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Doctoral thesis

NOVEL BIOMARKERS FOR THE MANAGEMENT OF GLUCOCORTICOID REPLACEMENT THERAPY IN PATIENTS WITH PRIMARY ADRENAL INSUFFICIENCY

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BASICS OF PHYSIOLOGY

REGULATION OF HYDRO-ELECTROLITIC METABOLISM IN HUMANS

The renin-angiotensin-aldosterone system (RAAS) and the antidiuretic system have a central role in the maintenance of hydro-electrolyte metabolism and cardiovascular regulation. They are also involved in several other processes, such as metabolism, stress, emotional disorders and inflammation. These homeostatic systems are characterized by multiple interactions, that could result in additive, synergic or antagonistic effects (1).

RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM (RAAS)

The renin-angiotensin-aldosterone system (RAAS) is a critical regulator of blood volume, electrolyte balance and systemic vascular resistance. While the baroreceptor reflex responds short term to decreased arterial pressure, the RAAS is responsible for acute and chronic alterations (2). Prorenin and renin are mainly produced by the juxtaglomerular (JG) cells, present within the afferent arterioles of the kidney, closely to macula densa (MD) cells, forming together with MD cells the iuxtaglomerular apparatus (JGA). Activation of JG cells causes the cleavage by enzymes like proconvertase 1 and cathepsin B of pre-prorenin and prorenin to renin, a 340 amino acid (aa) protein. The main stimuli able to determine renin release into circulation are a) reduction in renal perfusion perceived by the pressure transducer mechanism in afferent arterioles; b) delivery of sodium and chloride to the distal convoluted tubule (DCT); c) increased β-sympathetic flow acting through the β-1 adrenergic receptors; d) negative feedback from humoral factors like angiotensin II, vasopressin, atrial natriuretic peptide (ANP), nitric oxide (NO), prostaglandins and dopamine. Renin is the rate-limiting enzyme in RAAS (3). It is an aspartyl protease, and it acts cleaving the N-terminal of angiotensinogen, a 452 aa protein, primarily synthetised and constitutively secreted by the liver, leading to the formation of angiotensin I (AngI), a 10 aa peptide. The interaction of Ang I with angiotensin-converting enzyme (ACE) gives rise to angiotensin II (AngII), an active 8 aa peptide, that is the primary mediator of the physiologic effects of RAAS. The half-life of AngII in circulation is very short, less than 60 seconds, because it is degraded by peptidases into metabolites with lower systemic activity. ACE is a transmembrane protein that acts as peptidase. The enzyme has two subtypes (ACE and ACE2), expressed in different tissues. ACE converts AngI into AngII in the pulmonary tissue, but it also interacts with the bradykinin system to degrade bradykinin into inactive peptides. However, other metabolites produced by the action of ACE2, and other peptidases have also been described. ACE2 promotes the conversion of AngI to angiotensin 1-9 (Ang1-9) and the conversion of AngII to angiotensin 1-7 (Ang1-7). Other angiotensin molecules include Ang2-10,

derived from AngI and produced by the action of aminopeptidases. Ang2-10 gives rise to Ang2-8 (also called AngIII) and it is produced through the action of ACE. Ang3-8 (or AngIV) is produced from AngIII through the action of aminopeptidase (4).

The main physiological effects of AngII on extracellular volume and blood pressure regulation are mediated in five ways: a) vasoconstriction by contraction of the vascular smooth muscle in the arterioles; b) aldosterone secretion from the *zona glomerulosa* of the adrenal cortex; c) increase sodium reabsorption through increased activity of the Na-H antiporter in the proximal convoluted tubule (PCT); d) increasing sympathetic outflow from the central nervous system; e) release of vasopressin from the hypothalamus.

The physiological and pathophysiological effects of AngII are mainly mediated by two types of receptors: type 1 and type 2. AngII type 1 receptor (AT1-R) is a G-protein coupled receptor, widely distributed in many cell types, including heart, vasculature, kidney, adrenal glands, pituitary and central nervous system. AT-1 R is the primary target of AngII, but it also binds AngIII. In pathogenic states, the activation of the AT1-R leads to inflammation, fibrosis, oxidative stress, tissue remodelling and increased blood pressure. AngII type 2 receptor (AT2-R) is a G-protein coupled receptor, mainly expressed in fetal tissues, whose expression progressively decreases during adulthood. In adults, AT-2 R is distributed in the heart, kidney, adrenal glands, and brain, and mediates the opposing and protective effects of AngII via the AT1-R, leading to vasodilatation and natriuresis and inhibiting inflammation and fibrosis (5). AT2-R also binds Ang1-7, Ang1-9 and AngIII. Another related receptor is AT4, which binds AngIV and Ang3-7. Moreover, MAS receptors are transmembrane proteins coupled to G proteins, whose activation is able to decrease the sympathetic tone, blood pressure and fibrosis. Ang1-7 is the natural ligand of MAS receptors.

Apart from the systemic renin-angiotensin system (RAS), the modern view also includes the local (tissue) RAS, an AngII-producing system that is recognized for its role in hypertrophy, inflammation, remodelling and apoptosis (5). **Figure 1** synthetizes the main components of classical and alternative RAS.

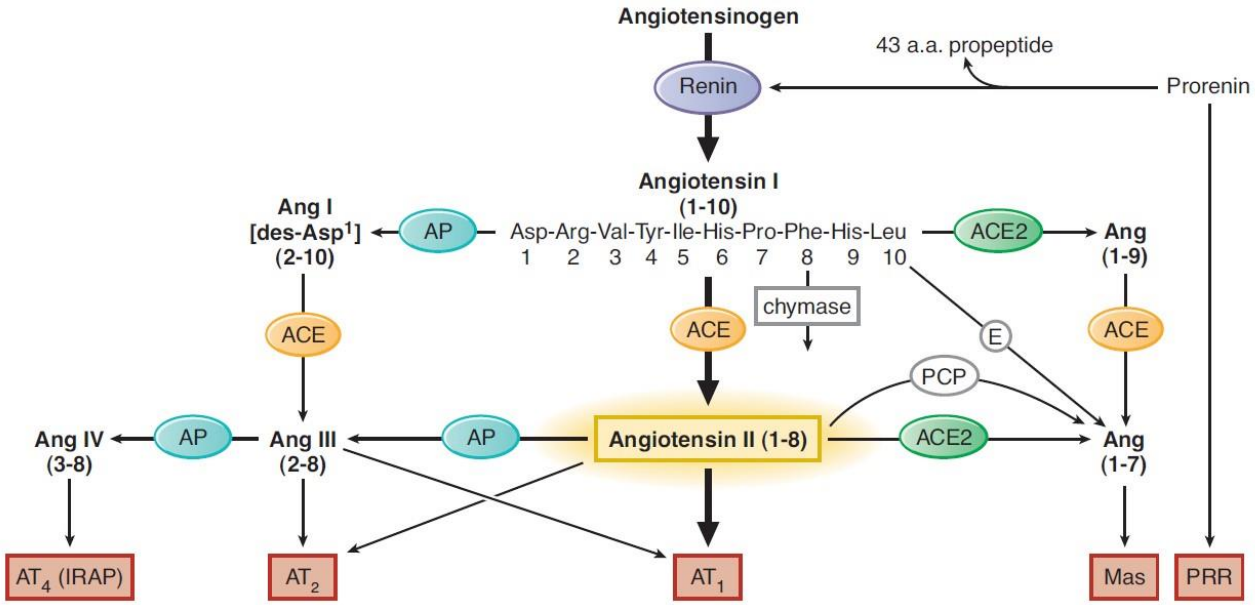


Figure 1: Components of Renin-Angiotensin system (RAS)

Abbreviations: ACE, angiotensin-converting enzyme; Ang, angiotensin; AP, aminopeptidases; E, endopeptidases; IRAP, insulin-regulated amino peptidases; PCP, prolylcarboxylpeptidase; PRR, (pro) renin receptor; AT, angiotensin receptor

From "Goodman & Gilman's. The Pharmacological Basis of THERAPEUTICHS. 12th edition"

AngII also induces the transcription of aldosterone synthase (CYP11B2) at the zona *glomerulosa* of the adrenal cortex, thus stimulating the synthesis of aldosterone, and increasing responses to other stimuli (e.g. ACTH, potassium). CYP11B2 belongs to the family of cytochrome P450 (CYP) family and shares 93% homology with its isoenzyme 11-β-hydroxylase (CYP11B1), the enzyme catalysing synthesis of glucocorticoids. Aldosterone is a mineralocorticoid, a class of steroid hormones. Aldosterone's primary effects regard electrolyte and renal homeostasis by binding to its specific receptor, namely mineralocorticoid receptor (MR), a cytosolic nuclear receptor, mainly expressed on principal epithelial cells in the renal cortical collecting duct. After the binding of aldosterone to MR, the receptor is dimerized, translocates into the nucleus, and acts as a ligand-activated transcription factor. Consequently, aldosterone enhances concentration of "epithelial sodium channel" (ENaC) at the apical membrane, resulting in increased sodium and water reabsorption. Aldosterone also activates Na-K ATPase at the basolateral membrane of apical cells, leading to sodium transport in the extracellular space and increases potassium uptake in the apical cells (6). Aldosterone also influences salt and water homeostasis by regulating thirst and salt appetite via the MRs present in various regions of the brain. Apart from these classical genomic effects, aldosterone is also able to elucidate non-genomic actions, through the activation of MR-dependent and MR-independent pathways. These biological rapid effects seem to play a significant role in the

hippocampus and the brainstem, as well as in the cardiomyocytes, endothelial cells and in the vascular smooth muscle cells (7). MRs are expressed in many organs involved in cardiovascular homeostasis: brain, heart, kidneys and vessels. The excessive activation of MRs has deleterious effects on the cardiovascular system through sympatho-excitation, elevated salt appetite, and renal retention of salt with consequent positive sodium balance, fibrosis and remodelling of the heart and arteries, as synthetized in **Figure 2**.

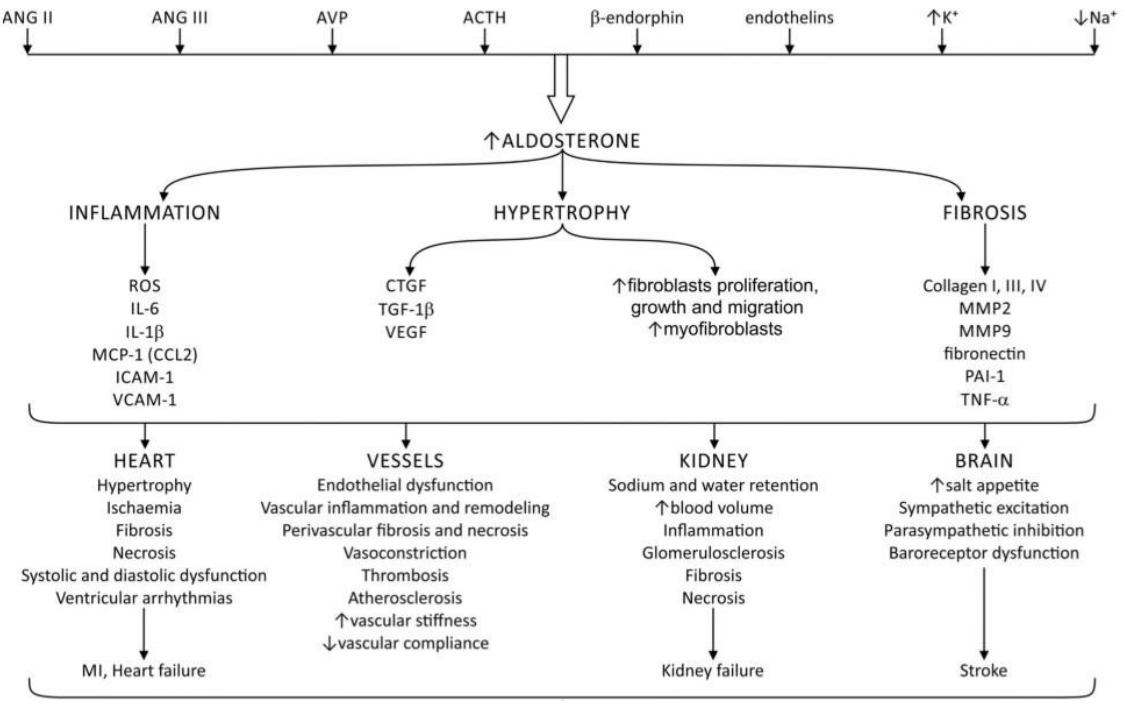


Figure 2: Role of Aldosterone in the pathological remodelling of cardiovascular system

Abbreviations: ANG, angiotensin; AVP, arginine vasopressin; ACTH, adrenocorticotropic hormone; ROS, reactive oxygen species; IL-6, interleukin-6; IL-1β, interleukin-1β; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; CTGF, connective tissue growth factor; TGF-1β, transforming growth factor-1β; VEGF, vascular endothelial growth factor; MMP2, matrix metallopeptidase 2; MMP9, matrix metallopeptidase 9; PAI-1, plasminogen activator inhibitor-1; TNF-α, tumor necrosis factor-α; MI, myocardial infarction.

From "Sztechman D, et al. Aldosterone and mineralocorticoid receptors in regulation of the cardiovascular system and pathological remodelling of the heart and arteries. J Physiol Pharmacol. 2018 Dec;69(6)"

ANTIDIURETIC SYSTEM

The hormone arginine vasopressin (AVP) or antidiuretic hormone (ADH) plays a central role in the control of body water homeostasis and related disorders. Other important functions are related to the modulation of pituitary hormonal secretion, stress, immune response and behaviour. AVP is a

neuropeptide characterized by 9 aa ring structure, synthetized by hypothalamic magnocellular neurons of the paraventricular (PVN) and supraoptic nuclei (SON), and stored in the neurohypophysis. The precursor molecule (pre-proAVP) is modified and cleaved during its course in the pituitary stalk, firstly converted into pro-AVP and, subsequently, into AVP, released in the blood with neurophisin 2 (NF2) and copeptin, in equimolar amounts. NF2 seems to act as a carrier protein, while copeptin as a chaperone protein, allowing the correct folding of the AVP precursor (8). **Figure 3** points out the structure of the precursor molecule.

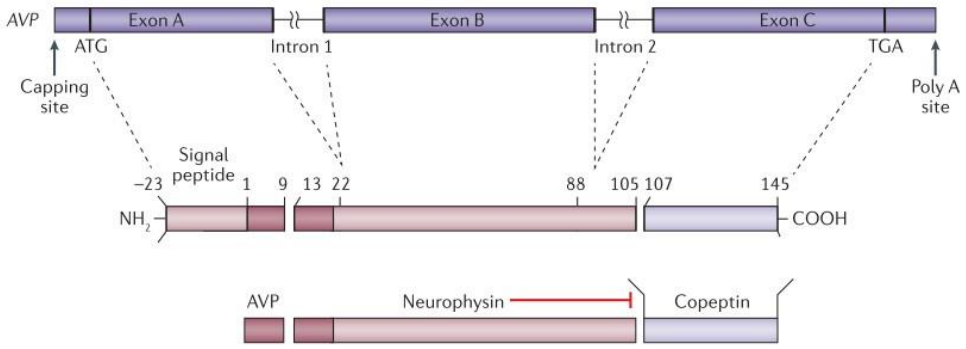


Figure 3: Arginine Vasopressin (AVP) and its protein product

From "Christ-Crain M, Fenske W. Copeptin in the diagnosis of vasopressin-dependent disorders of fluid homeostasis. Nat Rev Endocrinol. 2016 Mar;12(3):168-76"

AVP has a very short biological half-life (about 3 minutes), and it is cleared mostly by filtration in the kidneys. Moreover, the plasma level of AVP in the usual range is very low, and even the best assays are unable to quantify AVP in the low range of physiological values (9). The main stimuli for AVP release are the increase in plasma osmolality (p-Osm) and the reduction in the effective circulating volume (ECV), whereas hypoosmolality and plasma volume expansion inhibit its secretion (8). The control of p-Osm is guaranteed by the presence of osmoreceptors, strategically located in the central nervous system at the level of PVN and SON. Other osmoreceptors are present in the mesenteric and portal vessels. Instead, receptors located at the level of the carotid sinuses, aortic arch, cardiac atria, and pulmonary veins are responsible for controlling the ECV. There are many other secretory and inhibitory stimuli involved in the control of AVP secretion: AngII and endothelin-1 act in a stimulatory way, while atrial natriuretic peptide (ANP) as inhibiting factor. AVP receptors are expressed in many organs and tissues, as shown in **Figure 4**.
Three different receptors subtypes have been described: a) V2 receptors (V2R) whose effect is mediated by cyclic AMP (cAMP); b) V1a and V1b receptors (V1aR and V1bR, also called V3R)

whose activation are mediated by calcium signals. Kidney is one of the most important target organs for AVP effect. V2R are localized in the principal cells of the collecting duct (CD) and, to a lesser extent, in the thick ascending limb. Along the entire CD, vasopressin increases water permeability by promoting the insertion of aquaporin 2 (AQP2) in the luminal membrane of the CD cells, as well as AQP2 synthesis, allowing an increase in water absorption. Moreover, AVP exerts two other effects on the CD through V2R: a) in the cortical and outer medullary CD, it stimulates sodium reabsorption through the luminal sodium channel ENaC; b) in the terminal inner medullary CD, AVP increases the permeability to urea by activating the facilitated urea transporters UT-A1 and UT-A3. However, V1aR, whose activation in the kidney requires higher AVP concentration, partially counteract the V2R-mediated effects. Moreover, V1aR are expressed a) in the liver, with stimulation of multiple metabolic processes, such as glycogenolysis, gluconeogenesis and ureagenesis; b) in the vascular smooth cells, with the activation of vasoconstriction; c) in many other tissues, like platelets, adipocytes, adrenal glands and brain. Instead V2R are also expressed in the endothelial cells, where they mediate the release of von Willebrand factor (vWF) and factor VIII. V1bR are predominantly present in corticotroph cells of the anterior pituitary for the stimulation of ACTH release in response to chronic stress (10).

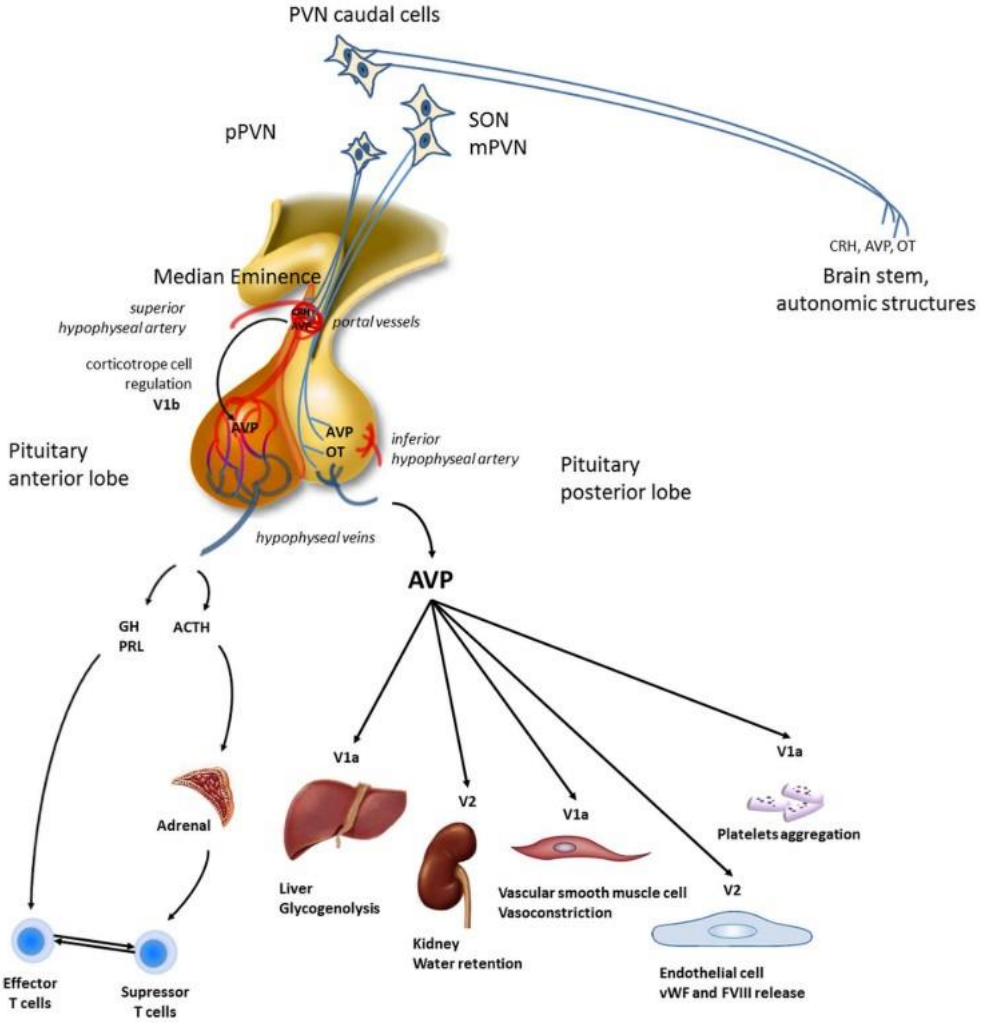


Figure 4: The spectrum of Arginine Vasopressin (AVP) pathophysiological effects and its central regulation

Abbreviations: PVN, paraventricular nuclei; SON, supraoptic nuclei; mPVN, magnocellular nuclei; pPVN, parvocellular nuclei; AVP, arginine vasopressin; CRH, corticotrophin releasing hormone; ACTH, adrenocorticotrophin hormone; GH, growth hormone; PRL, prolactin; OT, oxytocin; V1a, V1b, V2, AVP receptors.

From "Rotondo F, et al. Arginine vasopressin (AVP): a review of its historical perspectives, current research and multifunctional role in the hypothalamo-hypophysial system. Pituitary. 2016;19(4):345-55"

For the aforementioned reasons, AVP measurement is characterized by many critical issues, making it unreliable, mainly because of the high pre-analytic variability of the sample, together with the long laboratory processing time (11). So, research aimed to look for a more stable molecule reflecting AVP concentration. Copeptin, also known as the C-terminal part of pre-provasopressin (CT-proAVP), is 39-aa glycopeptide with leucine-rich core segment, that responds as rapidly as AVP to osmotic, haemodynamic, and stress-related stimuli (12). Since Morgenthaler et al (13) described an assay for copeptin, it becomes a surrogate marker for AVP production, both in healthy and ill

subjects, due to its stoichiometric synthesis with AVP. Copeptin itself has a role in the proteolysis of pre-proAVP, through its interaction with the calnexin/calreticulin system, involved in the monitoring of protein folding. However, its main function in the circulation is still unknown. Concerning elimination, copeptin is rapidly cleared from the blood stream, both inactivated by tissue-bound proteases (14) and directly eliminated via the kidney. In fact, in patients affected by chronic kidney disease plasma levels of copeptin and AVP are inversely correlated to the estimated glomerular filtration rate (eGFR). Nevertheless, copeptin values are also chronically elevated in patients affected by the autosomal dominant form of polycystic kidney disease (ADPKD) and by nephrogenic diabetes insipidus (DIN), due to the alteration of the architecture of the renal medulla in the former and a peripheric mechanism of AVP resistance in the latter. The main advantages of copeptin measurement are its higher ex-vivo stability, elevated sensitivity, longer half-life, no significant circadian rhythm, and the lack of pre-analytical procedures required (11,13). Roussel et al. (15) have recently confirmed a good correlation between copeptin and vasopressin in a large study of general population. Moreover, there is growing evidence concerning the potential prognostic and diagnostic role of copeptin in both cardiovascular and non-cardiovascular conditions (16,17), as summarized in **Table 1**.

Disease	Diagnostic biomarker	Prognostic biomarker
Autosomal dominant polycystic kidney disease	+	–
Acute myocardial infarction	+	+
Heart failure	+/–	++
Hypertension	–/+	–
Preeclampsia	+/–	+
Stroke	–	++
Aneurysmal subarachnoid hemorrhage	–	+/–
Intracerebral hemorrhage	–	+/–
Diabetes mellitus	–	++
Metabolic syndrome	–	++
Lower respiratory tract infections	+	–
Community acquired pneumonia	+	+
Ventilator associated pneumonia	–	+
Chronic obstructive pulmonary disease	–	+
SIADH	+	–
Diabetes insipidus	+	–
Acute pancreatitis	+/–	++

Table 1: Potential role of Copeptin as diagnostic and prognostic marker.

Abbreviations: SIADH, syndrome of inappropriate antidiuretic hormone hypersecretion.

From "Łukaszyk E, et al. Copeptin: Pathophysiology and potential clinical impact. Adv Med Sci. 2015 Sep;60(2):335-41"

In more detail, copeptin has been proposed as an unspecific marker potentially useful in the first phase of myocardial infarction, in which other conventional biomarkers are still undetectable, due to its early increase as a consequence of drop in cardiac output as well as of endogenous stress (18). Similarly, some authors have evaluated copeptin as a prognostic marker in hearth failure. Stoiser at al (19) showed that copeptin was a novel excellent predictor of outcome in advanced heart failure patients, even superior of brain natriuretic peptide (BNP) and N-terminal pro-BNP (NT-proBNP) in the prediction of death and cardiovascular events. Based on the assumption that copeptin is able to correlate with vasopressin concentrations in healthy subjects during iso-, hypo-, and hyperosmolar states (20), in the last years, several authors have highlighted the utility of copeptin measurement in the differential diagnosis of water metabolism disorders. Specifically, literature data suggested the evaluation of stimulated copeptin as a new suitable tool in the differential diagnosis of polyuria-polydipsia syndrome (21,22) and further, a recent multicentre study demonstrated that copeptin could be a promising predictor of post-surgical central diabetes insipidus (23).

REGULATION OF HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS IN HUMANS

The HPA axis is the major neuroendocrine system regulating homeostasis and coordinating adaptive response of the organism to stressors (24), playing a central role in the dynamic interactions between neuroendocrine and immune systems (25). Under basal conditions, the HPA axis exhibits both a circadian and ultradian rhythm of hormonal secretion (26). The central circadian oscillator is the hypothalamic suprachiasmatic nucleus (SCN), and its predominant input is the light-dark cycle. The main regulator of glucocorticoid secretion is the corticotropin releasing hormone (CRH), a neuropeptide released from the parvocellular neurons of hypothalamic PVN. CRH, through its binding to G-protein coupled receptor (corticotroph releasing hormone receptor 1 – CRHR1-) on corticotroph cells of adenohypophysis, controls the synthesis and post-translational changes of pro-opiomelanocortin (POMC), the precursor molecule of adrenocorticotrophic hormone (ACTH), leading to ACTH release. In addition to CRH, AVP, also released by PVN, acts through its V1bR in the pituitary corticotroph cells, leading to ACTH secretion without an effect on POMC transcription. Therefore, AVP is a potent synergic factor with CRH, although with little ACTH secretagogue activity alone (25). Catecholamines and neuropeptide Y (NPY) seem to have a stimulatory effect on CRH secretion, and this interaction highlights the close relationship between HPA axis and the sympathetic adrenergic system (25). ACTH is a polypeptide hormone that, released in systemic circulation, reaches adrenal cortex acting on melanocortin thype-2 receptors (MC2R) in the *zona fascicolata* of the adrenal gland. Consequently, it induces increased cholesterol biosynthesis, activation of steroidogenic pathway and cell proliferation, leading therefore to

synthesis and release of glucocorticoids, mainly cortisol in humans (24). Glucocorticoids are the final effectors of the HPA axis, participating in the control of whole-body homeostasis and of the organism's response to stress. Moreover, glucocorticoids are involved in many other physiological functions, including glucose, fat, and protein metabolism, as well as in inflammatory response, mood and cognitive function. Glucocorticoids also regulate the activity of the HPA axis, and thus their own production, through negative feedback mechanisms, acting at the level of the pituitary gland where they inhibit ACTH release and at the level of the hypothalamic PVN where they inhibit the synthesis and release of CRH and AVP (24). In addition, glucocorticoids indirectly regulate HPA axis activity via modulation of other brain structures, including the hippocampus, the amygdala, and the prefrontal cortex, which in turn regulate the activity of the PVN (26). Their effects are mediated by their ubiquitous cytoplasmic receptors, belonging to the superfamily of nuclear receptors. On ligand binding, the glucocorticoid receptors translocate into the nucleus, where they interact as homodimers with specific glucocorticoid responsive elements (GREs) within the DNA to activate appropriate hormone-responsive genes. Two receptors for glucocorticoids are known: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). The affinity of cortisol for MR is approximately 10-fold higher than the affinity for GR. GR is ubiquitously expressed in the periphery and in the brain, while the distribution of MR is more localized to specific organs, such as kidney and heart. The specificity of MR for aldosterone is guaranteed by the pre-receptorial metabolism of cortisol by the enzyme 11β- hydroxysteroid dehydrogenase type 2 (11-βHSD2) that converts cortisol in cortisone, thus preventing its binding to MR (27). In addition to their genomic effects, glucocorticoids could also modulate cellular activity through rapid nongenomic effects, mediated by G-protein coupled membrane-associated receptors. The inhibition of HPA axis at the level of the pituitary and of the hypothalamus seems to be related to a rapid nongenomic effect. Cortisol has a distinct circadian rhythm, characterised by a constant and reproducible pattern under stable physiological conditions. Cortisol reaches the lowest level around midnight. Then its levels start to rise at 2.00-3.00 a.m. and reach a pick in the morning around 8.00-9.00 a.m. after waking. Following this, the levels slowly decrease back to the nadir.

Apart from the physiological HPA axis function, under stressful conditions, HPA axis activity increases as the results of different afferent neural pathways, mainly from limbic system or encephalic trunk (28), as shown in **Figure 5**. Acute stress efficiently drives HPA stress response, and feedback mechanisms effectively terminate the response after the stressor drops. Generally, the HPA response begins with a pulse of ACTH, whose duration depends on type of stimulus and feedback. Cortisol responds slowly and lasts substantially longer. Instead, chronic stress exposure

causes marked changes in both baseline HPA function and responsiveness to stress that are long-lasting in nature and invoke different regulatory mechanisms.

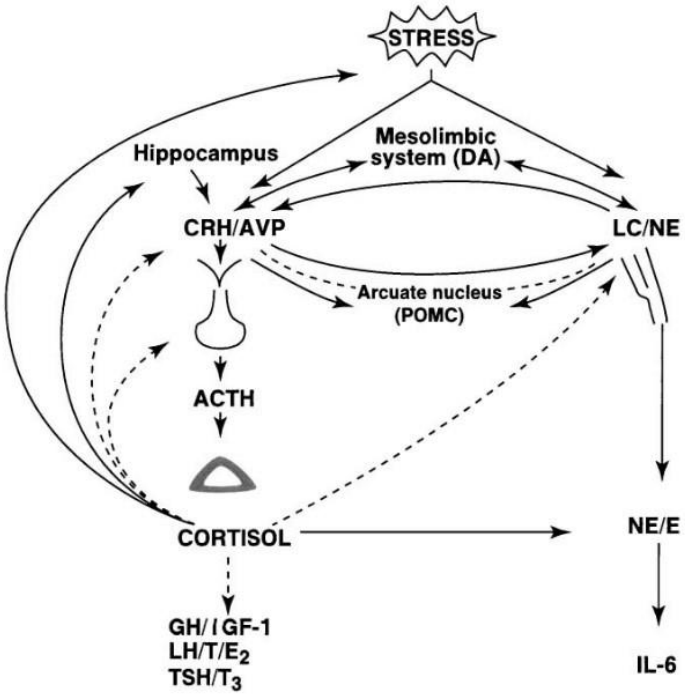


Figure 5: A schematic representation of the interrelations of the central and peripherical components of stress system.

Abbreviations: CRH, corticotropin releasing hormone; AVP, arginine vasopressin; ACTH, adrenocorticotropic hormone, POMC, pro-opiomelanortin; LC, locus coeruleus; NE, norepinephrine; E, epinephrine; IL-6, interleukin-6; GH, growth hormone; IGF-1, insulin-like growth factor 1; LH, luteinizing hormone; T, testosterone; E, estradiol; TSH, thyroid stimulating hormone; T3, triiodothyronine.

From "Tsigos C, et al. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress.J Psychosom Res. 2002 Oct;53(4):865-71"

PRIMARY ADRENAL INSUFFICIENCY (PAI)

DEFINITION AND ETIOPATHOGENESIS

Primary adrenal insufficiency (PAI) is a chronic disease characterized by insufficient production of glucocorticoids, mineralocorticoids and adrenal androgens due to the failure of the adrenal cortex to produce these hormones in sufficient amounts (29–31). PAI is also namely Addison disease (AD), after the description of eleven cases published by Dr. Thomas Addison of Gut's Hospital in London in 1856 (32). Although it is a rare disease, recent epidemiological studies showed that the incidence of AD is increasing, probably because of greater awareness of the disease, better diagnostic tools,

and longer life expectancy. In Europe, the estimated prevalence has doubled from 40–70 cases per million in the 1960s to the current estimate of 80–136 cases per million (30), higher in northern Europe than in central and southern Europe. Moreover, population studies have suggested that the estimated prevalence of PAI by 2030 will be between 220 and 285 per million per year (33). As summarized in **Table 2**, nowadays the most common cause in the Western countries is autoimmune adrenalitis (80-90% of cases), followed by tuberculosis and other infectious diseases (e.g. HIV/AIDS, candidiasis, histoplasmosis, CMV, and others) and metastatic malignant disease in about 10% of cases. Other rarer causes include bilateral adrenalectomy, genetic diseases (e.g. congenital adrenal hyperplasia, congenital adrenal hypoplasia, adrenoleukodystrophy in males, and others), adrenal haemorrhage (e.g. Waterhouse-Friderichsen Syndrome in sepsis) and drug-related adrenal insufficiency (e.g. ketoconazole, mitotane, metyrapone, and others) (34). Moreover, PAI could be related to various immune checkpoint inhibitors, a class drug widely used in the treatment of different types of malignant neoplasms (35). About 40% of autoimmune AD is isolated, with slight prevalence in males, while around 60% of cases presents as a part of autoimmune polyendocrine syndromes (APS), more frequently in females.

Etiology	Associated Features
Autoimmune	
Isolated	Not associated with other autoimmune disorders
APS type 1 (APECED)	Chronic cutaneous candidiasis, hypoparathyroidism
APS type 2	Autoimmune thyroid disease, type 1 diabetes
Adrenal—infiltration/injury	
Adrenal hemorrhage	Associated with sepsis, anticoagulants, anti-cardiolipin/lupus anti-coagulant syndrome
Adrenal metastases	Malignancies: lung, breast, colon, melanoma, lymphoma
Infections: adrenalitis	Tuberculosis, HIV/AIDS, CMV, candidiasis, histoplasmosis, syphilis, African trypanosomiasis, paracoccidioidomycosis (eg, in South America)
Infiltration	Hemochromatosis, primary amyloidosis
Bilateral adrenalectomy	Procedure for intractable Cushing's syndrome or bilateral pheochromocytoma
CAH: most forms can cause salt loss	Commonest cause of PAI in children (80%); may be diagnosed in older individuals
21-Hydroxylase deficiency	Commonest type of CAH is 21-hydroxylase deficiency, with associated hyperandrogenism
11β-hydroxylase deficiency	Hyperandrogenism, hypertension (in older children and adults)
3β-hydroxysteroid dehydrogenase II deficiency	Ambiguous genitalia in boys, hyperandrogenism in girls
P450 side-chain cleavage deficiency (CYP11A1 mutations)	XY sex reversal
P450 oxidoreductase deficiency	Skeletal malformations, abnormal genitalia
Congenital lipoid adrenal hyperplasia (StAR mutations)	XY sex reversal
Adrenal hypoplasia congenita	X-linked NROB1, Xp21 deletion (with Duchenne's muscular deficiency), SF-1 mutations (XY sex reversal), IMAGe syndrome
ACTH insensitivity syndromes	Type 1: ACTH receptor, melanocortin 2 receptor gene MC2R Type 2: MRAP Familial glucocorticoid deficiency (MCM4, NNT, TXNRD2) TripleA (Allgrove's) syndrome, achalasia, Addison's disease, alacrima, AAAS gene mutation
Drug-induced	Adrenal enzyme inhibitors: mitotane, ketoconazole, metyrapone, etomidate, aminoglutethimide, drugs that may accelerate cortisol metabolism and induce adrenal insufficiency T_4 also accelerates cortisol metabolism (at least in part through stimulation of 11β-HSD2) CTLA-4 inhibitors may enhance autoimmunity and cause PAI
Other metabolic disorders	Mitochondrial disease (rare) Adrenoleukodystrophy in males Wolman's disease

Table 2: Major aetiologies of PAI.

Abbreviations: APECED, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy; CMV, cytomegalovirus; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CYP, cytochrome P; HSD, hydroxysteroid dehydrogenase; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; IMAGe, intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia congenital, genital abnormalities; MC2R, melanocortin 2 receptor gene; MCM4, minichromosome maintenance-deficient 4; MRAP, melanocortin receptor accessory protein; NNT, nicotinamide nucleotide transhydrogenase; StAR, steroidogenic acute regulatory protein; TXNRD2, thioredoxin reductase 2.

From "Bornstain SR, et al. Diagnosis and Treatment of Primary Adrenal Insufficiency: An Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab, 2016;101(2):364-89"

Autoimmune polyendocrine syndromes (APS)

Autoimmune polyendocrine syndromes comprise a group of clinical conditions characterized by functional impairment of multiple endocrine glands due to loss of immune tolerance. These syndromes also frequently include conditions such as alopecia, vitiligo, celiac disease, and

autoimmune gastritis with vitamin B12 deficiency that affect nonendocrine organs (36). Autoimmune AD is a part of APS type 1, type 2 and type 4.

APS type 1 (APS-1)

APS-1, also named autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) is a rare autosomal recessive disease caused by mutations in the autoimmune regulator gene (AIRE). The estimated prevalence is roughly 1:80000 in most countries, with a higher prevalence in some countries such as Finland (1:25000) and Sardinia (1:14000) and among Persian Jews living in Israel (1:9000). APS-1 is characterized by the development of at least two of the three cardinal components during childhood: chronic mucocutaneous candidiasis, hypoparathyroidism and AD. Patients could also manifest other autoimmune diseases, such as premature ovarian insufficiency (POF), alopecia, chronic hepatitis and atrophic gastritis.

APS type 2 (APS-2)

APS-2 or Schmidt syndrome is a polygenic disorder, characterized by the development of at least two of the following three endocrinopathies: type 1 diabetes mellitus (in 69-82% of cases), autoimmune thyroiditis (in 30-52% of cases), and AD (in 100% of cases). The onset of APS-2 typically appears later than APS-1, in young adulthood, with a predominance in female gender. Many affected patients develop other autoimmune conditions, including celiac disease, alopecia, vitiligo, POF and pernicious anemia. APS-2 is a rare disease, with a prevalence of around 1.4-2 cases/100000 subjects. The pathogenesis of APS-2 is not completely clear. However, the available evidence hypothesizes an interaction between genetic and environment factors. Among many involved genes, those of the major histocompatibility system (HLA) and some HLA-related genes (TNF, MIC-A and CTLA-4) seem to play a central role in the pathogenesis.

APS type 4 (APS-4)

APS-4 is defined by the presence of at least two autoimmune manifestations not included in the other types of APS. Therefore, APS-4 could comprise forms of AD associated with other autoimmune diseases, different from thyroid disease and DM1, such as hypergonadotropic hypogonadism, atrophic gastritis, pernicious anemia, celiac disease, myasthenia gravis, vitiligo and alopecia.

PHYSIOPATHOLOGY AND NATURAL HISTORY

In autoimmune Addison disease (ADD), adrenal histology shows lymphocytic infiltration of all the layers of the adrenal cortex with plasma cells, macrophages and fibrosis. Islets of regenerating adrenocortical cells are also found. Immunohistochemistry shows infiltration by activated T lymphocytes. The adrenal medulla is spared. AAD is a T cell-mediated pathological condition due to cytotoxic effects of CD8+ T lymphocytes infiltration, rather than an autoantibody-mediated disease. The most relevant autoantibodies involved in AAD are adrenal cortex autoantibody (ACA) and autoantibody against 21-idrossilase (21-OHAbs). ACA and 21-OHAbs have good sensitivity and specificity, being positive in the 81% of patients with AAD, and in 0.013% of subjects with normal adrenal function. 21-OHAbs are excellent markers of adrenal autoimmunity; however, they do not appear to be involved in the pathogenesis of AAD. For example, although 21-OHAbs inhibit 21-OH enzyme activity in vitro, they do not have an effect in vivo. The presence of these autoantibodies is also correlated with an increased risk of developing adrenal insufficiency in patients with APS. This risk seems to be related to antibody titre, patient's age and type of coexisting autoimmune disease. Progression rate towards clinical form of PAI in subjects with ACA positivity is around 48.5%, further increased in case of infantile age, male gender, high antibody titre, and association with hypoparathyroidism and/or candidiasis. Moreover, in AAD, hypothetical pathogenic environmental factors (viral infections, stress, cigarette smoking, pollutants or other not yet defined agents) have been postulated (37). Concerning natural history, AAD is characterized by a long prodromal period marked by the presence of ACA/21-OHAbs. Therefore, we can distinguish five stages of adrenal impairment, as shown in **Figure 6**. Stage 0 is characterized by a normal response to ACTH (potential AAD). Stage 1 is revealed by high plasma renin levels alone. Stage 2 is characterized by increased renin, low aldosterone, normal basal cortisol and ACTH levels, but low cortisol response in ACTH stimulation test. Stage 3 is indicated by elevation of ACTH with normal/low basal cortisol levels. Finally, stage 4 is denoted by very high renin and ACTH levels with markedly low cortisol and aldosterone levels, and it is associated with the overt symptoms of adrenal failure. These observations suggest that the *zona glomerulosa* is the most susceptible to the autoimmune damage. The *zona fasciculata* is damaged later, probably protected by local release of glucocorticoids or by its greater thickness.

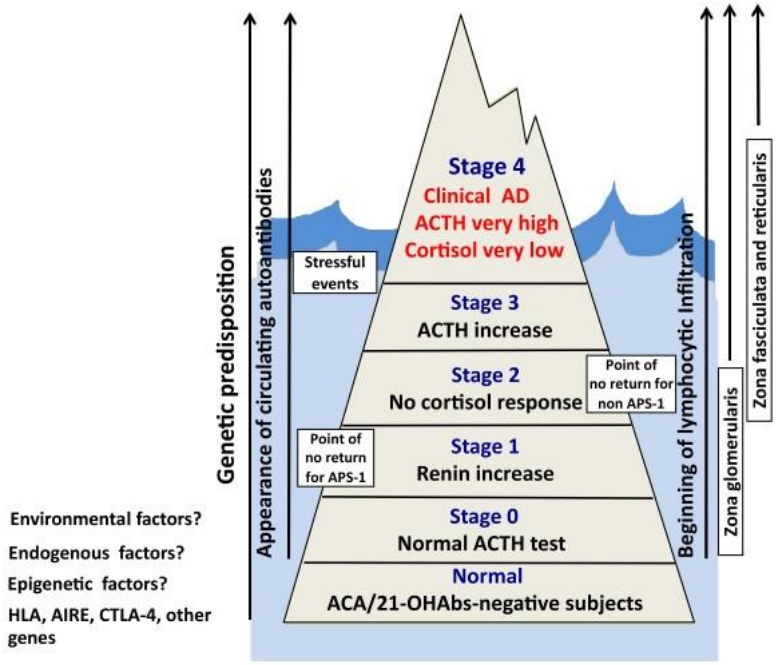


Figure 6: The natural history of autoimmune Addison disease (AAD)

Abbreviations: HLA, human leukocyte antigen; AIRE, AutoImmune Regulator; CTLA-4, cytotoxic T-lymphocyte antigen-4; APS, autoimmune polyendocrine syndrome; AD, Addison disease; ACTH, adrenocorticotrophic hormone; ACA, adrenal cortex autoantibody; 21-OHAbs, autoantibodies against 21-idrossilase

From "Betterle C, et al. Epidemiology, pathogenesis, and diagnosis of Addison's disease in adults. J Endocrinol Invest. 2019;42(12):1407-1433"

CLINICAL MANIFESTATIONS

Clinical signs and symptoms of PAI are often nonspecific and, therefore, the disease could by misdiagnosed for many years. In fact, about 60% of affected individuals are seen by various clinicians before the diagnosis is made (29,38). Typical manifestations are weakness and fatigue, weight loss, with failure to thrive in children, orthostatic hypotension and tachycardia due to dehydration, skin and mucosal hyperpigmentation, nausea, vomiting, diarrhea or recurrent abdominal pain, amenorrhea or libido reduction, depression, and salt-craving. In infants PAI often presents with seizures following hypoglycemic crisis. Rarely PAI could manifest as potentially life-threatening acute adrenal insufficiency or Addisonian crisis (29,30). The most frequent symptoms of adrenal crisis are malaise, fatigue, hypotension, nausea, vomiting, abdominal pain mimicking acute abdomen, muscle pain, mental confusion, somnolence, until hypovolemic shock and coma.

Typical laboratory findings are hypoglycemia, low plasma sodium and increased potassium levels, high plasma calcium concentrations, pre-renal insufficiency with high urea and creatinine.

DIAGNOSIS

The diagnosis of PAI requires two steps. First, the function of the adrenal cortex should be assessed. Once PAI is confirmed, it is mandatory to establish the aetiology. In case of suspected adrenal crisis, immediate therapy is mandatory, prior to the availability of the results of diagnostic tests (39,40). In most cases, the diagnosis is highly likely if the morning cortisol (between 6.00 and 10.00 a.m) is < 140 nmol/L (5 μg/dL) in combination with a plasma ACTH concentration elevated more than 2-fold above the upper limit of the reference interval for the specific assay (39,40). Plasma cortisol is 80% bound to cortisol-binding globulin (CBG) and 10–15% to albumin, so disorders that reduce (inflammation, rare genetic disorders) or increase (estrogens, pregnancy, mitotane) CBG levels need to be considered in interpretation of plasma cortisol levels. For confirmation, if basal tests are not unequivocal, a stimulation test should be performed. The conventional ACTH test, performed by an intravenous injection of 250 μg of synthetic corticotropin in adults (tetracosactide), is the gold standard for assessing adrenal function. A peak cortisol concentration of less than 500 nmol/L (18 μg/dL) at 30–60 minutes after ACTH administration is considered diagnostic for adrenal insufficiency. Another variation of the cosyntropin test uses a low-dose 1 μg of tetracosactide for adrenal stimulation. However, based on the currently available data, the 1 μg corticotropin test does not provide better diagnostic accuracy than the 250 μg corticotropin test in PAI (39).

The simultaneous measurements of plasma renin and aldosterone levels have a diagnostic role especially in the first phase of PAI, in which the presence of mineralocorticoids deficiency may predominate. The finding of elevated renin plasma levels in combination with inappropriately normal or low aldosterone concentration, is suggestive of PAI. Concerning laboratory abnormalities, adrenal insufficiency could be associated with anemia, lymphocytosis, eosinophilia, hypercalcemia and increased transaminase levels. Thyroid-stimulating hormone (TSH) levels may be slightly increased, because of the lack of the inhibitory effect of cortisol on TSH production or due to coexistent hypothyroidism with positive thyroid autoantibodies.

When PAI is confirmed at hormonal evaluation, the aetiology should be established. In adults the measurements of ACA and/or 21-OHAbs should be performed. In contrast, children should be first screened for baseline serum 17-hydroxyprogesterone (17-OHP) levels. ACA/21-OHAbs-negative young or adult males with PAI and normal 17-OHP should be tested for very long-chain fatty acids

(VLCFA) for adrenoleukodystrophy. Adrenal imaging is required for patients with PAI negative for ACA/21OHAbs. The algorithm for diagnostic workup is represented in **Figure 7**.

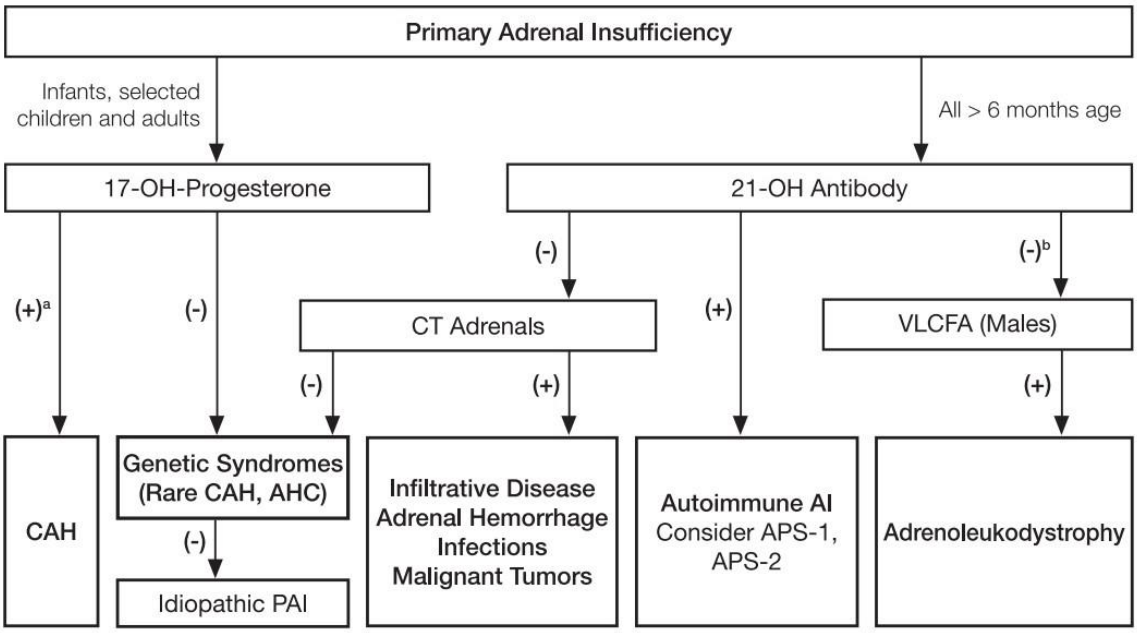


Figure 7: Algorithm for the diagnostic approach to primary adrenal insufficiency (PAI)

Abbreviations: CT, computed tomography; VLCFA, very long-chain fatty acids; CAH, congenital adrenal hyperplasia; AHC, adrenal hypoplasia congenital; AI, adrenal insufficiency; APS, autoimmune polyendocrine syndromes.

From "Bornstain SR, et al. Diagnosis and Treatment of Primary Adrenal Insufficiency: An Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab, 2016;101(2):364-89"

THERAPY

If untreated, PAI is a lethal condition and, before the availability of synthetic steroid hormones, most patients with PAI died within two years from diagnosis. The milestone of PAI treatment is based on glucocorticoids (GCs) and mineralocorticoids (MCs) replacement therapy, while androgen substitution is still debating.

GLUCOCORTICOID RAPLACEMENT THERAPY

Primary aim of GC replacement therapy is to reproduce as closely as possible the physiological circadian pattern of cortisol, avoiding both the supraphysiological peaks in the immediate post-treatment phase and the subsequent rapid decline (41,42). Several studies have demonstrated that, although properly managed, GC therapy is not able to mimic the physiological cortisol secretion rhythm (42).

The 2016 Endocrine Society Guidelines and the 2020 Italian Position Statement (39,43) recommend the use of short half-life GCs, such as hydrocortisone (HC) and cortisone acetate (CA). Instead, longer half-life GCs, such as prednisone or prednisolone, could be used only in selected cases, avoiding dexamethasone, due to the risk of Cushingoid side effects (39,43). The most common replacement regimen is based on immediate-release HC (15-25 mg daily), or CA (20-35 mg daily) divided into two or three doses. The highest dose should be given in the morning at awakening, the next either in the early afternoon (2 hours after lunch; two-dose regimen) or at lunch and afternoon (three-dose regimen). If a three-dose daily regimen is chosen, the last dose should be administered 5–6 hours before bedtime, to avoid an overexposure to cortisol during the night (43). Improved assay techniques have demonstrated that the normal daily production of cortisol is around 5–6 mg/m2 body surface area (BSA), lower than once estimated. Consequently, the current recommendations for oral replacement doses of HC are also lower, at 10–12 mg/m2 BSA (39,43). HC is an active GC that, thanks to its high intestinal permeability, has an oral bioavailability of around 96%, reaches peek plasma concentration within one hour after administration, but, due to its short half-life (approximately 90 minutes), is detectable in plasma only for about two hours. Instead, CA is an inactive precursor GC that requires activation via hepatic 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) enzyme before exerting biological activity. For this reason, therapy with CA may result in broader interindividual variability of pharmacokinetics parameters. Compared to HC, CA shows a lower serum cortisol peak and possible delayed clearance of cortisol. CA has a GC activity 0.8 times that of HC. For these reasons, both HC and CA are not able to perfectly mirror the endogenous cortisol rhythm (43). Therefore, scientific research has focused on the study of novel preparations. The dual-release hydrocortisone (DR-HC, Plenadren®) is a preparation characterized by an immediate-release coating combined with an extended-release core to obtain a more natural cortisol exposure-time profile and improve the outcome of GC replacement therapy (44). Cortisol exposure in term of "area under the curve" with DR-HC is 20% lower to that obtained with conventional HC. The first multicentric prospective randomized trial showed that once-daily DR-HC provided a more circadian-based cortisol profile, with reduced body weight, blood pressure and improved glucose metabolism compared to thrice-daily immediate HC (45). These results were confirmed in a more recent study performed by Quinckler et al (46) on patients affected by primary and secondary adrenal insufficiency. The authors found that DR-HC therapy was associated with reduction in body mass index (BMI) and glycated haemoglobin (HbA1c) and improvement in health-related quality of life (HR-QoL). Similar findings were obtained in an Italian prospective study performed by Giordano et al (47) with the demonstration that in AD DR-HC could be more effective than conventional HC in reducing central adiposity, improving glucose and

lipids metabolism, as well as quality of life. Moreover, a recent randomized controlled trial conducted on AD patients documented that DR-HC was also associated with a better immune cell response and consequently lower recurrent infections (48). Chronocort® is another modified release HC currently under development. It was designed to mimic the overnight rise in cortisol levels, delaying and sustaining HC absorption. A twice daily regime (15-20 mg at 11. p.m. and 10 mg at 7.00 a.m.) could reproduce physiological cortisol levels (49). Its use is under study for patients with congenital adrenal hyperplasia (CAH). A phase II study with patients with CAH treated with a single dose of 30 mg Chronocort® demonstrated a single cortisol peak at 6.00 a.m. and 17OH-progesterone (17OH-P) concentrations significantly lower than those observed in patients treated with conventional HC preparation. However, high concentrations of 17OH-P were observed in the afternoon, which confirms the need for a second smaller dose in the morning (50).

Figure 8 offers a schematic representation of the different pharmacokinetics properties of some GCs.

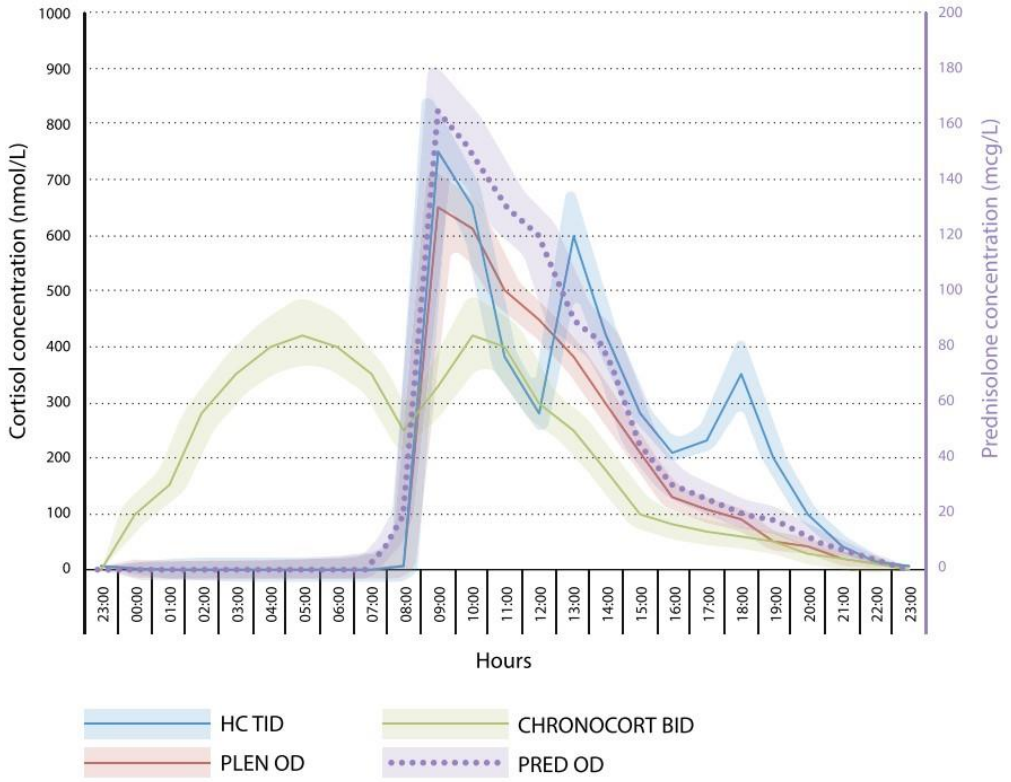


Figure 8: Schematic representation of cortisol levels in relation to different type of GCs.

Abbreviations: HC, hydrocortisone; PLEN, plenadren; PRED, prednisone; TID, thrice-daily; BID, twice-daily; OD, once-daily

From "Isidori AM et al. Towards the tailoring of glucocorticoid replacement in adrenal insufficiency: the Italian Society of Endocrinology Expert Opinion. J Endocrinol Invest. 2020 May;43(5):683-696"

A novel strategy of administration of GCs therapy is provided using infusion pumps for subcutaneous deliver of HC. This infusion could reconstitute normal cortisol serum levels and reproduce circadian rhythm, thus allowing a significative reduction of total daily dosage in most patients. However, at present, it is exclusively experimental and requires larger clinical studies before to be included among the therapeutic strategies in clinical settings for patients poorly controlled with conventional therapy (51).

MINERALOCORTICOID REPLACEMENT THERAPY

Mineralocorticoid replacement therapy is based on the use of synthetic mineralocorticoid 9α-fludrocortisone at starting dose of 50-100 μg/daily in adults, without any salt intake restriction (39,40). Children and young adults may need higher doses. During pregnancy, due to the increase of progesterone levels, particularly in the third trimester, dose of fludrocortisone needs to be increased. Fludrocortisone is routinely taken once daily in the morning because aldosterone level is typically highest at this time because it follows a circadian rhythm similar to cortisol. The fludrocortisone dose is related to individual fluid and electrolyte intake and losses, anyway a daily dose of 0.05-0.2 mg is generally sufficient in adults and adolescents with PAI.

ADRENAL ANDROGEN REPLACEMENT THERAPY

In women, adrenal production of the androgen precursors dehydroepiandrosterone (DHEA) and androstenedione is a major source of androgen production. Consequently, PAI is frequently associated with androgen deficiency in female patients. A systematic review and meta-analysis of randomized placebo controls of DHEA treatment have not shown any substantial clinical benefit, suggesting that the current evidence is insufficient to support routine use of DHEA in women with PAI (52). DHEA replacement in PAI has been shown to restore circulating levels of androgen precursors and androgens back to the normal range. Moreover, some studies have shown an improvement in HR-QoL and a reduction in depression and anxiety score. Therefore, DHEA replacement (25–50 mg as a single oral dose in the morning) may be considered in premenopausal women with PAI and in the presence of reduced or absent libido, depression, anxiety, and reduced energy levels despite optimized glucocorticoid and mineralocorticoid replacement (39).

TREATMENT OF ADRENAL CRISIS

Adrenal crisis could develop when the adrenal glands cannot produce a sufficient amount of cortisol in response to increased need. This medical emergency may be prevented through adequate patients'

education on the modalities to adapt GC dosage in these situations related to increased demand in adrenal steroids, such as trauma, surgery or intercurrent illnesses. The frequency of adrenal crisis is about 6.6 cases per 100 patients for year. Precipitating factors are mainly gastrointestinal infections and fever (45%) but also other stressful events, like surgery, emotional distress, major pain and pregnancy (53). In addition, some medications could trigger an adrenal crisis through their interaction with cortisol clearance. Initiation of l-tiroxine treatment may induce acute adrenal insufficiency due to increased cortisol metabolism. Similarly, drug that induce the drug-metabolizing enzyme CYP3A4 (e.g. carbamazepine, phenytoin, hypnotic agents, antifungal therapies, and others) are able to increase cortisol clearance, necessitating higher replacement dosage. An Addisonian crisis requires immediate parenteral administration of 100 mg HC intravenously together with fluid replacement with physiological saline solution (NaCl 0.9%), followed by HC 200 mg/24 hours (continuously intravenously or 6 hourly injection) (39). Depending on the clinical setting, the steroid dosage may be progressively reduced and then tapered until patient's individual daily dosage.

MONITORING OF REPLACEMENT TREATMENT

The correct tailoring of replacement strategy remains challenging (43). Under-replacement can result in significant impairment of well-being and could lead patients to serious consequences in case of increased steroid requirement due to intercurrent illness. Conversely, over-replacement of GCs may lead to metabolic and cardiovascular morbidity including impaired glucose tolerance, obesity, osteoporosis and sleep disturbance (54,55). In fact, patients affected by PAI present higher mortality rate compared to general population (54,56). Likely explanations could be supraphysiological GC maintenance doses, poor diurnal GC exposure-time profile, and inadequate rescue therapy in response to intercurrent illnesses. Moreover, even with treatment, the HR-QoL in patients with PAI is often impaired. There is not universal agreement regarding appropriate monitoring strategies of GC replacement therapy, due to the lack of objective parameters (57). The 24-h urine cortisol (urinary free cortisol -UFC-) have been used as an indicator of overall cortisol replacement, but this method is not able to detect cortisol fluctuations throughout the day, so it is not suitable for identification of undertreatment. Beyond being extremely variable according to renal function, the rapid saturation of CBG after GC absorption may result in transient increase in renal cortisol excretion, invalidating any comparisons with normal reference ranges (57).
ACTH is not recommended for adjust GC treatment (39), because patients who receive appropriate replacement therapy often have elevated ACTH levels, due to disturbance of the normal close relationship between ACTH and cortisol secretion and negative feedback. Conversely, other

subjects without signs and symptoms of overtreatment could present reduced ACTH levels, because of increased pituitary sensibility to GC inhibitory feedback. For these reasons, ACTH could not be considered an accurate parameter of replacement quality, and, in general, finding of ACTH in the normal range may mean an overtreatment condition. Some authors suggested the potential use of serum cortisol day curves in the monitoring of GC replacement, but literature data are debating. Nevertheless, the disadvantages of serum cortisol measurements lie in the need for frequent blood samples and patients' hospitalization and in the variations related to CBG alterations (39,40,57). Therefore, in recent years, some authors have investigated salivary cortisol daily curves as a promising new tool to evaluate cortisol profiles. This method is useful for the measurement of the free hormonal amount in a non-invasive and stress-free sampling for outpatients (58). The literature evidence is not completely in agreement because some studies have showed wide inter-individual variability in plasma and salivary profiles of cortisol, and a poor correlation between salivary and plasma measurements in patients with PAI (59). However, other studies have proposed the evaluation of salivary cortisol as an interesting tool for the management of GC replacement in adrenal insufficiency, both secondary (60) and primary (61). Moreover, other authors demonstrated a lower daily cortisol exposure in patients treated with DR-HC compared to subjects treated with the same dose of conventional HC, especially in the afternoon and in the evening (62). For the abovementioned reasons, still today, monitoring of GC replacement therapy predominantly relies on clinical assessment. The presence of weight gain, recurrent infection, insomnia, peripheral oedema is a clinical sign of over-replacement, while nausea, loss of appetite, fatigue, hyperpigmentation and weight loss are typical signs of under-replacement (63). Also concerning mineralocorticoid replacement, the monitoring is primarily assessed from the clinical point of view, by inquiring about salt craving, measuring blood pressure in the sitting and standing position, evaluating electrolyte profile, and identifying the presence of peripheral oedema. In addition, plasma renin activity in the upper reference range has been found to be a useful marker for a correct mineralocorticoid dose (39).

THE EXPERIMENTAL STUDY

Novel biomarkers for the management of glucocorticoid replacement therapy in patients with primary adrenal insufficiency.

ABSTRACT

Background

Primary adrenal insufficiency (PAI) is a rare endocrine disorder characterized by secretory deficit of glucocorticoids (GC) and mineralocorticoids (MC). Until now, management of replacement therapy is mainly based on clinical assessment of signs and symptoms of over- and undertreatment, due to the lack of objective markers for the biochemical monitoring. In recent years the evaluation of salivary cortisol and cortisone profile has been proposed as a novel tool to manage GC replacement therapy. Copeptin (CT-proAVP) has emerged as a promising marker in many cardio-cerebrovascular diseases, as well as in some electrolyte disorders, but its potential role in adrenal insufficiency has not yet been established.

Objectives

The aim of the present study is to investigate novel potential markers for the biochemical monitoring of GC replacement therapy in patients with PAI.

Methods

A case-control, cross sectional study was performed, enrolling nineteen adult patients affected by PAI in replacement therapy with conventional hydrocortisone (HC) or dual-release hydrocortisone (DR-HC) and MC (fludrocortisone) at stable dosage for at least three months. Before taking their replacement therapy, patients were evaluated for a) anthropometric and biometric data; b) 30-item questionnaire for the assessment of health-related quality of life (AddiQol-30); c) biochemical and hormonal parameters: fasting glucose, glycated hemoglobin (HbA1c), serum creatinine, serum lipids, serum electrolytes, serum and urinary osmolality, spot urinary sodium and potassium, plasma renin, ACTH and copeptin levels. Moreover, plasma renin, ACTH and copeptin levels were also measured 120 minutes after taking GC and MC replacement therapy. These parameters were measured at baseline (T0) and after twelve months of follow-up (T1), during stable replacement therapy. The week before every evaluation time, patients collected six saliva samples in a routine day for the quantification of salivary cortisol (F) and cortisone (E) profile. Forty-three matched healthy subjects served as controls for F and E profile. We compared the "area under the curve" (AUC) for cortisone ($AUC^{E0\rightarrow E5}$) of PAI patients to $AUC^{E0\rightarrow E5}$ of healthy controls to quantify the endogenous exposure to GC.

Results

Six patients at T0 (31.6%) and seven patients at T1(36.8%) had $AUC^{E0\rightarrow E5}$ >90^{th} percentile of $AUC^{E0\rightarrow E5}$ calculated in healthy controls, therefore in the range of likely GC overtreatment (Group A). The other thirteen patients at T0 (68.4%) and twelve patients at T1 (63.2%) were defined in adequate GC replacement therapy (Group B). Concerning biometric and biochemical parameters, we observed that Group A compared to Group B was characterized by: a) alteration of blood pressure (BP) profile with higher diastolic blood pressure (DBP) values (p=0.0422) and lower differential BP values (p=0.0182) at T0; b) impairment of glucose metabolism, regardless of the presence of diabetes mellitus, with higher HbA1c levels both at T0 (p=0.0258) and T1 (p=0.0018) and higher fasting glucose levels at T1 (p=0.0034). We also found a different distribution of many hormonal markers, in fact Group A compared to Group B was characterized by: a) lower ACTH levels before replacement therapy both at T0 (p=0.0009) and T1 (p=0.0449); b) lower ACTH levels 120 minutes after therapy both at T0 (p=0.0014) and T1 (p=0.0283); c) lower ACTH/Copeptin ratio before therapy both at T0 (p=0.0132) and T1 (p=0.0441); d) lower ACTH/Copeptin ratio 120 minutes after therapy at T0 (p=0.0350); e) higher Copeptin/u-Na ratio before therapy at T1 (p=0.0436); f) lower Renin/Copeptin ratio 120 minutes after therapy at T1 (p=0.0028). No significant differences according to electrolytic assessment and renin levels were found.

Conclusions

Using salivary cortisone profile as indicator of GC overexposure, our study provides further evidence that the evaluation of copeptin and, particularly, the ratio ACTH/copeptin could represent a novel biomarker of therapeutic quality in PAI patients. However, these preliminary results must be confirmed in subsequent larger studies.

BACKGROUND

Primary adrenal insufficiency (PAI) or Addison disease (AD) is a rare endocrine disorder characterized by chronic glucocorticoid and mineralocorticoid deficiency due to failure of the adrenal cortex (29–31). Nowadays 80-90% of PAI is caused by autoimmune adrenalitis (30). Treatment of PAI is aimed to restore electrolyte and metabolic balance using replacement dosages of glucocorticoids (GC), mainly conventional hydrocortisone (HC) or cortisone acetate (CA), and mineralocorticoids (MC), 9α-fludrocortisone (39,40,43). GC replacement therapy is essential for life. In fact, inadequate management of therapy according to intercurrent needs could lead patients to very serious consequences, like acute and potentially life-threatening adrenal crisis (53). On the other hand, PAI patients present, as compared to general population, increased morbidity and mortality (54), mainly due to cardiovascular and metabolic alterations, related to supraphysiological GC maintenance dosages (55,56,64). Recent studies showed that dual-release hydrocortisone (DR-HC) could be efficacious in reducing disease morbidity and improving quality of life (QoL) (44,46,47,65–67). Regardless of formulation type, monitoring and management of replacement therapy is primarily based on clinical signs and symptoms of over- and undertreatment, because of the lack of objective assessment hormonal markers (39–41,63,68). Urinary free cortisol measurement could be useful to underline an overreplacement, but this marker is not able to represent the daily fluctuation of cortisol and it is extremely variable according to renal tubular function (39,40). Some authors suggested the potential use of serum cortisol day curves in the monitoring of GC replacement, but the disadvantages lie in the need for patients' hospitalization and in the variations related to cortisol binding globulin (CBG) alterations (39,40,57). Evaluation of salivary cortisol has been proposed as a novel tool to manage GC replacement therapy in patients with PAI, because salivary cortisol day curves can be easily performed also in outpatients and its measurement through mass spectrometry (MS), or liquid chromatography-mass spectrometry (LC-MS) is not affected by CBG levels (58,62,69). Furthermore, the analysis of salivary cortisone, a cortisol's metabolite, was proposed, but literature data are still debating (70–73). ACTH cannot be used as a criterion for GC dose adjustment, because in PAI patients values in the normal range are often associated with a supraphysiological dosage of GC therapy. Anyway, since ACTH synthesis and secretion are physiologically regulated by CRH and AVP (24) the evaluation of these neurohormones could allow to estimate the adequacy of replacement therapy. Copeptin, the C-terminal part of the precursor pre-provasopressin (CT-proAVP), is a reliable surrogate marker for AVP production, stable and easy-to-measure (12). Several studies have underlined the advantages of copeptin as diagnostic and prognostic marker in many cardio-cerebrovascular diseases (16,17,74), as well as in some electrolyte disorders, such as in the differential diagnosis of hyponatremia and in

polyuria-polydipsia syndrome (14,21,22). However, its potential role in adrenal insufficiency has not yet been established. Concerning MC replacement therapy, the measurement of renin or plasma renin activity (PRA) has been proposed as a helpful tool, suggesting PRA levels in the upper reference range as biochemical indicator of replacement quality (75,76). However, as showed in previous studies (76), the complexity of the physiological system of PRA regulation makes it difficult to use it for titration of MC replacement therapy. Therefore, to date, GC treatment surveillance is mainly guided by clinical judgment assessing daily performance, subjective health status and signs and symptoms of GC overtreatment (weight gain, hypertension, hyperglycemia, dyslipidemia, osteoporosis) or undertreatment (fatigue, nausea, myalgia, joint stiffness, hypotension, melanodermia, weight loss) (39,40,43,68,77). Similarly, management of MC replacement therapy is mainly based on clinical parameters like salt craving, oedema, blood pressure in combination with lab parameters like serum sodium and potassium levels (39,40,43).

AIM

Based on these assumptions, the primary outcome of this study was to investigate new potential markers of GC replacement adequacy in patients affected by PAI.

Secondary outcome was the definition of copeptin, ACTH and renin levels 120 minutes after taking replacement therapy.

SUBJECTS AND METHODS

Design and study population

We designed a case-control, cross sectional study, conducted on adult patients affected by PAI in replacement therapy, referred to the Division of Endocrinology, Diabetes and Metabolism of the City of Health and Science University Hospital of Turin, enrolled between January 2021 and January 2022. Inclusion criteria were a) age ≥ 18 years old; b) diagnosis of PAI according to the Endocrine Society Guidelines (39) and the Italian Society of Endocrinology Position Statement (43); c) replacement therapy with conventional HC/DR-HC and 9α-fludrocortisone at stable dosage for at least 3 months.

Exclusion criteria were a) age < 18 years old; b) presence of severe cardio-cerebrovascular, respiratory, hepatobiliary or pancreatic diseases, renal dysfunction, intestinal or gastric motility disorders and autoimmune systemic disease in steroid suppressive treatment.

The diagnosis of autoimmune AD was based on the presence of circulating adrenal autoantibodies against the steroidogenetic enzyme 21-hydroxylase (21OHAbs).

All women with POI were under appropriate hormonal replacement therapy (HRT).

The study was characterized by two observation times: a) T0: at time of enrolment; b) T1: after 12 months of follow-up.

As a control group, we investigated a population of 43 healthy subjects, whose data were provided from the Endocrinology Unit, University Hospital of Padua. The healthy controls were all voluntary adults recruited among hospital employees and their family members; none of them were taking exogenous glucocorticoids or drugs that might interfere with the HPA axis; female volunteers were not taking oral or transdermal contraceptives and were investigated in the early follicular phase of the menstrual cycle. The control group was matched for age (44.74 ± 17.17, p=0.7948) and gender (19 males and 24 females, p=0.7876).

The study was performed in accordance with the guidelines in the Declaration of Helsinki and approved by the Ethics Committee of City of Health and Science University Hospital of Turin (N° 108444; 10/11/2020). Written informed consent was obtained from all enrolled subjects.

Clinical investigation

Body weight, height, waist circumference, blood pressure and heart rate were measured using standard methods at time T0 and T1. Body mass index (BMI) was calculated (weight divided per height squared, kg/m2). Body surface area (BSA) was calculated with the DuBois and DuBois formula (60). Health-related quality of life (HR-QoL) was evaluated by a 30-item questionnaire (AddiQoL), purposely developed and validated in patients with AD in European subjects (77,78). AddiQoL-30 contains 30 questions made for assessment of four subdimensions (fatigue, emotions, symptoms, and miscellaneous which involves intercurrent disease, sleep and sexuality) in patients with PAI. A score was provided for each item, ranging from point 1 (negative statement) to 4 (positive statement). The algebraic sum of the various item scores was calculated. A higher score indicated a batter quality of life.

Biochemical evaluation

Both PAI patients and healthy controls performed a multiple daily saliva collection during normal routine day, for the assessment of salivary cortisol (Kendall's compound F) and cortisone (Kendall's compound E) profile. Concerning PAI patients this collection was undertaken the week before their evaluation at time T0 and T1. The times of collection were a) on awakening/before taking replacement therapy; b) 90 minutes after therapy; c) 6 hours after therapy/before lunch; d) 8 hours and 30 minutes after therapy; e) 12 hours after therapy/before dinner; f) before sleeping. Patients were advised to soak the absorbent cotton for 2 or 3 minutes, then the saliva sample was placed in a syringe and kept at +4°C. Samples were collected at least 30 minutes before eating or drinking, to

avoid any source of food contamination; patients brushed their teeth at least 30 minutes before collecting their saliva. Smoking or eating liquorice was forbidden. The protocol was described in detail to the patients through a written form, to ensure a correct salivary sampling.
At time T0 and T1, blood samples were collected from all patients fasting in the morning between 7.00 and 9.00 a.m. before and 120 minutes after replacement therapy. We measured before taking replacement therapy the following lab parameters: copeptin, ACTH, renin, sodium (Na), potassium (K), creatinine, glucose, glycated hemoglobin (HbA1c), plasma osmolality (p-Osm), total cholesterol, HDL-cholesterol, triglycerides. Copeptin, ACTH and renin were also measured 120 minutes after taking GC and MC replacement therapy. Urine sample was collected for the measurement of urine osmolality (u-Osm), spot urinary sodium (U-Na) and spot urinary potassium (U-K).

Analytical methods

Salivary cortisol and cortisone (ng/ml) were determined on salivary samples collected by patients using a cotton-based sampling device called Salivette® (SARSTEDT, Nümbrecht, Germany) through MassChrom® Cortisol, Cortisone in Saliva- LC–MS/MS (Chromsystems Instruments & Chemicals GmbH, Gräfelfng, Germany). The quantification was performed on Nexera UHPLC system (Shimadzu, Kyoto, Japan) associated with a triple quadrupole mass spectrometry 4500MD (AB Sciex, Framingham, MA, USA). The assay sensitivity was 0.28 ng/ml for cortisol and 0.55 ng/ml for cortisone, intra- and inter-assay coefficients of variation were, respectively, below 5.1% and 8.8% for cortisol and below 4.9% and 8.8% for cortisone.
To assess endogenous daily cortisol exposure, we calculated the “Area Under the Curve” (AUC) for salivary cortisol and cortisone levels according to the trapezoidal formula proposed by *Pruessner* (79).
Plasma ACTH levels (ng/L) were measured by chemiluminescent immunometric assay (CLIA) using LIAISON Analyzer (DiaSorin, Saluggia, Italy), with an analytical sensibility of 1.6 ng/l, and intra- and inter-assay coefficients of variation below 4.9 and 8.8 %, respectively.
Plasma copeptin (pmol/L) concentrations were determined with the B.R.A.H.M.S. KRYPTOR compact PLUS® (Thermo Fisher Scientific, Hennigsdorf, Germany) automated method using the TRACE (Time-Resolved Amplified Cryptate Emission) technique, an immunofluorescent analysis, which measures the delayed fluorescent signal transferred from donor molecules when bound in an immunocomplex. The detection limit of the assay was 0.9 pmol/L, while intra- and inter-assay coefficients of variation were below 7% and below 12%, respectively.

Serum creatinine, glucose, total cholesterol, HDL-cholesterol, and triglycerides levels (mg/dL) were measured by enzymatic colorimetric method (AU5800, Beckman Coulter Inc, USA). HbA1c (mmol/mol) were measured by method D-100 HbA HPLC (BioRad Lab, USA). Serum Na (mmol/L) and K (mmol/L) were evaluated with impedance measured method (AU5800, Beckman Coulter Inc, USA). P-Osm (mOsm/kg) and u-Osm (mOsm/kg) were measured by automatic osmometer (Osmo Station OM-6050, ARKRAY Global, Kyoto, Japan) using freezing point depression method.

Statistical analysis

Categorical data were expressed as absolute or relative frequencies (n, %). Continuous variables were reported as median [25th-75th percentile] or mean ±standard deviation (SD), according to their distribution, evaluated with Kolmogorov-Smirnov test. Comparison of continuous variables between groups was performed by T Student test or ANOVA for independent samples in case of normal distribution, or by Mann-Whitney U test or Kruskal-Wallis test if not normally distributed. Chi-square test or Fisher's exact test were used for categorical variables, as appropriate. Wilcoxon signed-rank test was used for the analysis of paired samples. The correlation between continuous variables was evaluated with Pearson test. Because this study could be considered as proof-of-concept, we didn't perform the quantification of the statistical power and we didn't define a minimum sample size. A p-value < 0.05 was considered statistically significant. Data analysis was performed by GraphPad Prism (version 10.1.0).

RESULTS

PAI patients

Nineteen PAI patients, approximately equally represented in males (47.4%) and females (52.6%), of young adult age (mean age 47.95 ± 12.69 years, range 21-70) were enrolled. Six patients were affected by isolated autoimmune AD, twelve patients by autoimmune polyendocrine syndromes (APS) of various subgroups (10 APS2, 1 APS1 and 1 APS4) and one patient affected by idiopathic AD. Among the APS group, eight patients were affected by autoimmune primary hypothyroidism in treatment with levothyroxine at adequate replacement dosage and one patient was affected by primary hypoparathyroidism in therapy with calcium carbonate and calcitriol in good compensation. The other associated endocrine and non-endocrine autoimmune pathological conditions are reported in **Table 1**. Three patients presented type 1 diabetes mellitus (DMT1) in insulin therapy and two patients type 2 diabetes mellitus (DMT2) in treatment with antidiabetic agents and diet therapy. All

patients were treated with 9α-fludrocortisone (median dosage 0.075 [0.05-0.1] mg daily) and with HC. None of them were treated with CA. Concerning GC replacement therapy, seven patients were treated with conventional HC (median dosage 20 [20-20] mg daily), whereas eleven patients received DR-HC (median dosage 20 [20-20] mg daily). One patient was treated with HC 10 mg added to DR-HC 20 mg daily. Conventional HC was administered as a thrice- or twice-daily regimen. The main clinical patients' features are summarized in **Table 1**.

In PAI patients, anthropometric and biometric characteristics were in normal range, in particular weight, BMI, waist circumference, as well as blood pressure and heart rate. Concerning glucose metabolism, we must distinguish between diabetic subject (n=5) and non-diabetic subjects (n=14). Electrolyte assessment was overall within the normal range, both in serum (139.1 ± 4.50 nmol/L for Na and 4.13 ± 0.39 nmol/L for K) and in spot urine (115.7 ± 44.81 nmol/L for u-Na and 29.89 ± 13.52 nmol/L for u-K), as well as p-Osm and u-Osm (284.0 ± 7.99 mOsm/kg and 619.3 ± 221.1 mOsm/kg, respectively). Renal function was preserved, and lipid profile showed mean LDL cholesterol levels of 95.29 ± 29.11 mg/dL. The evaluation of electrolyte-free water clearance with Furst index (u-Na + u-K / s-Na) showed reduced values (1.049 ± 0.38), in association with normal u-Na spot. Regarding *sodium to urinary sodium ratio divided by the (serum potassium)2 to urinary potassium (SUSPPUP) ratio* as markers of aldosterone activity, its median value was reduced in the entire population (1.97 [1.35-3.05]). The evaluation of HR-QoL through AddiQol-30 administration showed that mean total score was 91.94 ± 8.87 /120, with a mean of pathologic items of 4.59/30 items. The hormonal evaluation performed before taking replacement therapy documented that renin and copeptin median values were within the normal laboratory range, instead ACTH median levels were above upper laboratory limit. **Figure 1** represents the variations of hormonal values according to the assumption of replacement therapy at time T0 and T1. At T0 we observed, 120 minutes after therapy, a significant decrease in ACTH levels (56.20 [45.40-196.0] vs 439.4 [154.6-562.3] ng/L; $p<0.0001$) and copeptin concentrations (4.70 [3.90-9.80] vs 6.10 [4.73-13.18] pmol/L; $p< 0.0001$), without any significant difference concerning renin levels (24.93 [13.12-81.59] vs 33.37 [12.67-95.82] mUI/L; p=0.2288). This trend was also observed at follow-up. In fact, at T1 the comparison between hormonal values 120 minutes after therapy and before taking therapy showed a significant reduce in ACTH (75.40 [35.90-224.9] vs 200.1 [122.2-1046] ng/L; p=0.0002) and copeptin levels (5.50 [3.80-9.80] vs 8.30 [5.00-15.30] pmol/L; p=0.0014), without any statistical significant variation in renin concentrations (52.10 [4.30-88.90] vs 58.30 [23.30-99.90] mUI/L; p=0.3465). The data are summarized in **Table 2**.

According to the quality of GC replacement therapy

To evaluate the endogenous exposure to GC, we calculated the "area under the curves" (AUCs) for cortisol ($AUC^{F0\rightarrow F5}$) and cortisone ($AUC^{E0\rightarrow E5}$) for both PAI patients and healthy controls. **Table 3** represents median values of F0→F5, E0→E5 and of their ratio F/E 0→5. **Figure 2** and **Figure 3** depict salivary cortisol and cortisone rhythm profile in PAI patients at T0 and T1.

Both cortisol and cortisone AUCs were characterized by elevated variability, especially AUC $^{F0\rightarrow F5}$ with a coefficient of variation (CV) of 97%, instead CV of AUC $^{E0\rightarrow E5}$ was lower (36%).

Apart from less dispersion of E values from median value, we can also observe that AUC $^{F0\rightarrow F5}$ descended more rapidly around 8 hours and 30 minutes after therapy, unlike AUC $^{E0\rightarrow E5}$ was characterized by more stability. For both F and E values there was a marked pick around 1 hours and 30 minutes after replacement therapy. We decided to compare cortisone AUCs of PAI patients with cortisone AUCs of healthy controls, in order to evaluate patients' exposure to GC replacement therapy. We compared the 90th percentile of AUC $^{E0\rightarrow E5}$ calculated in the healthy group of subjects (90th percentile: 21521) with that of the 19 PAI patients included in the study. The analysis showed that six patients at T0 (31.6%) and seven patients at T1 (36.8%) had AUC $^{E0\rightarrow E5}$ >90th percentile, in the range of excess of GC therapy. Therefore, we defined this group of patients in likely GC overtreatment condition (Group A). The others 13 patients at T0 (68.4%) and 12 patients at T1 (63.2%) were defined in adequate GC replacement therapy (Group B), because none of patients had a baseline AUC $^{E0\rightarrow E5}$ < 10th percentile of AUC $^{E0\rightarrow E5}$ calculated in the healthy group of subjects (10th percentile: 8768), thus in the range of undertreatment. Subsequently, we tested the distribution of the anthropometric, biometric, biochemical and hormonal values in these two different cohorts of subjects. We didn't find any substantial differences between these categories according to most anthropometric and biometric parameters, as shown in **Table 4**, except for, at T0, blood pressure profile. In fact, DBP values were significantly higher (p=0.0422) in group A (82.00 ± 7.58 mmHg) compared to group B (73.85 ± 6.82 mmHg). Conversely, differential BP values were significantly lower (p=0.0182) in group A (31.00 ± 7.42 mmHg) than in group B (41.92 ± 8.05 mmHg).

The analysis of biochemical parameters is indicated in **Table 5**. The two groups of patients were significantly different regarding glucose metabolism. Median levels of HbA1c were higher in group A than group B at T0 (46.50 [36.00-53.50] mmol/mol vs 34.00 [30.50-36.50] mmol/mol, respectively; p=0.0258). This difference also appeared at follow-up (51.00 [40.00-62.00] mmol/mol vs 33.50 [31.25-36.25] mmol/mol, respectively; p=0.0018). We must underline that these results were even confirmed after the exclusion of diabetic patients from the analysis. Instead, the difference regarding glucose fasting levels was observed only at T1 with median levels of group A (100.0 [80.00-195.0] mg/dL) significantly higher (p=0.0034) than median levels of group B (75.00

[68.00-79.75] mg/dL). Group A and group B were not statistically different concerning electrolyte assessment, renal function, plasma and urine osmolality. We found a statistically significant difference between group A and group B according to HDL cholesterol, whose levels were higher in group A both at T0 (88.00 ± 15.13 mg/dL vs 64.62 ± 20.24 mg/dL, p=0.0225) and at T1 (77.71 ± 16.42 mg/dL vs 61.17 ± 13.08 mg/dL, p=0.0267). **Table 6** represents the distribution of the hormones evaluated both basal and 120 minutes after replacement therapy between the two cohorts according to therapeutic adequacies. We can observe that group A and group B differed according to many hormonal values. At T0, median ACTH levels were significantly lower in group A than in group B considered both at baseline (146.7 [13.53-155.5] ng/L vs 552.7 [383.6-942.7] ng/L; p=0.0009) and 120 minutes after replacement therapy (35.80 [5.33-52.43] ng/L vs 157.8 [55.10-324.2] ng/L; p=0.0014). Moreover, this statistically significant difference persisted at T1, with lower median ACTH levels in group A compared to group B before (122.6 [51.40-182.6] vs 528.6 [185.3-1131] ng/L; p=0.0449) and after therapy (35.90 [14.80-51.00] vs 154.8 [56.20-367.9] ng/L; p=0.0283). As shown in **Figure 4**, group A and group B were also significantly different according to ACTH/Copeptin ratio, with lower median values in case of GC excess both before (6.41 [1.30-44.46] vs 109.1 [40.73-198.2; p=0.0132) and after replacement therapy (2.13 [1.08-20.74] vs 33.57 [10.94-65.75], p=0.0350). This difference was also observed at T1 only for baseline ACTH/Copeptin ratio. We also found a significant difference between the two groups concerning ratio Copeptin/u-Na at T1, resulting in higher levels of median Copeptin/u-Na values in Group A than Group B (10.43 [8.47-14.59] vs 4.46 [2.99-8.24]; p=0.0436], as illustrated in **Figure 5**. Moreover, at T1, the ratio Renin/copeptin measured 120 minutes after replacement therapy showed a different distribution among the two groups, with reduced value in case of GC excess (5.41 [1.17-12.14] vs 34.43 [16.60-59.18]; p=0.0028), as reported in **Figure 6**.

DISCUSSION

In the present study we aimed to explore the potential role of novel biochemical markers in the therapeutic monitoring of patients affected by PAI. We decided to quantify the extent of GC exposure through the comparison of salivary cortisone AUCs detected in PAI patients with those obtained from healthy controls, distinguishing a group of patients in likely GC excess and another group of patients in adequate GC replacement therapy. Therefore, we analysed the distribution of anthropometric, biometric, biochemical and hormonal variables among these groups.

The main results documented that the cohort of subjects in GC excess was characterized by: a) impairment of glucose metabolism, regardless of the presence of diabetes mellitus; b) alteration of blood pressure profile; c) reduced levels of ACTH and of its ratio with Copeptin measured both

fasting in the morning before replacement therapy and 120 minutes after taking GC treatment; c) higher baseline Copeptin/u-Na ratio; d) lower Renin/Copeptin ratio evaluated after replacement therapy. It's well known from the literature data that the main disadvantages of GC replacement therapy lie in the lack of ability to replicate properly the physiological cortisol circadian rhythm and in the difficulty of monitoring replacement quality (39–41,68) . Mah et al (80) proposed a nomogram based on the evaluation of serum cortisol 2.5-5 hours after the first administration of GC compared with reference percentiles, for individual adjustment of replacement therapy. However, this nomogram is inconvenient because it requires multiple blood sample collection, being expensive, invasive and not suitable for outpatients. For this reason, salivary cortisol day curves have been proposed for the monitoring strategy, since saliva collection is non-invasive, easily repeatable and stress-free (58,62). Although an excellent correlation between salivary and serum concentrations of cortisol was generally observed, a major concern remains the wide variability of salivary cortisol concentration which strongly limits the use of this analysis with the aim of adjusting glucocorticoid substitutive dose (59). Ross and al (81) found significantly higher salivary cortisol levels evaluated through AUCs for cortisol in a population of 31 patients with AD in replacement HC therapy compared to healthy controls. Ceccato et al (62), in a study performed on 18 AD patients and 43 healthy controls, suggested that salivary cortisol, apart from its usefulness of assessing cortisol profile, could also correlate with different therapeutic pharmacokinetics, achieving lower daily cortisol exposure, especially in the afternoon-evening, in case of DR-HC treatment. Moreover, there is growing literature evidence that salivary cortisone profile may provide a better reflection of serum cortisol levels than salivary cortisol (70). In serum cortisol levels exceed cortisone, and the ratio of cortisol to cortisone is approximately 4:1. Instead, in saliva the ratio of cortisol to cortisone is reversed with more than 4:1 salivary cortisone to cortisol, due to the elevated expression in salivary glands of 11βHSD type 2, the enzyme involved in the conversion of cortisol into cortisone. Therefore, salivary cortisone has been shown to reflect serum cortisol concentrations and can be used as a surrogate marker of serum cortisol. In the prospective cross-over study performed by Debono et al (71) on a group of 14 volunteers, the authors highlighted that salivary cortisone could be a more useful tool to assess the cortisol circadian rhythm under both physiological conditions and after oral HC replacement because it has not the same risk for drug contamination as observed when measuring salivary cortisol in this setting.

From these premises, we decided to use salivary cortisone AUCs of PAI patients for comparison with healthy subjects and therefore establish the quality of GC replacement therapy. In our population the analysis of salivary cortisol and cortisone profile performed by AUCs documented in case of salivary cortisol a higher dispersion of the variables and an early fall below detectable levels

during periods when serum cortisol concentrations are naturally low. Instead, cortisone levels remained within a range that could be continued to be easily measured. Furthermore, we have to underline that all PAI patients enrolled in the study were treated with HC, both conventional and dual release, consequently well evaluable through salivary cortisone profile, in accordance with the available evidence on this topic (70,71). However, the pharmacokinetics of immediate release HC and DR-HC are different, and this element could influence a different cortisol rhythm profile, according to the type of replacement therapy.

In our analysis, no patients presented cortisone AUC $<10^{th}$ percentile detected in healthy subjects, therefore in the range of undertreatment. This finding could be explained by the remark that an under-replacement could be potentially fatal for patients, exposing them to the risk of life-threatening adrenal crisis in case of augmented requirement of steroid therapy due to intercurrent factors. Moreover, an insufficient therapy is associated with poor quality of life. Therefore, to avoid these consequences, an over-replacement may occur in several patients with PAI. In addition, clinical features of overtreatment could be difficult to recognize, and clinicians traditionally consider such mild GC excess to have little clinical relevance (55). During the last years, several studies have provided convincing data that over-replacement of PAI may be associated with high risk of cardiovascular and bone complications, impaired QoL and increased mortality (56).

The results of this study confirmed the well-known effect of GC on glucose metabolism since diabetes and glucose intolerance are frequent complications of GC excess. This finding was independent from the presence of DMT1, that could be associated with autoimmune AD, recognizing a different pathogenesis. However, also in this context, GC replacement excess may influence the dosage of insulin required and the glucometabolic control. Our study also documented a correlation between GC excess and increase in DBP as well as a reduction in differential BP. The effects of GC therapy on blood pressure in PAI are still a matter of uncertainty, since a recent study provided evidence that genetic background more than HC dose may influence the risk of hypertension in this clinical setting (82). On the other hand, other studies showed that both systolic and diastolic BP increased after switching from low-dose to high-dose HC, and an improvement after switching from conventional to DR-HC (44,47). Concerning metabolic assessment, we also identified a different lipids profile, characterized by higher levels of HDL-cholesterol in the group of patients in likely GC excess. Literature data showed that GC have a complex, still not fully elucidated, effect on lipid metabolism, including direct and indirect action on lipolysis, free fatty acid production and turnover. A possible explanation of the difference observed could be the different, although not statistically significant, gender distribution among the two groups, with a predominance of females in patients likely overtreated, being female gender characterized by higher

HDL-cholesterol levels. As expectedly, the results of the study showed a strong correlation between GC excess and reduction in ACTH levels, both at baseline and after taking replacement therapy. Literature data agrees that the evidence of basal normal (or suppressed) ACTH concentrations are related to over-replacement and that, on the contrary, basal high ACTH levels could not drive an increase in GC dosage. The reasons why ACTH cannot be used as an accurate biomarker of glucocorticoid replacement are unclear. Some hypotheses rely in a reduced pituitary sensitivity to cortisol inhibition, or, more likely, in the inability of the standard HC/CA preparation to induce normal cortisol levels throughout the 24 hours. Copeptin represents a reliable biomarker of the activity of antidiuretic system, due to its stability in absence of analytical critical issues (11,16). There is growing literature data regarding its usefulness as both diagnostic and prognostic marker in several cardiovascular diseases (17,19,74), as well as in many electrolyte disorders (20,21). To our knowledge, no studies evaluated copeptin as a new tool useful for the management strategy of PAI, a very complex pathological model of fluid and electrolyte imbalance. Thus, our study suggested the potential role of this neurohormone in the assessment of replacement quality. From the physiological point of view, since ACTH secretion is regulated not only by CRH, but also by AVP, the evaluation of this neuropeptide and, particularly, of its surrogate marker copeptin could allow a further assessment of GC replacement therapy. Thus, AVP and copeptin secretion and synthesis are primarily related to many osmotic and hemodynamic stimuli, and mineralocorticoid therapy could interfere with fluid-electrolyte balance and blood volume. For these reasons, copeptin could correlate both to GC and to MC therapy. Our results showed that copeptin values tended to be higher in patients characterised by GC replacement excess, although not statistically significant. However statistical significance was reached if copeptin was considered in relation to u-Na, with the evidence of higher Copeptin/uNa ratio in case of GC overexposure. This result agrees with literature evidence that highlighted that Copeptin/u-Na ratio could be superior to the reference standard in discriminating volume-depleted from normovolemic hyponatremic disorders (83). Apart from absolute copeptin levels, the most interesting results concerned the ratio ACTH/copeptin and Renin/copeptin. We pointed out that ratio ACTH/copeptin was significantly reduced in patients in GC overtreatment at every observation time if considered before treatment assumption and only at time of enrolment if evaluated 120 minutes after therapy. Thus, it seems to be able to identify mainly GC treatment excess due to the simultaneous contribution of both lower morning ACTH levels and higher copeptin concentration. Instead, we found significantly lower Renin/copeptin ratio in GC excess therapeutic category, if measured 120 minutes after therapy at longer follow-up, but this evidence is partially in contrast with what was observed at time of enrolment. A possible explanation for this evidence relies in the extremely complex hormonal regulation of renin and in

the presence of various possible interferent factors, even drug-related, that could affect the results. The higher copeptin values found in patients likely in GC therapeutic excess would appear to be correlated to the higher level of natremia and plasma osmolarity that characterize these patients, although neither of them was statistically significant in distinguishing therapeutic quality. However, the significant reduction in glycopeptide after taking replacement therapy could suggest a more direct association with therapeutic compensation. This result may corroborate literature evidence regarding the inadequacy of the current GC replacement therapy in mimicking the circadian secretion of cortisol, not being able to reproduce the physiological morning peaks and nocturnal nadirs, responsible for higher levels of ACTH and lower levels of copeptin compared to normal subjects.

The main strengths of the study are related to a) cross sectional case-control design study; b) high quality of the laboratory that performs the measurement of the hormonal parameters, especially salivary cortisol and cortisone; c) two times of observations, at enrolment and after 12 months of treatment at stable dosage, for further data confirmation.

On the other hand, the main limitation of the study relies in the small number of patients recruited. However, we have to specify that the limited sample size is justified by some factors. First, the rarity of disease, and second the highly specific selection criteria that we establish. In fact, we selected only PAI patients in HC therapy, in order to avoid possible bias related to different formulation of short-term GC therapy, that may affect the analysis.

CONCLUSION

Until now, due to the lack of validated biomarkers for therapeutic monitoring (39,40,68), the evaluation of treatment quality in PAI is primarily empiric, based on clinical judgment of signs and symptoms of under- or overtreatment and on the assessment of patients' well-being through the administration of specific questionnaires (78). However, the definition of objective parameters for the biochemical monitoring is of primary importance.

According to literature evidence, we based our analysis on the comparison between salivary cortisone profile measured in PAI patients and those detected in healthy controls, as objective parameter for evaluating GC overexposure. We observed an impairment of glucose metabolism and blood pressure profile in the category of patients defined in overtreatment through this method. This finding, because these alterations are well-known complications of GC excess, further corroborate the significant usefulness of salivary cortisone in this context.

Moreover, our study provides further evidence that the evaluation of Copeptin and, particularly, the ratio ACTH/copeptin could represent a novel biomarker of therapeutic quality in PAI patients.

These results were obtained from a study performed on a small number of patients, due to rarity of disease, and therefore do not allow to drive definitive conclusion. So subsequent larger studies are required in order to confirm these preliminary observations.

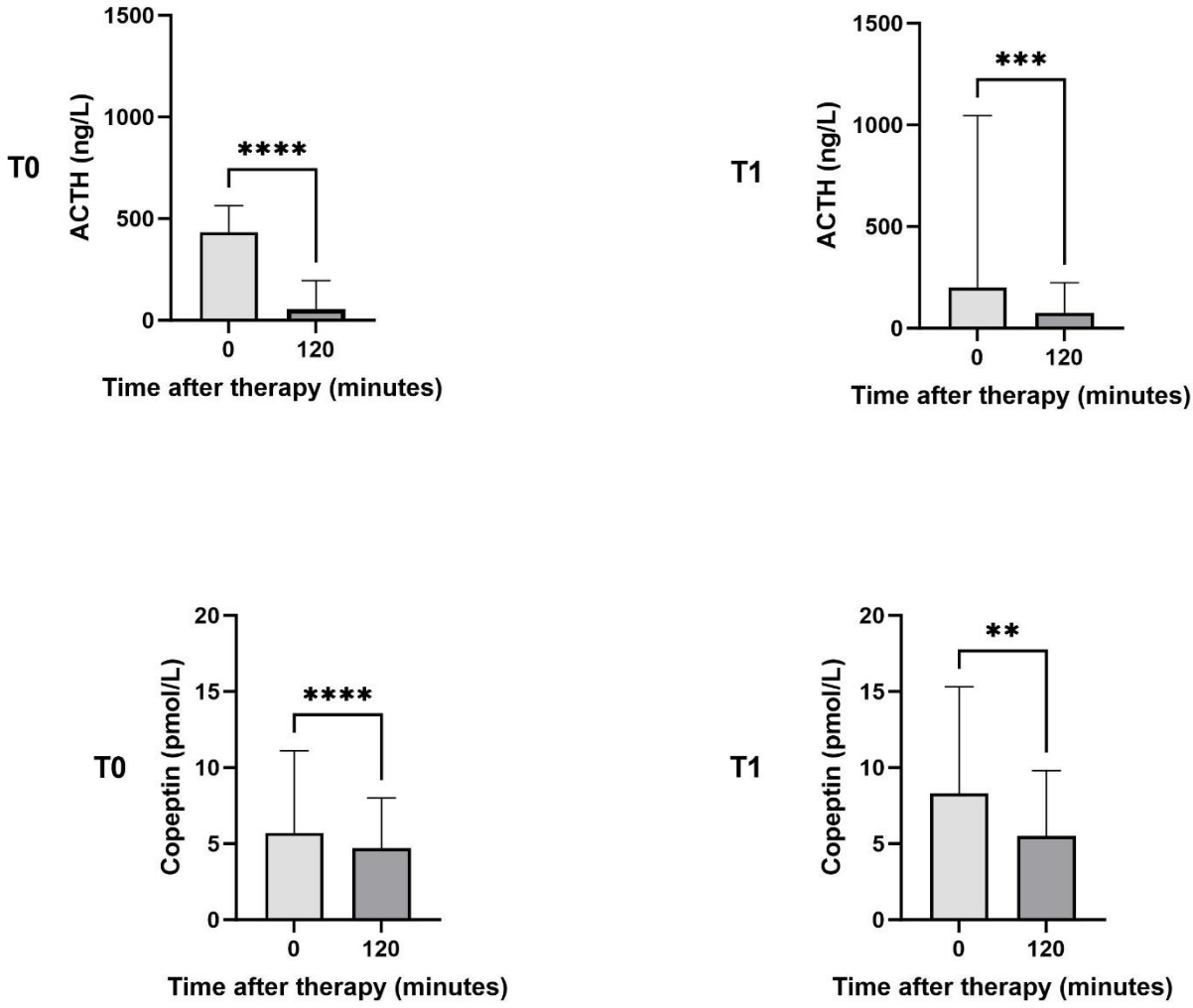
TABLES AND FIGURES

Table 1: Main clinical characteristics of PAI patients

Case	Gender	Age (years)	Aetiology	Associated diseases	Disease duration (years)	GC therapy (mg/daily)	MC therapy (mg/daily)
1	M	54	Idiopathic	HYPOTH	14	DR-HC 25	0.05
2	F	42	APS2	HYPOTH, CD	14	DR-HC 15	0.1
3	F	34	APS1	HYPOPTH, POI, Al	28	DR-HC 20	0.05
4	M	46	APS2	HT	36	DR-HC 20	0.15
5	F	21	AI	/	4	DR-HC 20	0.125
6	M	64	APS4	VIT, CAG	25	DR-HC 20	0.1
7	M	33	AI	HYPOTH	15	DR-HC 20	0.05
8	F	57	APS2	HT	18	DR-HC 20	0.05
9	F	52	APS2	HYPOTH, DMT1, POI	13	HC 20	0.15
10	F	54	APS2	HYPOTH, POI	6	DR-HC 20 + HC 10	0.05
11	M	62	AI	DMT2	23	DR-HC 25	0.1
12	F	57	APS2	HYPOTH, DM1	15	DR-HC 20	0.05
13	M	47	AI	/	11	HC 20	0.1
14	M	36	AI	/	18	DR-HC 20	0.075
15	M	61	APS2	HYPOTH	11	HC 20	0.075
16	F	45	AI	/	3	HC 20	0.1
17	M	38	APS2	HT, AT	7	HC 20	0.1
18	F	70	APS2	HYPOTH, CAG, DMT2	17	HC 20	0.05
19	F	38	APS2	DMT1, CAG	4	HC 17.5	0.05

Abbreviations: M, male; F, female; APS, autoimmune polyendocrine syndrome; AI, autoimmune isolated; HYPOTH, hypothyroidism; CD, coeliac disease; HYPOPTH, hypoparathyroidism; POI, primary ovary insufficiency; Al, alopecia; HT, Hashimoto's thyroiditis; VIT, vitiligo; CAG, chronic atrophic gastritis; DMT1, type 1 diabetes mellitus; DMT2, type 2 diabetes mellitus; AT, autoimmune thrombocytopenia; GC, glucocorticoid; MC, mineralocorticoid.

Figure 1: ACTH and copeptin levels before and 120 minutes after replacement therapy at T0 and T1



Data are expressed as median and interquartile range.

** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Table 2: ACTH, renin and copeptin levels before and 120 minutes after replacement therapy at T0 and T1.

	T0			T1		
	0	120	p-value	0	120	p-value
ACTH (ng/L)	439.4 [154.6-562.3]	56.20 [45.40-196.0]	*<0.0001*	200.1 [122.2-1046]	75.40 [35.90-224.90]	*0.0002*
Renin (mUI/L)	33.37 [12.67-95.82]	24.93 [13.12-81.59]	0.2288	58.30 [23.30-99.90]	52.10 [4.30-88.90]	0.3465
Copeptin (pmol/L)	6.10 [4.73-13.18]	4.70 [3.90-9.80]	*<0.0001*	8.30 [5.00-15.30]	5.50 [3.80-9.80]	*0.0014*

Data are expressed as median [25^{th}-75^{th} percentile]

Table 3: Salivary cortisol (F), cortisone (E) and cortisol/cortisone ratio (F/E) median values in healthy subjects and in PAI patients.

	Healthy subjects (n=43)	PAI patients (n=19)	
		T0	**T1**
F0 (ng/ml)	8.20 [5.30-11.60]	0.47 [0.47-2.25]	0.47 [0.47-1.50]
F1(ng/ml)	8.20 [4.50-15.00]	29.44 [12.60-102.4]	24.95 [19.24-74.21]
F2 (ng/ml)	4.30 [2.30-7.30]	7.67 [1.39-16.63]	4.50 [1.97-8.90]
F3 (ng/ml)	2.80 [1.70-4.40]	3.10 [0.64-4.88]	5.91 [1.43-22.04]
F4 (ng/ml)	1.70 [1.00-3.00]	1.13 [0.47-2.26]	2.26 [0.47-6.95]
F5 (ng/ml)	0.90 [0.60-1.20]	0.47 [0.47-1.02]	1.10 [0.47-2.48]
E0 (ng/ml)	25.00 [19.20-29.00]	2.00 [2.00-3.70]	2.00 [2.00-3.23]
E1 (ng/ml)	29.30 [19.40-36.00]	42.70 [33.77-60.22]	43.99 [36.88-49.54]
E2 (ng/ml)	18.30 [14.70-26.30]	20.23 [9.91-30.60]	21.96 [10.93-31.65]
E3 (ng/ml)	13.00 [11.10-17.80]	10.50 [4.48-20.18]	10.75 [7.04-18.72]
E4 (ng/ml)	11.00 [6.80-16.20]	6.59 [2.64-12.99]	11.46 [3.04-21.18]
E5 (ng/ml)	5.40 [4.10-8.70]	2.29 [2.00-7.50]	4.31 [2.00-9.14]
F/E0	0.34 [0.27-0.41]	0.24 [0.24-0.71]	0.24 [0.24-0.29]
F/E1	0.29 [0.21-0.41]	0.51 [0.34-1.77]	0.57 [0.41-1.50]
F/E2	0.23 [0.17-0.33]	0.28 [0.13-0.64]	0.22 [0.18-0.42]
F/E3	0.19 [0.15-0.26]	0.21 [0.16-0.30]	0.27 [0.16-1.65]
F/E4	0.18 [0.13-0.21]	0.22 [0.17-0.24]	0.22 [0.15-0.30]
F/E5	0.17 [0.13-0.21]	0.24 [0.18-0.24]	0.24 [0.21-0.25]

Data are reported as median [25th-75th percentile]

Abbreviations: PAI, primary adrenal insufficiency; F, cortisol; E, cortisone

Figure 2: Cortisol (F) and Cortisone (E) profile in PAI patients at T0

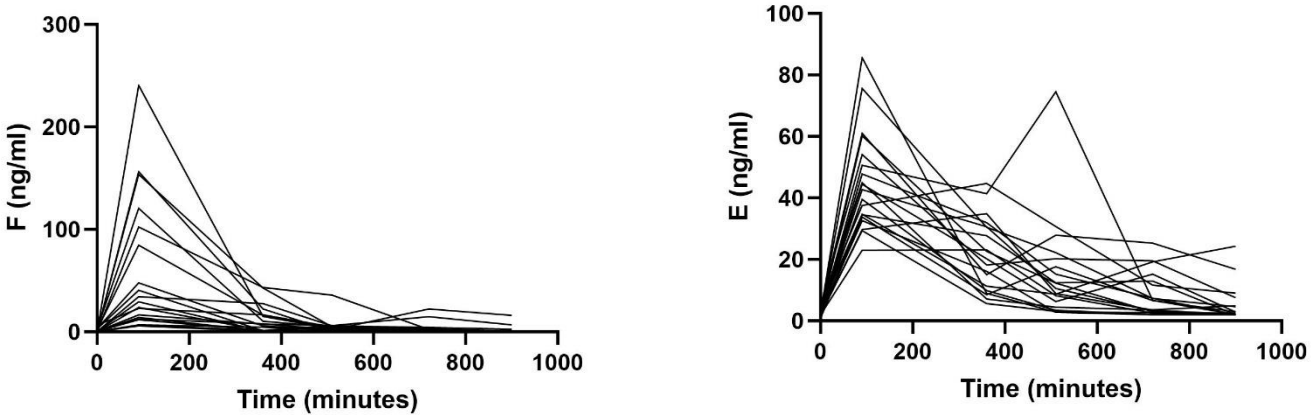


Figure 3: Cortisol (F) and Cortisone (E) profile in PAI patients at T1

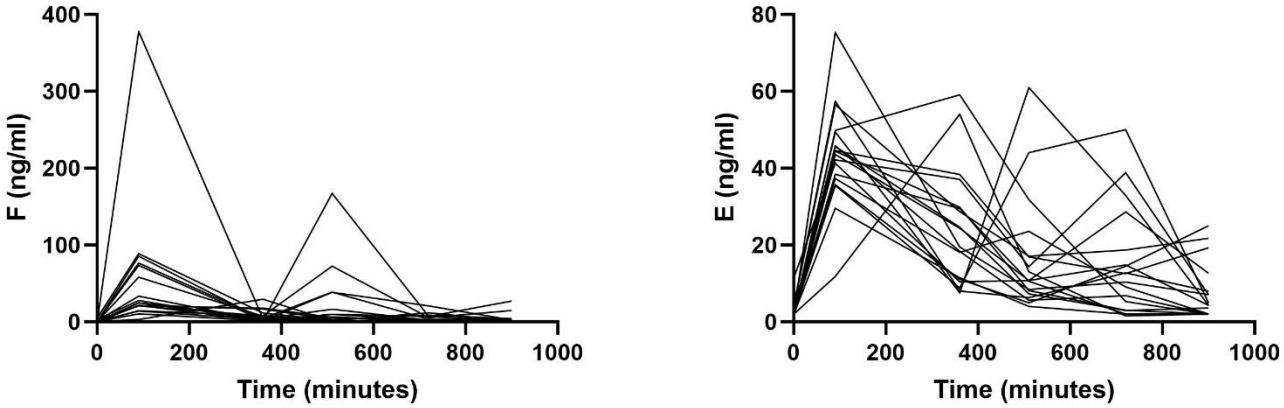


Table 4: Anthropometric and biometric parameters in all PAI patients, group A (GC excess) and group B (non-GC excess) at T0 and T1

	T0				T1			
	All patients (n=19)	**Group A (n=6)**	**Group B (n=13)**	**p-value A vs B**	**All patients (n=19)**	**Group A (n=7)**	**Group B (n=12)**	**p-value A vs B**
Age (years)	47.95 ± 12.69	50.83 ± 13.33	46.62 ± 12.71	0.5163	48.95 ± 12.69	52.14 ± 10.99	47.08 ± 13.69	0.4175
Male gender (n, %)	9, 47.4	1, 16.7	8, 61.5	0.1409	9, 47.3	2, 28.6	7, 58.3	0.3498
Weight (kg)	64.26 ± 11.17	62.17 ± 9.30	65.23 ± 12.17	0.5931	64.00 ± 10.48	63.71 ± 11.60	64.17 ± 10.31	0.9307
Height (m)	1.65 ± 0.07	1.60 ± 0.04	1.67 ± 0.07	0.0632	1.65 ± 0.07	1.63 ± 0.04	1.66 ± 0.08	0.5569
BMI (kg/m2)	23.46 ± 3.45	24.17 ± 3.49	23.14 ± 3.53	0.5614	23.53 ± 3.30	23.73 ± 3.47	23.41 ± 3.34	0.8424
Waist (cm)	81.50 ± 9.15	75.96 ± 7.16	83.81 ± 9.14	0.1090	82.78 ± 11.18	87.25 ± 11.13	79.93 ± 10.73	0.1833
HR (bpm)	68.21 ± 8.29	63.33 ± 4.50	70.46 ± 8.78	0.0805	68.37 ± 6.40	67.86 ± 5.52	68.67 ± 7.08	0.7987
SBP (mmHg)	115.0 ±10.57	113.0 ± 6.71	115.8 ± 11.88	0.6332	114.0 ± 13.08	117.7 ± 10.80	111.8 ± 14.15	0.3573
DBP (mmHg)	76.11 ± 7.78	82.00 ± 7.58	73.85 ± 6.82	*0.0422*	75.68 ± 9.30	80.86 ± 6.59	75.76 ± 9.53	0.0617
Diff. BP (mmHg)	38.89 ± 9.16	31.00 ± 7.42	41.92 ± 8.05	*0.0182*	38.32 ± 9.13	36.86 ± 6.26	39.17 ± 10.62	0.6090
HC (mg/day)	20.00 [20.00-20.00]	20.00 [18.75-21.25]	20.00 [20.00-20.00]	0.7317	20.00 [20.00-20.00]	20.00 [20.00-25.00]	20.00 [20.00-20.00]	0.2428
HC/BSA (mg/m2)	12.00 [10.63-13.23]	11.85 [10.74-13.51]	12.00 [10.45-13.34]	0.9636	12.14 [10.75-13.89]	12.60 [10.63-16.55]	12.09 [10.83-13.20]	0.5962

Data are reported as median [25th-75th percentile] or mean ±standard deviation, and as frequency count, as appropriate.

Abbreviations: BMI, body mass index; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; Diff. BP, differential blood pressure; HC, hydrocortisone; BSA, body surface area.

Table 5: Biochemical parameters in all PAI patients, group A (GC excess) and group B (non-GC excess) at T0 and T1

	T0				T1			
	All patients (n=19)	**Group A (n=6)**	**Group B (n=13)**	**p-value A vs B**	**All patients (n=19)**	**Group A (n=7)**	**Group B (n=12)**	**p-value A vs B**
HbA1c (nmol/l)	36.00 [33.00-48.00]	46.50 [36.00-53.50]	34.00 [30.50-36.50]	*0.0258*	34.00 [32.00-51.00]	51.00 [40.00-62.00]	33.50 [31.25-36.25]	*0.0018*
Glucose (mg/dl)	76.00 [67.00-88.00]	84.50 [69.75-94.75]	76.00 [66.00-82.50]	0.4797	79.00 [70.00-100.0]	100.0 [80.00-195.0]	75.00 [68.00-79.75]	*0.0034*
Total cholesterol (mg/dl)	188.4 ± 24.20	181.6 ± 20.06	191.5 ± 26.02	0.4254	215.3 ± 26.02	211.0 ± 14.59	217.8 ± 31.20	0.5999
HDL (mg/dl)	72.00 ± 21.48	88.00 ± 15.13	64.62 ± 20.24	*0.0225*	67.26 ± 16.18	77.71 ± 16.42	61.17 ± 13.08	*0.0267*
TG (mg/dl)	105.3 ± 54.97	90.17 ± 35.99	112.3 ± 61.85	0.4302	100.3 ± 50.47	89.71 ± 45.13	106.5 ± 13.08	0.5001
cLDL (mg/dl)	95.29 ± 29.11	83.40 ± 27.83	100.8 ± 29.07	0.2368	132.8 ± 30.51	129.3 ± 29.32	135.0 ± 32.45	0.7126
s-Na (mmol/L)	139.1 ± 4.50	138.7 ± 4.08	139.2 ± 4.83	0.8078	139.1 ± 2.62	139.3 ± 3.09	139.0 ± 2.45	0.8262
s-K (mmol/L)	4.13 ± 0.39	3.98 ± 0.47	4.20 ± 0.34	0.2672	4.04 ± 0.27	4.09 ± 0.13	4.02 ± 0.33	0.6098
Creatinine (mg/dl)	0.81 [0.71-0.95]	0.85 [0.78-1.09]	0.76 [0.69-0.92]	0.1464	0.87 [0.70-0.97]	0.87 [0.73-1.12]	0.83 [0.69-0.97]	0.5763
p-Osm (mOsm/kg)	283.6 ± 8.03	285.8 ± 6.91	282.7 ± 8.57	0.4815	286.7 ± 6.26	287.7 ± 4.79	286.0 ± 7.18	0.2359
u-Osm (mOsm/kg)	613.9 ± 226.7	658.6 ± 92.99	595.3 ± 265.2	0.6156	598.9 ± 215.1	505.1 ± 202.2	658.6 ± 209.3	0.1447

Data are reported as median [25th-75th percentile] or mean ± standard deviation, and as frequency count, as appropriate.

Abbreviations: Na, sodium; K, potassium, p-Osm, plasmatic osmolality; u-Osm, urine osmolality.

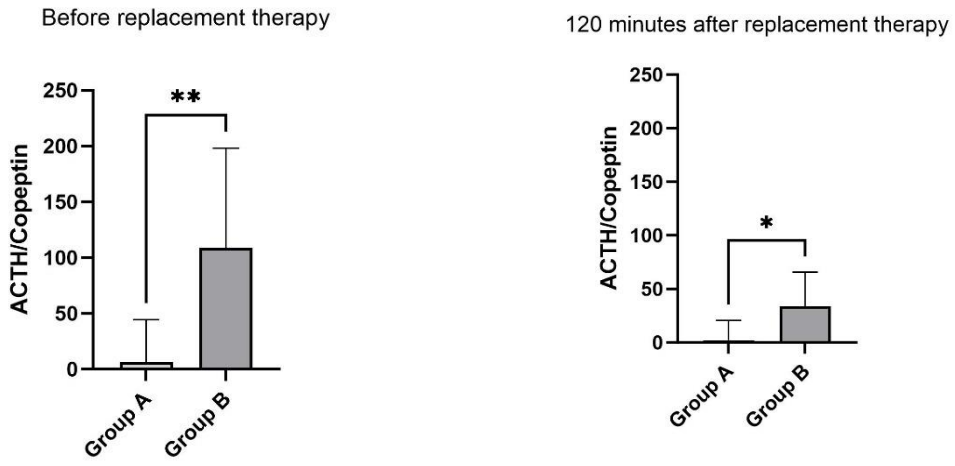
Table 6: Hormonal parameters in all PAI patients, group A (GC excess) and group B (non-GC excess) at T0 and T1.

	T0				T1			
	All patients (n=19)	**Group A (n=6)**	**Group B (n=13)**	**p-value A vs B**	**All patients (n=19)**	**Group A (n=7)**	**Group B (n=12)**	**p-value A vs B**
ACTH (ng/L)	431.4 [154.3-564.3]	146.7 [13.53-155.5]	552.7 [383.6-942.7]	*0.0009*	200.0 [122.2-1046]	122.6 [51.40-182.6]	528.6 [185.3-1131]	*0.0449*
Renin (mUI/L)	25.40 [12.55-41.17]	31.04 [9.89-47.17]	25.40 [12.75-119.7]	0.7654	58.50 [23.30-99.90]	30.50 [6.00-61.30]	67.40 [47.83-111.4]	0.2268
Copeptin (pmol/L)	5.70 [4.70-14.30]	16.60 [4.30-24.48]	5.40 [4.25-9.30]	0.2818	6.20 [5.00-15.30]	7.25 [3.33-15.75]	6.10 [5.40-12.10]	0.8369
Cop/u-Na	7.04 [3.79-12.95]	12.75 [3.49-22.80]	5.94 [3.64-11.12]	0.3676	8.24 [4.18-12.25]	10.43 [8.47-14.59]	4.46 [2.99-8.24]	*0.0436*
ACTH/Cop	58.89 [22.47-170.5]	6.41 [1.30-44.46]	109.1 [40.73-198.2]	*0.0132*	54.61 [16.71-165.5]	22.63 [3.14-29.93]	82.00 [36.90-169.6]	*0.0441*
Renin/Cop	4.49 [0.86-11.21]	2.68 [0.41-15.20]	4.70 [2.05-12.66]	0.5214	6.28 [1.11-21.23]	2.56 [0.60-12.26]	7.11 [4.12-21.88]	0.2614
ACTH 120 (ng/L)	56.20 [45.40-196.0]	35.80 [5.33-52.43]	157.8 [55.10-324.2]	*0.0014*	75.40 [35.90-224.9]	35.90 [14.80-51.00]	154.8 [56.20-367.9]	*0.0283*
Renin 120 (mUI/L)	24.93 [13.12-81.59]	25.05 [9.40-82.66]	24.93 [13.41-98.65]	0.5789	52.10 [4.30-88.90]	35.80 [3.50-69.10]	52.35 [15.00-91.15]	0.5918
Copeptin 120 (pmol/L)	4.70 [3.90-9.80]	11.40 [3.38-20.23]	4.70 [3.75-7.25]	0.5354	7.00 [3.80-9.80]	8.50 [4.20-12.50]	4.30 [3.15-9.63]	0.2896
ACTH/Cop 120	13.70 [8.65-50.36]	2.13 [1.08-20.74]	33.57 [10.94-65.75]	*0.0350*	7.08 [1.06-21.23]	2.86 [0.90-16.45]	9.77 [3.78-23.05]	0.1042
Renin/Cop 120	7.88 [1.78-15.88]	9.36 [0.59-44.37]	7.41 [2.71-14.34]	0.8490	18.55 [6.33-37.69]	5.41 [1.17-12.14]	34.43 [16.60-59.18]	*0.0028*

Data are reported as median [25th-75th percentile] or mean ± standard deviation, as appropriate.

Abbreviations: Cop, copeptin; Na, sodium.

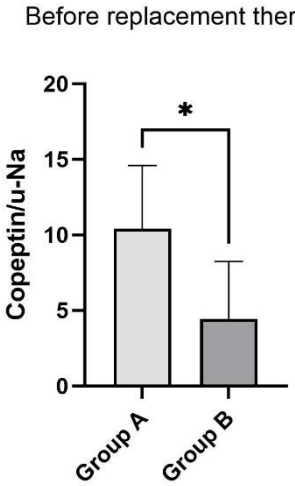
Figure 4: ACTH/Copeptin ratio evaluated in Group A (GC excess) and Group B (non-GC excess) before and 120 minutes after replacement therapy at T0.



Data are expressed as median and interquartile range.

*p<0.05, ** p < 0.01

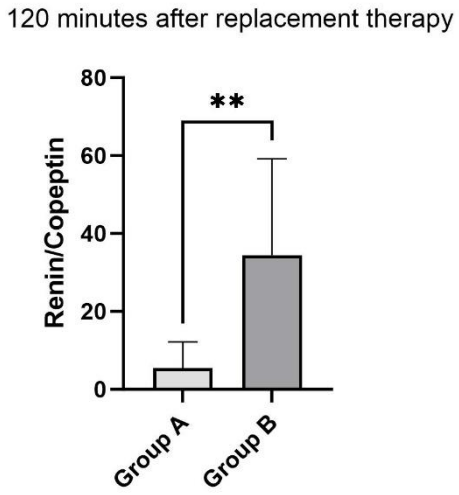
Figure 5: Copeptin/u-Na ratio evaluated in Group A (GC excess) and Group B (non-GC excess) before replacement therapy at T1.



Data are expressed as median and interquartile range.

*p<0.05

Figure 6: Renin/copeptin ratio evaluated in Group A (GC excess) and Group B (non-GC excess) 120 minutes after replacement therapy at T1.



Data are expressed as median and interquartile range.

** $p < 0.01$

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