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Potential applications of copeptin as a biomarker in the pathology of the hypothalamus-pituitary-adrenal axis: a focus on Cushing's syndrome

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SUMMARY

ABBREVIATIONS

1 mg-DST, 1 mg dexamethasone suppression test
17-OHP, 17-hydroxyprogesterone
95%CI, 95% confidence interval
ABPM, ambulatory blood pressure monitoring
ACS, adrenal Cushing's syndrome
ACTH, adrenocortoctropic hormone
ACTHD, ACTH deficiency
AI, adrenal insufficiency
AMI, acute myocardial infarction
aPTT, activated partial thromboplastin time
ATIII, antithrombin III
AUC, area under the curve
AVP, arginine-vasopressin
bALP, bone alkaline phosphatase
BIPSS, bilateral inferior petrosal sinus sampling
BMD, bone mineral density
BMI, body mass index
BP, blood pressure
cAMP, cyclic adenosine monophosphate
CD, Cushing's disease
cop/pNa, copeptin adjusted for plasma sodium
cop/pOsm, copeptin adjusted for plasma osmolality
CoV, coefficient of variation
CRH, corticotropin-releasing hormone
CS, Cushing's syndrome
CTX, C-terminal telopeptide (beta-crosslaps)
CV, cardiovascular
CVD, CV disease
DDAVP, desmopressin
DHEAS, dehydroepiandrosterone sulfate
DI, diabetes insipidus
DM, diabetes mellitus
EAS, ectopic ACTH secretion
eGFR, estimated glomerular filtration rate
FAI, free androgen index
FIB, fibrinogen
FG, Ferriman-Gallwey
FN, femoral neck
FSH/LHD, follicle-stimulating/luteinizing hormone deficiency
FVIII, factor VIII
GH, growth hormone
GHD, GH deficiency
HADS, hospital anxiety and depression scale
HbA1c, glycosylated hemoglobin
hCRH, human CRH
HDDST, high-dose dexamethasone suppression test
HDL, high-density lipoprotein
HOMA, homeostasis model assessment
HPA, hypothalamus-pituitary-adrenal
IFG, impaired fasting glucose
IGT, impaired glucose tolerance
IMT, intima-media thickness
INR, international normalization ratio
IQR, interquartile range
ITT, insulin tolerance test
LC-MS/MS, liquid chromatography-tandem mass spectrometry
LDL, low-density lipoprotein
LIA, immunoluminometric assay
MEN, multiple endocrine neoplasia
mLNSC, mean late-night salivary cortisol
MRI, magnetic resonance imaging
mRNA, messenger RyboNucleic Acid
mUFC, mean urinary free cortisol
NET, neuroendocrine tumor
NIH, National Institutes of Health
NPV, negative predictive value
OC, osteocalcin
oCRH, ovine CRH
OGTT, oral glucose tolerance test
OR, odds ratio
PAOD, peripheral artery obliterative disease
pC, functional protein C
POMC, pro-opiomelanocortin
pOsm, plasma osmolality
PPV, positive predictive value
pro-AVP, arginine-vasopressin precursor
pS, functional protein S
PT, prothrombin time
PTH, parathyroid hormone
RIA, radioimmunological assay
ROC, receiver operating characteristics
RS, radiosurgery
RT, radiotherapy
SD, standard deviation
SE, sensitivity
SHBG, sex hormone-binding globuline
SIAD/SIADH, syndrome of inappropriate antidiuresis/antidiuretic hormone secretion
SGLT2i, sodium-glucose transporter 2 inhibitors
SP, specificity
TBS, trabecular bone score
TH, total hip
TNS, transsphenoidal surgery
TSHD, thyreostimulating hormone deficiency
TT, total testosterone
UFC, urinary free cortisol
ULN, upper limit of normal
uOsm, urine osmolality
V1aR, type 1a vasopressin receptor
V1bR, type 1b vasopressin receptor
V2R, type 2 vasopressin receptor
vWF, von Willebrand factor
WC, waist circumference
WDT, water deprivation test
WHO, World Health Organization
Δ4A, delta-4 androstenedione

ABSTRACT

Background

Due to its nature of arginin-vasopressin (AVP) mirror-peptide, copeptin may be a novel biomarker in Cushing's syndrome (CS). This is a condition of chronic hypothalamus-pituitary-adrenal (HPA) axis hyperactivation, whose pathogenesis also involves a dysregulation of AVP system. Data on this topic are outdated and conflicting due to challenges in AVP determination, but the availability of such a reliable surrogate marker in recent years has led us reopen the debate through 3 studies.

Objectives

Study 1: to determine if copeptin differs within the spectrum of HPA axis states (CS *vs* healthy HPA, CS *vs* adrenal insufficiency (AI)). **Study 2:** to explore the relationship between copeptin and the keypoint diagnostic features of CS, as well as its comorbidity-derived burden. **Study 3:** to investigate the longitudinal intra-individual variations of copeptin at the different disease states after CS treatment.

Patients and methods

All the enrolled patients (71 active CS, 82 Healthy HPA and 36 AI in the cross-sectional **Study 1**; 80 active CS and 10 neuroendocrine tumors (NETs) in the cross-sectional **Study 2**; 30 adrenocorticotropic hormone (ACTH)-dependent CS in the prospective observational **Study 3**) referred to the Endocrinology Department of Ancona University Hospital between January 2016 and December 2022. Copeptin was measured with the ultra-sensitive, immuno-luminometric BRAHMS Copeptin assay on the KRYPTOR Compact Plus system.

Results

Study 1: CS and Healthy HPA had similar copeptin, but the former had hypercortisolism-induced higher plasma osmolality and sodium, thus a significantly lower adjusted copeptin. Diuretic-induced dehydration, either alone (p=0.006) or combined with diabetes mellitus (DM) (p=0.005), confounded the real copeptin in both groups: after correction, the cortisol-induced inhibition on copeptin in non-severely complicated CS patients became visible (3.7 *vs* 5.6, p=0.022). No differences emerged between CS and AI patients, provided

the confounders acted also on the latter (p=0.003 diuretics, p=0.006 combination DM/diuretics). **Study 2**: copeptin significantly differed among Cushing's disease (CD), adrenal CS (ACS) and ectopic ACTH secretion (EAS) (3.7 *vs* 5.1 *vs* 12.2 pmol/L respectively, p=0.002) with an epiphenomenal, rather than pathogenetical meaning. Within ACTH-dependent forms, copeptin mirrored ACTH trend and showed superior accuracy than the gold standard human corticotropin-releasing hormone (hCRH) test in discriminating EAS from CD at a cutoff of ≥9.1 pmol/L (AUC 0.993 p=0.001 *vs* AUC 0.889 p=0.029 hCRH test). Compared to NET, copeptin was lower in CD and higher in EAS. Copeptin reflected the systemic burden of CS in terms of number of comorbidities (ρ=0.332 p=0.003), presence of ipokalemia (16.4 *vs* 3.8, p<0.001) and the biomarkers of hypertension, central obesity, dyslipidemia (p<0.050). **Study 3:** copeptin had a 23% longitudinal intra-individual variability (CoV). Although not significantly, it increased after medical therapy (+2.6 pmol/L) and slightly decreased in the persistent/recurrent disease state (-0.1 pmol/L), as opposite to mean urinary free cortisol (mUFC). Copeptin itself had no prognostic value in predicting remission or persistence/recurrence of CD after pituitary surgery, but its CoV was negatively associated with persistent/recurrent disease (ρ-0.466, p=0.014).

Conclusion

This research project demonstrated that in the presence of a clinical suspicion for CS, copeptin assessment is unuseful in discriminating between patients with CS and those with a Healthy HPA, but once the diagnosis of CS has been made, it is both a powerful differential diagnostic biomarker and a reliable marker of the comorbidity-derived clinical burden. Despite a poor prognostic usefulness when assessed longitudinally, copeptin CoV can detect glucocorticoid inhibition on AVP system has reverted.

KEYWORDS

Cortisol; arginine vasopressin; Cushing's disease; ectopic ACTH.

INTRODUCTION TO THE RESEARCH PROJECT

Background

Copeptin is a 39 amino acid glycopeptide with a leucine-rich core which constitutes the C-terminal extremity of pro-AVP [1]. Copeptin is produced and released into the bloodstream in equimolar amounts with AVP, thus deserving the appellative of "AVP mirror peptide". The hypothalamic neurons hosting the synthesis of pro-AVP are located in the paraventricular and supraoptic magnocellular nuclei, as well as in some parvicellular nuclei. Molecular maturation occurs during axonal transport towards the pituitary via enzymatic cleavage. Following osmotic, hemodynamic stimuli and stressors, AVP and copeptin undergo a two-way release: on one hand, the posterior pituitary accounts for AVP systemic release aimed at preserving the body's homeostasis by binding to V1aR and V2R; on the other hand, the pituitary portal veins carry AVP to the anterior pituitary corticotrophs: here, AVP binding to its V1bR serves as a booster for CRH stimulation on ACTH and, in turn, cortisol production. This redundant mechanism ensures the stress response is properly mounted by the HPA axis.

Recent findings by Beglinger *et al.* support the existence, at least in physiological conditions, of a cross-talk between AVP system and HPA axis [2]. They found, indeed, circadian cortisol and copeptin rhythms overlapped so that the presence of a common circadian pacemaker, or the influence exerted by one over the other, could be hypothesized: in healthy individuals copeptin, thus AVP, peak and nadir (h 00-02 and 16-20, respectively) occur right before those of ACTH and some hours before those of cortisol. However, despite the high inter-individual variability, copeptin levels are more stable throughout the day as compared to ACTH and cortisol [3]. Further confirmation on this topic come from studies reporting the increase in copeptin levels during ITT [4] and after glucagon challenge, in this case alongside ACTH and GH, which also participates to the stress response [5].

Unfortunately, data on AVP system behavior when HPA axis is chronically hyperactivated (e.g., in CS) are scarce, outdated and partially conflicting. Harvey Cushing raised for the first time the assumption CRH and AVP are initiators or promoters of the corticotroph proliferation in CD [6]: his theory was later supported by

the anatomopathological finding of overexpressed V1bR in corticotroph tumors, which accounts for the use of the stimulation tests with CRH and DDAVP (a synthetic AVP analogue) in the differential diagnosis of CS in clinical practice. The first attempt to clarify the role of AVP in the pathogenesis of CS by means of its *in vivo* measurement dates back to the 1990s by Wittert *et al.*, who measured AVP, CRH, ACTH and other pituitary hormones on blood samples taken from the inferior petrosal sinuses of 9 CD patients [7]. Their work strengthened the assumption of a hypothalamic origin for CD Harvey Cushing had previously raised [6]: their demonstration of a strong correlation between ACTH and AVP, which were higher ipsilaterally to the adenoma in most patients despite a decrease in CRH levels, suggested the tumorigenesis might be supported by an increased transportation of AVP from the posterior to the anterior pituitary [7]. This, alongside the up-regulation of CRH receptors, would allow AVP to interplay with CRH, even in the case the latter is present at the smallest amounts. Conversely, the group from Naples supported the assumption of a pituitary origin, giving AVP a prognostic relevance. Colao *et al.*, indeed, simultaneously measured ACTH and AVP on blood samples from both the petrosal sinuses and the periphery before and after the stimulation with oCRH in 20 CD patients, 12 controls with other pituitary diseases and, just for the peripheral aliquot, 10 healthy volunteers [8]. Although significantly higher in hypercortisolemic patients than pituitary controls at both sites, peripheral AVP levels were similar between CD and healthy volunteers; moreover, only a subgroup of patients with CD showed a positive AVP response to oCRH stimulation. Patients with CD who had higher AVP levels at baseline and a non-responsive AVP (reduced, or not increased as far as is expected in hypercortisolemic states) to oCRH stimulation were those experiencing worse post-surgical outcomes. In the same years, the group from the NIH (Bethesda) also documented overlapping AVP levels between CS patients and healthy subjects. Particularly, a study conducted by Kalogeras *et al.* on 9 healthy volunteers undergoing BIPSS highlighted the existence of a dominant sinus in secreting ACTH and AVP both at baseline and after oCRH stimulation, thus placing the emphasis on a CRH-induced AVP secretion aimed in turn at enhancing the corticotropic stimulation of CRH itself [9]. This paper was followed by a comparison between the 9 healthy volunteers and 23 patients with CD (Friedman *et al.*) [10]: the dominance of one petrosal sinus over the other in terms of hormonal secretion was confirmed also in CD patients, both at baseline and after stimulation with

oCRH; moreover, when a lateralization gradient (>1,5) was identified, ACTH and AVP varied consistently. In line with Colao *et al.* [8], also Friedman *et al.* reported similar AVP levels between CD and healthy controls, both at the periphery and, limited to the baseline assessment, at the dominant sinus level [10]. However, the NIH group constantly reported an exaggerated response of AVP to oCRH stimulation in CD. Three hypothesis were raised in this regard: a) AVP hypersecretion is an initiator or promoter of the tumorigenesis (e.g. by triggering the functional autonomization of the corticotrophs alongside the loss of function of some oncosuppressor gene), thus preceding the onset of CD and persisting despite the expected glucocorticoid inhibition; b) the pituitary tumor simultaneously produces ACTH and AVP or, at least, some AVP releasing factor aimed at amplifying, with autocrine mechanisms, the corticotropic activity of AVP; c) the mechanisms prefixed to control the corticotropic activity are intrinsically dysregulated by hypercortisolism via the suppression of endogenous CRH, which makes both the corticotrophs and AVP-producing neurons hypersensitive to exogenous CRH stimulation via receptor up-regulation. This would explain why in baseline conditions AVP did not differ between healthy subjects and patients with CD, instead of being reduced in the latter as a consequence of hypercortisolism-induced hypervolemia. Clarifications on this topic came shortly after from Yanovski *et al.*, who compared the BIPSS results of 23 patients with CD, 16 patients with EAS and 7 patients with ACS [11]. The finding of overlapping AVP levels among the three forms both in baseline conditions and, unlike ACTH, after oCRH stimulation allowed them to exclude the assumptions a) and b) in favor of hypothesis c), which considered hypercortisolism itself as the primum movens for the CRH-induced AVP secretion. As a further confirmation of the crucial role hypercortisolism has in inhibiting the regulatory mechanisms of corticotropic activity, Yanovski *et al.* also reported an inverse correlation between UFC and the AVP increase after oCRH, which was much more consistent in mild hypercortisolism. Conflicting results were finally obtained by Knoepfelmacher *et al*. when comparing the responses of 13 patients with CD and 15 healthy controls to a 7.5-hour WDT in terms of plasma AVP, pOsm and uOsm, as well as their ratio [12]. In CD patients the test was completed with the assessment of uOsm after WDT was followed by the administration of 10 µg DDAVP. As compared to healthy individuals, patients with CD had higher post-WDT AVP levels and lower uOsm, despite similar pOsm. uOsm persisted lower after DDAVP administration, thus suggesting a renal

resistance to both endogenous and exogenous AVP in hypercortisolemic states. Novel contributions came ten years later from the Japanese group led by Tatsuno, who offered 27 patients with adrenal nodules and subclinical CS a combined 1 mg-DST-AVP test, with the aim of verifying if a different response to the test could be associated to a different metabolic pattern not (completely) clinically evident yet [13]. The 14 patients defined as AVP-responders showed a better hormonal pattern, which was characterized by lower midnight plasma cortisol and 1 mg-DST than the non-responders. Although this indicated a lower degree of autonomization in hormonal secretion, their clinical phenotype was much more impaired in terms of arterial hypertension and glucose metabolism. The molecular face of this scenario was characterized by an increased expression of mRNA encoding V1aR in the adrenals of the responders as compared to the non-responders. These results are not surprising if the paramount role of AVP in impairing glucose metabolism is considered: here, AVP actions both direct and mediated by the HPA axis [14-16], accounting for the CS-like phenotype found in 25% middle-aged people with high copeptin (DM, insulin-resistance, visceral obesity, arterial hypertension, microalbuminuria, chronic inflammation and CV risk [17], also known as Pseudo-Cushing.

Despite the non-consistent, as well as non-conclusive results, after these studies the interest on the interplay between AVP and HPA systems progressively decreased and this was likely due to the critical issues AVP assessment was vitiated by. The first assay for AVP determination was developed in the 1970s [18]. Due to the small dimensions of AVP molecule preventing the development of sandwich assays, it was a competitive RIA. Still today, the limitations it was vitiated by are a matter of fact and have rendered AVP unserviceable in clinical practice. Assessing the homone is, indeed, time-consuming and brings suboptimal results in terms of SE and SP. In order to attempt improving AVP diagnostic performance, high volumes of plasma and laborious pre-analytical procedures, such as extraction and ≥24 hour incubation are currently required, which render even more challenging the routine application of AVP assessment [19]. Further issues must be considered: since a high amount of circulating AVP is bound to platelets (which express V1aR), there is the risk of both an underestimation and an overestimation of its levels, the latter in case platelets are not completely removed from plasma or delays occur in sample processing [20-21]. The short half-life (<30 minutes) and high instability of the peptide persisting at low temperatures (-20°C) further complicate this scenario. However,

the fact that AVP precursor-derived fragments neurophysin II and copeptin are released in equimolar amounts with AVP accounts for the possibility to use them as surrogate biomarkers of AVP presence and activity. While assessing neurophysin II is also difficult, as the molecular structure contains 7 disulfide bonds and tends to bind AVP, copeptin proved to be the perfect mirror peptide. As shown in Table I, a sandwich LIA and its immunofluorecent automated evolution on the KRYPTOR platform are the currently available assays for copeptin measurement [22]. As compared to AVP, copeptin assessment has several advantages: a lower plasma volume is required, no pre-analytical procedures are needed (extraction, purification, protease addition), the analytic performance is good (high SE, minimal inter-laboratory variability), the assay is not time-consuming and molecular stability up to 7 days at ambient temperature (up to 14 days at 4°C) is guaranteed on different serum/plasma samples.

	Size (amino acids)	**Serum/plasma volume (µl)**	**Extraction**	**Molecular stability**	**Assay**	**SE**	**Analyical time (hours)**
AVP	9	400	yes (bound to platelets)	no (neither at 20°C)	competitive RIA	low	up to 48
Copeptin	39 + glycan	50	no	yes (up to 7-14 days in different samples)	sandwich LIA; automated immunofluorescence (KRYPTOR)	high	0.5-2.5

Table I – Laboratory assessment of AVP and copeptin: strenghts and pitfalls.

In the last years, the wider availability of rapid, high-sensitivity immuno-enzimatic assays for copeptin let clinicians overcome AVP-related pitfalls, so that copeptin assessment is positively contributing to both the clinical practice management of hydro-electrolitic homeostasis disturbances (diabetes insipidus above all), and the prognostic definition of those conditions finding (at least part of) their pathogenesis in the dysregulation of AVP system [23-25]. The latter include DM, arterial hypertension, obesity and the metabolic syndrome, thromboembolic disease, coronary artery disease and bone fractures, which are also the main systemic complications of CS [26].

With the exception of a recent post-operative evaluation aimed at exploring its predictive value for early disease remission [27], copeptin behavior as a surrogate marker of AVP in CS is still unexplored. Preliminary data on our experience show patients with CS had lower plasma copeptin levels than otherwise healthy individuals [unpublished data]; moreover, copeptin seemed to modulate the ACTH and cortisol response following stimulation with hCRH and DDAVP in a small cohort of patients with CD.

Aims of the research project

On these bases, we explored the potential applications of copeptin in the pathology of the HPA axis, particularly as a diagnostic and/or prognostic biomarker in CS.

The objectives of this research project were the following:

1) to determine whether plasma copeptin levels differ between patients with CS and healthy subjects, as well as between patients with CS and subjects with primary and secondary AI (investigated in **Study 1**);
2) to determine whether plasma copeptin levels are correlated with the key point diagnostic biochemical features of CS: severity of hypercortisolism, loss of cortisol circadian secretory rhythm, loss of negative glucocorticoid feedback and etiology of CS (investigated in **Study 2**);
3) to determine whether plasma copeptin levels are correlated with the clinical burden of CS with regards to hypercortisolism-related systemic comorbidities and their biomarkers (investigated in **Study 2**);
4) to determine whether plasma copeptin levels are subject to intra-individual variations in response to the treatment of CS and according to disease control status during longitudinal follow-up (investigated in **Study 3**).

STUDY 1 – Copeptin behavior in HPA axis dysfunctions

Purpose

To determine whether plasma copeptin levels differ between patients with CS and healthy subjects, as well as between patients with CS and subjects with primary and secondary AI.

Study design

Cross-sectional study.

Patients and methods

All the patients admitted to the Endocrinology and Metabolic Diseases Department of Ancona University Hospital (Azienda Ospedaliero-Universitaria delle Marche, Ancona, Italy) between January 2016 and December 2022 with CS, either suspected or diagnosed according to the criteria established by the Endocrine Society [28-29], or diagnosed AI were consecutively enrolled (n=223). Among them, those having at least one determination of plasma copeptin were selected (n=200). If a patient was admitted more than once in the very same clinical situation (e.g. newly diagnosed CS and re-evaluation of the same patient with active CS in naive conditions due to initial denial of any therapy, or reiterated clinical suspicion of CS in the same patient) during the observation period, the first admission in which plasma copeptin was available was chosen in chronological order. Adjunctive exclusion criteria were active DI (n=3) and Nelson's syndrome (n=2), which would bring issues in interpreting copeptin levels and HPA axis parameters, respectively. Based on the results of the HPA axis evaluation [28-29], the 195 selected patients were then divided into the following three groups: "CS" (n=89), "healthy HPA" (n=77), and "AI" (n=29). CS group – For the study purpose, we chose to maintain in the "CS" group the following patient profiles: (a) newly diagnosed, or known but treatment-naive CS (n=41); (b) surgery-/RT-naive CD admitted after a ≥6 month wash-out from any CS-directed drugs (n=6); (c) post-surgical recurrence of CD (n=10); (d) uncontrolled CS despite medical treatment (n=14). As widely

accepted, mUFC levels >ULN defined an uncontrolled disease [28]; however, patients treated with metyrapone were defined uncontrolled if worsening of both biochemical markers and clinical features was documented, since LC-MS/MS is no more available at our site. Patients with active, but controlled CS at their first admission during the study period (n=6) were excluded, whereas remission of CD following pituitary surgery or bilateral adrenalectomy made the patients shift into the "AI" (n=7) or the "healthy HPA" (n=5) group, depending on the development of iatrogenic AI. Healthy HPA group – Among the patients enrolled in the "healthy HPA" group after screening failure for endogenous CS (n=77), the following final diagnoses were included: (a) primary metabolic syndrome (defined as the co-existence of ≥3 among: central obesity, hypertriglyceridemia, low HDL cholesterol, arterial hypertension, elevated fasting glucose); (b) any combination of two components of the metabolic syndrome; (c) polycystic ovary syndrome; (d) non-functioning adrenal tumor or bilateral hyperplasia; (e) non-functioning pituitary tumor. AI group – In addition to the above-mentioned CS patients resulted in iatrogenic AI, the "AI" group was composed by 14 primary and 15 secondary AI patients. All of them had their plasma copeptin measured under adequate replacement conditions in terms of glucocorticoids and, where applicable, mineralocorticoids or other pituitary hormone deficiencies. Replacement regimens were defined adequate according to the currently accepted clinical and biochemical criteria, as established by the Endocrine Society [29-30]. The final structure of the study cohort (Study 1 Figure 1) included 189 patients (58 males and 131 females, mean age 50±15 years): of them, 71 were enrolled in the "CS", 82 in the "healthy HPA" and 36 in the "AI" groups. After obtaining patients' informed consent, the following data were collected: gender, age, body weight, height, BMI (weight/height^2), 1 mg-DST, mUFC (≥2 samples), mLNSC (≥2 samples), copeptin, fasting glucose, plasma sodium and plasma urea to calculate plasma osmolality ($pOsm = 2 \times pSodium + \frac{pGlucose}{18} + \frac{pUrea}{2.8}$), creatinine, eGFR (Cockroft), urine sodium (consistently available only for CS and AI patients), HbA1c, smoking habits, presence of diabetes mellitus (DM), use of diuretics (SGLT2i included), presence of hypoaldosteronism, presence of hypopituitarism, pituitary hormonal deficiencies, glucocorticoid replacement dose (expressed in equivalent hydrocortisone milligrams). Separate comparisons were made between the "CS" and each of the two control groups.

Plasma copeptin measurement

The ultra-sensitive, immuno-luminometric BRAHMS Copeptin assay on the KRYPTOR Compact Plus system (Thermo Fisher Scientific, Hennigsdorf, Germany) was used to measure copeptin levels. This assay has a lower limit of detection 0.7 pmol/L and a functional sensitivity <2 pmol/L.

Statistical analysis

Continuous variables are presented either as mean ± SD or as median (IQR), depending on their distribution (normal vs non-normal, respectively); categorical variables are expressed as number and percentage (%). The one sample t-test was used to verify if mean copeptin levels in the study group were the same as those documented in the general population. After verifying the normal distribution of quantitative variables with the Shapiro-Wilk test, comparisons between two groups were made with the Student's t-test (normal distribution), or the Mann-Whitney test (non-normal distribution). For comparisons concerning more than two groups the ANOVA (parametric), or the Kruskal-Wallis test (non-parametric) were run and the Bonferroni test was used for *post hoc* analyses. Categorical variables were compared with the chi-square (χ^2) or the Fisher's exact test, where appropriate. The Pearson's P and the non-parametric Spearman's Rho (ρ) were used, as appropriate, for the bi-varied correlation analyses, whereas the Beta (β) coefficient was used for the linear and logistic regression analyses. A P value of 0.050 was considered for the statistical significance. The IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. was used for the statistical analysis.

Results

Study 1 Table I illustrates the clinical and biochemical features of the 189 study participants and the three study groups. Despite an extreme interindividual variability, mean copeptin levels were significantly higher in the study cohort than the general population (6.8 (range 0.8-71.2) *vs* 3.8 (range 0.44-44.3) pmol/L [31], p<0.001). Copeptin levels were higher in males (9.0 *vs* 5.9, p=0.005; Study 1 Figure 2), patients with DM (8.7 *vs* 5.1, p=0.048) and diuretic users (9.9 *vs* 5.3, p=0.001), with an even more pronounced difference in case

DM and use of diuretics co-existed (4.9 pmol/L in neither DM nor diuretic use, *vs* 6.2 pmol/L in either DM or diuretic use, *vs* 11.1 pmol/L in both DM and diuretic use, p=0.003; Study 1 Figure 3); no significant difference was noted between smokers and non-smokers, as well as between patients with and without hypopituitarism or hypoaldosteronism. Copeptin levels were correlated with age (ρ=0.190, p=0.009), weight (ρ=0.318, p<0.001), BMI (ρ=0.305, p<0.001) and HbA1c (ρ=0.181, p=0.031), but neither with plasma osmolality and its determinants except for urea (ρ=0.205, p=0.007), nor with eGFR or urine sodium. Although weakly, male gender (β=-0.179, p<0.014), age (β=0.301 p<0.001), the presence of DM (β=0.230 p=0.001), the use of diuretics (β=0.269 p<0.001) as well as their co-existence (β=0.299 p<0.001), but not weight/BMI, separately influenced copeptin levels. A multiple regression was therefore run to predict copeptin from gender, age and the presence of DM combined with the use of diuretics (Combo DM/diuretic). The model significantly predicted copeptin levels with moderate quality: $F(3,185)=12.808$, $p<0.001$, $R^2=0.172$ (Study 1 Table II).

CS vs Healthy HPA

The members of the two groups were age- and gender-matched, but patients with CS had significantly lower BMI (28 *vs* 32 kg/m^2, p=0.016) and eGFR (118 *vs* 139 ml/min, p=0.022) (Study 1 Table I). The proportion of smokers was similar between groups, as well as the distribution of DM and use of diuretics, considered both separately and in combination. As expected, the two groups obtained significantly different results when tested for CS (p<0.001 for 1 mg-DST, mLNSC and mUFC) and the proportion of hypopituitary patients was higher among those with CS (37% vs 15%, p=0.001): TSHD was the most represented pituitary deficiency in this group, with a twice-higher prevalence as compared to the Healthy HPA patients (81% *vs* 42%, p=0.026). Among the latter, FSH/LHD was the preponderant pituitary deficiency (75%). Despite overlapping and extremely variable copeptin levels (3.8 (range 0.8-47.7) pmol/L in CS *vs* 5.0 (range 1.1-71.2) pmol/L in Healthy HPA, p>0.050), patients with CS had significantly higher plasma sodium and osmolality than controls (142 *vs* 141 mmol/L, p=0.022 and 302 *vs* 297 mOsm/kg, p<0.001, respectively), despite similar plasma fasting glucose and urea. After adjusting copeptin levels for plasma sodium and osmolality, a significant difference between CS and Healthy HPA was highlighted (0.046 *vs* 0.054 p=0.048 for cop/pNa, 0.021 *vs* 0.025 p=0.038 for cop/pOsm, respectively). Copeptin was correlated neither with plasma osmolality nor with plasma or, limited

to CS, urine sodium. Although weakly, copeptin was correlated with fasting glucose (ρ=0.208, p=0.010) and urea (ρ=0.177, p=0.032), which influenced its levels both separately (β=0.252 p=0.002 for fasting glucose, β=0.453 p<0.001 for urea) and in a multiple linear regression model (F(2-144)=29.243, p<0.001, R^2=0.289; β=0.291 p<0.001 for fasting glucose, β=0.478 p<0.001 for urea). As shown in Study 1 Figures 4 and 5, when patients with CS were splitted from those with a Healthy HPA, copeptin levels were significantly influenced by glucose only in the former (β=0.476, p<0.001), whereas urea had a predictive role on copeptin only in the latter (β=0.644, p<0.001). Therefore, the effect of diabetes mellitus and of diuretic-induced dehydration (likely reflected by urea) on copeptin was further explored by stratifying patients as shown in Study 1 Table III. CS and Healthy HPA patients differed in their copeptin levels not much for their DM status, but for the resetting of their AVP system determined by the use of diuretics (9.8 *vs* 5.4 pmol/L, p=0.003), or by the combination of the two parameters (4.8 *vs* 6.2 *vs* 10.9 pmol/L, p=0.006). In the absence of these factors, patients with CS exhibited the lowest copeptin (Study 1 Figure 6): however, when comparing the Combo status 0 (Healthy HPA, None) with the Combo status 3 (CS, None), the difference in copeptin levels was not statistically significant. Similar results emerged when comparing the Combo status 2 (Healthy HPA, Both) with the Combo status 5 (CS, Both), but not when comparing the Combo status 1 (Healthy HPA, Either) with the Combo status 4 (CS, Either): this was the only situation in which copeptin levels significantly differed between Healthy HPA and CS patients (p=0.022). A logistic regression was run to test copeptin as a predictor for CS in the presence of clinical suspicion, with no significant results. Nonetheless, no correlations were shown between copeptin and the three biochemical parameters tested for the screening of CS. Of note, plasma osmolality was correlated with mLNSC (ρ=0.315, p=0.001) and 1-mg DST (ρ=0.262 p=0.009, Study 1 Figure 7), the latter being one of its independent predictors in a good multiple linear regression model (F(5-72)=25.893, p<0.001, R^2=0.643; β=0.272 p=0.023 for 1 mg-DST, β=0.469 p<0.001 for plasma sodium, β=0.544 p<0.001 for urea). Moreover, despite the finding of overlapping copeptin levels between patients with and without hypopituitarism, copeptin was significantly lower in the subgroup of patients with TSHD as compared to their counterpart (6.7 *vs* 14.7 pmol/L, p=0.007).

CS vs AI

The members of the two groups were matched by age and BMI (Study 1 Table I). Gender distribution was almost equal in the AI group, whereas females were largely predominant among CS patients (56% *vs* 78%, p=0.026), who were also reported to have a higher eGFR (139 *vs* 93 ml/min, p=0.002). More patients were smokers (37% *vs* 17%, p=0.033) or had DM, either alone (59% *vs* 19%, p<0.001) or combined with the use of diuretics (69% *vs* 28%, p=0.017), in the CS group as compared to the AI group. Among AI members, hypoaldosteronemic patients were 16/36 (44%). The proportion of hypopituitary patients was higher among those with AI (58% vs 37%, p=0.032): apart from ACTH deficiency, which involved 22/36 (61%) AI members, the two groups differed in the proportion of GHD patients (48% AI *vs* 15% CS, p=0.025). Copeptin levels confirmed highly variable and similar between groups (3.8 (range 0.8-47.7) pmol/L in CS *vs* 5.0 (range 2.0-37.3) pmol/L in AI, p>0.050), and once again patients with CS had significantly higher plasma sodium and osmolality than controls (142 *vs* 140 mmol/L, p=0.048 and 302 *vs* 291 mOsm/kg, p<0.001, respectively), despite similar plasma fasting glucose and urea. However, no significant differences between CS and AI patients emerged after adjusting copeptin levels for plasma sodium (cop/pNa) and osmolality (cop/pOsm). Copeptin was correlated neither with plasma osmolality, nor with plasma or urine sodium, nor with plasma glucose. As shown above, copeptin levels were significantly influenced by glucose in CS patients (β=0.476, p<0.001), when splitted from those with AI (Study 1 Figure 8). A weak correlation was shown between copeptin and urea (ρ=0.252, p=0.014), but the linear regression analysis did not identify urea as a predictor for copeptin. Considering only patients with AI, no correlations between copeptin levels and the glucocorticoid replacement dose were found, nor differences in copeptin levels according to the presence of hypoaldosteronism (i.e. primary *vs* secondary AI) or hypopituitarism were seen. The analysis was then focused on the effects exerted by the presence of diabetes mellitus and diuretic-induced dehydration (once again likely reflected by urea) on copeptin and patients were stratified as previously done (Study 1 Table IV). When comparing patients with CS and AI, DM status did not affect copeptin levels, but the resetting of patients' AVP system determined by the use of diuretics (8.8 *vs* 5.2 pmol/L, p=0.003), or by the combination of diuretics and DM (4.7 *vs* 5.9 *vs* 10.4 pmol/L, p=0.006) did. In the absence of these factors, patients with CS persisted in exhibiting the lowest copeptin (Study 1 Figure 9). However, none of the three direct comparisons

(Combo status 0 vs Combo status 3; Combo status 1 vs Combo status 4; Combo status 2 vs Combo status 5) resulted in significantly different copeptin levels.

Discussion

Due to its nature of AVP "mirror peptide", copeptin may be a novel biomarker in CS. This is, indeed, a condition of chronic HPA axis hyperactivation whose pathogenesis also involves a dysregulation of AVP system [26, 32-35]. In this cross-sectional study, we investigated the potential application of copeptin as a diagnostic biomarker for CS in a large cohort of patients referring to the Endocrinology and Metabolic Diseases Department of Ancona University Hospital (Azienda Ospedaliero-Universitaria delle Marche, Ancona, Italy) over the last 6 years. Patients with either suspected or already diagnosed, but active and uncontrolled, CS, as well as patients with diagnosed primary and secondary AI were included and copeptin levels were compared between the CS group and each of the control groups. When running the analysis, confounding factors such as DM or CV comorbidities requiring the use of diuretics (SGLT2i included), which notably activate AVP system, were given the outmost importance [36-37]. Copeptin levels were higher in the study cohort than the general population, but exhibited the same large inter-individual variability [31]. A gender difference favoring males was highlighted for copeptin, which was also higher in patients with DM and diuretic users, but did not vary according to smoking habits and the presence of hypopituitarism or hypoaldosteronism [36-40]. As expected, copeptin levels correlated with age, weight, long-term glycemic control and urea as markers of DM and dehydration, respectively. Surprisingly, no relationships were observed concerning plasma osmolality, plasma or urine sodium, fasting glucose and kidney function. Male gender, age and the combination of DM and the use of diuretics were independent predictors for copeptin. Patients with CS and those whose HPA axis resulted healthy after screening were matched by age, gender, smoking habits and the presence of the above-mentioned confounders, considered both separately and in combination ("combo DM/diur"). As previously reported both for copeptin in healthy subjects (Bhandari et al. [41]) and for AVP in hypercortisolemic patients (Knoepfelmacher et al. [12]), we found an extreme inter-individual variability in copeptin levels, which however were similar between groups (3.8 vs 5.0 pmol/L $p>0.050$, respectively). This finding was in line with

previous results concerning AVP by Friedman [10] and Colao [8], but this study brings some additional contributions of great pathophysiological value. First, patients with CS had higher plasma osmolality and sodium, thus a significantly lower adjusted copeptin ($p=0.038$ for cop/pOsm, $p=0.048$ for cop/pNa), than controls, despite similar plasma fasting glucose and urea and a lower eGFR. This was likely due to hypercortisolism itself rather than diuretic-induced dehydration, which would have involved both groups equally ($p>0.050$ for diuretic use) and the finding of a significant correlation between plasma osmolality and some biomarkers of CS (mLNSC and 1 mg-DST, the latter being also an independent predictor for osmolality) supports this hypothesis. Second, the "real" copeptin levels were masked, or distorted as well, by the non-negligible effect exerted by DM [39] and, even more, by diuretic-induced dehydration. Both fasting glucose and urea were, indeed, independent predictors for copeptin, but their action differed when splitting the cohort into CS (where copeptin was influenced only by glucose, $\beta=0.476$, $p<0.001$) and Healthy HPA (whose copeptin was primarily influenced by urea, $\beta=0.644$, $p<0.001$). When stratified according to their disease status and, in turn, the presence of DM, the use of diuretics and the combination of the two confounders, CS and Healthy HPA patients differed in their copeptin levels for the resetting of their AVP system determined by the use of diuretics ($p=0.006$), or by the combination of diuretics and DM ($p=0.005$). In the absence of confounders, CS patients exhibited the lowest, albeit non statistically significant, copeptin. When the confounders acted in combination, their influcence on copeptin levels exceeded such as to mask that of hypercortisolism, which became visible when only one of the confounders existed (Combo status 1 vs Combo status 4, $p=0.022$). The lower BMI and the higher prevalence of hypopituitarism, albeit adequately replaced, among CS patients may have influenced copeptin. Specifically concerning the latter, the presence of a TSHD was a predictor for lower copeptin levels (data not shown): this finding was pretty unexpected, as central hypothyroidism is one of the exclusion criteria for the diagnosis of SIAD [42]. However, this unusual relationship may just reflect the presence of chronic hypercortisolism (cortisol-induced copeptin inhibition and functional hypothyroidism) in the absence of any direct relationships between copeptin and the biochemical markers of CS. Patients with CS and AI were matched by age and BMI. Female gender, smoking habits, DM and the use of diuretics, the latter either alone or in combination, were more represented in CS

group, whereas hypoaldosteronism and hypopituitarism prevailed among AI patients. Once again, plasma osmolality and sodium were higher in CS than AI patients, whereas fasting glucose and urea did not differ. Despite this, both copeptin (3.8 *vs* 5.0 pmol/L, p>0.050) and adjusted copeptin (cop/pOsm and cop/pNa) were similar between groups. When stratified according to their disease status and, in turn, the presence of DM, the use of diuretics and the combination of the two confounders, once again CS and AI patients differed in their copeptin levels for the resetting of their AVP system determined by the use of diuretics (p=0.003), or by the combination of diuretics and DM (p=0.006). However, none of the three direct comparisons we performed (Combo status 0 vs Combo status 3; Combo status 1 vs Combo status 4; Combo status 2 vs Combo status 5) resulted in significantly different copeptin levels. In this regard, one important selection bias must be considered among the others (different gender distribution, smoking habits, prevalence of the confounding factors): all AI patients were rather to be considered eucortisolemic, because the sample for copeptin was taken in the morning, 60 to 90 minutes after they took their replacement therapy, whose adequacy had been preliminary verified according to the management modalities established by the Endocrine Society [29-30]. Copeptin levels were neither correlated with glucocorticoid replacement dose, nor varied according to the etiology of AI in our cohort. In primary AI patients, mineralocorticoid replacement was also adequate [43]. This may have further masked the discrepancy in copeptin levels we expected between CS and AI favoring the latter: in AI, indeed, the free water clearance is impaired due to altered cAMP-mediated AVP signalling towards V2R in the collection duct, with subsequent increase in AVP levels [44-47]. In turn, AVP excess either determines an euvolemic, hyposmolar hyponatremia (SIAD) in secondary AI, or exacerbates the hypoaldosteronism-induced salt wasting with hypovolemic hyponatremia in primary AI. None of the enrolled patients had hyponatremia and no relationships between copeptin, plasma osmolality, plasma or urine sodium were observed.

Considering all, in the presence of a clinical suspicion for CS, copeptin assessment turned out unuseful. Moreover, copeptin behavior does not differ between the two opposite HPA axis dysfunctions, at least in the case of adequately replaced AI.

	All (n=189)	CS (n=71)	Healthy HPA (n=82)	AI (n=36)	p
Gender, female	131 (69)	55 (78)*	65 (68)	20 (56)*	0.026*
Age (years)	50±15 (16-89)	48±13 (19-78)	50±17 (17-89)	52±16 (16-82)	n.s.
BMI (kg/m2)	29 (11)	28 (9)•	32 (10)•	25 (8)	0.016•
eGFR, Cockroft (ml/min)	122±54 (33-386)	118±36 (33-211)•*	139±67 (38-386)•	93±35 (36-177)*	0.022• 0.002*
Diabetes mellitus	90 (48)	42 (59)*	41 (50)	7 (19)*	<0.001*
Diuretics	61 (32)	27 (38)	27 (33)	7 (19)	n.s.
Combo DM/diuretic use - **none** - **either** - **both**	 84 (45) 59 (31) 46 (24)	* 22 (31) 29 (41) 20 (28)	 36 (44) 24 (29) 22 (27)	* 26 (72) 6 (17) 4 (11)	0.017*
Smoke	52 (28)	26 (37)*	20 (24)	6 (17)*	0.033*
Copeptin (pmol/L)	4.6 (4.4)	3.8 (4.4)	5.0 (4.6)	5.0 (2.9)	n.s.
plasma Osmolality (mOsm/kg)	298±9 (267-321)	302±7 (289-319)•*	297±10 (275-321)•	291±9 (267-307)*	<0.001• <0.001*
plasma Sodium (mmol/L)	141±3 (130-147)	142±3 (135-147)•*	141±3 (132-147)•	140±4 (130-146)*	0.022• 0.048*
plasma Urea (mmol/L)	12.6 (5.4)	13.6±3.8 (7.9-29.2)	13.9±7.4 (4.7-56.2)	14.3±4.2 (8.3-21.2)	n.s.
plasma Glucose, fasting (mmol/L)	5.3 (1.6)	5.3 (2.0)	5.5 (1.5)	5.0 (0.8)	n.s.
	133.7 (103.7)	144.0 (103.6)	n.a.	129.9 (100.5)	n.s.

urine sodium (mmol/24h)					
HbA1c (mmol/mol)	41 (17)	41 (17)	42 (23)	39 (7)	n.s.
1 mg-DST (nmol/L)	115 (365)	375 (375)	30 (25)	n.a.	<0.001
mLNSC (nmol/L)	11 (19)	19 (14)	3 (6)	n.a.	<0.001
mUFC (nmol/24h)	2800 (6132)	8029 (8829)	1982±1083 (248-5104)	n.a.	<0.001
GC replacement (mg HC eq/day)	20.5±8.1 (6.7-40.0)	n.a.	n.a.	20.5±8.1 (6.7-40.0)	n.a.
Hypopituitarism - **n. deficiencies (except ACTH)** - **TSHD** - **FSH/LHD** - **GHD** - **PRLD**	59 (31) 2 (1) 38 (64) 35 (59) 18 (31) 7 (12)	26 (37)•* 2 (1) 21 (81)• 15 (58) 4 (15)* 4 (15)	12 (15)• 2 (1) 5 (42)• 9 (75) 4 (33) 1 (8)	21 (58)* 2±2 (0-4) 12 (57) 11 (52) 10 (48)* 2 (10)	0.001•, 0.032* 0.026• 0.025*
Hypoaldosteronism	16 (9)	n.a.	n.a.	16 (44)	n.a.

Study 1 Table I – Clinical and biochemical features of the study participants and the three study groups.

Model	Non-standardized coefficients		Standardized coefficients	t-test	p	95% confidence interval (CI) for B	
	B	Standard deviation error	Beta			Inferior limit	Superior limit
Constant	1.683	2.019		0.834	0.406	-2.300	5.667
Gender	-3.275	1.163	-0.190	-2.186	0.005	-5.569	-0.980
Age	0.110	0.037	0.211	2.977	0.003	0.037	0.182
Combo DM/diuretics	2.457	0.703	0.248	3.494	0.001	1.069	3.844

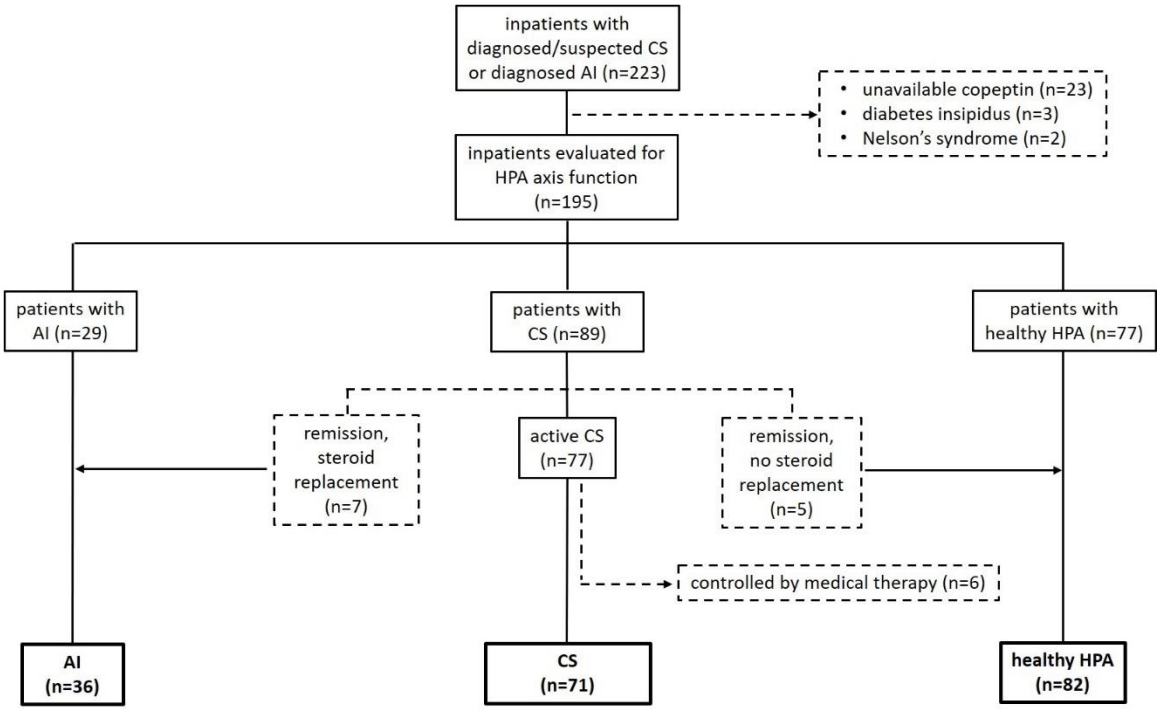
Study 1 Table II – Multiple regression model best predicting copeptin levels.

	Copeptin, median (IQR)	p
DM status 0 = HHPA, DM- 1 = HHPA, DM+ 2 = CS, DM- 3 = CS, DM+	 4.9 (3.7) 5.2 (7.2) 3.5 (4) 4.7 (5.7)	0.052
Diuretic status 0 = HHPA, diur- 1 = HHPA, diur+ 2 = CS, diur- 3 = CS, diur+	 4.6 (5.0) 6.3 (10.2) 3.4 (3.7) 6.3 (5.6)	0.006
Combo DM/diuretic status 0 = HHPA, None 1 = HHPA, Either 2 = HHPA, Both 3 = CS, None 4 = CS, Either 5 = CS, Both	 4.7 (3.7) 5.6 (6.5)* 6.5 (12.5) 3.4 (4.2) 3.7 (3.2)* 6.9 (7.2)	0.005 (*0.022)

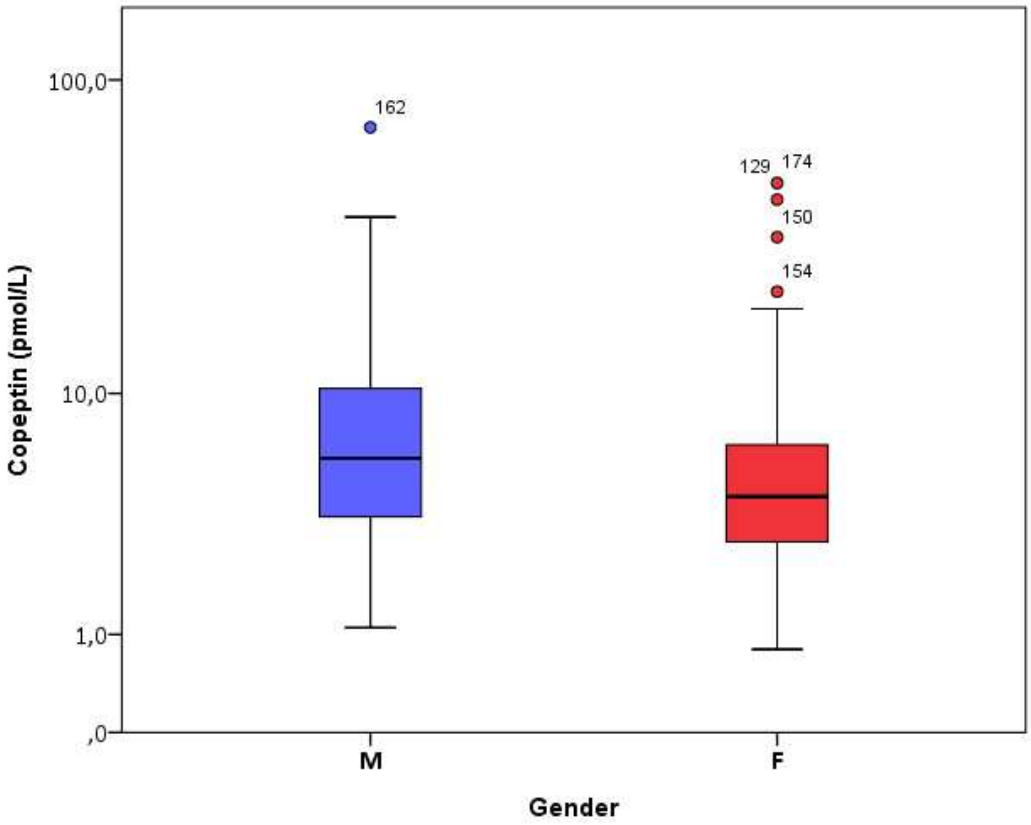
Study 1 Table III – Copeptin levels according to patients' status in terms of HPA axis pathology, DM, use of diuretics.

	Copeptin, median (IQR)	p
DM status 0 = AI, DM- 1 = AI, DM+ 2 = CS, DM- 3 = CS, DM+	 4.2 (3.6) 5.9 (2.6) 3.5 (4) 4.7 (5.7)	0.181
Diuretic status 0 = AI, diur- 1 = AI, diur+ 2 = CS, diur- 3 = CS, diur+	 4.1 (2.9) 6.2 (5.6) 3.4 (3.7) 6.3 (5.6)	0.013
Combo DM/diuretic status 0 = AI, None 1 = AI, Either 2 = AI, Both 3 = CS, None 4 = CS, Either 5 = CS, Both	 4.1 (3.1) 5.3 (3.3) 6.1 (25.6) 3.4 (4.2) 3.7 (3.2) 6.9 (7.2)	0.013

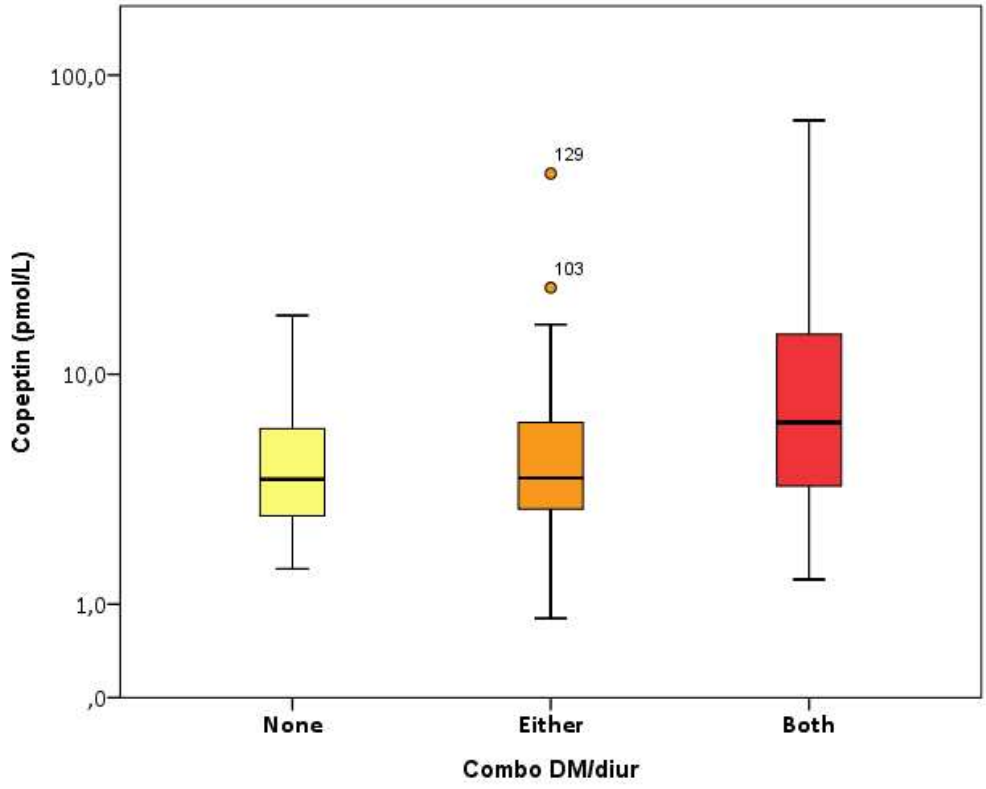
Study 1 Table IV – Copeptin levels according to patients' status in terms of HPA axis dysfunction, DM, use of diuretics.



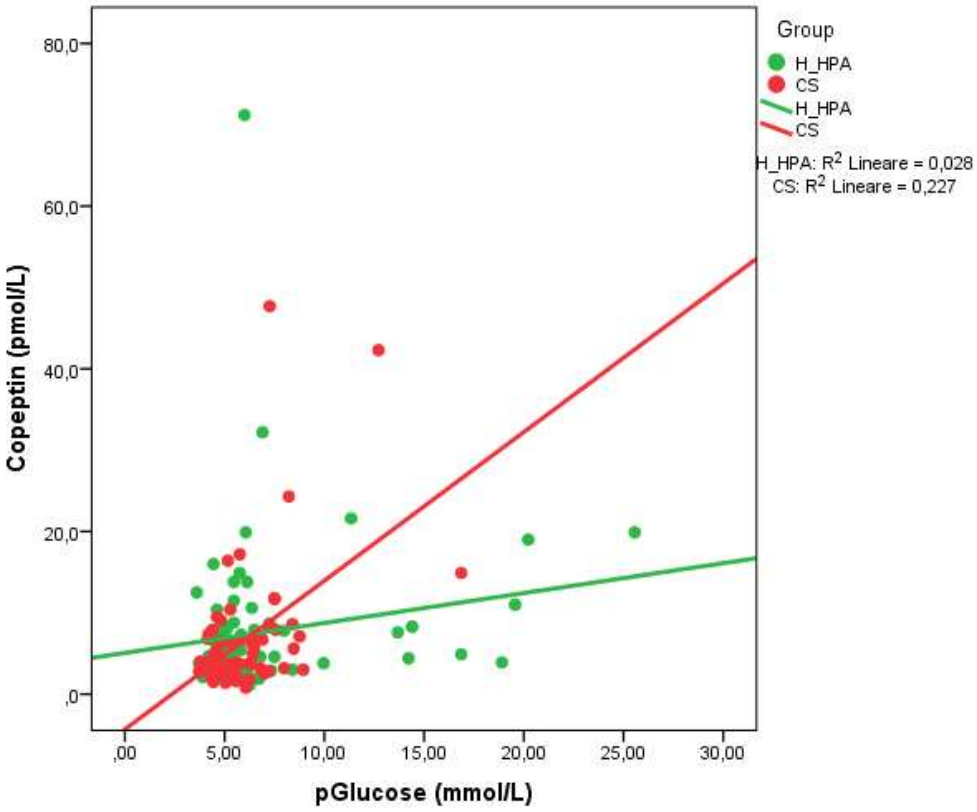
Study 1 Figure 1 – Enrollment strategy and creation of the study groups.



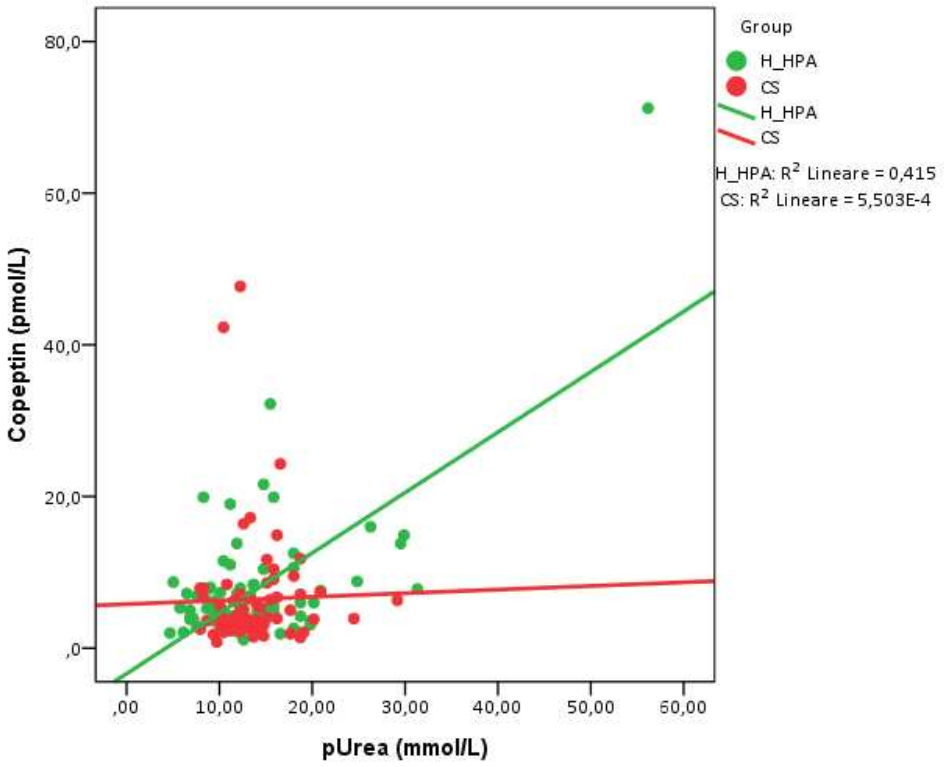
Study 1 Figure 2 – Gender distribution of copeptin levels (p=0.005).



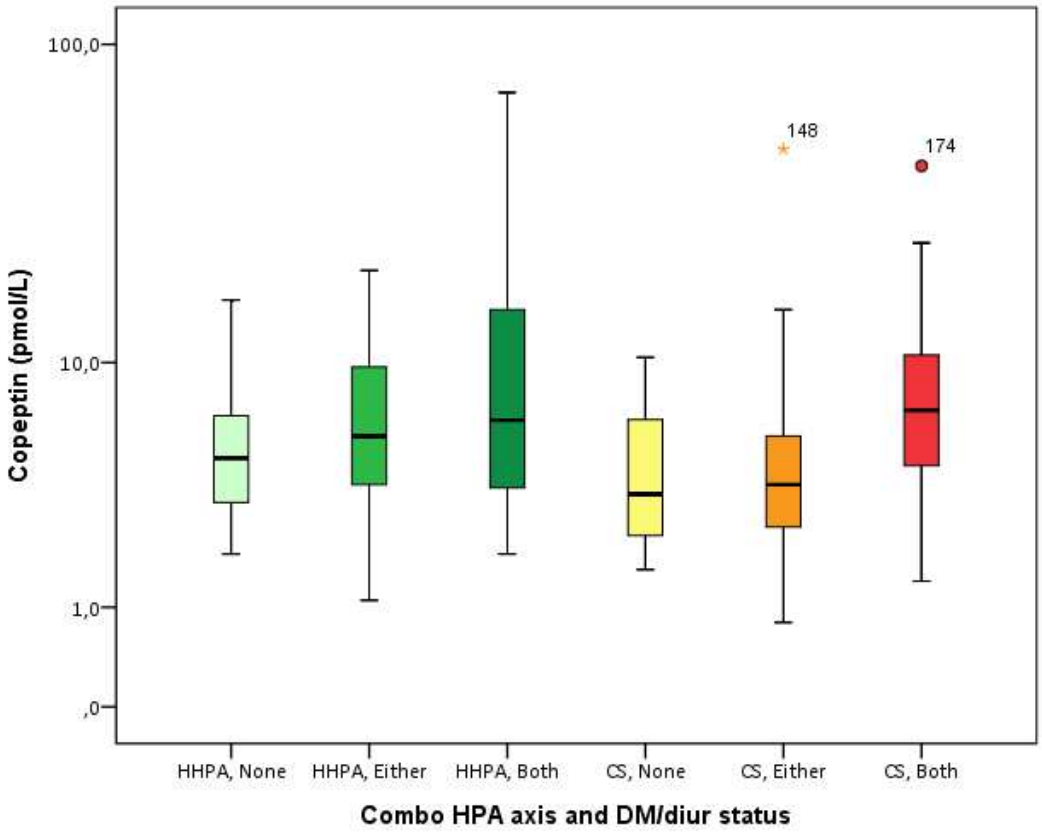
Study 1 Figure 3 – Distribution of copeptin levels according to the presence of DM and/or use of diuretics (p=0.003).



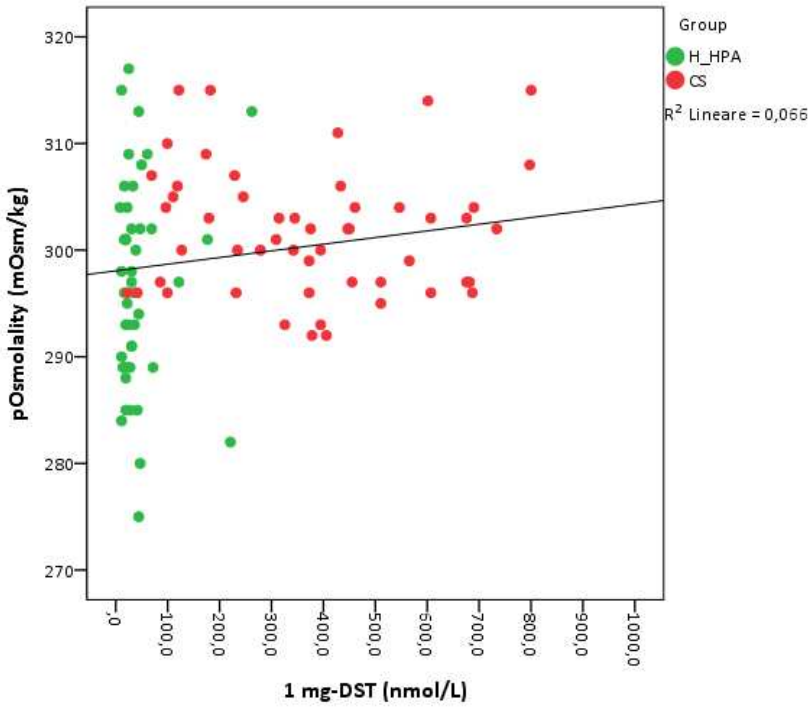
Study 1 Figure 4 – Linear regression between fasting glucose and copeptin (p<0.001 for CS, p>0.050 for Healthy HPA).



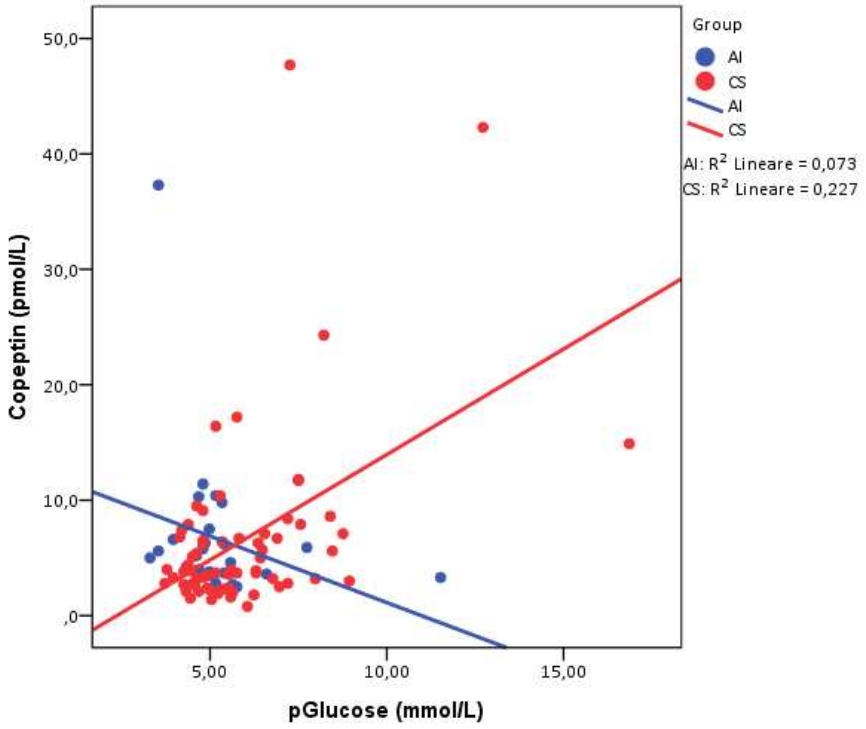
Study 1 Figure 5 – Linear regression between urea and copeptin (p>0.050 for CS, p<0.001 for Healthy HPA).



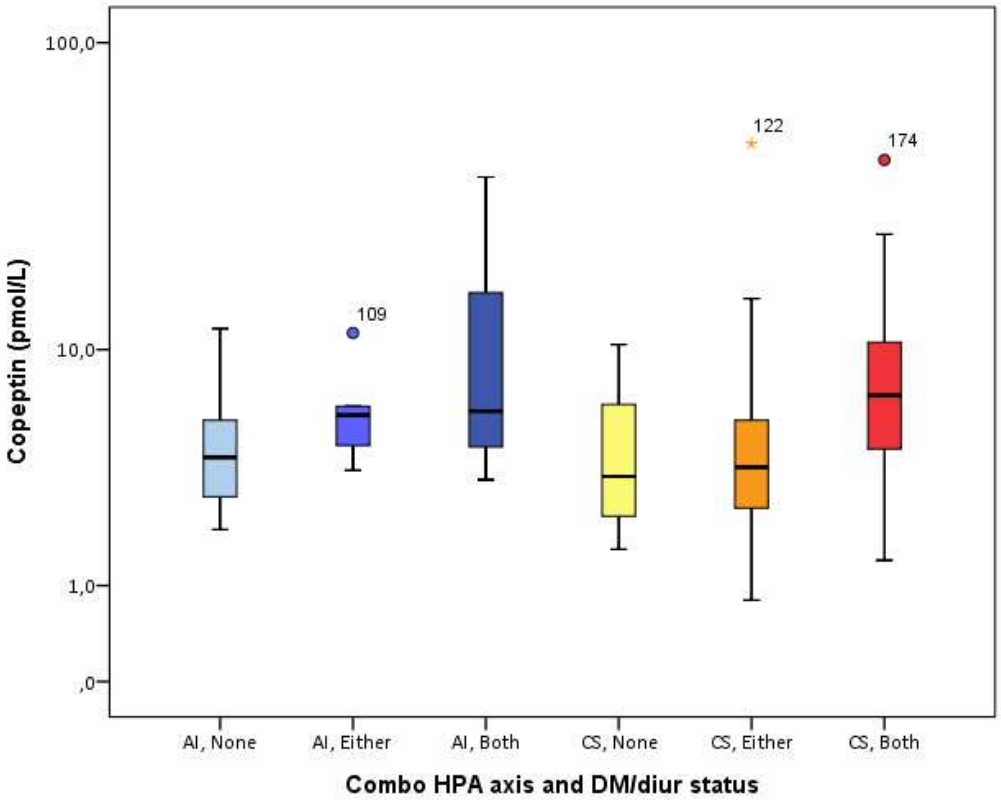
Study 1 Figure 6 – Copeptin levels according to patients' status (HPA axis pathology, combo DM/diuretics).



Study 1 Figure 7 – Relationship between 1-mg DST and plasma osmolality (ρ=0.262 p=0.009).



Study 1 Figure 8 – Linear regression between fasting glucose and copeptin (p<0.001 for CS, p>0.050 for AI).



Study 1 Figure 9 – Copeptin levels according to patients' status (HPA axis dysfunction, combo DM/diuretics).

STUDY 2 – Use of copeptin in the diagnostic work-up and prognostic assessment of Cushing's syndrome

Purpose

To determine whether plasma copeptin levels are correlated with the key point diagnostic biochemical features (severity of hypercortisolism, loss of cortisol circadian secretory rhythm, loss of negative glucocorticoid feedback and etiology), as well as with the clinical burden (hypercortisolism-related systemic comorbidities and their biomarkers) of CS.

Study design

Cross-sectional study.

Patients and methods

All the patients admitted to the Endocrinology and Metabolic Diseases Department of Ancona University Hospital (Azienda Ospedaliero-Universitaria delle Marche, Ancona, Italy) between January 2016 and December 2022 with a history of CS were consecutively enrolled (n=112). Among them, those having both active CS and at least one determination of plasma copeptin, provided the absence of both posterior pituitary dysfunctions and water intake/urine output impairment (a range of 500-3000 ml/day was considered normal), were selected (n=80). If a patient was admitted more than once during the observation period, the first admission in which plasma copeptin was available was chosen in chronological order. The study cohort included 64 (80%) females and 16 (20%) males, aged 50±14 (19-78) years. The diagnosis of CS and the subsequent etiological diagnosis were made according to the criteria established by the Endocrine Society and the Consensus Statement on the diagnosis and complications of CS [26, 28]. Among the dynamic tests we used for the differential diagnosis of ACTH-dependent forms, the stimulations with hCRH and DDAVP deserve a detailed mention. Each patient underwent both tests, which were performed on two different days

of hospital stay in standardized conditions (starting at 08:00 after a 8-hour fasting, supine patient). Plasma ACTH and cortisol levels were measured before (0') and 15', 30' and 60' after the intravenous administration of 100 µg hCRH (CRH Ferring®) or 10 µg DDAVP (DDAVP Ferring®): the magnitude of the change (Δ) in ACTH and cortisol levels from baseline to their peak was calculated according to the formula $\Delta = \frac{peak\ hormone - baseline\ hormone}{baseline\ hormone} \times 100$. A positive response was defined by ΔACTH ≥35% and ΔCortisol ≥20% [48-49]. For patients diagnosed with CS before 2016, thus enrolled during their follow-up, an active disease was defined as the simultaneous presence of mUFC >3034 nmol/24h (corresponding to 110 µg/24h, which is the local ULN; at least two measurements were required) and one among the following criteria: 1 mg-DST >50 nmol/L (corresponding to 1,8 µg/dl), mLNSC >8 nmol/L (corresponding to 0,30 µg/dl, which is the local ULN; at least two measurements were required), re-appearance or worsening of clinical features suggestive for hypercortisolism, provided the patient was not taking glucocorticoids for any reasons. Patients taking CS-targeted medications were also considered as harboring an active disease, which was defined as "controlled" on "uncontrolled" on the basis of mUFC levels (≤ or > ULN, respectively). The severity of hypercortisolism in naïve patients and those with an uncontrolled disease was quantified according to mUFC in mild (ULN < mUFC ≤ 2xULN) and severe (mUFC >2xULN). After obtaining patients' informed consent, the following data were collected: demography and physiological/family medical history (gender, age, ethnicity, family history for CVD and DM, smoking habits, menstrual history in women), anthropometric parameters (weight, height, waist circumference), clinical, biochemical and imaging disease indicators (disease duration from the onset of the first symptoms, 1 mg-DST, mUFC, mLNSC, plasma cortisol h 08:00, 17:00, 23:00, plasma ACTH, HDDST, hCRH test, DDAVP test, pituitary imaging, adrenal imaging, total body imaging), number of CS-related comorbidities and their indicators (*see following section*), prior and current therapies targeted to CS and concomitant medications (particularly, the use of diuretics and/or SLGT2i), plasma copeptin, fasting glucose, urea, plasma and urine sodium in order to calculate pOsm according to the formula: $pOsm = 2 \times pSodium + \frac{pGlucose}{18} + \frac{pUrea}{2.8}$ and adjust copeptin levels as reported in **Study 1**. The analysis was conducted on the whole group first. Then, patients were divided according to disease etiology in three groups: CD (n=58), ACS (n=12), EAS (n=10). Comparisons were made among the three groups and, more specifically, within the subset of patients with

ACTH-dependent CS (CD *vs* EAS). For this purpose, an ancillary evaluation was run on a small control group of patients with active NET (n=10, 3 males and 7 females, mean age 56±14 (30-76) years) attending the Department in the same timeframe, who agreed to have their copeptin measured.

Indicators of CS comorbidities

The interview patients underwent at their hospital admission included specific questions on the presence of the CS comorbidities mentioned below. If additional comorbidities with respect to what patients declared were identified during the hospital stay, they were included in the count. Patients taking comorbidity-targeted drugs were considered as harboring the comorbidity even if the related detected indexes were normal. Arterial hypertension: it was defined as systolic BP ≥140 mmHg and/or diastolic BP ≥90 mmHg. All patients underwent either multiple blood pressure measurements throughout the day or the ABPM during their hospital stay, a urine sample for albuminuria and an ophtalmologic evaluation to rule out hypertensive retinopathy. Data on additional known CVD such as structural cardiopathy and major CV events (stroke and AMI) were collected. CV risk was assessed by means of the WHO CVD laboratory-based (total cholesterol) and non-laboratory (BMI)-based risk charts, which according to our experience are the CVD risk scores performing best in patients with CS and correlate with all biochemical indexes of the disease [unpublished data]. Dyslipidemia: when present, it was classified into cholesterol-based dyslipidemia (which included high total cholesterol, low HDL cholesterol and high LDL cholesterol), hypertriglyceridemia and mixed dyslipidemia according to the local ULN of each parameter: this beacause each patient was at different CVD risk, thus individualized lipid targets would have been needed. Atheromatous disease was assessed by carotid arteries ultrasound, whose findings were classified into: normal IMT, high IMT, stenosis <30%, stenosis ≥30%. The presence of leucoencephalopathy was verified on pituitary or, where available, brain MRI. Data on known PAOD were also collected. Glycemic alterations: they were classified as IFG, IGT and DM as defined by the WHO criteria according to plasma fasting glucose, HbA1c and the results of the standardized OGTT. Insulin was also measured in order to calculate the HOMA index for insulin-resistance. The urine sample for albuminuria and the ophtalmologic evaluation for retinopathy served also to detect the same, but DM-related

complications. Overweight and obesity: BMI was calculated with the formula: $BMI = \frac{weight}{height^2}$. If present, obesity was graded according to the WHO criteria. Hypercoagulability: it was assessed by measuring PLT, PT, INR, aPTT, vWF, the clotting factors FIB and FVIII, and the reculators ATIII, pC and pS.

Hypokalemia: plasma and urine potassium were measured. Hypopituitarism: all pituitary axes were tested as recommended by the Endocrine Society [29]. All patients with non-functional pituitary deficiencies had their plasma copeptin measured under adequate replacement conditions, which were defined according to the currently accepted clinical and biochemical criteria, as established by the Endocrine Society [29-30]. Osteoporosis: it was defined according to the WHO criteria. Patients with osteoporosis were divided into non-severe and severe (fracturative) forms and the number of fractured sites was registered. The recorded biochemical indexes of phosphocalcic metabolism were: plasma and urine calcium and phosphorus, vitamin D, PTH, OC, bALP, CTX. The BMD at the lumbar site (L1-L4), FN and TH was registered. The TBS at the lumbar site was measured to assess bone quality. Hyperandrogenism: limited to women, it was assessed both clinically (FG score ≥8) [50] and by measuring 17-OHP, Δ4A, DHEAS, TT, SHBG and FAI.. Neuro-psychological disturbances: limited to a small subgroup of patients, they were screened through the HADS questionnaire administered by a psychiatrist, a standardized neurocognitive evaluation conducted by a neurologist and a psychologist, a voxel-based morphometry MRI performed and analyzed by a neuroradiologist and a medical physicist.

Plasma copeptin measurement

The ultra-sensitive, immuno-luminometric BRAHMS Copeptin assay on the KRYPTOR Compact Plus system (Thermo Fisher Scientific, Hennigsdorf, Germany) was used to measure copeptin levels. This assay has a lower limit of detection 0.7 pmol/L and a functional sensitivity <2 pmol/L.

Statistical analysis

Variables are presented depending on their distribution (normal vs non-normal, respectively) either as mean ± SD or as median (IQR) if continuous, as number and percentage (%) if categorical, as mean (95%CI) if ordinal.

After verifying the normal distribution of quantitative variables with the Shapiro-Wilk test, comparisons between two groups were made with the Student's t-test (normal distribution), or the Mann-Whitney test (non-normal distribution). For comparisons concerning more than two groups the ANOVA (parametric), or the Kruskal-Wallis test (non-parametric) were run and the Bonferroni test was used for *post hoc* analyses. Categorical variables were compared with the chi-square (χ^2) or the Fisher's exact test, where appropriate. The Pearson's P and the non-parametric Spearman's Rho (ρ) were used, as appropriate, for the bi-varied correlation analyses, whereas the Beta (β) coefficient was used for the linear and logistic regression analyses. The ROC curve was used to assess copeptin performance with respect to the gold standard (hCRH test) in distinguishing between CD and EAS. The optimal copeptin cutoff for this purpose was calculated with the Younden's Index (YI) according to the formula ($YI = SE + SP - 1$), where SE indicated the proportion of CD patients with a positive test, and SP indicated the proportion of EAS patients with a negative test. The 95%CI for SE and SP were calculated with the formulae: $95\%CI(SE) = \pm 1.96^2\sqrt{\frac{SE(1-SE)}{n\ CD}}$ and $95\%CI(SP) = \pm 1.96^2\sqrt{\frac{SP(1-SP)}{n\ EAS}}$, respectively. The PPV indicated the proportion of patients tested positive and truly having CD, the NVP indicated the proportion of patients tested negative and truly having EAS. The 95%CI for PPV and NPV were calculated with the formulae: $95\%CI(PPV) = \pm 1.96^2\sqrt{\frac{PPV(1-PPV)}{n\ positive\ test}}$ and $95\%CI(NPV) = \pm 1.96^2\sqrt{\frac{NPV(1-NPV)}{n\ negative\ test}}$, respectively. A P value of 0.050 was considered for the statistical significance. The IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. was used for the statistical analysis.

Results

Of the 80 enrolled patients, 85% had an ACTH-dependent CS. CD was the most represented ACTH-dependent form, with a 73% prevalence in the whole cohort. The proportion of naïve and already treated patients were almost equal (51% *vs* 49%) and 6 patients were enrolled due to disease recurrence. Prior non-medical therapies included TNS, adrenalectomy and RT/RS, which were performed by 26%, 5% and 13% patients, respectively and were variably combined each other, as well as with pharmacological options. Among the

latter, the most frequently used at patients' admission were metyrapone (6%), pasireotide and ketoconazole (5% each). Other pharmacological options incuded cabergoline, osilodrostat, long-acting octreotide for EAS and combined therapy. Median disease duration was 46(110) months. Pituitary imaging was performed in 71 patients, resulting negative in 29 and showing a micro- or macroadenoma in 35 and 7 patients, respectively. Adrenal imaging was performed in 72 patients, resulting negative in 21 and showing mono- or bilateral hyperplasia/nodules in 23 and 26 patients, respectively. A metastatic disease was detected in the remaining 2 patients, one with ACS due to adrenocortical carcinoma and one with occult EAS. The source of EAS was identified in only 3 of 10 patients, who harbored a pancreatic NET, a pheochromocytoma and an extra-pituitary, intrasellar tumor.

Copeptin and the biochemical markers of hypercortisolism

The 1mg-DST was performed in 57 patients: of them, 93% exhibited loss of the negative glucocorticoid feedback, with mean plasma cortisol levels after 1 mg-DST of 392±381 nmol/L. Loss of circadian cortisol secretion rhythm was tested in 76 patients and documented in 87% of them, with a median mLNSC of 19(14) nmol/L besides the lacking of plasma cortisol halving from h 08:00 to h 23:00 (mean Δcortisol = -21.0±25.2%). Median mUFC was 7518(9677) nmol/24h. After stratifying patients according to mUFC adjusted for ULN, 14 (17%) patients had a controlled disease, 19 (24%) a mild disease and 47 (59%) a severe disease. Already treated patients had significant lower mUFC and 1-mg DST than the naïve ones (6346 *vs* 10705 nmol/24h p=0.020 and 254 *vs* 467 nmol/L p=0.009, respectively), whereas mLNSC was similar between groups. A gender difference favoring males was highlighted for mUFC (12691 *vs* 6401 nmol/24h, p=0.002), but not mLNSC and 1 mg-DST. Of the three disease indicators, mUFC was the only correlated with age (ρ=-0.491, p<0.001) and, alongside 1 mg-DST, disease duration (ρ=-0.387 p<0.001 for mUFC, ρ=-0.377 p<0.001 for 1 mg-DST). Median copeptin was 3.9(4.4) pmol/L. Mean plasma osmolality, plasma sodium and eGFR were 302±6 mOsm/kg, 142±3 mmol/L and 113±39 ml/min, respectively. Median plasma fasting glucose, urea and urine sodium were 5.4(1.9) mmol/L, 6(2.5) mmol/L and 121(94) mmol/24h, respectively. A gender difference favoring males was highlighted for copeptin (6.6 vs 3.7 pmol/L, p=0.030), which was also correlated with age (ρ=0.343, p=0.002). No significant correlations were found between copeptin and plasma osmolality and its determinants except

for urea (ρ=0.249, p=0.027), as well as between copeptin and eGFR or urine sodium. Copeptin did not vary according to the 1 mg-DST responsiveness, the disruption of cortisol circadian rhythm, or the severity of hypercortisolism. Neither the three diagnostic biomarkers (1 mg-DST, mLNSC, mUFC), nor disease duration were significantly correlated with copeptin. Overlapping results were obtained when adjusted copeptin (cop/pOsm, cop/pNa) was considered. Naïve and already treated patients, as well as those with primary disease and recurrence had similar copeptin, but a significant difference was found concerning CS etiology (p=0.002): as shown in Study 2 Figure 1, patients with CD and EAS exhibited the lowest (3.7(4.2) pmol/L) and highest copeptin (12.2(24.1) pmol/L), respectively, whereas patients with ACS had intermediate levels (5.1(4.8) pmol/L). No significant data on copeptin were found in the subgroup of patients with ACS. Conversely, copeptin was correlated negatively with mUFC in CD and positively, albeit not significantly, in EAS (ρ=-0.316 p=0.016 and ρ=0.633 p=0.067, respectively; Study 2 Figure 2). Here, copeptin was inversely correlated with, and predicted by, disease duration (ρ=-0.818 p=0.004; β=-0.686 p=0.029, Study 2 Figure 3).

Copeptin and the etiological differentiation of CS

Study 2 Table I illustrates the distribution of hormonal pattern and copeptin levels according to the etiology of CS. Median ACTH significantly differed between patients with ACS and those with an ACTH-dependent form (2 *vs* 47 pg/ml, p<0.001), EAS accounting for the highest levels in the latter (143 *vs* 46 pg/ml of CD, p=0.003). The results of all dynamic tests currently used for the differential diagnosis of CS also significantly differed among the three groups (p<0.001 for the HDDST, p=0.001 for the hCRH test, p=0.008-0.016 for the DDAVP test). When only patients with ACTH-dependent CS were considered, neither gender nor recruiting (naïve *vs* already treated) differences were found in ACTH levels, which were also independent from age and disease duration. However, ACTH varied according to the severity of hypercortisolism (24 *vs* 46 *vs* 62 pg/ml in controlled, mild and severe hypercortisolism, respectively; p=0.004) and was correlated with mUFC (ρ=0.310, p=0.012), plasma cortisol h23 as an indicator of dysrupted circadianity (ρ=0.316, p=0.012), as well as with the results of dynamic tests suggesting EAS (ρ=0.444 p=0.003 for Δcortisol during HDDST, ρ=-0.331 p=0.019 for ΔACTH during hCRH test). Copeptin and ACTH were moderately correlated (ρ=0.357, p=0.003): in this regard,

the former's trend quite overlapped the latter's behavior with threefold higher levels in EAS than CD (p=0.001; Study 2 Table I and Study 2 Figure 1). This set the basis for the etiological differentiation of CS by means of the simultaneous ACTH and copeptin assessment, assuming copeptin as a potential biomarker of EAS: as evidenced with immediacy by Study 2 Figure 4, patients with ACS had low ACTH and low-medium copeptin, patients with CD had medium-high ACTH and low-medium copeptin, whereas patients with EAS had both high-very high ACTH and copeptin. Of note, the unique case of EAS sustained by an extra-pituitary, intrasellar tumor slightly deviated from this trend, exhibiting an ACTH of 140 pg/ml and a copeptin of 5.0 pmol/L. An additional analysis was then conducted in the subgroup of naïve patients with ACTH-dependent CS (n=32), aimed at comparing copeptin performance with that of the currently used dynamic test best performing in differentiating CD from EAS, thus chosen as the gold standard (Study 2 Table II). Based on its accuracy, which was represented by an AUC of 0.889 at a cutoff of <35%, the Δ%ACTH during hCRH test was chosen as the gold standard for the diagnosis of EAS (p=0.029, Study 2 Figure 5). With an AUC of 0.993 at a cutoff of ≥9.1 pmol/L, copeptin performance was superior to that of the gold standard in identifying EAS (p=0.001, Study 2 Figure 6).

Copeptin as a biomarker of extra-pituitary neuroendocrine cell-derived tumor

The NET group included: two patients with pancreatic NET (of them, one had MEN 1, four patients with pheochromocytoma and three patients with medullary thyroid carcinoma, plus one patient with both of the latter in the context of a MEN 2. None of them had paraneoplastic SIAD; mean plasma sodium and osmolality were 141±2 mmol/L and 300±10 mOsm/kg, respectively. Median copeptin was 5.1(4.1) pmol/L. Only two patients, both harboring isolated pheochromocytoma, had copeptin levels ≥9.1 pmol/L. When comparing patients in the NET group with those having ACTH-dependent CS, no significant data concerning copeptin were highlighted. However, a significant difference in copeptin levels emerged among groups after splitting the latter into CD and EAS (see Study 2 Table I; p=0.002). More in detail, copeptin was significantly lower in CD than NET patients (p=0.045), who in turn had lower, albeit non-significantly, copeptin levels than EAS (Study 2 Figure 7). When patients with EAS and NET were considered together as patients harboring an extra-

pituitary neuroendocrine cell-derived tumor, their median copeptin confirmed almost twofold higher than that of patients with CD (6.9 vs 3.7 pmol/L, p=0.001; Study 2 Figure 8).

Copeptin and the clinical burden of CS

Study 2 Figures 9 and 10 illustrate the number (median 5(3)) and type of hypercortisolism-related systemic comorbidities in the study cohort. None of the patients was free from comorbidities. When patients were stratified according to the number of comorbidities, both biomarkers of CS (1 mg-DST, mLNSC, mUFC and ACTH) and copeptin were similar among groups and no gender differences were found; however, older patients and those with higher copeptin levels had more comorbidities (ρ=0.283 p=0.011, ρ=0.332 p=0.003). Arterial hypertension – it was the most represented comorbidity in the study cohort, involving 79% patients and requiring polypharmacological therapy in >60% cases. Mean systolic and diastolic BP were 130±15 and 82±10 mmHg, respectively. A non-dipper or *reverse* dipper ABPM profile was found in 58% cases (median ΔBP day→night -5(12)%). Retinopathy, structural cardiopathy and microalbuminuria involved 30%, 19% and 16% hypertensive patients, respectively. All CS biomarkers were similar between hypertensive and non-hypertensive patients, but the former had significantly higher copeptin (4.4 *vs* 3.5 pmol/L, p=0.041); within the hypertension group, diuretic users had significantly higher copeptin levels with respect to their counterpart (6.5 *vs* 3.7 pmol/L, p=0.043). No correlations were found between BP values and copeptin. Dyslipidemia – nearly totally in the cholesterol-based (65%) and mixed forms (33%), dyslipidemia involved 68% patients. Mean total cholesterol, HDL, LDL and triglycerides were 5.0±1.2, 1.6±0.4, 2.8±0.9 and 1.4±0.7 mmol/L, respectively. In almost two-third cases, the carotid ultrasound revealed a high IMT or the presence of carotid stenosis, whereas leucoencephalopathy was documented in 43% patients undergoing brain MRI. The prevalence of PAOD was low (4%) in the study cohort. All CS biomarkers, as well as copeptin, were similar between dyslipidemic and non-dyslipidemic patients. Although weakly, copeptin was correlated specularly with HDL (ρ=-0.246, p=0.028) and triglycerides (ρ=0.246, p=0.028). Overweight/obesity – this clinical feature of CS represented one of the most frequent comorbidities with CV impact, accounting for a 68% prevalence. Overweight was slightly less represented than obesity (44% vs 45%), whose grades were distributed as follows: 53% grade I, 20% grade II, 27% grade III. Mean weight, BMI and WC were 78±20 kg, 29±kg/m^2 and

104±16 cm. The biochemical indexes of CS and copeptin levels were similar between overweight/obese patients and their counterpart, as well as among the three obesity classes. Copeptin levels correlated directly with weight, BMI and, particularly, WC (ρ=0.318 p=0.004, ρ=0.315 p=0.005 and ρ=0.612 p<0.001 (Study 2 Figure 11), respectively). Glycemic alterations – they involved 59% patients with the following distribution: 4% IFG, 21% IGT, 75% DM. Almost half of the latter was on antidiabetic therapy with one or more active principles: of them, SGLT2i were used by 3 patients, whereas 9 patients needed insulin. Mean HbA1c, fasting glucose and insulin were 44±11 mmol/mol, 6.0±2.1 mmol/L and 9.9±6.7 mU/L, respectively, with a mean HOMA index of 3.4±2.0. Microalbuminuria was detected in 21% patients, whereas the prevalence of diabetic retinopathy was 4%. Patients with DM and their counterpart were similar concerning all CS biomarkers except for mUFC, which was significantly higher in DM than non-DM patients (p=0.044); copeptin did not vary significantly among groups, but was weakly correlated with both fasting glucose and HbA1c (ρ=0.301 p=0.007 and ρ=0.240 p=0.037). CVD risk and CV events – in the study cohort, 2 patients had a history of heart failure, 4 had prior AMI and 2 had prior ischemic stroke. Median CVD risk was 5(11)% and 4(8)% according to laboratory-based and BMI-based WHO CVD risk charts, respectively. Most patients fell into the low to intermediate CVD risk categories, as shown in Study 2 Figure 12. Both risk scores correlated with copeptin (ρ=0.352 p=0.007 for laboratory-based and ρ=0.341 p=0.009 for BMI-based CVD risk chart, respectively), but not with the biomarkers of CS. Osteoporosis – it involved 54% patients, irrespective of gender and age (post-menopausal in women/>50 years in men). The majority of osteoporotic patients (70%) had a severe form, where fractures at the vertebral site predominated, but specific anti-reabsorbitive or anabolic drugs were taken in addition to calcium and vitamin D supplements only in 25% cases. Mean BMD (and median Z-score) were 1.056±0.146 g/cm^2 (-0.7) at L1-L4, 0.828±0.128 g/cm^2 (-1.1) at FN and 0.892±0.159 g/cm^2 (-1.1) at TH, respectively. Mean TBS L1-L4 was 1.088±0.183. Biochemical markers of hypercortisolism and copeptin were similar between osteoporotic and non-osteoporotic patients, as well as between the fractured osteoporotic patients and their counterpart. The only significant correlation was the negative one highlighted between mUFC and the Z-score L1-L4 (ρ=0.396, p=0.002); no significant correlations involved copeptin and the bone mineral density indexes, as well as the biochemical markers of phosphocalcic metabolism, whose behavior in

relationship to CS indexes is summarized in Study 2 Table III. Neuropsychological disturbances – reported by 41% patients, psychiatric disorders were represented by an anxious-depressive syndrome, either alone (73%) or psychosis-associated (27%) and required psychopharmacological therapy in almost two-third of cases. In the sugroup of patients (n=13) undergoing screening with the HADS questionnaire, the median scores for anxiety and depression were 6(6) and 6(5), respectively; the neurocognitive evaluation highlighted an attentive-dysexecutive syndrome mainly impairing short- and long-term memory, cognitive flexibility, abstract reasoning and problem solving. A patient with extremely severe EAS due to pheochromocytoma exhibited the worst neurocognitive performance despite the young age (33 years old). At the same time, the voxel-based morphometry MRI detected some clusters of grey matter atrophy, which were located in pre-frontal cortex areas accounting for the impaired neurocognitive results. The biochemical markers of CS and copeptin neither differed, nor correlated with the presence and severity of neuropsychological disorders in terms of number of needed drugs, HADS scores, neurocognitive scores. Hypopituitarism – anterior pituitary deficiencies involved 35% patients. In most cases they were of functional nature, requiring replacement treatment only in one third cases. The 1 mg-DST, mLNSC and mUFC were significantly higher ($p<0.001$ to $=0.045$) in patients with hypopituitarism as compared to their counterpart; no significant data emerged on copeptin in this regard, as well as when considering separately FSH/LHD, TSHD and GHD. Study 2 Table IV illustrates the status of the anterior pituitary axes and the relationship their biomarkers encountered both with CS indexes and copeptin. Hypercoagulability – a thromboembolic disease was documented in 11% patients, still requiring medical treatment in half of them at hospital admission. Neither the three biomarkers of CS nor copeptin significantly varied according to the presence of hypercoagulability. Copeptin did not correlate with any of the coagulation indexes; conversely, significant data emerged for 1 mg-DST and mUFC (Study 2 Table V). Hypokalemia – this disorder was reported by 9% patients, mainly -but not exclusively- with EAS. Median copeptin levels were four-fold higher in hypokalemic than normokalemic patients (16.4 *vs* 3.8, $p<0.001$) and a similar trend was shown concerning the disease biomarkers ($p=0.001$ to 0.035), which unlike copeptin were also correlated with potassium levels ($\rho=-0.310$ $p=0.019$ for 1 mg-DST, $\rho=-0.328$ $p=0.003$ for mUFC). Hyperandrogenism – this disorder involved 50% women in the study cohort. Mean FG score was 11±3;

median androgen levels were: 3.8(4.8) nmol/L for 17-OHP, 4.0(6.7) µmol/L for DHEAS, 8.3(13.8) nmol/L for Δ4A, 0.2(0.3) nmol/L for TT, 5.2(15.5)% for FAI. Copeptin was similar between hyperandrogenic women and their counterpart; mUFC was the only biomarker of hypercortisolism significantly differing between groups, being threefold higher among hirsute than non-hirsute patients ($p<0.001$) and significantly correlating with almost all clinical ($p=0.055$) and biochemical parameters of hyperandrogenism ($p<0.001$-0.049).

Discussion

Despite the historically proven contribution of AVP system dysregulation to the pathogenesis of CS, or at least of its pituitary form (CD) system [26, 32-35], in Study 1 we demonstrated copeptin assessment is not useful to diagnose CS in the presence of a clinical suspicion. Starting from this assumption, we wondered if copeptin could be either a reliable tool in the etiologic differential diagnosis, or a biomarker of prognostic usefulness once the diagnosis of CS has been made. Eighty patients with active CS referring to the Endocrinology and Metabolic Diseases Department of Ancona University Hospital (Azienda Ospedaliero-Universitaria delle Marche, Ancona, Italy) over the last 6 years were enrolled in this cross-sectional study. Copeptin behavior was assessed first with respect to disease activity biomarkers, then with regards to the dynamic evaluations for the differential diagnosis of ACTH-dependent forms (CD *vs* EAS) and, lastly, from a prognostic point of view, according to the presence and severity of hypercortisolism-related comorbidities. We confirmed that, even if assessed after the diagnostic completion, copeptin is not a reliable biomarker of hypercortisolism itself: its behavior, indeed, did not vary according to the 1 mg-DST responsiveness, the disruption of cortisol circadian rhythm expressed by mLNSC and Δ% cortisol h8→23, or the severity of hypercortisolism expressed by mUFC. A pretty exciting result emerged, however, when the attention was focused on disease etiology: after splitting patients in the three etiologic subgroups, indeed, a significant difference was highlighted concerning copeptin levels, which were the lowest and the highest in CD and EAS, respectively, stooding at the intermediate in ACS (3.7 *vs* 12.2 *vs* 5.1 pmol/L, $p=0.002$). Despite unclear underlying reasons, the finding of a higher copeptin in patients with ACS than CD suggests copeptin release is rather an epiphenomenon of hypercortisolism than a promoter of ACTH hypersecretion. This is in line with the finding by Yanovski *et al.* of overlapping AVP levels,

both at baseline and after stimulation with oCRH during BIPSS, in all CS forms [11], as well as with Knoepfelmacher's assumption of a partial renal resistance to AVP in CS [12]. We could not explore this theory due to the lack of information on urine osmolality; however, the finding copeptin was not correlated with plasma osmolality made us raise the hypothesis of an impaired osmotic response to AVP in CS. Moreover, the appearance of an opposite correlation between copeptin and mUFC when CD and EAS patients were compared (Study 2 Figure 2) supported the assumption raised in **Study 1** that the burden of the disease and its complications may skew the inhibition exerted by hypercortisolism on AVP system, further tightening over copeptin release the attribute of hypercortisolism-related epiphenomenon. Copeptin levels were correlated with ACTH (ρ=0.357, p=0.003) and their trends overlapped within the ACTH-dependent forms: both parameters were, indeed, threefold higher in EAS than CD (mean ACTH EAS *vs* CD: 143 *vs* 46 pg/ml, p=0.001; *see above for copeptin*). Additionally, ACTH was correlated with the results of dynamic tests suggesting an EAS. These findings set the basis for the etiological differentiation of CS by means of the simultaneous ACTH and copeptin assessment, assuming copeptin as a novel, peculiar biomarker of EAS (Study 2 Figure 4). The performance of copeptin in distinguishing the EAS from CD was then compared with that of Δ%ACTH during hCRH test in the naïve, ACTH-dependent subgroup of patients. If our gold standard differentiated the two forms with a 96% SE and a 100% SP, a 100% PPV and a 67% NPV (AUC=0.889, p=0.029), copeptin showed the best accuracy for the purpose, with a 100% SE, a 96% SP, an 83% PPV and a 100% NPV at a cutoff of 9.1 pmol/L (AUC=0.993, p=0.001). This innovative contribution is of particular clinical significance when dynamic tests are poorly practicable, due to either the scarce venous heritage of hypercortisolemic patients or their systemic compromission (e.g. heart failure, acute psychosis, severe mlignant hypercortisolism), or even technical issues, such as the limited availability of hCRH (CRH Ferring®) we will encounter worldwide in the near future [51]. If copeptin behavior is an epiphenomenon of hypercortisolism rather than the sole, or contributory cause of ACTH hypersecretion, the following assumption could be raised to explain its consistent increase in patients with EAS: is copeptin a biomarker of the neuroendocrine tumor cell, whatever the ability of the latter to produce ACTH? We know ACTH-producing NET cells express the mRNA encoding V1bR and POMC receptors in abnormal amounts and quality as compared to the corticotrophs [52-53], as well as

paraneoplastic SIAD/SIADH can be theoretically found out by assessing copeptin [54]; however, we know nothing about the relationship between copeptin and NET in the absence of a SIAD. The additional analysis we ran on a small group of NET patients demonstrated those exhibiting the highest copeptin levels (≥9.1 pmol/L) had pheochromocytoma: this is not surprising, as catecholamines both stimulate AVP system [32] and impair CV health and glycometabolic control [55]. Patients with NET had higher copeptin levels than those with CD and lower, albeit non significantly, than patients with EAS: of these findings, the former is consistent with the inhibition of AVP system exerted by glucocorticoids; whether the latter depends on the different molecular profiles exhibited by the ACTH-producing and the non-ACTH-producing NET cells is currently unknown. Further clarification is needed on this topic and performing immunostaining/molecular biology analyses on patients' histological samples could serve to this purpose. The peculiar behavior of copeptin among patients with EAS likely finds an additional explanation in the pattern of systemic complications they exhibited. Recently, copeptin assessment has proven prognostically useful in all systemic conditions whose pathogenesis involves, at least in part, AVP: these are DM, arterial hypertension, obesity and the metabolic syndrome, thromboembolic disease, ischemic heart disease and bone fractures, which also represent the main systemic complications of CS [23]. In the study cohort, copeptin levels were significantly correlated with the number of documented comorbidities. More in detail, copeptin was either higher among patients harboring those comorbidities at strongest impact on the clinical phenotype of EAS patients (arterial hypertension above all, particularly when diuretics were required), or correlated with the biomarkers suggestive of worsening control of comorbidities (fasting glucose and HbA1c for DM [13, 39], HDL (inversely) and triglycerides (directly) for dyslipidemia, weight, BMI and WC for overweight/obesity [56]). When CVD risk was assessed, both the chosen scores were directly correlated with copeptin. Irrespective of the confounding factors highlighted in **Study 1**, the highest copeptin reflected the heaviest burden of EAS, thus proving an ancillary biomarker favoring EAS in the differential diagnostic work-up of ACTH-dependent forms. Further confirmation of this assumption comes from the demonstration of both four-fold higher copeptin and disease indexes among hypokalemic patients, mainly harboring an EAS. Conversely, no relevant relationships were

found between copeptin and neuro-psychological disturbances, hypercoagulability, hyperandrogenism, hypopituitarism and osteoporosis, neither simple nor severe.

Considering all, rather than promoting ACTH hypersecretion, copeptin release is a hypercortisolism-related epiphenomenon reflecting the systemic burden of the disease, which is typical of EAS. ACTH and copeptin are closely related: when simultaneously measured, their trend likely orientates towards the etiologic diagnosis of CS. In the subgroup of patients with ACTH-dependent CS, copeptin performance in distinguishing between CD and EAS is superior than that of Δ%ACTH during hCRH test.

	CD (n=58)	ACS (n=12)	EAS (n=10)	p
mUFC (nmol/24h)	7063 (8463)	5518 (8884)	21354 (80356)	n.s.
mLNSC (nmol/L)	19.3 (15.2)	16.6 (8.2)	20.7 (114.5)	n.s.
1 mg-DST (nmol/L)	345 (367)	385 (458)	1592 (1142)	n.s.
cortisol h 08→23 (Δ%)	-21.4 (41.3)	-9.4 (48.0)	-13.1 (70.4)	n.s.
ACTH (pg/ml)	46 (41)	2 (6)	143 (173)	<0.001
hCRH test, ACTH (Δ%)	188.0 (192.5)	0.0 (180.0)	5.0 (52.5)	0.001
hCRH test, cortisol (Δ%)	53.0 (65.0)	-1.0 (30.3)	0.0 (30.6)	0.001
DDAVP test, ACTH (Δ%)	102.0 (380.5)	43.0 (43.0)	5.0 (9.0)	0.008
DDAVP test, cortisol (Δ%)	46.5 (62.5)	3.0 (6.0)	7.0 (13.0)	0.016
HDDST, cortisol (Δ%)	-86 (64.4)	-28.5 (47.9)	-25.0 (350.5)	<0.001
copeptin (pmol/L)	3.7 (4.2)	5.1 (4.8)	12.2 (24.1)	0.002
plasma osmolality (mOsm/kg)	301±6	305±7	304±8	n.s.
adjusted copeptin (Cop/pOsm)	0.012 (0.014)	0.017 (0.016)	0.040 (0.076)	0.002

Study 2 Table I – Distribution of hormonal pattern and copeptin according to CS etiology.

	Spearman's ρ with source of ACTH secretion	SE% (95%CI)	SP% (95%CI)	PPV% (95%CI)	NPV% (95%CI)	AUC	p
HDDST, cortisol (nmol/L)	0.474 (p=0.014)	96 (±0.08)	67 (±0.53)	96 (±0.08)	67 (±0.53)	0.826	0.071
hCRH test, ACTH (Δ%)	**-0.404 (p=0.027)**	**96 (±0.07)**	**100**	**100**	**67 (0.53)**	**0.889**	**0.029**
hCRH test, cortisol (Δ%)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
DDAVP test, ACTH (Δ%)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
DDAVP test, cortisol (Δ%)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Copeptin (pmol/L)	**0.620 (p<0.001)**	**100**	**96 (±0.07)**	**83 (±0.30)**	**100**	**0.993**	**0.001**

Study 2 Table II – Copeptin performance vs the currently used tests for the etiologic diagnosis of ACTH-dependent CS.

		Spearman's ρ, p		
	Mean±SD or Median (IQR)	**1 mg-DST**	**mLNSC**	**mUFC**
plasma Calcium (mmol/L)	2.3±0.1	n.s.	-0.326; 0.011	-0.286; 0.012
plasma Phosphorus (mmol/L)	1.0±0.2	n.s.	n.s.	-0.246; 0.032
urine Calcium (mmol/24h)	34 (32)	n.s.	n.s.	0.448; 0.001
urine Phosphorus (mmol/24h)	211±104	n.s.	n.s.	0.417; 0.003
vitamin D (nmol/L)	52.9 (45.2)	-0.287; 0.034	n.s.	n.s.
parathyroid hormone (pmol/L)	6.6 (6.3)	n.s.	n.s.	n.s.
osteocalcin (ng/ml)	12.5 (11.2)	n.s.	-0.320; 0.017	-0.335; 0.005
beta-crosslaps (pg/ml)	358 (350)	n.s.	n.s.	n.s.
bone alkaline phosphatase (mcg/L)	9.3 (7.0)	n.s.	n.s.	n.s.

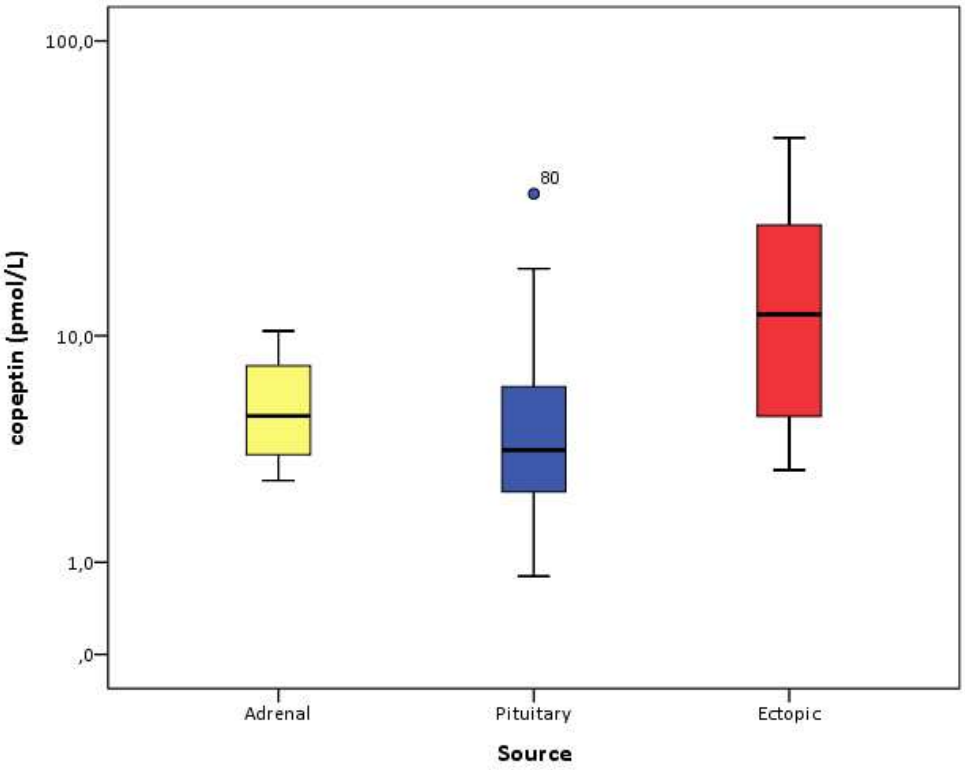
Study 2 Table III – Biomarkers of phosphocalcic metabolism and their correlation with CS biochemical indexes.

			Spearman's ρ, p			
		Mean±SD or Median (IQR)	**1 mg-DST**	**mLNSC**	**mUFC**	**copeptin**
	TSH (mIU/L)	0.9±0.7	n.s.	n.s.	-0.264; 0.020	n.s.
	fT3 (pmol/L)	3.5±0.7	n.s.	n.s.	-0.286; 0.015	n.s.
	fT4 (pmol/L)	11.9±2.8	n.s.	-0.408; 0.001	-0.463; <0.001	**0.330; 0.003**
	GH (ng/ml)	0.4 (0.9)	n.s.	n.s.	n.s.	**-0.293; 0.011**
	IGF-1 (xLLN)	2.2 (1.4)	n.s.	n.s.	n.s.	n.s.
	IGFBP3 (µg/ml)	4.4±1.3	n.s.	n.s.	n.s.	n.s.
	PRL (µg/L)	12.3±8.2	n.s.	n.s.	n.s.	n.s.
	FSH (IU/L)	5.8 (25.0)	n.s.	n.s.	-0.462; <0.001	n.s.
	LH (IU/L)	4.6 (11.1)	n.s.	n.s.	-0.466; <0.001	n.s.
males	**total testosterone (nmol/L)**	1.1±0.6	n.s.	n.s.	n.s.	n.s.
	SHBG (nmol/L)	48.4±44.5	n.s.	n.s.	n.s.	n.s.
	FAI (%)	28.7±22.4	n.s.	n.s.	n.s.	n.s.
females	**estradiol (pmol/L)**	12.7 (28.3)	n.s.	n.s.	n.s.	n.s.
	progesterone (nmol/L)	0.1 (0.3)	n.s.	n.s.	n.s.	n.s.

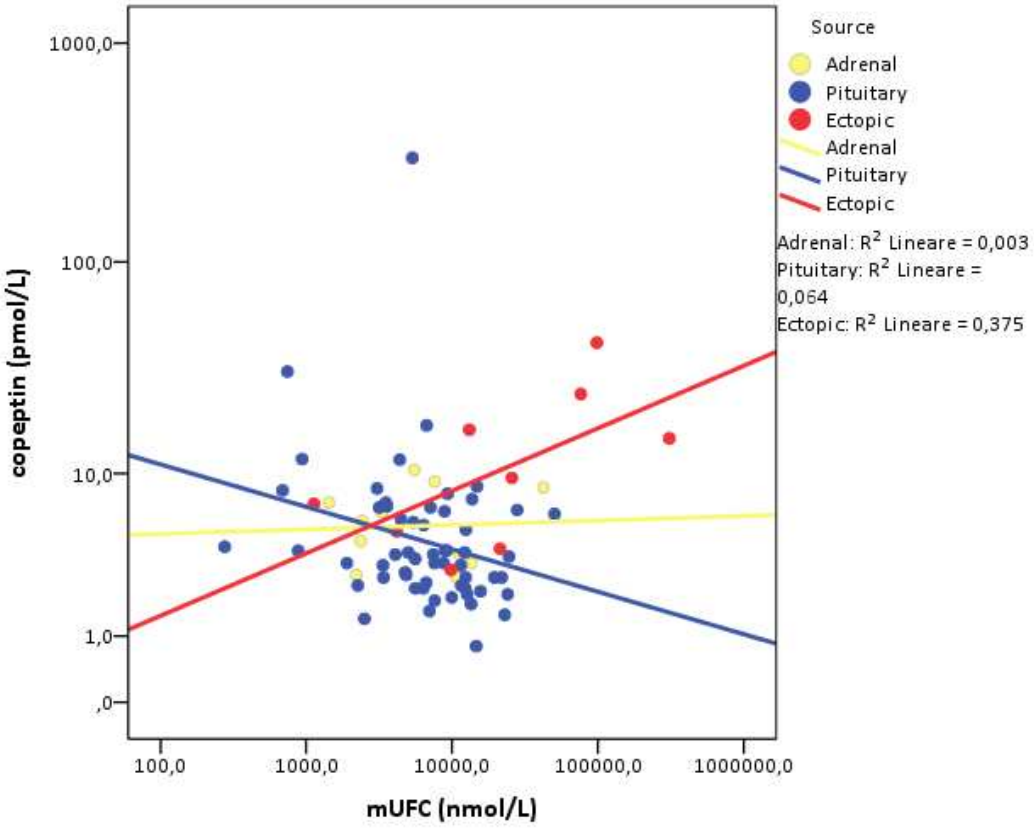
Study 2 Table IV – Hormonal axes of the anterior pituitary and their their correlation with CS biochemical indexes and copeptin.

		Spearman's ρ, p		
	Mean±SD or Median (IQR)	**1 mg-DST**	**mLNSC**	**mUFC**
PT (%)	99±14	n.s.	n.s.	n.s.
INR	1.0±0.2	n.s.	n.s.	n.s.
aPTT (sec)	28±4	n.s.	n.s.	-0.322; 0.005
Platelets (x10^3/mm^3)	264±84	-0.305; 0.021	n.s.	n.s.
Fibrinogen (µg/dl)	418±100	n.s.	n.s.	-0.550; 0.002
FVIII (%)	146±37	n.s.	n.s.	n.s.
vWF (%)	148±45	n.s.	n.s.	n.s.
AT3 (%)	118±18	n.s.	n.s.	0.447; 0.006
Functional pC (%)	135±29	0.763; 0.001	n.s.	n.s.
pS (%)	105±18	0.561; 0.004	n.s.	0.416; 0.013

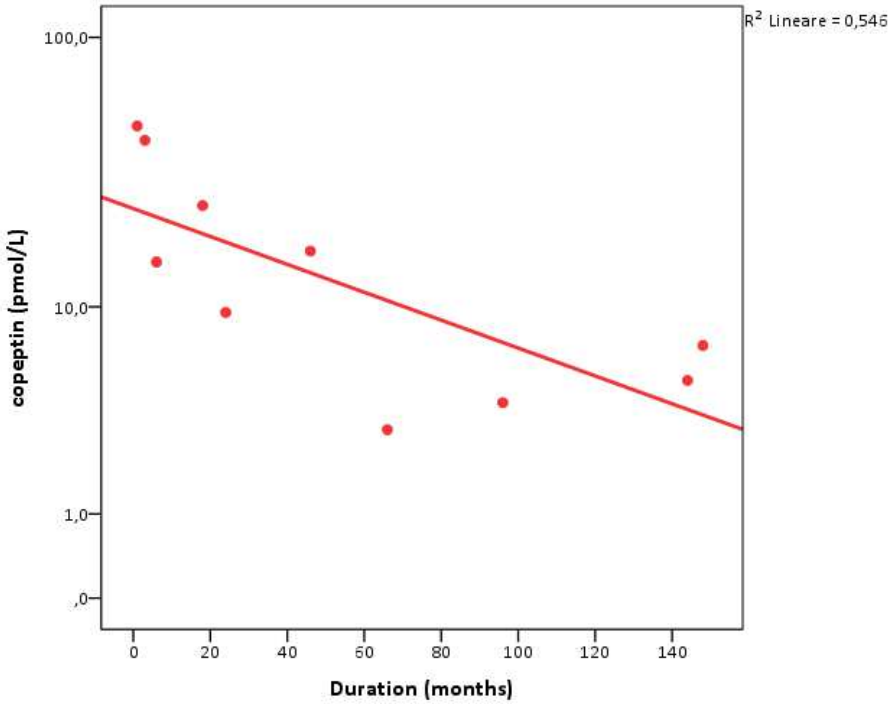
Study 2 Table V – Coagulation indexes and their correlation with CS biochemical indexes.



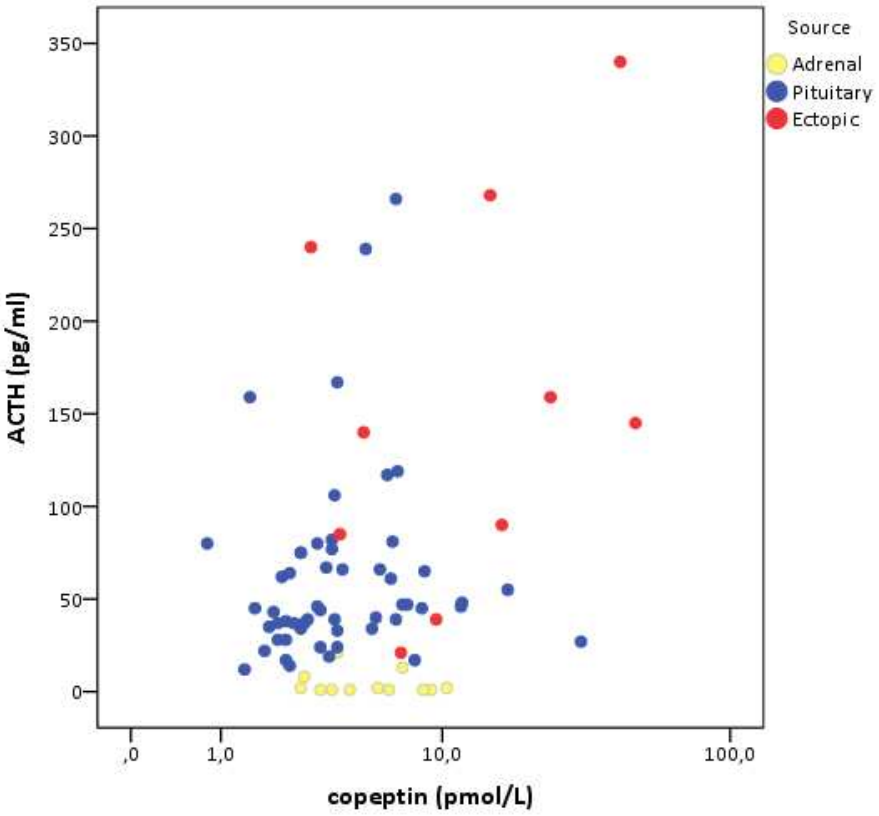
Study 2 Figure 1 – Median copeptin levels according to CS etiology (p=0.002).



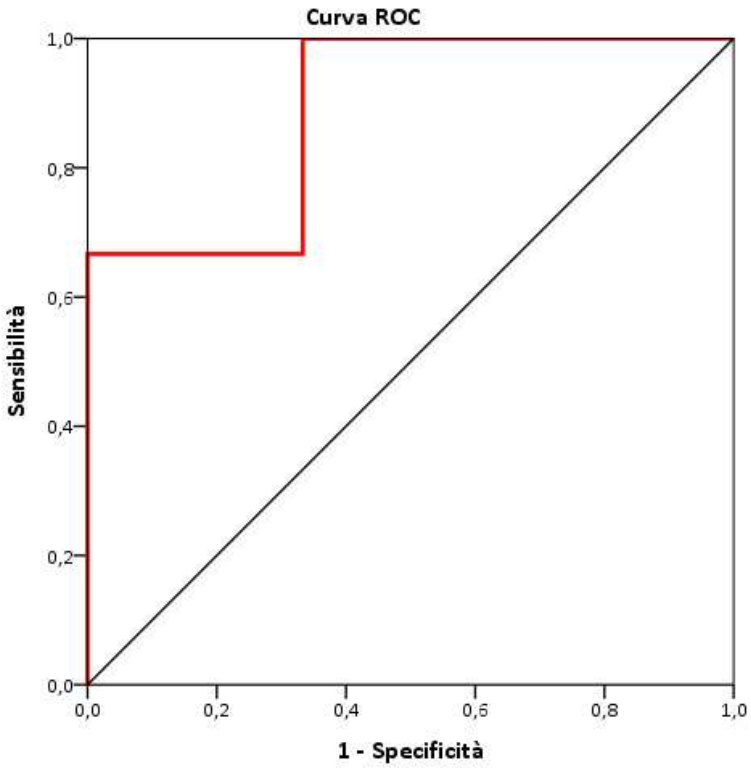
Study 2 Figure 2 – Correlation between mUFC and copeptin according to CS etiology (p=0.016 for CD, p=0.067 for EAS, p=0.979 for ACS).



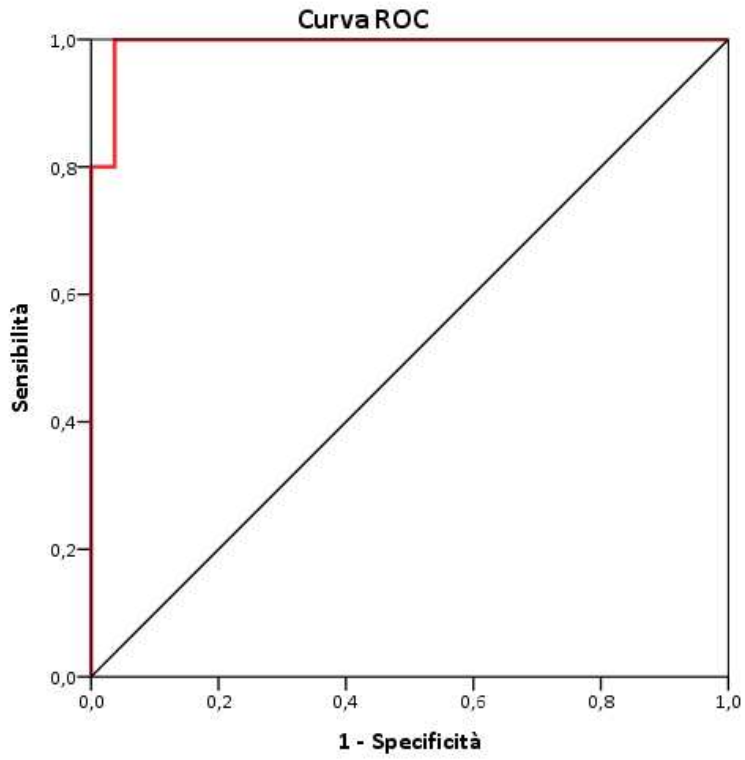
Study 2 Figure 3 – Linear regression between disease duration and copeptin levels in EAS (p=0.029).



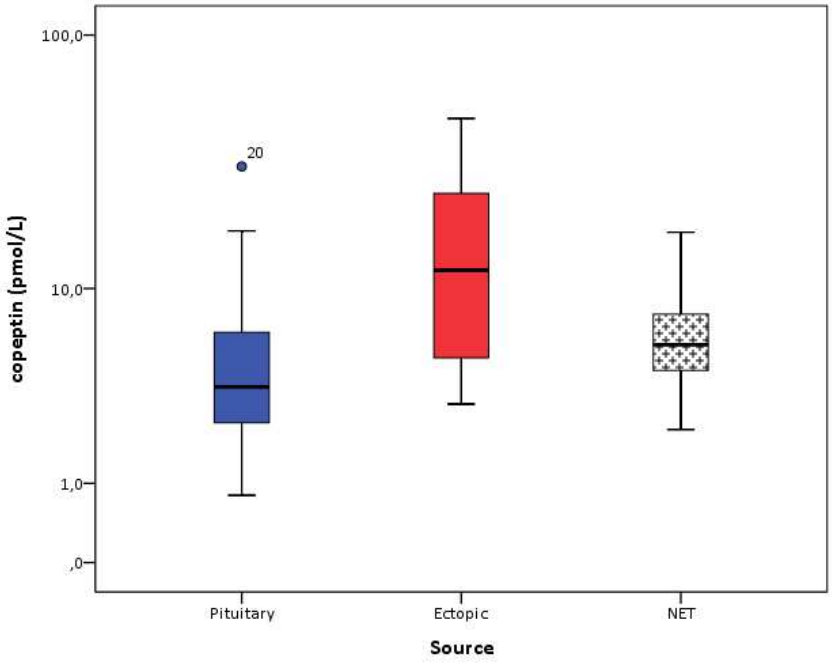
Study 2 Figure 4 – Etiologic classification of CS based on copeptin and ACTH levels.



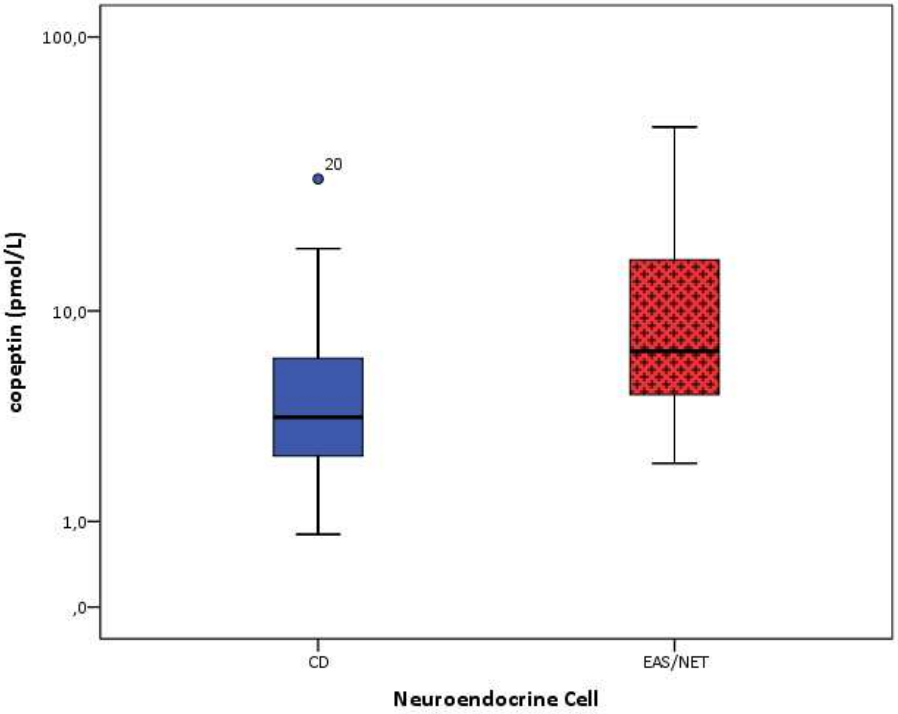
Study 2 Figure 5 – ROC curve for Δ% ACTH during hCRH test in discriminating EAS from CD (AUC 0.889, p=0.029).



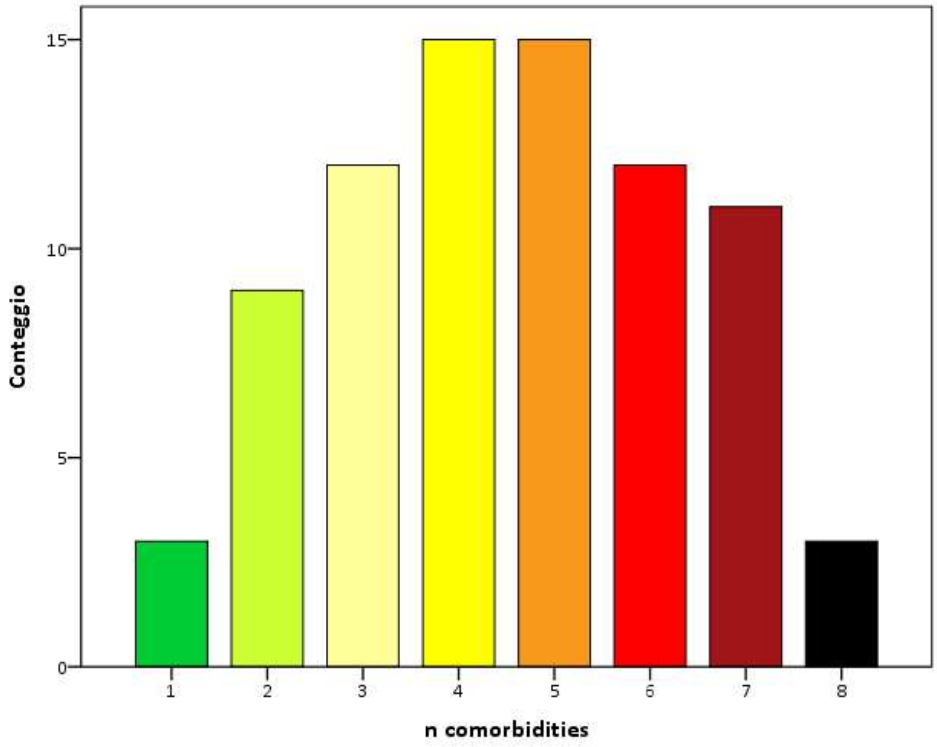
Study 2 Figure 6 – ROC curve for copeptin in discriminating EAS from CD (AUC 0.993, p=0.001).



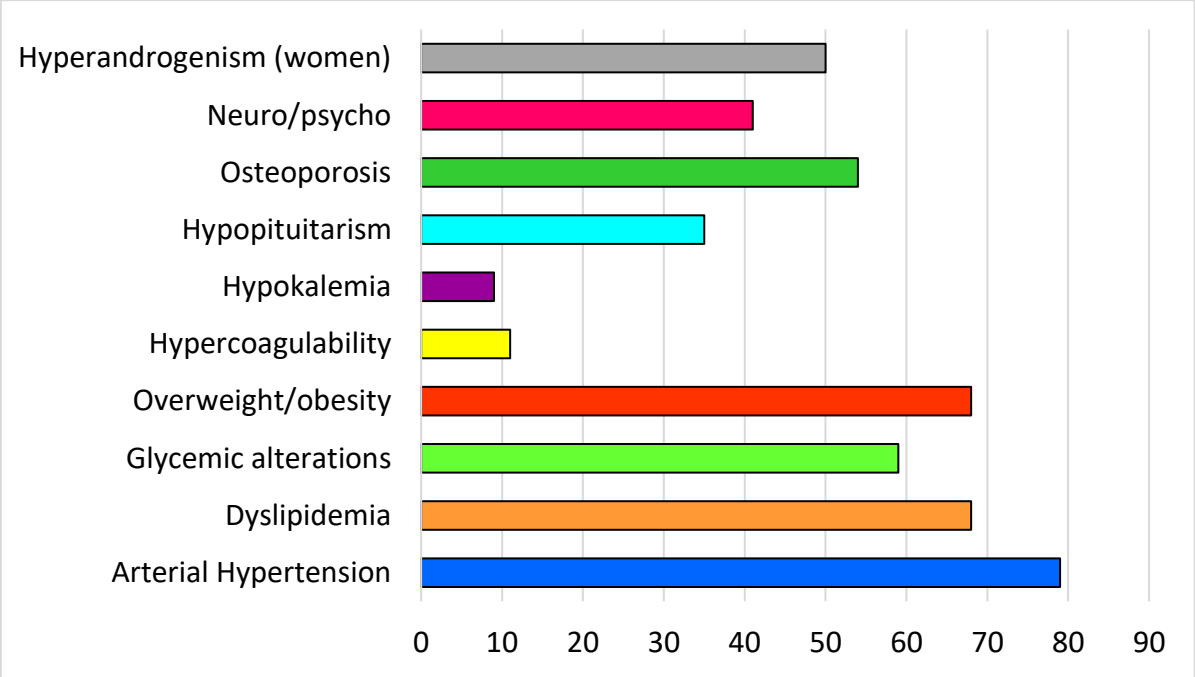
Study 2 Figure 7 – Distribution of copeptin levels in patients with NET as compared to CD (p=0.045) and EAS (p>0.050).



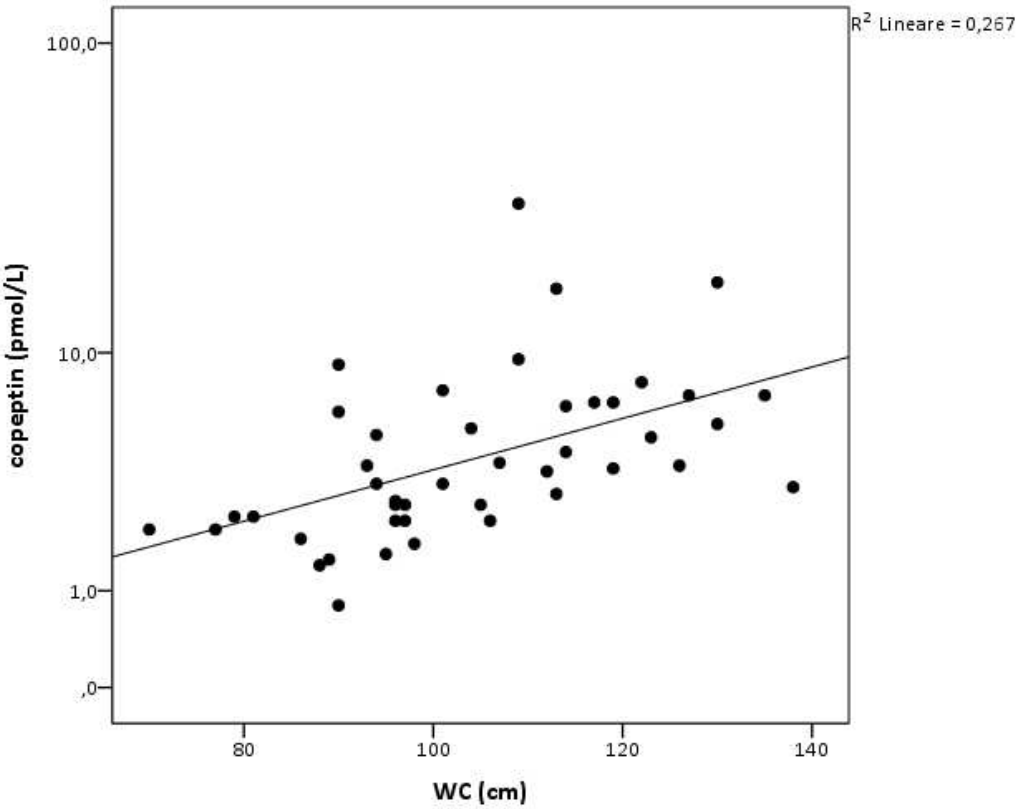
Study 2 Figure 8 – Distribution of copeptin levels in patients with CD as compared to those harboring a neuroendocrine cell-derived extra-pituitary tumor (p=0.001).



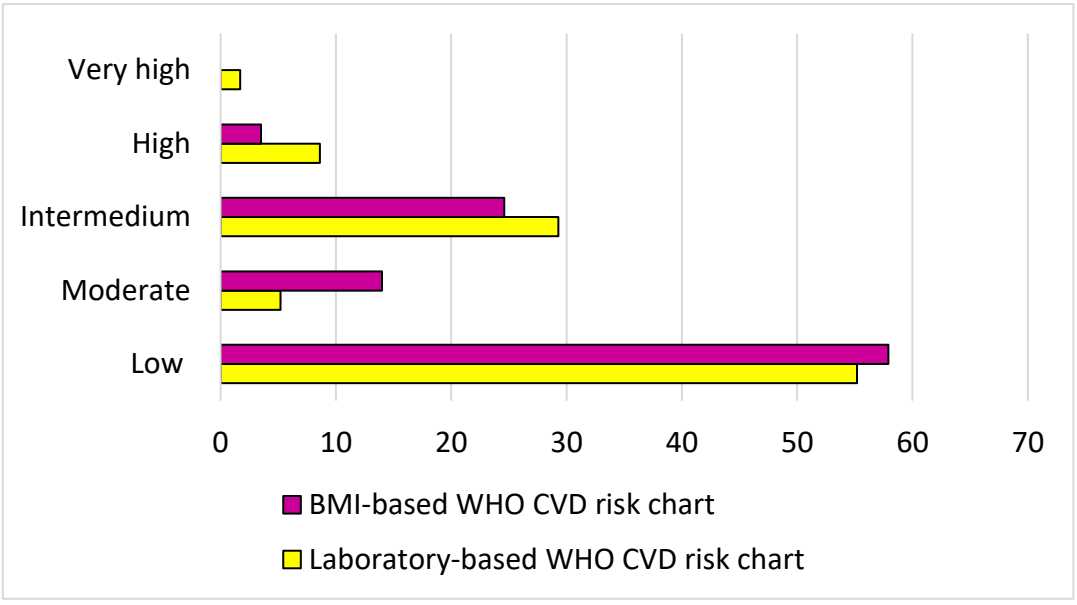
Study 2 Figure 9 – Prevalence of hpercortisolism-related systemic comorbidities in the study cohort, classified by number (underlying disease burden).



Study 2 Figure 10 – Prevalence of hypercortisolism-related systemic comorbidities in the study cohort, classified by type.



Study 2 Figure 11 – Correlation between waist circumference and copeptin (ρ=0.620, p<0.001).



Study 2 Figure 12 – Distribution of CVD risk in the study cohort according to the laboratory-based and BMI-based WHO CVD risk charts.

STUDY 3 – Use of copeptin in the follow-up of Cushing's syndrome

Purpose

To determine whether plasma copeptin levels are subject to intra-individual variations in response to the treatment of CS and according to disease control status during longitudinal follow-up.

Study design

Prospective cohort study.

Patients and methods

All the patients admitted to the Endocrinology and Metabolic Diseases Department of Ancona University Hospital (Azienda Ospedaliero-Universitaria delle Marche, Ancona, Italy) between January 2016 and December 2022 with a history CS were consecutively enrolled (n=112). Among them, those harboring an ACTH-dependent disease and having at least two determinations of plasma copeptin under different disease states, provided the absence of both posterior pituitary dysfunctions and water intake/urine output impairment (a range of 500-3000 ml/day was considered normal), were selected (n=30). The study cohort included 22 (73%) females and 8 (27%) males, aged 44±12 (19-74) years. Of them, 90% had CD and 10% EAS. Patients were followed-up for 35±20 months. The diagnosis of CS and the subsequent etiological diagnosis were made according to the criteria established by the Endocrine Society and the Consensus Statement on the diagnosis and complications of CS [26, 28]. Each time patients underwent the treatment option(s) best fitting with their clinical, biochemical and morphological profile based on both the Endocrine Society guidelines and our experience [57-58].

The following disease states were identified: (a) active disease, (b) active disease_TP, (c) remission_ACTHD, (d) remission, (e) persistent/recurrent, (f) persistent/recurrent_TP. (a) An active disease was defined as the simultaneous presence of mUFC >3034 nmol/24h (corresponding to 110 µg/24h, which is the local ULN; at

least two measurements were required) and one among the following criteria: 1 mg-DST >50 nmol/L (corresponding to 1,8 µg/dl), mLNSC >8 nmol/L (corresponding to 0,30 µg/dl, which is the local ULN; at least two measurements were required), clinical features suggestive for hypercortisolism, provided the patient was not taking glucocorticoids for any reasons. Patients taking CS-targeted medications as the first-line option at enrollment were also considered as harboring an active disease, which was defined as "controlled" on "uncontrolled" on the basis of mUFC levels (≤ or > ULN, respectively). The severity of hypercortisolism in naïve and uncontrolled patients was quantified according to mUFC in mild (ULN < mUFC ≤ 2xULN) and severe (mUFC >2xULN). (b) An active disease_TP was defined as the active disease re-evaluated after either the starting of medical therapy, or a change in the previous treatment regimen (daily dose, type and/or number of drugs). (c) The remission_ACTHD state was defined as the iatrogenic secondary adrenal insufficiency following transphenoidal surgery, radiotherapy/radiosurgery or even their combination to treat CD. The need for cortisol replacement at hospital admission was the essential inclusion criterion: this was assessed either clinically or by means of the dynamic stimulation test with 250 µg ACTH (Synacthen®) [29]. (d) The remission state was defined as the recovery of the HPA axis function following transphenoidal surgery (performed ≥3 months before the assessment), radiotherapy/radiosurgery or their combination to treat CD, irrespective of a prior remission_ACTHD state. The recovery of the HPA axis function implied cortisol replacement was not, or no longer, needed at hospital admission and was assessed either clinically (resolution of CS stigmatae) or biochemically (serial post-operative plasma cortisol between 150-300 nmol/L), as well as by means of the dynamic stimulation test with 250 µg ACTH (Synacthen®) in the case of a prior remission_ACTHD state [26, 29]. (e) A persistent/recurrent state was defined as an active disease following transphenoidal surgery, radiotherapy/radiosurgery or their combination to treat CD. Although falling within the same study group, each of the two adjectives refers to a specific category of patients: those harboring a persistent disease either never experienced remission or had an active disease back within 6 months from surgery, whereas those harboring recurrent disease had been in a remission state (either with or without ACTHD) for ≥6 months after surgery. (f) A persistent/recurrent_TP state was defined as the persistent/recurrent disease re-evaluated after the starting of second-line medical therapy. After obtaining patients' informed consent, gender, age and the

etiology of CS were recorded at baseline and the following data were collected at each admission: disease state, copeptin, mUFC to quantify disease control and number of comorbidities, which expressed the clinical burden derived from the history of CS. If a patient was admitted more than once in the same disease state, mean copeptin and mUFC levels were calculated and registered for the study purpose. The intra-individual variability of copeptin during longitudinal follow-up was calculated. Comparisons between copeptin levels at subsequent disease states were made (active *vs* active_TP, active *vs* remission, active *vs* remission_ACTHD, active *vs* persistent/recurrent, persistent/recurrent *vs* persistent/recurrent_TP). Copeptin and mUFC variations (Δ) according to disease state were calculated and their correlation was tested. After excluding patients with EAS, the influence of copeptin levels on the occurrence of remission (either with or without ACTHD) and post-surgical persistence/recurrence was verified.

Plasma copeptin measurement

The ultra-sensitive, immuno-luminometric BRAHMS Copeptin assay on the KRYPTOR Compact Plus system (Thermo Fisher Scientific, Hennigsdorf, Germany) was used to measure copeptin levels. This assay has a lower limit of detection 0.7 pmol/L and a functional sensitivity <2 pmol/L.

Statistical analysis

Variables are presented depending on their distribution (normal vs non-normal, respectively) either as mean ± SD or as median IQR if continuous, as number and percentage (%) if categorical, as mean (95%CI) if ordinal. The intra-individual variability of copeptin was calculated with the CoV formula: $CoV = \frac{SD}{mean} \times 100$. After verifying the normal distribution of quantitative variables with the Shapiro-Wilk test, comparisons between two dependent groups were made with the paired-samples t-test. The Pearson's P and the non-parametric Spearman's Rho (ρ) were used, as appropriate, for the bi-varied correlation analyses, whereas the Beta (β) coefficient was used for the linear and logistic regression analyses. The OR was used to verify outcomes. A P value of 0.050 was considered for the statistical significance. The IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. was used for the statistical analysis.

Results

The median intra-individual variability of copeptin during longitudinal follow-up was 23(34)%. At the end of follow-up, patients had been treated according to the options listed in Study 3 Figure 1. Study 3 Table I illustrates copeptin and mUFC behavior, as well as the number of comorbidities according to the different disease states thus obtained. The hormonal trend throughout the follow-up is also visible at glance in Study 3 Figure 2 (mUFC is in log scale for convenience). The severity of CS among patients with active disease was distributed as follows: 5% controlled, 11% mild, 84% severe. The re-evaluation after treatment start/change showed 38% controlled patients, 50% patients with mild hypercortisolism and 12% patients with severe hypercortisolism. Although similar between the two timepoints, mean copeptin increased by 2.6 pmol/L; the 7421 nmol/24h decrease in mUFC was statistically significant between the two timepoints ($t(7)=3.015$ $p=0.020$). The rate of post-surgical remissions, either with or without ACTHD, was 30%. Neither copeptin (Δ: -0.07 pmol/L) nor mUFC (Δ: -6278 nmol/24h) varied significantly from the active state to the remission state, but when comparing the active state with the remission_ACTHD state, for similar copeptin levels (+0.6 pmol/L), both mUFC (ΔmUFC: -12326 nmol/24h) and the number of comorbidities (Δn comorbidities: -2) significantly improved ($t(3)=3.563$ $p=0.038$ for mUFC, $t(5)=6.325$ $p=0.001$ for number of comorbidities). The persistence/recurrence rate after surgery was 50%. Of the patients in persistent/recurrence state, mild and severe hypercortisolism were found in 53% and 47% cases, respectively. From the active to the persistent/recurrence state, neither copeptin nor mUFC exhibited significant variations (Δcopeptin: -0.1 pmol/L, ΔmUFC: -9822 nmol/L). Not enough data were available to run a comparison between the remission or remission_ACTHD states and the persistent/recurrent state. When patients with persistent/recurrent disease were treated in second-line, the rate of severe hypercortisolism decreased to 33%, that of mild hypercortisolism fell down to 22% and 45% patients became controlled. No significant differences were higlighted for copeptin (Δ: +0.6 pmol/L) and for mUFC (Δ: +956 nmol/24h), but an inverse correlation was found between copeptin and mUFC ($\rho=-0.585$, $p=0.046$). No significant correlations were found between copeptin and mUFC at any other timepoints. Neither copeptin levels during active disease, nor mean copeptin

throughout the follow-up were correlated with the outcomes of remission and persistence/recurrence after pituitary surgery in patients with CD. However, patients experiencing a persistent/recurrent disease after surgery had significantly lower intra-individual variability in copeptin levels than their counterpart (20% *vs* 38%, p=0.017, Study 3 Figure 3) and mean copeptin CoV was inversely correlated with that outcome (ρ-0.466, p=0.014). A logistic regression was then run to ascertain the effects of copeptin CoV throughout follow-up on the likelihood patients encountered a post-surgical persistence or recurrence of CD, but the identified model was not statistically significant. One male patient died during the follow-up due to cardiovascular disease. He had CD with negative pituitary imaging, for which explorative neurosurgery was unfeasible due to concomitant malignancy and a hereditary cardiomyopathy requiring the implantation of a defibrillator/pacemaker. He exhibited the highest mean copeptin levels of the study cohort throughout the follow up (12.8 pmol/L).

Discussion

Findings from **Studies 1 and 2** pointed out copeptin release is a hypercortisolism-related epiphenomenon reflecting the systemic burden of CS, particularly in terms of CV and glycometabolic impairment. In this prospective study, we followed-up 30 patients with ACTH-dependent CS (90% CD) for a mean of 35 months in order to assess whether copeptin is subject to intra-individual variations in response to the best therapeutic options each patient could be offered according to both the Endocrine Society guidelines and our experience [57-58]. Copeptin levels showed a 23% median intra-individual variability throughout the follow-up. Although the t-paired analysis showed its levels did not vary significantly, observing copeptin behavior from one disease state to another was interestingly informative. Study 3 Figure 2 simulates one of the possible natural histories of CD by using the mean copeptin and mUFC values (expressed in a log scale) in the study cohort at each specific timepoint. When an active disease was re-evaluated after either the starting of a new medical therapy, or the changes brought in the ongoing medical treatment, copeptin levels showed an increasing trend which was opposite to that of mUFC, likely reflecting the progressive restoration of the secretory dynamics of both HPA and AVP system or, alternatively, a state of relative glucocorticoid deprivation. Of note,

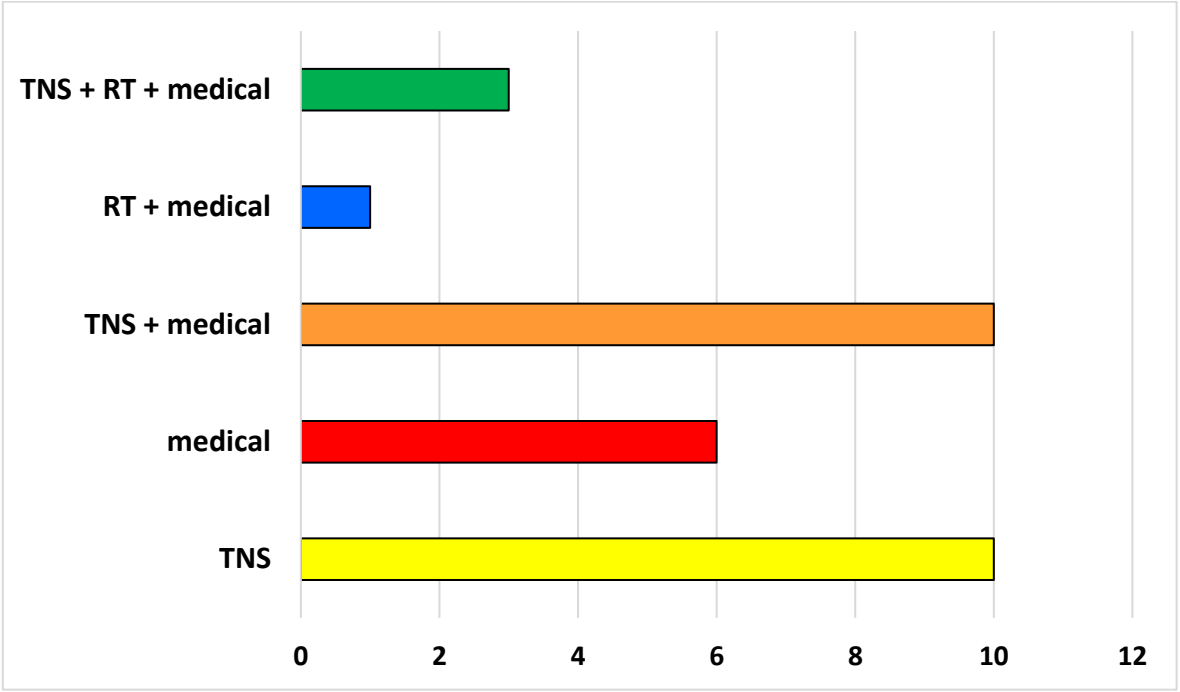
this finding was in line with data from our previous experience [unpublished data], as well as with the results by Yanovski *et al.* concerning AVP during BIPSS [11]. When disease remission with ACTHD was encountered after surgery, both mUFC and copeptin levels fell down to the physiologic range and remained quite stable once ACTHD had resolved. This finding is only appearently in contrast with the raise in copeptin during medical treatment, as it can be explained by the fact that an adequate glucocorticoid replacement is usually promptly initiated in case of remission with ACTHD, thus rendering patients eucortisolemic (see **Study 1**). Disease recurrence was associated to a further decrease in copeptin levels, which once again was opposite to that of mUFC and reflected the findings of the previous **Studies 1 and 2** on copeptin inhibition by hypercortisolism. Once a second-line medical treatment was resumed, the observed raise in copeptin levels was slight, and this was likely due to the lack of variations in mUFC observed in this specific study cohort. The only significant correlation between copeptin and mUFC was highlighted at this timepoint: it was of moderate intensity and, as expected, inverse (ρ=-0.585, p=0.046). No significant correlations were found between copeptin and mUFC at any other timepoints: at least partly, this finding may be explained by both the small number of patients some disease state categories hosted and the lack of necessity to assess UFC in some disease state categories, which accounted for the non-consistent availability of mUFC in the study cohort at specific disease states. Moreover, our cohort was too small to attempt highlighting the influence of each specific treatment option on copeptin levels. Another limitation of this study is the lack of information on copeptin levels immediately after surgery, which may have served as predictors for remission or persistence of CD in the absence of post-operative fluid balance disorders, as recently hypothesized, albeit not robustly demonstrated, by Flippo *et al.* [27]. If copeptin itself had no prognostic value in predicting remission or persistence/recurrence of CD after pituitary surgery, its intra-individual variability was negatively associated with, but was not a predictor of, persistent/recurrent disease. This finding further supports the assumption copeptin release is not, or not solely, implicated in the pathogenesis of CD, and of its recurrence as well. Fascinating points of reflection on the primary role of copeptin as an epiphenomenon come from the observation the only patient who died had both a very severe CD, which was unoperable due to systemic

multiple complications and refractory to all the tested medical options, and the highest copeptin in the study cohort.

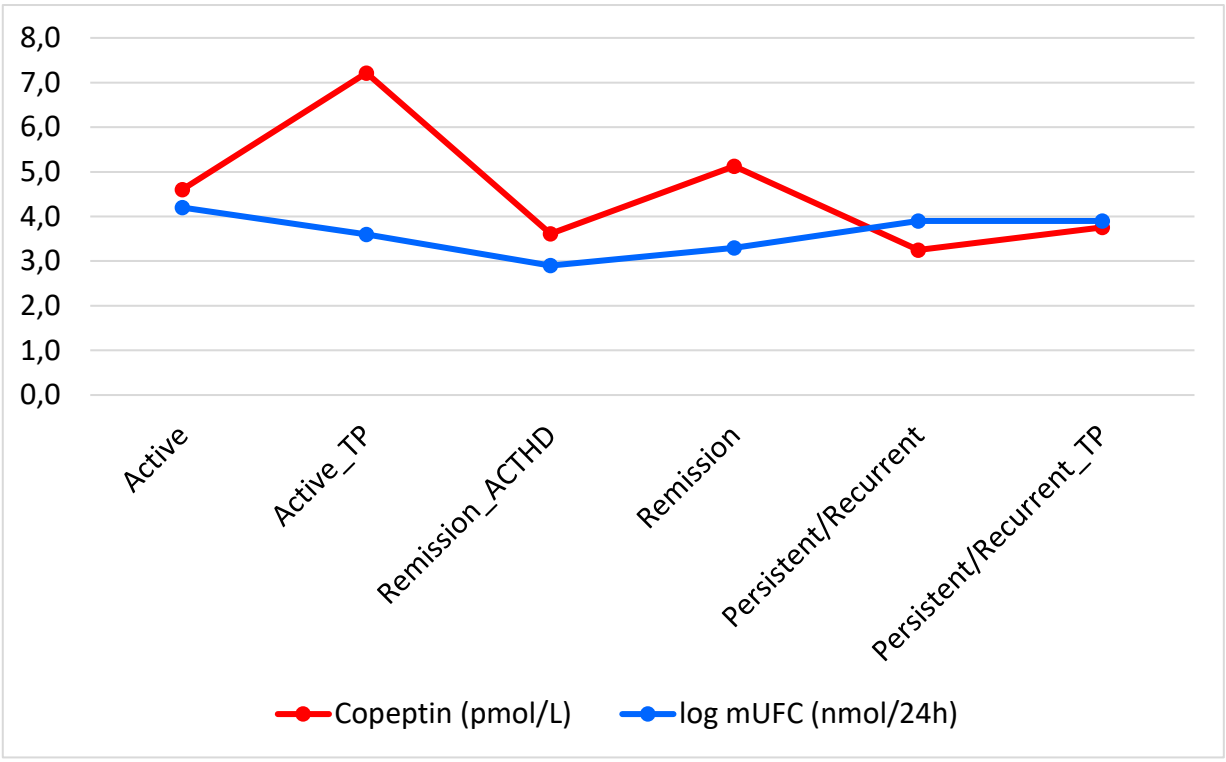
Considering all, longitudinal assessment of copeptin seems to be of scarce prognostic usefulness in patients with CS. However, once medical therapy is started, an increase in copeptin levels is expected alongside the reduction in mUFC; moreover, a higher variability is observed among patients with CD not encountering post-surgical persistence or recurrence: these findings likely imply the restoration of a physiological secretory dynamics of the AVP system occurs during and after an effective treatment.

	Active disease	Active disease_TP	Remission	Remission_ACTHD	Persistent/recurrent	Persistent/recurrent_TP
Copeptin (pmol/L)	3.9 (4.2)	6.0 (6.6)	3.5 (7.9)	3.2 (3.5)	2.7 (1.8)	3.5 (2.9)
mUFC (nmol/24h)	11505 (16334)	3697 (3628)	1876 (1214)	566 (1338)	5601 (9050)	3890 (7160)
number of comorbidities	5 (3)	5 (3)	4 (4)	4 (3)	4 (2)	4 (4)

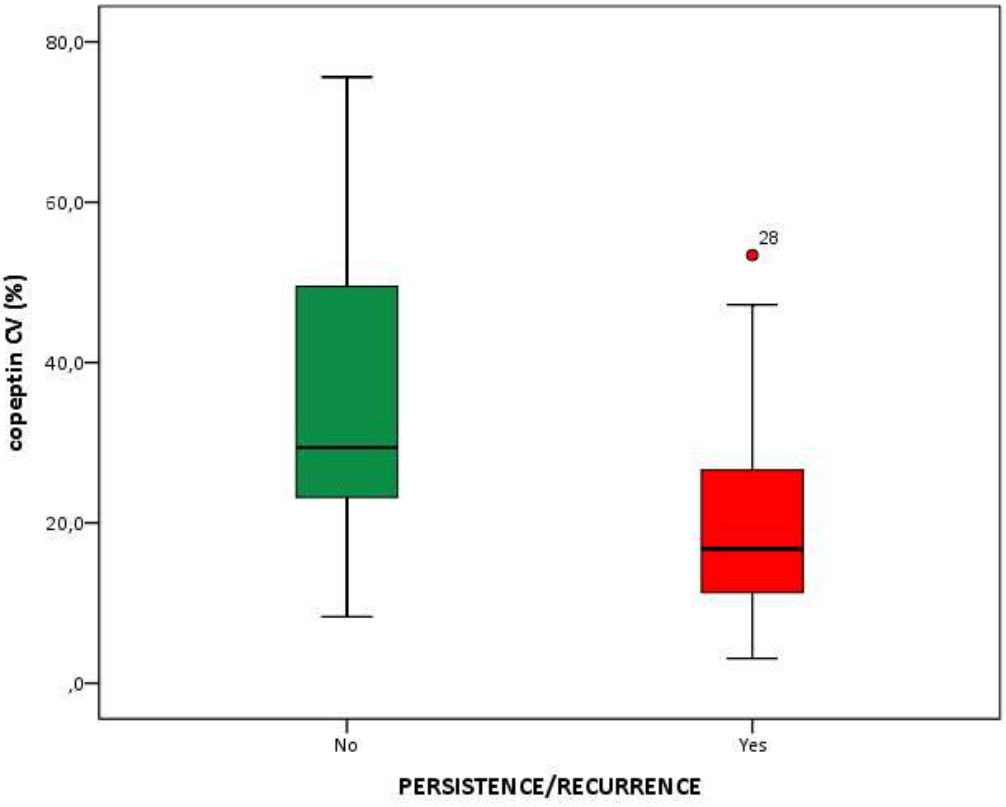
Study 3 Table I – Distribution of copeptin, mUFC and number of comorbidities according to the different disease states.



Study 3 Figure 1 – Distribution (%) of the treatment options offered to the patients in the study cohort.



Study 3 Figure 2 – Simulation of one of the natural histories of CD: copeptin and mUFC trend according to the subsequent disease states.



Study 3 Figure 3 – Distribution of copeptin intra-individual variability (CV) according to the persistent/recurrent disease outcome in patients with CD (p=0.017).

CONCLUSIONS

Due to its nature of AVP mirror-peptide, copeptin may be a novel biomarker of diagnostic and prognostic value in CS. This is, indeed, a condition characterized by the chronic hyperactivation of the HPA axis, whose pathogenesis involves, among the other mechanisms, also a dysregulation of the AVP system.

In **Study 1**, the potential usefulness of copeptin for the diagnosis of CS was verified on a cohort of 71 patients with active CS (CS group) referring to the Endocrinology and Metabolic Diseases Department of Ancona University Hospital (Azienda Ospedaliero-Universitaria delle Marche) over the last 6 years, whose copeptin levels were compared first with those of 82 controls, who were not hypercortisolemic but had been referred for clinical suspicion of CS (Healthy HPA group), then with those of 36 patients with primary or secondary adrenal insufficiency (AI group). The study demonstrated that, in the presence of a clinical suspicion for CS, copeptin assessment turned out unuseful in discriminating between patients with CS and those with a Healthy HPA. Moreover, copeptin behavior did not differ between the two opposite HPA axis dysfunctions, at least in the case of adequately replaced AI. However, two keypoints of outmost pathophysiological importance emerged from this study concerning CS: first, an hypercortisolism-induced dysruption of the osmotic response to AVP, which was responsible for the higher plasma osmolality and sodium in CS than Healthy HPA patients; second, the masking or distorting effect exerted on “real” copeptin levels by specific confounding factors notably activating AVP system: DM, CV comorbidities requiring the use of diuretics (SGLT2i included) and their combination. In the absence of confounders, CS patients exhibited the lowest copeptin; when the confounders acted in combination, their influcence on copeptin levels exceeded such as to mask that of hypercortisolism, which became visible when only one of the confounders existed.

In **Study 2**, the etiologic differential diagnosis and the prognostic evaluation of 80 patients with CS (58 with CD, 12 with ACS and 10 with EAS) referring to the Endocrinology and Metabolic Diseases Department of Ancona University Hospital (Azienda Ospedaliero-Universitaria delle Marche, Ancona, Italy) over the last 6 years were integrated with copeptin assessment in order to clarify if this biomarker could be of additive value. The study demonstrated that copeptin release was a hypercortisolism-related epiphenomenon reflecting the

systemic burden of the disease, which is typical of EAS, rather than primarily implicated in the pathogenesis of the disease by promoting ACTH hypersecretion. However, due to their close relationship, the simultaneous determination of ACTH and copeptin trend suggested at a glance the etiologic diagnosis of CS (low ACTH and low-medium copeptin in ACS, high ACTH and medium-high copeptin in CD; very high ACTH and high-very high copeptin in EAS) and, in the subgroup of patients with ACTH-dependent CS, the comparison between the performance of copeptin and that of the gold standard (Δ%ACTH during hCRH test, AUC=0.889, p=0.029) in distinguishing between CD and EAS stated the superiority of copeptin (AUC=0.993, p=0.001), proposing it as a novel, peculiar biomarker of EAS. This finding is of outmost importance today as never before, since the lacking of hCRH in the near future has been recently foreseen worldwide. Of note, an ancillary analysis involved a small group of patients with NET and was aimed at exploring, despite the non-conclusive results as yet, the different copeptin behavior in EAS *vs* non-ACTH-producing NETs. Additional support to the above-mentioned proposal came from the information copeptin yielded on the heavy systemic burden in terms of number and type of hypercortisolism-related comorbidities: arterial hypertension, particularly when diuretics were required, worsening glycometabolic control, central obesity and hypokalemia profoundly impacted the clinical picture of EAS and were associated to, or correlated with, higher copeptin levels.

In **Study 3**, 30 patients with ACTH-dependent CS (27 CD and 3 EAS) were prospectively followed-up for a mean of 35 months with the purpose of exploring the intra-individual variations in copeptin levels in response to treatment, which was 23%. The study demonstrated longitudinal assessment of copeptin in patients with CS was of poor prognostic usefulness, as copeptin levels did not change significantly according to the different disease states each patient could encounter. However, once medical therapy was started, the reduction in mUFC was accompanied by an increase in copeptin levels, whose variability was significantly higher in patients not encountering post-surgical persistence or recurrence of the disease. An effective treatment seemed therefore able to reverse the inhibition exerted by the elevated cortisol levels on the secretory dynamics of the AVP system.

Further contributions will help clarify better this fascinating, as well as still quite unexplored chapter of Neuroendocrinology.

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