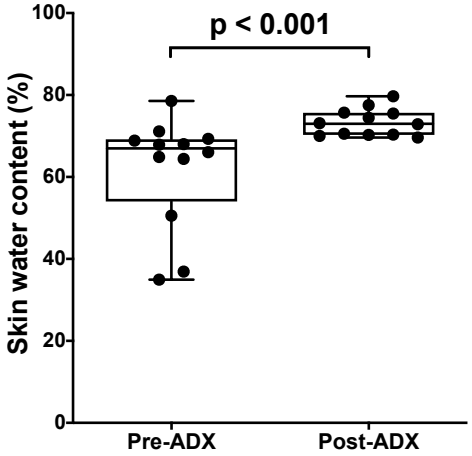
filtration rate significantly decreased after adrenalectomy from 82 ± 21 mL/min/1.73 m2 to 67 ± 18 mL/min/1.73 m2 ($p=7x10^{-3}$).

2.2 Changing in skin sodium, potassium and water after unilateral adrenalectomy

To verify if adrenalectomy might affect skin electrolytes and water content, we compared skin-Na^+, K^+ and water content of unilateral PA patients at baseline and one-month after AVS-guided unilateral adrenalectomy (**Figure 6** and **7**). Interestingly, we have observed that water content increased significantly one month after adrenalectomy ($p<10^{-4}$; **Figure 6**) without any changes in skin Na+ content relative to dry weight (p=0.4; **Figure 7a**). However, as expected, there was a significant decreased of skin Na^+ content relative to water content ($p=9x10^{-3}$; **Figure 7c**).

Regarding skin-K^+ content, it has been observed a significant increase of skin-K^+ content relative to dry weight (**Figure 7b**) and water content (**Figure 7d**) one-month after adrenalectomy ($p=4x10^{-2}$).

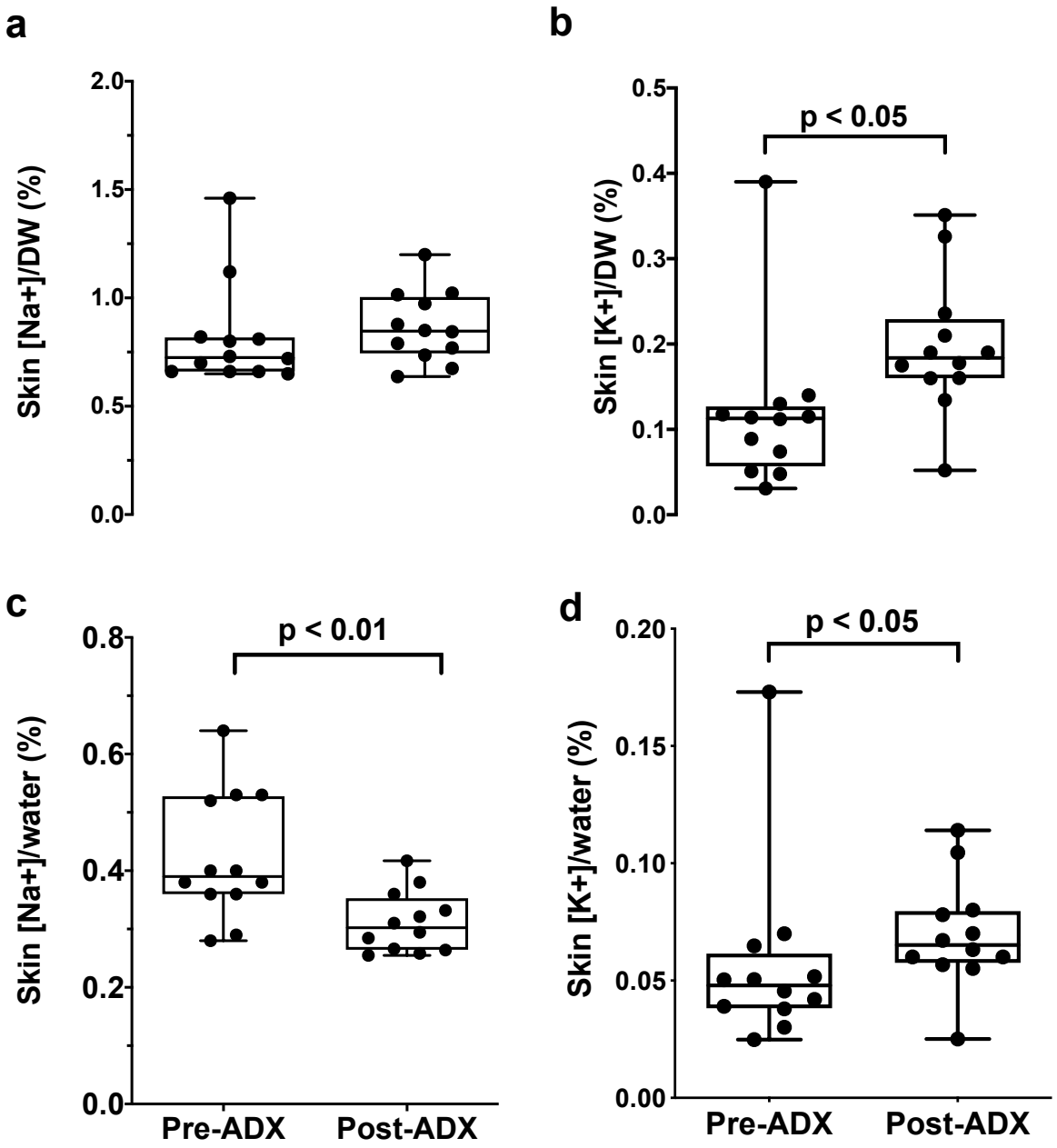
Figure 6. Skin water content in unilateral PA patients (n=12) before and one-month after unilateral adrenalectomy.



Individual values of skin water content expressed as percentage relative to wet weight, before adrenalectomy (**pre-ADX**) *and one-month after* (**post-ADX**).

Statistical analysis was performed on log-transformed data by paired t-test.

Figure 7. Skin-Na^+ and K^+ accumulation in unilateral PA patients (n=12) before and one-month after unilateral adrenalectomy.

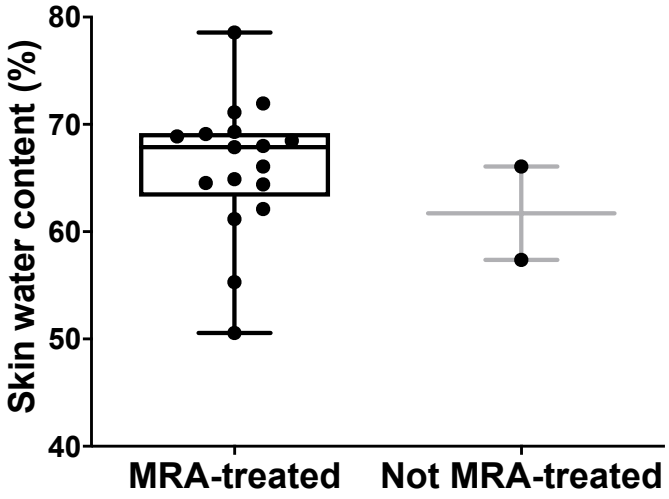


a,b *Individual values of Skin-Na^+ and K^+ content expressed as percentage relative to dry weight (DW)* **c,d** *Individual values of skin Na^+ and K^+ content relative to skin water.*
Statistical analysis was performed on log-transformed data by paired t-test.
***Pre-ADX**=before adrenalectomy; **post-ADX**=one-month after adrenalectomy.*

2.3 Skin sodium, potassium and water in unilateral PA depending on MRA treatment

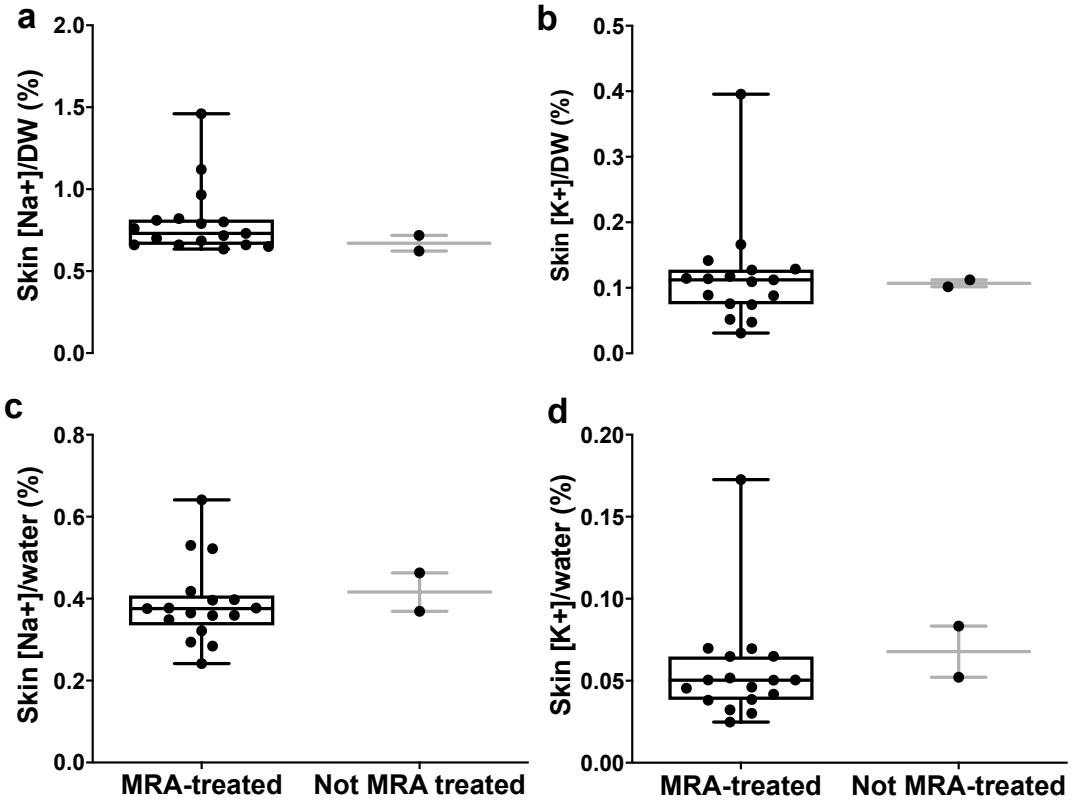
Seventeen unilateral PA patients were on mineralocorticoid receptor antagonist (MRA) treatment at the time of baseline skin biopsy. The remaining 2 unilateral PA patients were in wash-out of MRA treatment from at least 4 weeks before skin biopsy. Skin water content did not differ significantly between MRA treated vs non-MR treated (**Figure 8**; p=0.1). Moreover, no significant difference was observed between the two groups in terms of Na^+ content relative to dry weight (**Figure 9a**) as well as Na^+ content relative to water (**Figure 9c**) in PA patients treated with MRA vs non treated (p=0.29 and p=0.84, respectively). No significant differences in terms of K^+ skin content relative to dry weight or water content were observed (**Figure 9b** and **9d** p>0.05).

Figure 8. Skin water content in unilateral primary aldosteronism patients treated with mineralocorticoid receptor antagonists (n=17) or in absence of mineralocorticoid receptor antagonists treatment (n=2).



*Individual values of skin water content expressed as percentage relative to wet weight, in patients with primary aldosteronism treated with mineralocorticoid-receptor antagonists (**MRA-treated**) or without MRA treatment (**Not MRA-treated**).*
Statistical analysis was performed on log-transformed data by unpaired t-test.

Figure 9. Skin-Na^+ and K^+ accumulation in unilateral primary aldosteronism patients treated with mineralocorticoid receptor antagonists (n=17) or in absence of treatment (n=2).



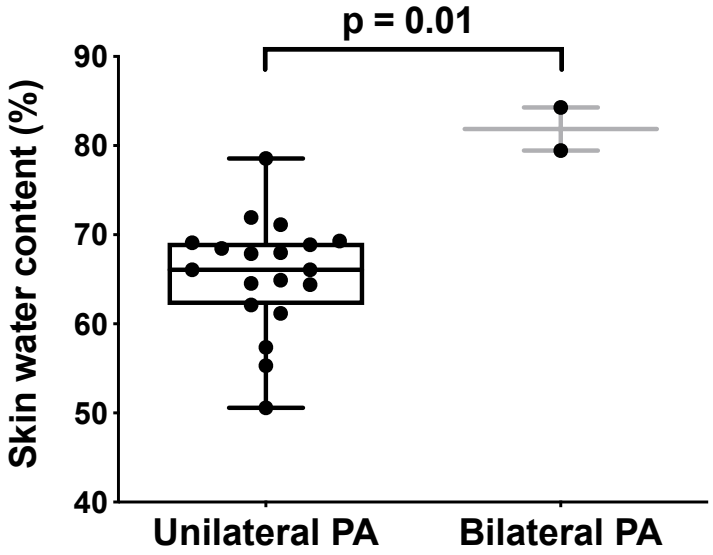
a,b *Individual values of Skin-Na^+ and K^+ content expressed as percentage relative to dry weight (DW)* **c,d** *Individual values of skin Na^+ and K^+ content relative to skin water.*
Statistical analysis was performed on log-transformed data by unpaired t-test.
***MRA-treated**= treated with mineralocorticoid receptor antagonists;* ***Not MRA-treated**= absence of mineralocorticoid receptor antagonist treatment.*

2.3 Changing in skin sodium, potassium and water in unilateral PA vs bilateral PA

We explored whether PA patients with unilateral hypersecretion of aldosterone differ in terms of skin electrolytes and water content from those who had a bilateral hypersecretion. The biopsies were performed in bilateral PA at the time of AVS and in wash-out of interfering drugs while biopsies in unilateral PA were obtained before surgery and on MRA treatment.

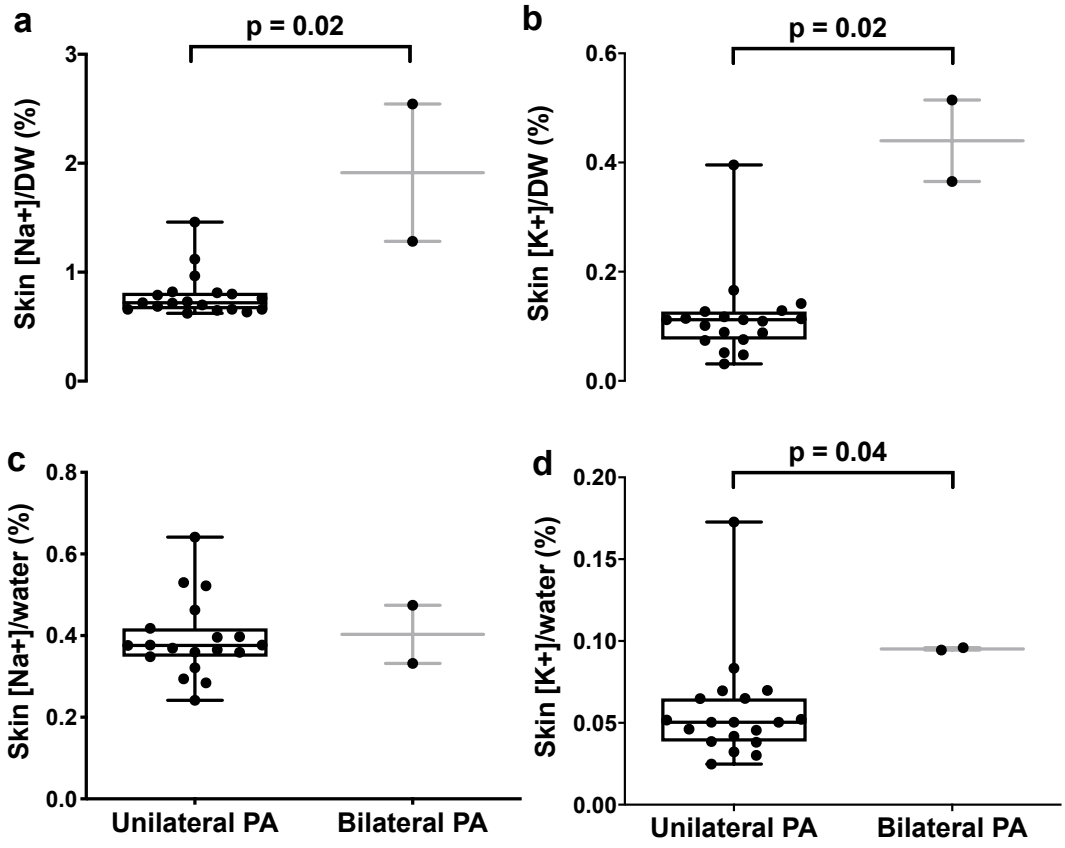
Figures 10 and **11** showed the results of skin Na^+, K^+ and water in the two groups. Skin water content and skin-Na^+ content relative to DW were significantly different between the two groups (p=0.01 and p=0.02, respectively) even if skin-Na^+ content relative to water content did not differ (p=0.9). Skin K^+ content relative both to dry weight or water content was significantly higher in bilateral PA compared to unilateral PA (p=0.02 and p=0.04, respectively).

Figure 10. Skin water content in unilateral (n=19) vs bilateral primary aldosteronism (n=2).



*Individual values of skin water content expressed as percentage relative to wet weight, in patients with unilateral primary aldosteronism (**Unilateral PA**) or bilateral primary aldosteronism (**Bilateral PA**). Statistical analysis was performed on log-transformed data by unpaired t-test.*

Figure 11. Skin-Na^+ and K^+ content in unilateral (n=19) and bilateral primary aldosteronism (n=2).



a,b *Individual values of Skin-Na^+ and K^+ content expressed as percentage relative to dry weight (DW)* **c,d** *Individual values of skin Na^+ and K^+ content relative to skin water.*
Statistical analysis was performed on log-transformed data by unpaired t-test.
Unilateral PA*=unilateral primary aldosteronism;* ***Bilateral PA****= bilateral primary aldosteronism.*

Discussion

This is the first study, to the best of our knowledge, to directly measure by chemical analysis skin-Na^+, K^+ and water accumulation in humans affected by PA. We investigated a sizable group of PA (n=21); 19 of whom had unilateral PA and therefore were cured after AVS-guided adrenalectomy. Our data showed that Na^+ is stored in the skin of PA patients without apparent accompanying water retention, thus indicating the concept that a certain amount of Na^+ is osmotically inactive and implying that tissue-specific regulatory mechanisms might control the release and storage of Na^+ from a kidney-independent reservoir. Interestingly, in the few PA patients who had a bilateral aldosterone hypersecretion, skin-

Na^+ content seems to be even higher, although this finding needs to be further investigated in a larger cohort.

Using ^{23}Na-MRI technique, Titze and coauthors have previously suggested that Na^+-skin would accumulate in muscle and skin in 5 patients affected by PA[13]. They observed that PA patients had higher levels of Na^+ in muscle and that Na^+ decreased significantly four weeks after adrenalectomy, although they observed little or no effect on body weight after medical or surgical treatment of PA. A similar raise of Na^+ content was seen experimentally with mineralocorticoid receptor activation in deoxycorticosterone acetate (DOCA) salt-treated rats, indicating that skeletal muscle is a relevant sodium reservoir in this experimental model of secondary hypertension[16].

Since non-invasive ^{23}Na-MRI has scant resolution in estimating Na^+-skin content, it cannot provide precise quantitative measurements of tissue electrolytes content. In addition, ^{23}Na coils are expensive and not widely available thus precluding a diffusion of this technique to measure Na^+ noninvasively and repeatedly[11–13].

Thus, to verify if the skin-Na^+ storage is increased in humans affected by PA and is corrected by cure of PA, chemical-physical analysis of skin water and electrolytes was necessary. We set up a protocol to recruit consecutive PA patients and to measure Na^+, K^+ and water content in skin biopsies from abdomen and/or gluteus obtained by a biopsy-punch. Chemical analysis of tissue electrolytes and water content was performed as described in previous animal studies[4].

Of much interest, our present results showed that the Na^+ accumulation in the skin was reversible after curative unilateral adrenalectomy (**Figure 12**). The limited data in those PA of ours with bilateral disease, who underwent medical treatment, suggested that the Na^+ skin content remained high at follow-up. This was not in line with previous analysis of tissue sodium content performed by 23Na-MRI in patients with refractory essential hypertension showing that patients with spironolactone treatment had significantly reduced muscle sodium content[11].

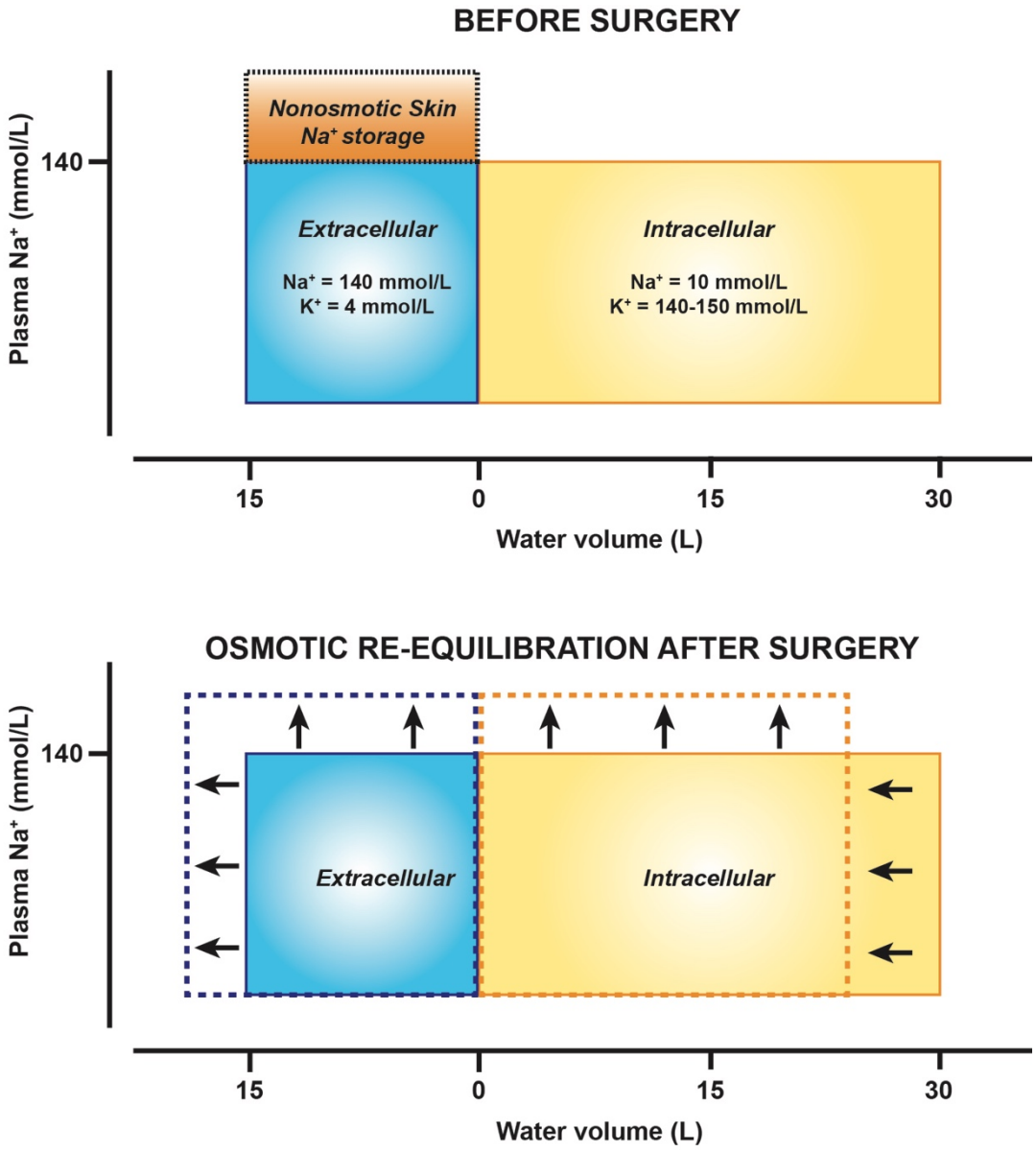
The present study includes also skin-K^+ data, which were not investigated in humans so far. The skin-K^+ content was similar in PA and in Controls, suggesting that skin-Na^+ retention in excess over water was not parallel by K^+ losses, indicating interstitial hypertonicity. Moreover, skin-K^+ content increased significantly after adrenalectomy, suggesting a strict relation between serum K^+ and skin-K^+ levels. Based on these observations, we postulated that Na^+ might be “non-osmotically” stored in the skin while K^+ is only “osmotically” regulated and influenced by water loss or retention.

Finally, these data were obtained by a well-characterized group of PA subjects, with a limited variability in terms of tissue electrolytes and water content and with an unequivocal diagnosis of PA. Moreover, PA subjects were well-matched for age, sex, and BMI with controls and EH patients. Thus, even if previous studies demonstrated by ^{23}Na-MRI an increased Na^+ storage in skin and muscle with age and in male sex, our data clearly suggested that aldosterone might play an independent key role in the regulation of non-osmotic skin-Na^+ storage. In this regard, we speculate that tissue sodium storage might represent an independent cardiovascular risk factor and that it might be responsible of the target organ damage in PA patients.

In conclusion, this study demonstrates that Na^+ and water homeostasis is far more complicated than the widely accepted two-compartment model. Moreover, the molecular mechanisms by which aldosterone regulates nonosmotic tissue Na^+ storage should be further elucidated.

Thus, more research is needed to understand the (patho)physiology of nonosmotic sodium storage, that might lead to a more targeted treatment not only for PA patients but also in salt-sensitive hypertensive patients.

Figure 12. Schematic representation of Na+ skin storage before adrenalectomy and its osmotic re-equilibration after unilateral adrenalectomy.

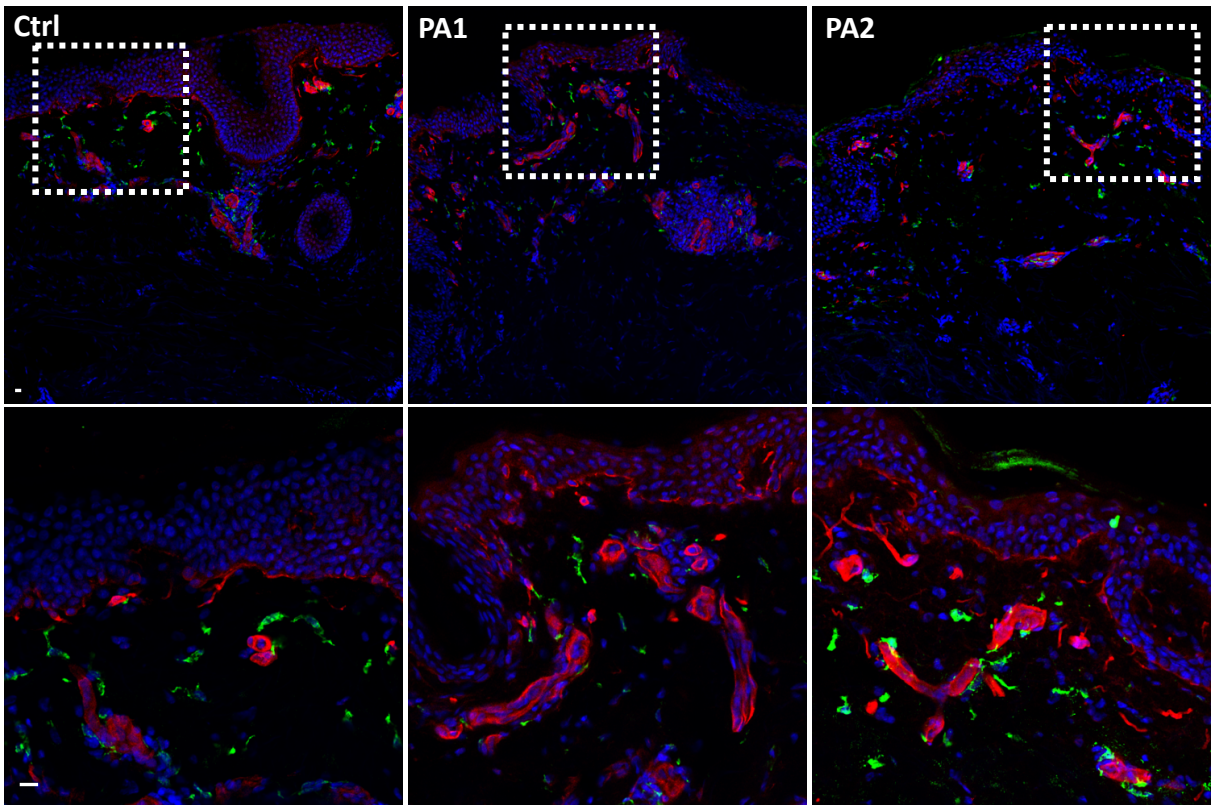


Perspectives

Previous experimental studies have identified glycosaminoglycans as the principal Na^+-binding site. This local hypertonicity triggers mononuclear phagocyte system (MPS) cells, mainly macrophages, to exert their activity and modulate interstitial electrolyte composition by TonEBP/VEGFC, resulting in modulation of cutaneous lymphatic capillary function.

Following these previous studies and our promising data, we also collected biopsies for immunohistochemistry, and we validate a immunofluorescence protocol in order to determine the skin macrophages infiltration and their activation by the expression of TonEBP (**Figure 13**).

Figure 13. Immunofluorescence of skin biopsies in a control patient (n=1) and PA patients (n=2).



Representative confocal images of human skin cryosections showing the merge of the stainings immunolabeled with anti-laminin (red) to distinguished epidermis from derma, anti-CD163 (green) to mark macrophages population, and anti-TonEBP (red) to mark monocyte in the derma. Nuclei (blue) are stained with Hoechst. A higher magnification of a selected area of each image are also showed on the bottom of the panel. The scale bar represents 20µm. **Ctrl** = control normotensive patient, **PA** = patient with primary aldosteronism.

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Chapter 5

The skin-sodium storage in Fibromuscular Dysplasia and its relationship with Blood Pressure Variability

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SUMMARY

Rationale: Renal fibromuscular dysplasia (RFMD) accounts for > 10% of cases of renovascular hypertension (HT). The mechanisms involved are mainly represented by inappropriately high aldosterone secretion due to the increased renin-angiotensin-aldosterone system (RAAS) activity, secondary to the reduction of renal flow, with ensuing excess sodium intake and body fluid volume expansion. High blood pressure (BP) is the most important cardiovascular risk factor and it has been demonstrated that, besides the office and home BP values, also the short-term blood pressure variability (BPV) can increase the cardiovascular comorbidities in hypertensive patients. Despite higher short-term BPV markers have been associated to other forms of secondary hypertension, in FMD patients there are no data. Moreover, the precise molecular mechanisms whereby sodium retention raises BP remained poorly defined until recent years when accumulating evidences pointed to a role of extra-renal regulatory mechanisms involving a salt-skin storage compartment as a reservoir of free extracellular Na^+. The hypothesis of this study is that FMD patients may have higher BPV markers compared to primary hypertensive subjects and that this finding might be correlate to higher skin-sodium storage.

Objective: The main objective of the study is to evaluate the BPV profile and skin sodium content in hypertensive patients with diagnosis of RFMD, before and one-month after renal revascularization. The second endpoint is to correlate skin sodium content and BPV markers, to explore the potential pathogenic role of sodium in hypertension and cardiovascular risk.

Study design: A multicenter study will be carried out by the Universities of Maastricht, Padua, and Rome, and it consists of two main phases. In the first part of the study, BPV markers in FMD and essential hypertensive patients will be retrospectively compared. In the second part of the study a prospective analysis on BPV profile at baseline and after angioplasty, simultaneously to the collecting data on skin-sodium and water retention, will be performed.

Study population: Essential hypertensive patients and hypertensive patients affected by RFMD, of both of sex and aged between 18 and 70 years old, able to sign the Informed Consent and to continue the follow-up, will be enrolled.

Intervention: In the prospective part of the study, only patients affected by RFMD will undergo clinical evaluation, laboratory blood samples, 24-h ambulatory blood pressure monitoring, and 4mm-skin biopsy, before and after renal angioplasty.

Main study parameters/endpoints: In the retrospective phase, data about 24h ABPM short-term-derived BPV (e.g. 24-h systolic and diastolic standard deviation, weighted standard

deviation, average real variability, etc.) in FMD patients compared to essential hypertensive subjects will be collected. In the second part of the study, we will prospectively evaluate the differences in terms of 24h ABPM short-term-derived BPV and skin-sodium and water content in skin biopsy, before and after specific renal artery revascularization.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: Our project plan is straightforward and rationally designed and furthermore we possess all the expertise and the required models to perform the planned experiments.

In the retrospective part of the research project, hypertensive FMD patients, with renal stenosis likely to revascularization, should undergo the first ambulatory visit during which they will be informed about the study purposes. If patients will agree to participate, we will collect data on present and past medical history, current drug therapy, anthropometric evaluation (e.g. weight, height, waist circumference, BMI, systolic and diastolic blood pressure, etc.), 24-h ambulatory blood pressure monitoring, blood samples, [e.g. blood count, glycemia, lipid profile, plasma aldosterone concentration (PAC), direct renin (DRC), serum creatinine, serum ions (Na^+, K^+, Ca^{2+}), 24-h urinary albumin excretion (UAE)], and angio-CT scan measurements, such as stenosis percentage, kidneys diameters, etc. During the renal angiography, in which the endoscopic revascularization will be performed, the first skin biopsy will be performed. At the follow-up evaluation, it will be analyzed the anthropometric and BP values, including the BPV through 24h ABPM, and the biochemical and hormonal profile after treatment. In this follow-up visit a second skin biopsy will be collected to examine the reduction of sodium storage in the skin, due to the reduction of RAAS activity. As makers of hypertension-mediated organ damage we will use measurements of intima media thickness at Doppler ultrasonography study, the UAE, and the echocardiographic parameters, before and after renal revascularization.

The endoscopic renal vascularization is a part of management of RFMD, as specified in the guidelines, and therefore there are no additional risks related to the procedure for patients enrolled in the present study. There are few risks related to skin biopsy: (i) small risk of bleeding (controlled in most cases with simple pressure on the site), (ii) bruising, (iii) wound infection (relatively uncommon, but this would require antibiotic treatment, topic or systemic), (iv) allergic reaction to local anesthesia Lidocaine cream and pain, (v) small scar (3 to 4 millimeters fine line, which sometimes heals as a circular indentation) or keloids.

The potential benefits of this study concern a better understanding of the cardiovascular risk profile and the physiopathological role of interstitial sodium in RFMD hypertension, its correlation with organ damage and the development of new more rational and targeted treatments.

1. INTRODUCTION AND RATIONALE

Fibromuscular dysplasia (FMD) is a non-atherosclerotic, non-inflammatory angiopathy of unknown cause affecting medium-sized (most commonly renal 60-75%) arteries, characterized by circumferential deposition of collagen in the intima with duplication or disruption of internal elastic lamina and development of long, irregular (tubular) or focal, smooth (concentric band) stenosis and macroaneurysms. It is more common in women and younger individuals and it has a multifactorial etiology, including vessel wall ischemia and smoking, hormonal and genetic factors[1].

The pathological classification is based on the histological pattern:

• Intimal fibroplasia (less than 10%)

• Medial dysplasia:

  – Medial fibroplasia (80%)

  – Perimedial fibroplasia (10-15%)

  – Medial hyperplasia (1-2%)

• Adventitial (periarterial) fibroplasia.

Moreover, FMD has been described in almost every vascular bed, including:

– Renal arteries (60-75%) (the most common form) bilaterally,

– Cervico-cranial arteries (25-30%),

– Non-renal visceral arteries (9%),

– Arteries in the extremities (5%),

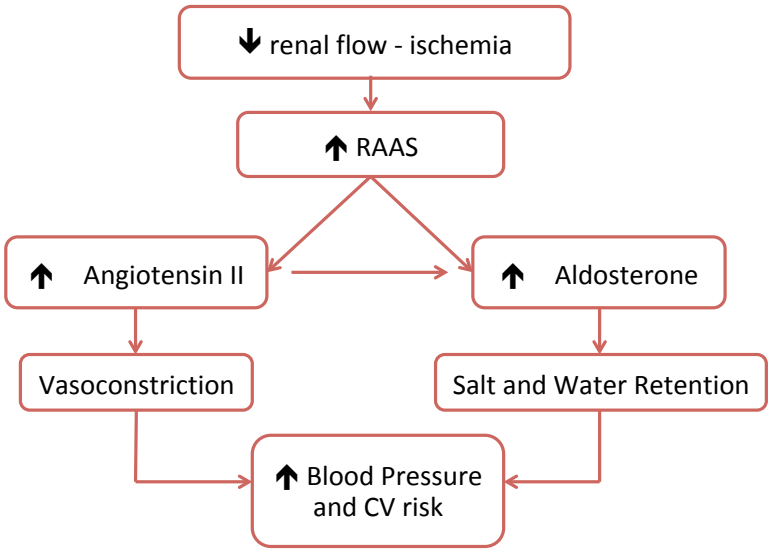
– Others including pulmonary and coronary arteries and multiple vascular beds have been observed in about 28% of patients.

"String of beads" is the classical angiographic pattern. The gold standard for treatment is represented by percutaneous renal angioplasty (PTRA), associated, in limited cases, to stent placement.

FMD accounts for more than 10% of cases of renovascular hypertension (HT), and it should be considered in a young person, usually female, who presents with severe hypertension and headaches in the absence of obesity, use of contraceptives, and history of parenchymal renal disease. Early diagnosis and treatment is very important for good long-term results. In these patients, the treatment of choice is PTRA (with stent placement only in selected cases) ± medical therapy, which frequently leads to very good control of hypertension[2].

The pathogenic mechanism involved can be summarized in the **Figure 1**.

Figure 1. Schematic flow-chart that summarizes the pathogenic mechanism of association between FMD and HT.



RAAS, renin – angiotensin – aldosterone – system; CV, cardiovascular.

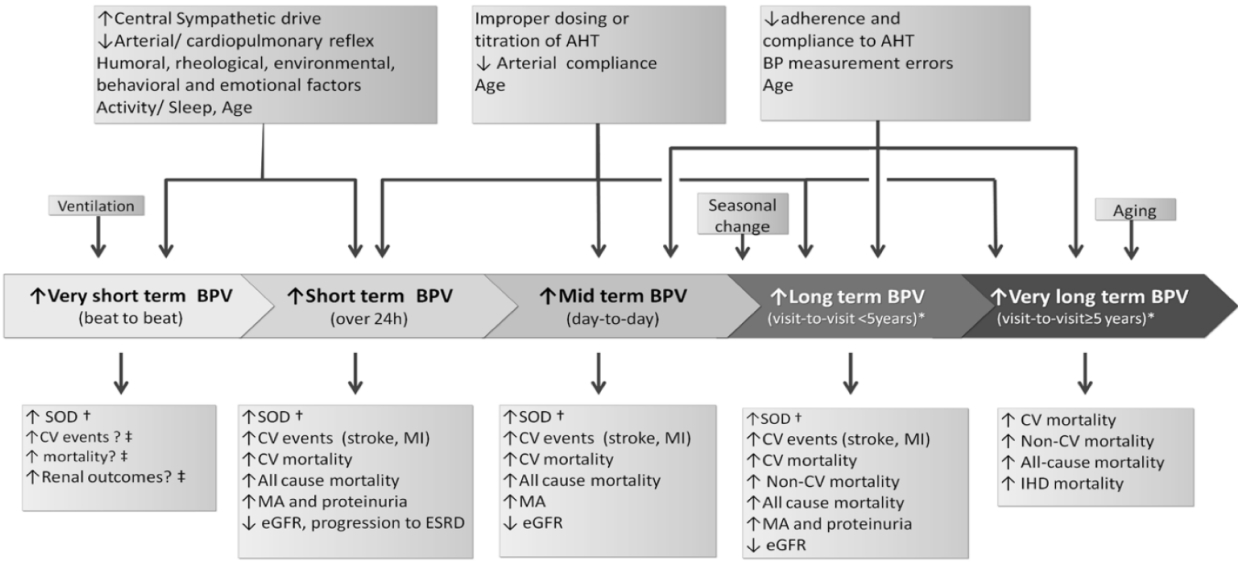
Especially when the stenosis leads to a critical restriction of renal arteries, the renal flow can be reduced and the multifocal ischemia of parenchyma determines a over and compensatory activation of renin-angiotensin-aldosterone system (RAAS) with increasing of BP values, due to vasoconstriction and salt and water retention.

Despite office blood pressure (O-BP) measurement remains the gold standard for diagnosis of HT[3], it has been demonstrated that the assessment and quantification of BP variability (BPV), is of both physiopathological and prognostic importance. Sustained increases in BPV over time may reflect alterations in cardiovascular (CV) regulatory mechanisms[4].

Non-invasive, intermittent, reading-to-reading over 24 hours BP measurements provided evidence that BPV may contribute independently to CV events prediction, over and beyond average BP (**Figure 2**)[5].

Moreover, some studies reported an increasing of short-term BPV indexes in patients affected by others forms of secondary hypertension, such as primary aldosteronism (PA)[6], Cushing's syndrome[7], Obstructive Sleep Apnea[8]. However, in subjects with renal FMD, BPV profile have not been evaluated yet and it has not been analyzed whether BPV markers are increased in these patients neither if, after revascularization treatment (PTRA – percutaneous renal angioplasty), one or more markers are reduced.

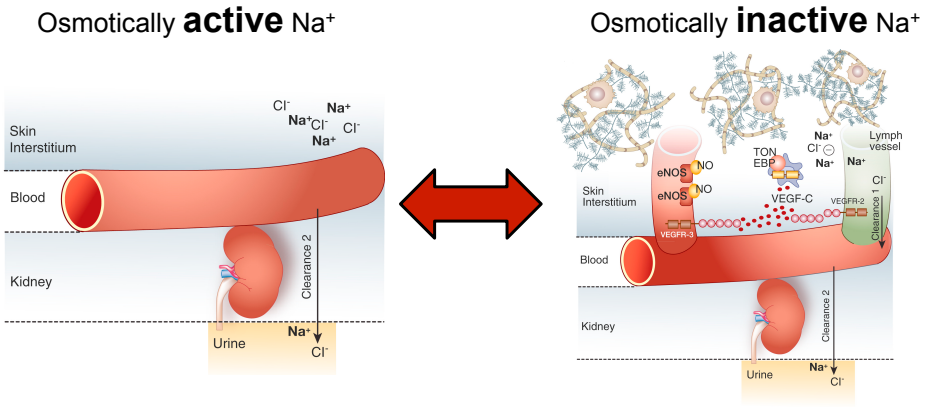
Figure 2. Schematic representation of different types of BPV and their effect on CV morbidity and mortality[4].



Furthermore, it has not been studied whether salt and water retention could be involved in BPV changes, as potential pathogenic mechanism, in patients with Renal FMD.

Salt and water skin storage could represent a feasible and additional system influencing the response to salt load and BP in humans. The skin acts as a third compartment for sodium[9] (**Figure 3**), capable of non-osmotic sodium storage and mediating a vasodilator response via VEGF-C. This appears to constitute an extra-renal mechanism controlling BP, mainly during high salt intake. Incorporating this model into the traditional paradigm of sodium homeostasis, the skin may act as a buffer as well as a reservoir for sodium, while the kidney controls sodium excretion and reabsorption, controlling serum osmolality and total body water. It is conceivable that people predisposed to salt-sensitive hypertension have defects in the pathways of skin sodium and water homeostasis[10]. It is widely recognized that excess sodium intake, with ensuing body fluid volume expansion, in the setting of inappropriately high aldosterone secretion plays a key role in many forms of HT, not only in PA, but also in essential HT in over-weight-obese subjects in the metabolic syndrome. However, the precise molecular mechanisms whereby sodium retention raises BP remained poorly defined until recent years when accumulating evidences pointed to a role of extra-renal regulatory mechanisms involving a salt-skin storage compartment as a reservoir of free extracellular sodium and macrophages were identified as important modulator of lymphatic vessels formation and draining of interstitial electrolytes. Thus, in patients with excessive production of aldosterone, such as renal arteries FMD, which determines sodium-retention *per se*, this mechanism has not been analyzed yet and, in this context, a lot of mechanisms should be clarified.

Figure 3. Sodium skin storage



In summary:

- FMD, especially if associated with high values of renal artery stenosis, determine the increasing of BP values, hypertension-mediated organ damage (HMOD), and CV complications, but pathogenic mechanisms underlying this association are multiple and not completely discovered.
- We hypothesis that the hormonal and vascular changes related to FMD might have a role not only in the HT but also in the BPV modifications and skin-Na+ and water content.

2. OBJECTIVES

2.1 Primary Objective: to analyze the 24h ABPM-derived short-term BPV profile in renal FMD and to correlate it with sodium and water skin storage.

2.2 Secondary Objective(s):

- To compare BPV markers amongst renal FMD patients and essential hypertensive subjects (in the retrospective phase of the study).
- To compare skin sodium and water content in patients affected by FMD, primary aldosteronism (PA Group), essential hypertension (EH Group) and patients with normal arterial blood pressure (Control Group).
- To estimate the effectiveness of the revascularization treatment (e.g. PTRA) in terms of 24h ABPM-derived short-term BPV and water and sodium-skin content.
- To assess the RAAS in FMD patients and to correlate it with 24h ABPM-derived short-term BPV variables and skin water and sodium content.

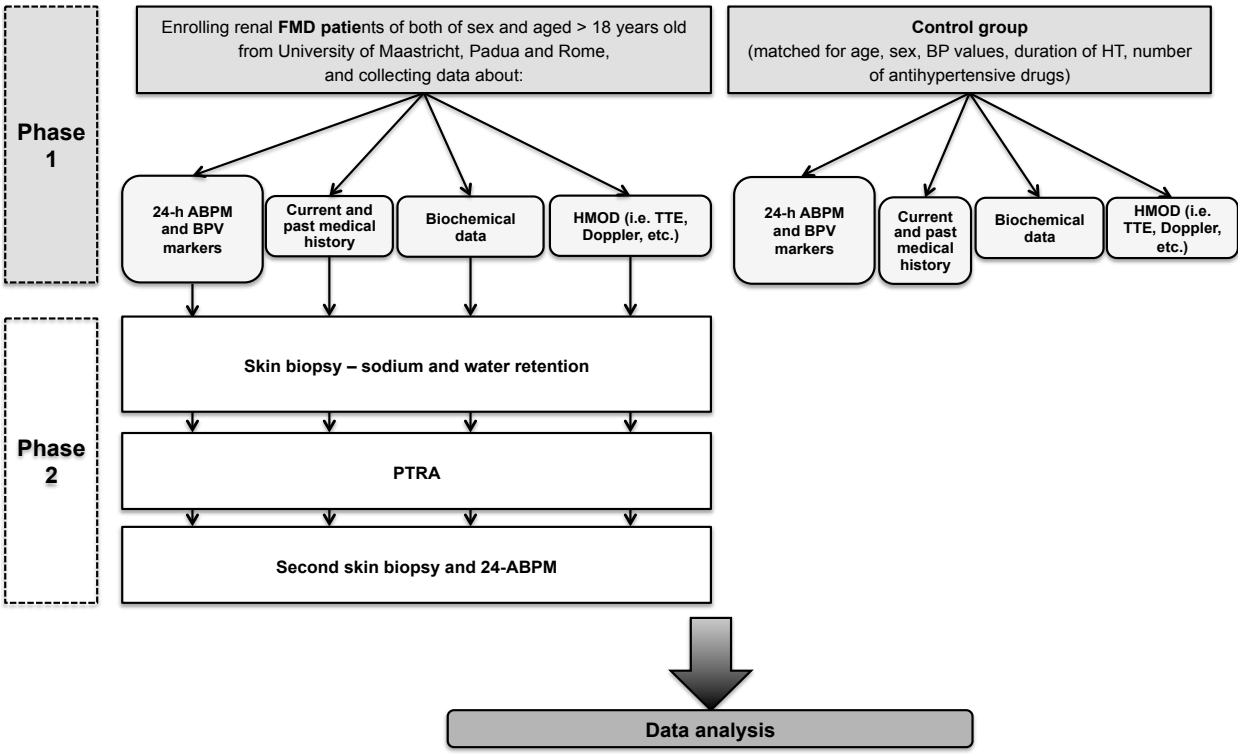
- To correlate BVP markers and skin water and sodium content with signs of hypertension mediated organ damage (HMOD; e.g. cardiac remodeling, renal and vascular damage).

3. STUDY DESIGN

The present study is a multi-center observational study, which involves the University of Maastricht, University of Rome "Sapienza", and University of Padua. The study includes two phases (**Figure 4**):

1. The first one is retrospective, during which we will collect data from 24h ABPM-derived short term BPV markers in patients affected by renal FMD and essential hypertensive subjects.
2. The second phase is prospective. In patients affected by RFMD, we will analyze skin sodium and water performing a 4mm skin biopsy before and 1-month after PTRA (see Methods describing the technique); the data obtained will be compared to other three groups of patients that have been already analyzed in Padua (patients affected by PA, patients with normal arterial blood pressure and essential hypertensive patients), where the study has been already approved by local Ethical Committee (see Flow-chart, **Figure 5**). Lastly, in this second part of the study we will repeat a 24h APBM to evaluate potential changes in BPV markers after revascularization.

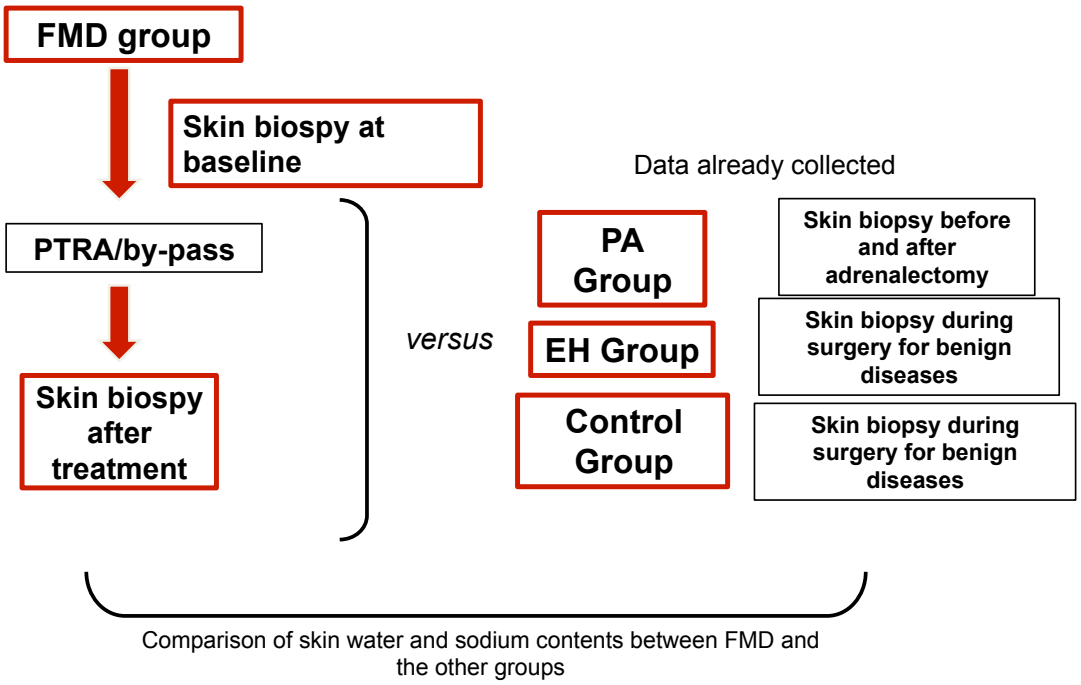
Figure 4. Study flow-chart.



During these two periods, all data will be collected in a single database, including each significant variable for the study.

The putative duration of both of phases is three months and the analysis of data about 1 month.

Figure 5. Flow-chart of the second phase of the study.



4. STUDY POPULATION

4.1 Population

- Patients affected by Renal FMD and essential hypertensive (EH) patients;
- Renal FMD patients submitted to renal angioplasty (e.g. PTRA), selected from the abovementioned Institutions (***Renal FMD Group***).
- EH patients, selected from those where a secondary HT was excluded by biochemical/radiological/clinical findings (***EH Group***).

Written informed consent will be obtained from all participants.

4.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

Renal FMD Group

✓ Patients of both sex, aged ≥ 18 years old;

- ✓ A signed and dated informed consent form;
- ✓ Diagnosis of HT (O-BP ≥ 140/90 mmHg or 24h ABPM ≥ 135/85 mmHg or patients taking antihypertensive drugs) according the ESC/ESH guidelines[3];
- ✓ Documented renal artery FMD (uni- and/or multifocal disease) with Doppler renal ultrasound and/or by CT scan/MRI angiography.

EH Group

- ✓ Aged from 18 to 75 years old;
- ✓ A signed and dated informed consent form;
- ✓ A diagnosis of essential hypertension defined either as:
  - Use of antihypertensive drug(s)

  - Arterial hypertension: in untreated patients this must be confirmed by 24h ambulatory blood pressure monitoring (24h ABPM), or home blood pressure monitoring, with blood pressure higher or equal to 135 mmHg for systolic blood pressure and/or higher or equal to 85 mmHg for diastolic blood pressure.
  - Exclusion of secondary hypertension by hormonal biochemical screening (plasma aldosterone, direct renin concentration, ARR < 2.06 ng/dL:mIU/L, ACTH, 24h free urine cortisol, morning plasma cortisol level, 24h urine metanephrines and catecholamines).

4.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

- ✓ Atherosclerotic renal artery stenosis;
- ✓ CKD with eGFR CKD-EPI <60 ml/min/1.73m2;
- ✓ Diabetes type I and II;
- ✓ Pregnancy;
- ✓ Patient has any serious medical condition, which in the opinion of the investigator, may adversely affect the safety of the participant;
- ✓ Known history of unresolved drug use or alcohol dependency;
- ✓ Current enrolment in another investigational drug or device trial;
- ✓ Life expectancy < 2 years;
- ✓ History of allergy/intolerance to local anesthesia;
- ✓ Refusal of the patient to undergo renal angioplasty;
- ✓ Refusal of the patient to participate in research.

4.4 Sample size calculation

For the prospective phase of the study, we have calculated that with 300 consecutive hypertensive patients we expect to have 6 cases of renal FMD (2% of prevalence). Using a specific software (R Core Team (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.), we have calculated that a sample size of 6 patients for each group (Renal FMD and the control Group) will have 98% power to detect a difference in means of 7 nmol/L (the difference between a RFMD Group mean, μ1, of 26 nmol/L and the control Group mean, μ2, of 19 nmol/L) assuming that the common standard deviation is 3 nmol/L using a two group t-test with a 0.05 two-sided significance level.

A sample size of 6 will have 99.84% power to detect a difference in means of 6 nmol/L (the difference between Renal FMD pre-angioplasty skin sodium content mean, μ1, of 26 nmol/L and Renal FMD post-angioplasty skin sodium content mean, μ2, of 20 nmol/L) assuming that the common standard deviation is 3 nmol/L using a paired two group t-test with a 0.05 two-sided significance level.

5. NON-INVESTIGATIONAL PRODUCT

5.1 Name and description of non-investigational product(s)

- **For the 24h ABPM we will use the SpaceLabs 90127®** (Redmond, WA, USA)[11]. This is a portable, noninvasive recorder used to perform 24h ABPM and from which it is possible derive BPV indexes. The between measurement intervals were 15 min (daytime) and 20 min (night-time). During each recording, subjects were required to attend at their usual daily activities, only refraining from unusual physical exercise or behavioral challenges. Only recordings rated as of sufficient quality, i.e. including at least 70% of valid readings over the 24 h and at least two valid readings per hour during daytime and one valid reading per hour during night-time, were considered for the final analysis. Day and night periods were defined and corrected according to what reported by the patient in the diary. The average daytime period was finally identified as the interval from 0800 h to 2300 h and the night period as the interval from 23:00 h to 08:00 h. All procedures were performed according to current guidelines[12].
- **Dermopunch** (DERMO-PUNCH® STERYLAB, Milano, Italy). It is a very common disposable for Skin Biopsy. Punch biopsies are simple to perform, have few complications, and if small, can heal without suturing. Product has razors made of stainless steel with fine cutting edge. It is available in broad range of sizes. For the present study, a 4 mm size punch will be used.

6. METHODS

6.1 Study parameters/endpoints

6.1.1 Main study parameter/endpoint

The **main endpoint** is to analyze the 24h ABPM-derived short-term BPV profile in Renal FMD and to correlate it with sodium and water skin storage.

The 24h ABPM-derived short-term BPV indexes that will be analyzed are:

- Systolic and diastolic BP standard deviation (SD) of 24h, daytime and night-time period;
- Degree of nocturnal BP fall (dipping pattern), calculated as [(daytime SBP x night-time SBP)/daytime SBP x 100%] for SBP and [(daytime DBP x night-time DBP)/daytime DBP x 100%] for DBP. Patients were classified as dippers if BP falls x10% and <20% of daytime average BP or non-dippers (fall <10%). Nocturnal BP falls > or equal 20% and <0% identified "extreme" dipper and "reverse" dipper subjects;
- Average of daytime and night-time SD, each weighted for the duration of the day and night periods [24h "weighted" SD of BP (wSD)], which allows for removing the mathematical interference from night-time BP fall;
- Average Real Variability (ARV) for 24h SBP and DBP, i.e., the average of the absolute differences between consecutive BP measurements over 24h, according to a mathematical algorithm:

$$ARV = 1^{N-1} \sum_{k=1}^{N-1} (BP_{k+1} - BP_k)$$

where N denotes the number of valid BP measurements, and k is the order of measurements;

- Coefficient of variation (CV, %) of SBP and DBP, calculated as SD/mean pressure × 100%.

In the first part of the study all these variables will be obtained in Renal FMD before PTRA and compared with those calculated in EH control group. We expected that the overactivation of RAAS in FMD patients determines a higher BPV. In the second phase of the protocol the same BPV indexes will be correlated with water and sodium skin storage with the aim to understand the pathogenic mechanism involved in the altered BPV profile in Renal FMD patients.

In this context, the skin biopsy will be performed in fewer cases. In more details, in informed consenting patients the epidermis and superficial dermis will be pierced by a sterile punch, the punch removed and hemostasis obtained with sterile gauze. The biopsy will be placed in a sample pot for bench preparation and immediate frozen in liquid nitrogen. The biopsy site will be closed with steri-strips. Wound care information sheet and materials will be provided to the participant.

- Skin sample analysis for water and electrolytes content. All frozen skin samples will be weighted to determine total (WET) weight and then desiccated at 90°C for 72 hours to determine the DRY weight. We found in preliminary experiments that after this period the weights remained unchanged with further drying. The difference between total WET and DRY weight will estimate the tissue water content. Na^+ and K^+ concentration in the samples will be measured by atomic adsorption spectrometry (SpectrAA 50/55 Varian, Agilent).
- Immunohistochemical analysis of the skin tissue. To investigate the crosstalk between skin Na^+, macrophages activation and lymphatic vessel density modulation in patients with FMD and HT, we will perform immunohistochemistry in the collected skin biopsies from FMD patients (FMD Group) and HT patients (EH Group), using antibodies to mark the specific transmembrane glycoproteins (anti-CD14/CD40) expressed by human monocytes and by the lymphatic endothelial cells (anti-Lyve).
- Primary skin fibroblast preparation. Skin primary fibroblasts will be prepared from skin biopsies obtained during surgery from FMD Group and EH Group patients and cultured for the biochemical and molecular experiments.
- Biochemical and molecular analysis of primary skin fibroblasts. We will evaluate the expression level of the tonicity responsive enhancer-binding protein TonEBP and the vascular endothelium growth factor C VEGF-C, which are two fundamental players in the regulation of the interstitial electrolyte homeostasis, in primary skin fibroblasts obtained from PA Group, EH Group and Control Group patients. In detail, we will directly evaluate TonEBP/VEGF-C expression level both by real-time quantitative PCR and Western Blot analysis. We will also perform biochemical analysis of intracellular pathways, focusing on the activation of MAPK cascade, which are upstream in the activation of TonEBP[13]. We will also check the expression level of the ion channels/pumps of the PM, with a particular interest in the plasma membrane Na^+/Ca^{2+} exchanger (NCX), by real-time quantitative PCR, Western Blot analysis and immunofluorescences. We will monitor the intracellular Ca^{2+} signaling using the ratiometric fluorescent dye Fura-2 and genetically encoded probe (Cytosolic-targeted Aequorin), to determine how high Na+-water skin content can alter the ion balance of the plasma membrane.
- Assessment of intracellular Ca^{2+} dynamics in primary skin fibroblasts. We will perform

biochemical analysis and intracellular Ca^{2+} measurements in primary skin fibroblasts obtained from FMD Group and EH Group, after Na^{+} or aldosterone treatment.

- Analysis of immune cell-lymphatic vessel crosstalk. We will explore the crosstalk between skin-cells and macrophages using a co-culture system. To address this issue, a cell co-culture system involving human macrophages from donors/U937 human cell-line and fibroblasts from patients with FMD, will be set up. Cell-cell signaling systems will be monitored between these cell types, including macrophage/fibroblast by: 1) culturing one cell type in the conditioned media taken from the other cell culture; 2) co-culturing the two cell types in transwell inserts to physically separate them (paracrine signaling); 3) co-culturing the two cell types in physical contact (juxtacrine signaling). Cell culture media and cell images will be collected during time and changes in soluble protein secretion, cytokine production, cellular behavior and morphology will be assessed.

6.1.2 Secondary study parameters/endpoints

1. In the retrospective phase of the study we will compare BPV markers amongst renal FMD patients and a control group of EH subjects, as described above.

2. To estimate the effectiveness of the revascularization treatment (e.g. PTRA) in terms of 24h ABPM-derived short-term BPV and water and sodium-skin content. The BPV indexes and the sodium and water skin retention will be performed after PTRA with the same procedures described for the baseline and data compared with the baseline before treatment.

3. To assess the RAAS in FMD patients and to correlate it with 24h ABPM-derived short-term BPV variables and water and sodium-skin content. In more details, we will analyze the levels of plasma aldosterone concentration (PAC) and direct renin (DRC) using a validated dual aldosterone and direct active renin concentration chemiluminescent commercially available assay in an automated analyser (Aldo LIAISON® MOME; DRC LIAISON® MOME; DiaSorin, Saluggia, Italy). Measurements will be performed on a LIAISON XL Analyser[14]. The reduction of renin is defined as a level < 2 mU/L and that the elevation of aldosterone as >0.45 nmol/L. The aldosterone-renin ratio (ARR) will be calculated in nmol/dL/mUI/L.

4. To correlate BVP markers and water and sodium-skin content with signs of hypertension mediated organ damage (HMOD) (e.g. cardiac remodeling, renal and vascular damage).

- Cardiac remodeling. To correlate BPV indexes and water and Na^{+} skin content of FMD Group (before and after PTRA) and EH Group patients with HMOD, we will measure left ventricular mass index (LVMI) and left ventricular diastolic function parameters[15].

M-mode and 2D echocardiography were performed in all patients with a 3.5-MHz transducer by a cardiologist blind to the cause of hypertension and ongoing medical therapy. All measurements were performed on the average of ≥3 cardiac cycles according to the American Society of Echocardiography guidelines[15]. LV wall thickness and internal dimensions were measured from 2-D-guided M-mode echocardiographic tracings obtained at mid-chord level in the parasternal long axis view. The LV mass (LVM) was estimated according to Devereux et al.2 LV mass index (LVMI) was calculated by indexing LV mass to height$^{2.7}$. Relative wall thickness (RWT) was calculated at end-diastole to estimate LV geometry, as RWT= (inter-ventricular septum thickness + posterior wall thickness)/LV diameter.

The criteria for LV hypertrophy (LVH) were LVMI>50 g/m2.7 and >47 g/m2.7 for men and women, respectively. LVH was classified as concentric or eccentric using a cut off for RWT> 0.45 or < 0.45, respectively; an RWT > 0.45 along with a normal LVMI identified LV concentric remodeling. LV end-diastolic and end-systolic volumes were calculated with the Teicholz's correction of the cube formula. Ejection fraction was calculated by standard methods.6 Stroke work (SW) was estimated as systolic BP (measured after the echocardiographic study) times stroke volume and converted into gram-meters by multiplying by 0.0144.

- Renal damage Markers of renal function that will be collected are: serum creatinine () the estimated glomerular filtration rate (GFR), and urinary albumin excretion (UAE). GFR was measured by using the so called "abbreviated equation," which takes into consideration serum creatinine, age, gender, and race (is available at www.kdoqi.org) for easy computation: estimated GFR (mL×min−1×1.73 m−2)=186×(serum creatinine)−1.154×(age)−0.203× (0.742 if female)×(1.210 if black)[16,17]. UAE rate was analyzed as milligrams×24 hours×$^{-1}$ and also after normalization for milligrams of urinary creatinine. The normal range of UAE rate was 30 to 300 mg×g^{-1} of creatinine (or 30 to 300 mg×24 hours^{-1}). According to the timed 24-hour UAE rate, samples were divided into 3 groups: normoalbuminuric (UAE <28.8 mg×24 hours^{-1}, e.g., <20 μg×min−1), microalbuminuric (UAE 28.8 to 288 mg×24 hours^{-1}, e.g., 20 to 200 μg×min^{-1}), and macroalbuminuric (UAE >288 mg×24 hours^{-1}, e.g., >200 μg×min−1).
- Vascular damage. The carotid Doppler ultrasonography will perform in each patient[18]. High resolution B mode, color Doppler, and pulse Doppler ultrasonography of both carotid arteries were performed with an ultrasound machine equipped with a 7.5 MHz

linear array transducer. Patients were examined in the supine position with the head tilted backwards. After the carotid arteries were located by transverse scans the probe was rotated 90° to obtain and record a longitudinal image of the anterior and posterior walls. The maximum IMT was measured at the near and far walls of the common carotid artery, the bifurcation, and the internal carotid arteries and was expressed as a mean aggregate value. The IMT was assessed as normal if it did not exceed 1 mm. With regard to the incidence of plaque (defined as a focal thickening of the intima-media complex greater than 1.3 mm), its maximum diameter was assessed and included in further analysis. Furthermore, the grade of stenosis in the carotid and vertebral arteries was assessed through the increase in the peak systolic and end diastolic velocities (according to the criteria of Hood at al. [19]). The carotid and vertebral atherosclerosis was considered severe when the grade of stenosis was $\geqslant$ 70%. When Doppler ultrasound indicated severe stenosis of the carotid or vertebral artery, the actual grade of stenosis was confirmed by standard angiography. All scans were obtained by the same experienced sonographer, who had no prior knowledge of the patients' clinical and angiographic characteristics.

6.1.3 Other study parameters

We will obtained anthropometric data and baseline habits, like age (years), BMI (kg/m^2), waist circumference (cm), number of drugs, comorbidities (i.e. past history of acute or chronic coronary syndrome, diabetes, cerebrovascular accidents, dyslipidemia), smoking, etc. that might intervene with the main study parameter (confounders).

6.2 Study procedures

We included below a schematic scheduled assessments.

1. Enrollment of Renal FMD and EH patients. From all patients, after complete and exhaustive explanation of the risks linked to the procedures, we will obtain the informed consent for the prospective phase of the study.
2. Baseline clinical evaluation. The first ambulatory evaluation will include the determination of:
   - Name and Surname (which will be replaced with an I.D. number in the dataset);
   - sex (M/F);
   - age (years);
   - time to onset of hypertension (months);
   - weight (kg), height (m), and BMI (kg/m^2);

- office SBP and DBP (mmHg) according to last ESC/ESH Guidelines 2018[3] and heart rate (mmHg);

- past medical history, including past cardiovascular accidents (e.g. coronary syndrome, cerebrovascular diseases, arrhythmias, heart failure, peripheral artery diseases), diabetes, dyslipidemia, chronic kidney disease, etc;

- current and past habits, such as smoking, alcohol and or drugs abuse;

- current medications.

3. Confirm diagnosis of FMD with biochemical and instrumental assessment.

   In this phase enrolled patients will undergo to measure of the main biochemical routinely examination and hormonal assessments for secondary forms of hypertension: blood count, glycaemia, HbA1c, total cholesterol, LDL, HDL, triglycerides, PTH e vitamin D, TSH, serum cortisol, ACTH, PAC, DRC, plasma catecholamine will be measured with conventional methods; serum creatinine, BUN, serum ions (Na^+, K^+, Ca^{2+}) will be measured with ELISA. Urinary tests: UAE, urinary catecholamine and metanephrine, 24h urinary ions (Na+, K+, PO43-, Ca2+), 24h UFC will be measured with conventional methods. The diagnosis of Renal FMD will be suspected with high values of PAC and DRC (and a lower ARR) and confirmed by abdominal CT scan with contrast, as routinely occur.

4. HMOD. Patients with Renal FMD will undergo to the assessments of HMOD with Doppler echocardiography, carotid Doppler ultrasonography (as described in the previous section – 8.1.2).

5. 24h ABPM. To determine the BPV profile all patients will perform a 24h ABPM (as described above).

6. Skin biopsy. The skin biopsy will be performed in each Renal FMD and EH Group to evaluate the sodium and water skin storage. The detailed technique is described in the section 8.1.1.

7. Percutaneous transluminal renal angioplasty (PTRA). Several studies have evaluated the efficacy of revascularization by PTRA in patients with FMD, also in terms of better BP values control and renal function[20]. Therefore, according to literature[21], the revascularization procedure will be indicated in:

   - uncontrolled blood pressure, despite three antihypertensive medications at maximal doses,

   - individuals are intolerant to the medications or when the compliance is an issue,

   - as an alternative to lifelong dependency on a medication in a relatively young

individual.

The possible complications are related to the puncture site (e.g. atero-venous fistula, pseudoaneurysm, bleeding/hematoma, femoral nerve injury, infection), catheter-related (dissection, perforation/rupture, balloon rupture, thrombosis, renal artery spasm), contrast mediated (e.g. anaphylaxis).

8. 3-months of Follow-up with clinical re-evaluation, biochemical assessment, HMOD, 24h ABPM, skin biopsy.

6.3 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

6.4 Premature termination of the study

The criteria for terminating the study prematurely are:

- Serious adverse reactions, including abnormalities in laboratory analytes, vital signs, or outcomes,
- Inability to recruit or adequately enroll an adequate number of patients,
- Financial considerations,
- Protocol found to be impractical or unworkable,
- Investigator(s) loses interest,
- Problem, arise in the medicine's stability or manufacture,
- Failure of the investigator and/or staff to follow either good clinical practice standards or to adhere to protocol requirements,
- Unacceptable change in personnel or facilities at the investigator's site,
- Determination that no statistical significant result can be obtained.

7. SAFETY REPORTING

7.1 Adverse events (AEs)

The present study presents few risks related to skin biopsy:

- Small risk of bleeding (controlled in most cases with simple pressure on the site),
- Bruising,
- Wound infection (relatively uncommon, but this would require antibiotic treatment,

topic or systemic),

- Allergic reaction to local anesthesia Lidocaine cream,
- Pain,
- Small scar (3 to 4 millimeters fine line, which sometimes heals as a circular indentation) or keloids.

7.1.1 Serious adverse events (SAEs)

Serious adverse events are not expected in the present study, since no new treatment or drugs will be tested. Thus, no major measures for adverse effects are necessary. For the prospective phase of the study, the sterility during biopsies is ensured by preparing the surgical site that is re-cleaned with alcohol swabs and draped.

7.1.2 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.
SAEs need to be reported till end of study within the Netherlands, as defined in the protocol.

8. STATISTICAL ANALYSIS

Results will be expressed as mean ± SD, or median and interquartile range, as appropriate. Continuous variables will be tested for normal distribution with Kolmogorov-Smirnov test. Parametric and non-parametric statistics will be used for log-transformed data and variable with a skewed distribution, respectively. Within-patient comparison of paired t-samples test will be used for PA patients before and after surgery; Pearson's $\chi 2$ test will be used for categorical variables.
Significance will be set at $p < 0.05$. Since several comparisons will be undertaken for primary and secondary objectives, the post-hoc Scheffè test will be used to warrant against false positive findings. Missing data, if applicable, will be replaced as mean.
The statistical analysis will be performed with t-test and one-way ANOVA followed by post hoc Scheffè test. Specific licensed softwares, including GraphPad (vers 8.1.2) and SPSS (for Mac vers 25) are already available for these tasks.

9. ETHICAL CONSIDERATIONS

9.1 Regulation statement

All the procedures performed in the present study involving human participants are in accordance with the ethical standards and the principles *of the Declaration of Helsinki* and its later amendments (7th revision, 2013 World Medical Association, 64th WMA General Assembly, Fortaleza, Brazil, October 2013) and in accordance with the Medical Research Involving Human Subjects Act (WMO).

9.2 Recruitment and consent

Hypertensive patients referred to a tertiary care unit are evaluated and screened for secondary hypertension. Patients that meet the inclusion criteria mentioned above will be recruit for the study. Patients will be recruited only after a signed informed consent. Before signing the consent, the patients are verbally carefully educated by an experienced medical doctor or researcher about the purpose of the study, the procedures, the risks and benefits. A potential research subject will have the opportunity to read the consent document and ask questions about anything they do not understand. If a patient is considering participating in the study, he or she may take the consent document home to discuss with family and friend. The patient has to communicate the acceptance or refusal to participate in the study within the next outpatient visit. The patient information letter and informed consent form is attached as a separate document. The consent document is clearly written and understandable to subjects, with a non-technical language.

9.3 Objection by minors or incapacitated subjects

Patients not able to sign the informed consent are excluded from the study. This statement is also specified in the informed consent.

9.4 Benefits and risks assessment, group relatedness

Our project plan is straightforward and rationally designed and furthermore we possess all the expertise and the required models to perform the planned analysis. We think that our research workflow does not present significant difficulties and that we have good chance to achieve all the proposed aims. However, a potential risk endangering this project entails the limited amount of biopsies to be used for the experiments/studies/analysis. Having well in mind this potential problem we will reinforce the existing collaboration between the three Units (Maastricht, Padua and Rome) to have as much as possible materials available and will prove to be highly efficient

in selecting patients with all forms of hypertension as required for the study.
Given the large population of HT patients, this proposal has the potential of exerting a strong impact both on the development of theoretical knowledge of one of the most pervasive pathologies of the century, and also on the introduction of new concepts for the management of HT patients which is of major concern for the public health. The major plus of this project is the relevant number of RFMD cases treated in the Units, including renal angioplasty which offers reversibility to the model; the expertise of the team not just in clinical management of RFMD but also in the characterization of the clinical and molecular phenotype, will provide a more comprehensive knowledge applicable to salt-sensitive HT.

9.5 Compensation for injury

The investigator has a liability insurance which is in accordance with article 7 of the WMO.
The investigator has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study.
The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

10. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

10.1 Handling and storage of data and documents

All data will be collected in a SPSS database, which will be available only for the nominated investigators. Patients' privacy will be guarantee by the anonymity and name and surname will be replaced with an ID code.

The biopsies will be stored at -80 degree (each patient will be identify by an ID code) and processed to obtain skin sodium and water content. Subsequently, the specimens will be eliminated.

10.2 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given. All amendments will be notified to the METC that gave a favourable opinion.

10.3 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited

METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

10.4 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit.
The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.
In case the study is ended prematurely, the sponsor will notify the accredited METC within 15 days, including the reasons for the premature termination. Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

10.5 Public disclosure and publication policy

This study has no sponsors. The investigators have not to disclosure.

If feasible, when the study will terminate, data will be published in a scientific journal after a proper analysis.

11. STRUCTURED RISK ANALYSIS

Biopsies will be performed using a registered disposable dermo-punch (4 mm) device.
There are few risks related to skin biopsy using dermo-punch:

- Small risk of bleeding (controlled in most cases with simple pressure on the site),
- Bruising,
- Wound infection (relatively uncommon, but this would require antibiotic treatment, topic or systemic),
- Allergic reaction to local anaesthesia Lidocaine cream,
- Pain,
- Small scar (3 to 4 millimeters fine line, which sometimes heals as a circular indentation) or keloids.

Even if the risks of the procedure are limited, the biopsies will be performed by experienced medical doctor in sterile conditions.
All the above-mentioned risks are acceptable.

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Chapter 6

General discussion

GENERAL DISCUSSION

Albeit vastly underdiagnosed, primary aldosteronism (PA) is the most common cause of arterial hypertension (HT) and particularly of drug-resistant arterial hypertension (RH), a high-risk condition with a poor prognosis [1, 2]. A timely diagnosis of PA followed by targeted treatment is pivotal to prevent the cardiovascular morbidity and mortality induced by aldosterone excess [3, 4].

The screening for PA in patients with RH is often precluded since, by definition, these patients are on a complex therapeutic regimen entailing three or more drugs including a diuretic, which deeply affect the renin-angiotensin-aldosterone system [1, 2]. Therefore, many PA patients are mislabeled as being affected by essential HT because they are never worked-up for PA. As a result, they remain exposed to the long-term, and often lifelong, detrimental effects of hyperaldosteronism and a decreased quality of life.

In the setting of inappropriately high aldosterone secretion, excess sodium intake and secondary body fluid volume expansion play a key role in causing HT and hypertension-mediated organ damage. The link between salt and primary aldosteronism is clear: the detrimental consequences of high plasma aldosterone do not cause cardiovascular damage when sodium intake is restricted, thus ruling out deleterious effects of aldosterone acting alone [5]. However, the precise mechanisms whereby sodium retention raises BP remained incompletely defined until recently, when accumulating evidences pointed to a role of extrarenal regulatory mechanisms involving salt-skin storage as a reservoir of free extracellular Na^+, and macrophages-mediated modulation of lymphatic vessels drainage of water and electrolytes from interstitium [6, 7].

Accordingly, this doctoral work had three main aims:

1. to ascertain if unilateral surgically curable PA can be diagnosed in patients with RH.

HT is defined as “resistant” to treatment when BP remains above goal (SBP >140 and/or DBP >90mmHg, confirmed by ABPM or HBPM) despite appropriate lifestyle measures and the concurrent adherence to therapy with optimal or best-tolerated doses of three or more drugs that should include a diuretic [1, 2]. Patients with RH are characterized by distinct demographics, comorbidities, and metabolic abnormalities, such as nondipping or reverse

dipping BP and sympathetic nervous system overactivity, visceral obesity, and aldosterone excess.

The majority of studies indicate that PA is a common cause of RH, with a prevalence rate of ≈20% [2]. Therefore, the Endocrine Society Guidelines recommend the screening for PA in all such patients [3].

The work presented in my thesis starts from the identification of PA in resistant hypertensive patients, who by definition are on multiple drugs including diuretics, angiotensin-converting enzyme inhibitors, angiotensin-type 1 receptors blockers, and beta-blockers, that deeply affect the renin-angiotensin-aldosterone system and, therefore, can preclude identification of PA. At variance with non RH patients, in whom BP can be controlled with agents that do not influence the renin-angiotensin-aldosterone system, such as slow-release verapamil, hydralazine, or an α1-adrenergic receptor antagonist (prazosin, doxazosin, or terazosin) [2, 8], in patient with RH withdrawal of the above confounding medications is not safe.

Considering these clinical challenges posed by the patients with "difficult-to-treat" HT, we set up a protocol for their evaluation which was routinely used with clinical success and constituted the basis of a prospective study. From September 2011 to September 2018, 1016 patients were evaluated at our specialized Center for "difficult-to-control" HT. Of the 1016 patients, 71% did not fulfill the RH definition either because they were on < 3 medications, or were not on optimal medical treatment (18%), or were judged to be not fully adherent to prescribed treatment and, therefore, they were excluded from this study. The remaining 110 patients fulfilled the ESC/ESH definition of RH in that they exhibited BP values (both systolic and diastolic) above 140/90 mmHg in spite of an average of 3.6 of drugs at baseline, which corresponded to 4.8 ± 2.1 Defined Daily Doses (DDD). Owing to their high-risk, their wish to pursue surgical cure, and the lack of contraindications to general anesthesia and surgery, they were offered AVS, which was performed after verification that their plasma renin levels were not overtly elevated, but because of their high BP values and the associated high risk, without withdrawal of interfering drugs.

The procedure was performed with no intra- and post-procedural complications and was bilaterally selective in 82% of the patients. Unambiguous evidence of a unilateral form of aldosteronism was achieved in 25 of patients who, therefore, underwent AVS-guided laparoscopic adrenalectomy. In conclusion, this proof-of-concept study demonstrated that AVS is feasible and allows identification of unilateral PA in RH patients, a challenging PA phenotype owing to the need of multiple confounding antihypertensive drugs.

2. to determine if unilateral adrenalectomy can resolve RH, thus improving quality of life

Unilateral laparoscopic adrenalectomy is the best treatment that can be offered when a lateralized cause of PA is timely identified, because it cures PA and can prevent and/or regress its cardiovascular complications. AVS-guided transperitoneal or retroperitoneal laparoscopic adrenalectomy has become the standard surgical method in term of safety and feasibility for unilateral PA, since it offers many advantages over conventional open surgery, such as reduced postoperative pain, less postoperative ileus, shorter hospitalization stay after surgery, lower wound-related complication rates, less operative blood loss, and lower postoperative complication rates[9, 10].

Previous studies have documented biochemical cure, and cure or improvement of HT with adrenalectomy and target medical treatment in PA. A complete biochemical success in terms of correction of hypokalemia and hyperaldosteronism after unilateral adrenalectomy occurs in more than 98% of cases. As regards the blood pressure outcome, it was differently stratified in the different studies as cure, marked improvement, mild improvement or no improvement in the AVIS-2 Study or as complete, partial, and absent success, in the PASO Study [11, 12]. Identified predictors of a good clinical response were age, sex, short duration of HT, and high number of antihypertensive medications.

However, none of the previous studies focused on patients with RH. Therefore, we sought to determine if AVS-guided adrenalectomy can be useful to resolve BP resistance to treatment in RH patients. In our study we showed that AVS-guided unilateral laparoscopic adrenalectomy allows biochemical cure of PA in 96% of the patients, and also, more importantly, resolution of RH in all. Not only the BP values were brought down to target levels after surgery, but a highly significant decrease of antihypertensive medications burden, in terms of both number and DDD needed to control HT, was also seen. These changes were accompanied by regression of cardiovascular damage, indicating that the correction of hyperaldosteronism and the ensuing favorable hemodynamic changes were instrumental in lowering the overall cardiovascular risk in these patients.

Besides leading to increased cardiovascular morbidity and mortality, PA was claimed to be associated to higher prevalence of anxiety and depression, with potential impact on health-related quality of life (QoL). In 2010, Sukor et al. examined health-related QoL in patients with unilateral PA, before and after unilateral adrenalectomy, and found a significant improvement in post-surgical QoL of these patients both in physical and mental condition [13]. The same authors later confirmed that PA patients had subnormal QoL scores compared to normal population [14].

In Chapter 3 of the thesis, we assessed the impact of surgery on health related QoL (both in Mental and Physical components) and, for the first time, depression status of patients suffering from PA, to compare them with a control group of patients with non-secreting adrenal tumor who also underwent adrenalectomy. In agreement with previous studies, we confirmed that patients with PA have an impaired health-related QoL compared with normal population. PA affects the QoL by worsening the mental component and the depression status. Adrenalectomy improves the mental component of health-related QoL and depression status at 1 month and at long term. In the long term, surgery determines an improvement also in the physical component of health-related QoL of PA patients, confirming the beneficial effect of adrenalectomy.

3. to investigate if extracellular skin Na^+- storage occurs in humans affected by PA, the main curable cause of human salt-sensitive endocrine hypertension, which can be a suitable model to investigate the changes in skin-Na^+ content in relation to aldosteronism and its surgical correction

The role of salt in the pathogenesis of HT is not well understood and is much debated. According to the classical physiology, the central mechanism linking sodium and BP balance has been described by Guyton and Borst, who placed the kidney at the very center of long-term BP regulation and demonstrated that long-term control of BP is closely related with salt and body fluid homeostasis[15]. Recently, Titze et al. introduced a new paradigm about sodium homeostasis, inspired by previous investigations that have challenged the accepted concept of the two-compartment model[16]. In the last decade, the skin has emerged as a new player in the control of BP through local homeostasis of Na^+, but neither the mechanisms underlying excess extracellular interstitium skin-Na^+ deposition, nor its impact on the surrounding cellular environment are currently known. To date only very scant studies in humans, where the Na^+-skin content has been quantified indirectly by ^{23}Na-magnetic resonance imaging (MRI), are available. They suggested that Na^+ accumulation in skin and muscle is associated with ageing and HT, particularly in patients with poorly controlled BP or PA, where skin-Na^+ accumulation was reversed after surgery[17, 18].

In Chapter 4 we proposed to use PA, a paradigm of salt-dependent low-renin HT and the most common cause of secondary HT, as a model to investigate the changes in skin-Na^+, K^+ and water content in HT. Our results show and confirm that Na^+ is stored in the skin of PA subjects without concomitant water retention, suggesting that a certain amount of Na^+ is osmotically inactive and implying that tissue-specific regulatory mechanisms might control the release and storage of Na^+ from a kidney-independent reservoir. Importantly, I

found that Na^+ accumulation in the skin was reversible after unilateral adrenalectomy, but not medical treatment. Finally, skin-Na^+ content seems to be even higher in those PA patients who had a bilateral aldosterone hypersecretion, although more data are needed to prove this preliminary evidence. Our study includes also skin-K^+ data, which were not investigated so far. In this regard, skin-K^+ content, although not differing significantly between PA and Controls, increased significantly after adrenalectomy, suggesting a strict relation between serum K^+ and skin-K^+ levels.

In conclusion, this thesis demonstrates that i) AVS is feasible and allows identification of unilateral PA in RH patients, a challenging PA phenotype owing to the need of multiple antihypertensive drugs potentially confounding AVS; ii) unilateral AVS-guided adrenalectomy allows biochemical cure of PA, resolution of RH with a prominent clinical benefit in spite of severity of arterial hypertension, presence of hypertension-mediated organ damage, and documented resistance to treatment, and improves QoL; iii) sodium homeostasis is more complicated than the widely accepted two-compartment model. More research is needed to understand the pathophysiology of nonsomotic sodium storage and clarification of the exact physiology of sodium homeostasis may improve treatment of PA patients and hypertensive patients in the future.

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Addendum

SUMMARY

CHAPTER 1 provides an introduction for this thesis in which the relevance of adequate blood pressure (BP) control is discussed. The screening for secondary causes of arterial hypertension (HT) is mandatory since it allows i) the identification of the underlying HT pathophysiology ii) cure of HT or a significant improvement of BP control and iii) a better prevention of target-organ damage and cardiovascular events by a specific pharmacological treatment.

Primary aldosteronism (PA) is the most common and potentially curable cause of HT and it should always be suspected in hypertensive patients. The diagnosis of PA and its targeted treatment are of crucial importance since aldosterone excess, independently from the degree of BP, induces fibrosis, generation of reactive oxygen species, and inflammatory changes; patients with PA have higher cardiovascular morbidity and mortality than age- and sex-matched patients with essential HT and the same degree of BP elevation. Subtyping of PA should be performed at third level referral centers that can adequately perform and interpret adrenal vein sampling (AVS) results. When a unilateral cause of PA is discovered, hyperaldosteronism and hypokalemia are curable with adrenalectomy in almost all patients, and BP can be normalized or considerably reduced in a substantial proportion of the patients. Screening for PA is particularly beneficial when HT is severe and/or resistant to treatment, because target treatment and/or surgery allow to bring blood pressure under control and the withdrawal, or a prominent reduction in the number and dosage of antihypertensive medications, and permit to prevent or regress organ damage.

Finally, the current views on the physiology of sodium (Na^+) homeostasis and BP have been discussed. High Na^+ intake is causally related to high BP and high BP is known to increase the risk for cardiovascular events and death considerably. The effect of Na^+ intake on BP varies considerably among individuals, discriminating sodium sensitive individuals, who are characterized by a BP increase after an increase in Na^+ intake, from Na^+ resistant individuals, who do not develop a BP increase. According to the two-compartment model, the kidney is solely responsible for matching Na^+ excretion and Na^+ intake, thereby preventing Na^+ and water retention, extracellular volume expansion and an increase in BP. The kidney is therefore thought to play a pivotal role in determining the effects of Na^+ intake on BP (i.e. Na^+ sensitivity).

This theory has been questioned by long-term Na^+ balance studies that have shown that Na^+ can accumulate in the human body without concurrent retention of water. This is not

in line with the current physiological concept that Na^+ retention is always accompanied by water retention. Experimental studies have demonstrated that negatively-charged polysaccharides, called glycosaminoglycans (GAGs), are able to bind and osmotically inactivate Na^+ in the skin interstitium. As a result, Na^+ storage by GAGs will not be accompanied by water retention. Skin Na^+ accumulation has been observed in patients that are prone to volume expansion such as patients with HT, hyperaldosteronism and dialysis patients and may affect BP regulation.

In **CHAPTER 2** we have investigated if AVS, the key test recommended by current guidelines to demonstrate a unilateral surgically curable form of PA, can identify PA even in resistant hypertensive patients (RH), who by definition are on multiple drugs. Moreover, we sought for determining if AVS-guided adrenalectomy can be useful to resolve BP resistance to treatment in these patients. We identified 77 patients with RH and additional clues of PA among 1016 consecutive patients referred for "difficult-to-treat" hypertension to our ESH Center of Excellence. As they wished to pursue surgical cure of PA, they underwent AVS, which showed unilateral PA in 25 patients; these 25 patients were submitted to AVS-guided laparoscopic unilateral adrenalectomy. After surgery, BP fell from 161/99±26/14 mmHg at baseline, to 133/84±14/9 mmHg at 6 months ($p<10^{-4}$ and $p=10^{-2}$, for systolic and diastolic, respectively), notwithstanding the fall of antihypertensive drugs required to achieve blood pressure control (from 3.6±0.2 agents at baseline to 1.2±0.2 at 6 months; and from 4.8±2.1 to 1.2±1.4 defined daily doses; $p <10^{-4}$ for both). RH was resolved in all and 20% of the patients were clinically cured in that they no longer needed antihypertensive treatment; moreover, 96% were biochemically cured and cardiac and renal organ damage regressed. Together, these data demonstrate that AVS allows identification of unilateral PA in patients presenting with RH and, more importantly, AVS-guided adrenalectomy effectively resolved RH in these patients.

In **CHAPTER 3** we assessed the impact of surgery on health-related quality of life (QoL) and depression status of patients suffering from PA, which is associated with an increased prevalence of anxiety and depression.

Data on QoL and depression status were prospectively collected before, early after surgery (at 1 month) and at late follow up (at least 6 months) in patients with unilateral PA (n=26) and in a control group (n=15) with non-secreting adrenal tumors submitted to unilateral

laparoscopic adrenalectomy. QoL was assessed using the Short Form 36 (SF-36) Health Survey for Physical (PCS) and Mental Component (MCS); the depression status by a 20-item depression scale (DS) questionnaire. Biochemical cure of the disease was achieved following surgery in all PA patients; HT was cured in 31% of cases and improved in the remaining 69% of cases. No morbidity occurred in both groups. We found no significant differences between PA patients and controls concerning demographics, preoperative PCS, MCS and DS values. In patients with PA, MCS values improved at early (42.72±13.68 vs 51.56±9.03, p=0.0005) and late follow up (42.72±13.68 vs 51.81±7.04, p<0.0001); also DS values improved at early (15.92±11.98 vs 8.3±8.8, p=0.0002) and late follow up (15.92±11.98 vs 4.57± 6.11, p<0.0001). In PA patients PCS values significantly improved at late follow up (51.02±8.04 vs 55.85±5.1, p=0.013). An improvement of MCS and DS scores was found at early and late follow-up also in controls, while no significant differences in PCS were found.

In **CHAPTER 4** we introduce our main hypothesis that nonosmotic Na^+ storage has a significant impact on sodium and water homeostasis. We have proposed to use PA, a paradigm of salt-dependent low-renin HT and the most common cause of secondary HT, as a model to investigate the changes in skin-Na^+ content in HT. Our results show and confirm that Na^+ is stored in the skin of PA subjects without apparent accompanying water retention, proving the concept that a certain amount of Na^+ is inactive from a fluid balance view-point and implying that tissue-specific regulatory mechanisms might control the release and storage of Na^+ from a kidney-independent reservoir. More interestingly, skin-Na^+ accumulation seems to be reversible after unilateral adrenalectomy but remains unchanged after medical treatment. Finally, skin-Na^+ content seems to be even higher in those PA patients who had a bilateral aldosterone hypersecretion, even if more data are needed to prove this evidence.

The present study includes also skin-K^+ data, which were not investigated so far. In this regard, skin-K^+ content seems to be similar in PA and in Controls; however, it increased significantly after adrenalectomy, suggesting a strict relation between serum K^+ and skin-K^+ levels.

Based on these observations, we postulated that Na^+ can be “non-osmotically” stored in the skin while K^+ is “osmotically” regulated and influenced by water loss or retention.

In **CHAPTER 5** we present a protocol of a multicenter study, that will be carried out by the Universities of Maastricht, Padua, and Rome, aimed to analyze blood pressure variability (BPV) and skin electrolytes and water content in patients affected by fibromuscular dysplasia (FMD), a non-atherosclerotic, non-inflammatory angiopathy of unknown cause affecting medium-sized (most commonly renal 60-75%) arteries. The hypothesis of the study is that renal FMD patients may have higher BPV markers compared to primary hypertensive subjects and that this finding might be correlate to the higher skin-sodium and -water retention.

The study consists of two main phases. In the first part of the study, we have retrospectively compared BPV markers in FMD and essential hypertensive patients.

In the second part of the study a prospective analysis on BPV profile at baseline and after angioplasty, simultaneously to the collecting data on skin-sodium and water retention, will be performed. We will prospectively evaluate the differences in terms of 24h ABPM short-term-derived BPV and skin-sodium and water content in skin biopsy, before and after specific renal artery revascularization. We will collect data on present and past medical history, current drug therapy, anthropometric evaluation (e.g. weight, height, waist circumference, BMI, systolic and diastolic blood pressure, etc.), 24-h ambulatory blood pressure monitoring, blood samples, [e.g. blood count, glycemia, lipid profile, plasma aldosterone concentration (PAC), direct renin (DRC), serum creatinine, serum ions (Na+, K+, Ca2+), 24-h urinary albumin excretion (UAE)], and angio-CT scan measurements, such as stenosis percentage and kidneys diameters. During the renal angiography, in which the endoscopic revascularization will be performed, the first skin biopsy will be performed. At the follow-up evaluation, the anthropometric and BP values, including the BPV thought 24h ABPM, and the biochemical and hormonal profile after treatment will be analyzed. In this follow-up visit a second skin biopsy will be collected to examine the feasible reduction in sodium and water storage in the skin, due to the reduction of renin–angiotensin–aldosterone system (RAAS) activity. As makers of hypertension-mediated organ damage we will use measurements of intima media thickness at Doppler ultrasonography study, the UAE, and the echocardiographic parameters, before and after renal revascularization.

The potential benefits of this study concern a better understanding of the cardiovascular risk profile and the physiopathological role of interstitial sodium in renal FMD hypertension, its correlation with organ damage and the development of new more rational and targeted treatments.

IMPACT PARAGRAPH

As Max Planck stated in 1923, “Science does not recognize national borders; its limit is simply the limit of human knowledge.” Knowledge is universal in nature, and it is critical in order to stay competitive in an ever-changing world. Academic research, the most important source of cutting-edge knowledge, along with government and industry, is the critical pedal to build a knowledge-based economy in any country. In this respect, basic research is the basis for economic innovation.

Even if they do not offer an immediate commercialized solution to cardiovascular problems, the findings presented within this thesis provide novel insights that may have potential implications for clinical practice and future research in cardiovascular diseases.

Because of the high prevalence and poor rate of blood pressure control, arterial hypertension (HT), and in particular drug-resistant hypertension (RH), is the major cause of mortality and early disability worldwide. It is a major risk factor for stroke, coronary heart disease and heart failure with an estimated cost of €169 billion in the European Union[1]. The disease burden and related costs of HT are thus substantial and call for continuous effort to control this condition. In this respect, the identification of the cause of HT and the underlying pathophysiology is crucial since it allows achieving cure of the HT, especially in younger patients, or, when this is not feasible, a better control of blood pressure and a better prevention of specific target-organ damage and cardiovascular events by a more targeted pharmacological treatment.

Previously considered a rare disease, recent prevalence studies demonstrate that PA is a very common and vastly underdiagnosed etiology of HT, particularly RH[2–4]. It is caused by inappropriately high aldosterone production, relatively autonomous of renin-angiotensin system and non-suppressible by sodium loading. Such inappropriate production of aldosterone causes HT, cardiovascular damage, sodium retention, suppression of plasma renin, and increased potassium excretion that (if prolonged and severe) may lead to hypokalemia[3]. Lack of mechanistic knowledge has impaired the development of effective preventing strategies and timely diagnostic strategies. This results in late, or even missed diagnoses with raising development of RH and cardiovascular complications.

The first objective of this thesis was to demonstrate the crucial importance of identification of PA in resistant hypertensive patients, a well-characterized subgroup of HT patients with distinct demographics, comorbidities, and metabolic abnormalities. Our findings suggest that adrenal vein sampling (AVS), the key procedure for PA subtyping, is feasible and

allows identification of unilateral PA in RH patients, a challenging PA phenotype owing to the need of multiple antihypertensive drugs potentially confounding AVS results. Moreover, AVS-guided adrenalectomy allows biochemical cure and resolution of RH in those with underlying PA, with a prominent clinical benefit in spite of severity of HT and presence of hypertension-mediated organ damage.

To further understand the positive effect of biochemical cure of PA, we assessed the impact of surgery on health-related quality of life (both in Mental and Physical components) and, for the first time, depression status of patients suffering from PA. In agreement with previous studies, we confirmed that patients with PA have an impaired health-related quality of life compared with normal population and that PA affects the quality of life by worsening the mental component and the depression status. The biochemical cure of PA by surgery improves the mental component of health-related quality of life and depression status at 1 month after adrenalectomy and at long term. In the long term, surgery determines an improvement also in the physical component of health-related quality of life of PA patients, confirming the beneficial effect of adrenalectomy.

Finally, since the pathophysiology of HT is not always clear, elucidation of the role of environmental influences, especially the role of dietary salt intake and the salt sensitivity of BP, is urgently needed. Recent studies have demonstrated that sodium and water homeostasis is far more complicated than previously assumed and emphasized the role of sodium storage and the immune system in sodium balance[5–7]. In this respect, to gain further insight into the mechanisms by which salt increases BP, we have investigated if extracellular skin Na^+- storage occurs in humans affected by PA, a suitable model to explore the changes in skin-Na^+ content in relation to aldosteronism and its surgical correction. Our results suggested that Na^+ is stored in the skin of PA subjects without concomitant water retention, suggesting that a certain amount of Na^+ is osmotically inactive and implying that tissue-specific regulatory mechanisms might control the release and storage of Na^+ from a kidney-independent reservoir. Importantly, Na^+ accumulation in the skin seems to be reversible after unilateral adrenalectomy, but not medical treatment.

The presence of a third compartment, in which sodium can be stored without concurrent water retention, is of a crucial importance in HT and even more in PA. In fact, given the currently disappointing status of blood-pressure control worldwide, which derives from both imprecise knowledge of the underlying mechanisms and the pathophysiologic diversity of hypertensive patients, it is evident that mechanistic investigation of extracellular tissue Na^+ storage, is front-of-the-edge research that can have a huge impact

from multiple standpoints, including identification of novel diagnostic and prognostic markers and more specific therapeutic targets for pharmacologic interventions.

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Dr. Torresan has presented her research to national and international conferences. She is a junior member of the European Society of Endocrine Surgery (ESES), and Junior Delegate of the Società Italiana Unitaria di Endocrinochirurgia (SIUEC).

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2018, Padova, Italy	"Triveneta Society of Surgery" Meeting, Second Best Clinical Case Award under 40 with the oral presentation "Cancro virilizzante del surrene in gravida di feto femmina: dilemmi diagnostici e terapeutici"

2017, Basel, Swiss	47th World Congress of Surgery, oral presentation "Long-Term Outcome After Surgery for Medullary Thyroid Carcinoma: A Single-Center Experience" and two posters "*Effect of unilateral adrenalectomy on the quality of life of patients with lateralized primary aldosteronism*" and "*Surgery for adrenal metastases: a single center experience*"
2017, Padova, Italy	"Triveneta Society of Surgery" Meeting, oral presentation "Una ipercalcemia sospetta"
2016, Padova, Italy	"Triveneta Society of Surgery" Meeting, oral presentation "Carcinoma midollare della tiroide"

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