Clinical and biochemical data for the diagnosis of endogenous hypercortisolism: the "Cushingomic" approach

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SUPPLEMENTARY MATERIAL

The full database is available in the repository data of the University of Padova[1].

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Inclusion criteria in the selected groups

Patients were divided in groups, according to the final diagnosis:

- ✓ Cushing's syndrome (CS, n = 40): patients with a new diagnosis of hypercortisolism. Endogenous hypercortisolism was confirmed with histology in 36 cases (positive ACTH staining in pituitary adenoma, or cortisol-secreting adrenal adenoma), surgical remission of CS (n=4).
- ✓ Non-CS: patients without CS (n = 615), further divided in:
  - Adrenal incidentaloma (n = 319): patients with adrenal lesion discovered accidentally by abdominal imaging (computed tomography or magnetic resonance), without any suspicion of adrenal-related disorder [2]. Patients with active malignancies or clear signs/symptoms of overt hypercortisolism (recent-onset easy bruising, facial plethora, proximal muscle weakness, or reddish-purple striae >1 cm wide) or with a final diagnosis of CS were excluded. Radiological characteristics were consistent with a benign adrenal incidentaloma (largest diameter <40mm and high lipid content with Hounsfield Unit <10 in all cases); primary aldosteronism and pheochromocytoma were ruled out in all subjects, if clinically indicated[3].
  - Suspected-CS (susp-CS, n = 263): Patients in whom endogenous CS has been excluded. In all cases, non-neoplastic hypercortisolism (NNH, formerly known as pseudo-Cushing state) has been ruled out with long-term observations and dynamic tests [4]. Moreover, a sufficient follow-up of at least one year enabled us to exclude cyclic CS [5].
  - Pituitary incidentaloma (n = 33): patients with pituitary lesion discovered serendipitously by cerebral imaging (magnetic resonance in all cases), without any suspicion of pituitary-related disorder. A functioning pituitary adenoma was ruled out in all subjects.

Clinical features and chief complaints of hypercortisolism collected

- Arterial hypertension: Blood pressure (BP) levels were measured during the outpatient clinic visit and considered normal according to the European Society of Hypertension practice guidelines for office and out-of-office blood pressure measurement [6]. Three categories of hypertensive patients were considered: normal BP (<130/85 mmHg without medications), hypertension with one or two drugs, and hypertension with at least three drugs used to control BP levels.
- Glucose metabolism alterations (GMAs): were defined according to the international criteria in impaired fasting glucose (IFG: basal glucose 5.5 – 7.0 mmol/L), impaired glucose tolerance (IGT: glucose levels 120 min after OGTT 7.8-11.1 mmol/L), and diabetes mellitus (DM: basal glucose ≥7 mmol/L, glucose levels 120 min after OGTT ≥11.1 mmol/L or HbA1c ≥48 mmol/mol, or use of anti-diabetic drugs), according to the American Diabetes Association guidelines[7].
- Weight: Body mass index (BMI) was calculated dividing weight by height squared (kg/m^2). Participants were weighed using a scale wearing light clothing and no shoes, recorded to the nearest 0.5 kg, and were measured using a vertical ruler, recorded to the nearest 0.5 cm. Three weight classes were considered: normal, overweight (BMI 25-30 kg/m^2) and obesity (BMI ≥30 kg/m^2).
- Skeletal disease: Bone mineral density (BMD) at lumbar spine (L1–L4) and femur (neck and total) was determined by DXA, using Hologic QDR 4500 C densitometer (Hologic Inc., Waltham, MA, USA). The instrument was calibrated daily according to the manufacturer's instructions (coefficient of variation was 0.6% and 1.2% for instrument and in vivo, respectively). The data were analyzed by the same operator using the same software. Spinal radiographs of the vertebrae T1–L5 were used to define vertebral fractures on visual inspection using the semiquantitative method described by Genant (on lateral X-ray a fracture is defined as >20% reduction in anterior, middle or posterior vertebral height). According to bone disease, patients were divided into normal DXA and no fractures, osteopenia (BMD t score –1 to –2.5), or osteoporosis (t score < –2.5) with or without vertebral or hip fractures.

- The presence of mood disorders, hyperandrogenic symptoms, as well as the signs that best discriminate CS were collected as a dichotomous variable yes/no: moon face, unusual fat pads (defined as the detection of dorsocervical fat pad and/or supraclavicular fat pad), purple striae >1 cm wide, easy bruising, and muscle weakness.

***Supplementary Table 1*:** Technical remarks of first-line screening tests.

	Test protocol	***Written instructions and nurse education to patients before the collection***	***Laboratory assay***
Serum cortisol after 1 mg 1-mg overnight dexamethasone suppression test	Oral intake of 1mg dexamethasone (two 0.5 mg tablets) at 23.00, blood collection the next morning between 08.00 and 09.00. Performed after UFC and LNSC collection.	yes	Serum cortisol levels were measured by a commercial CE-IVD chemiluminescence immunoassay, with declared intra- and inter-assay coefficient of variation (CV) <7% and <9%, respectively (Immulite 2000, Diagnostic Products Co., CA, USA) until September 2020; then serum cortisol was determined by a commercial CE-IVD chemiluminescence immunoassay, with declared intra- and inter-assay coefficient of variation (CV) <7% and <8%, respectively (Beckman Coulter, Inc., CA USA). Patient sample comparability was verified: no significant bias was observed between methods. All patients presented adequate dexamethasone levels after overnight 1mg dexamethasone suppression test, and those that assumed interfering drugs were excluded. Dexamethasone levels were measure with a LC/MS-MS method [8].
Late night salivary cortisol (LNSC)	Saliva collection was performed between 11.00 and 12.00 pm using a Salivette® device (Sarstedt, Numbrecht, Germany)	yes	LC-MS/MS method, with an intra-assay and inter-assay CV of 6% for cortisol. LNSC range 0.5-2.5 nmol/L. Clinical practice cutoff for overt CS: 2.6 nmol/L, considered as upper limit of normality (ULN) in the manuscript [9].
Urinary free cortisol (UFC)	Patients were instructed to discard the first morning urine void and to collect all urine for the next 24 h, so that the morning urine void on the second day corresponds to the conclusion of the collection.	yes	LC-MS/MS method, with calculated intra/inter-assay CV <6% and <8%. UFC range 16-169 nmol/24h. Clinical practice cutoff for overt CS: 170 nmol/24h, considered as ULN in the manuscript [10].

***Supplementary Table 2*:** Sensitivity (SE), specificity (SP), negative likelihood ratio (LR^{neg}), positive likelihood ratio (LR^{pos}), Diagnostic Odds Ratio (DOR) and respective 95% of confidence interval (95% CI). CS: Cushing's Syndrome.

Adrenal incidentaloma vs CS					
	SE % (95% CI)	**SP % (95% CI)**	**LR^{neg} (95% CI)**	**LR^{pos} (95% CI)**	**DOR (95% CI)**
Hypertension	77.5 (62.5-87.7)	22.4 (18.1-27.3)	1 (0.55-1.85)	1 (0.84-1.19)	1 (0.45-2.18)
Glucose metabolism alteration	30 (18-45)	42.3 (36.7-48)	1.66 (1.3-2.1)	0.52 (0.32-0.84)	0.31 (0.15-0.64)
Overweight	65 (49.5-77.9)	21.1 (16.4-26.6)	1.66 (1.02-2.71)	0.82 (0.65-1.04)	0.5 (0.24-1.02)
Skeletal disease	88 (70-95.8)	16.5 (11-24.2)	0.73 (0.23-2.59)	1.05 (0.89-1.24)	1.45 (0.4-5.32)
Mood disorders	22.5 (12.3-37.5)	90 (86.2-92.8)	0.86 (0.73-1.02)	2.24 (1.15-4.35)	2.6 (1.14-5.95)
Dyslipidemia	55 (39.8-69.3)	37.5 (32-43.4)	1.2 (0.82-1.75)	0.88 (0.66-1.18)	0.73 (0.38-1.43)
Moon face	40 (26.4-55.4)	99.7 (98.2-99.9)	0.6 (0.48-0.78)	127.6 (17.4-936)	212 (27-1667)
Unusual fat pads	25 (14.2-40.2)	97.5 (95.1-98.7)	0.77 (0.64-0.92)	9.97 (4.18-23.79)	12.96 (4.76-35.3)
Purple striae	17.5 (8.8-32)	99.7 (98.2-99.9)	0.83 (0.72-0.96)	55.83 (7.05-442)	67.5 (8-565.2)
Easy bruising	27.5 (16.1-42.8)	99.7 (98.2-99.9)	0.73 (0.6-0.88)	87.73 (11.63-662)	121 (15.1-968)
Muscle weakness	2.5 (1-12.88)	99.7 (98.2-99.9)	0.98 (0.93-1.03)	7.96 (0.51-125)	8.15 (0.5-133)
Suspected CS vs CS					
	SE % (95% CI)	**SP % (95% CI)**	**LR^{neg} (95% CI)**	**LR^{pos} (95% CI)**	**DOR (95% CI)**
Hypertension	77.5 (62.5-87.7)	53.4 (0.48-0.6)	0.42 (0.23-0.75)	1.68 (1.36-2.09)	4.04 (1.85-8.83)
Glucose metabolism alteration	30 (18-45)	59.5 (53.1-65.6)	1.18 (0.94-1.48)	0.74 (0.45-1.22)	0.63 (0.3-1.3)
Overweight	65 (49.5-77.9)	16.8 (12.3-22.4)	2.09 (1.24-3.51)	0.78 (0.62-0.99)	0.37 (0.18-0.79)
Skeletal disease	88 (70-95.8)	7 (29.2-17.5)	1.62 (0.39-6.7)	0.95 (0.81-1.12)	0.59 (0.12-2.84)
Mood disorders	22.5 (12.3-37.5)	86.3 (81.6-89.9)	0.9 (0.76-1.07)	1.64 (0.86-3.15)	1.83 (0.8-4.16)
PCOS	50 (21.5-78.5)	49.1 (36.6-61.7)	1.02 (0.48-2.14)	0.98 (0.47-2.06)	0.97 (0.22-4.24)
Dyslipidemia	55 (39.8-69.3)	56.4 (49.7-62.9)	0.8 (0.56-1.15)	1.26 (0.92-1.74)	1.58 (0.8-3.12)
Moon face	40 (26.4-55.4)	95.4 (92.2-97.4)	0.63 (0.49-0.81)	8.77 (4.48-17.14)	13.94 (5.9-32.9)
Unusual fat pads	25 (14.2-40.2)	92.8 (89-95.3)	0.81 (0.67-0.97)	3.46 (1.74-6.9)	4.28 (1.82-10)
Purple striae	17.5 (8.8-32)	95.8 (92.7-97.7)	0.86 (0.74-0.99)	4.18 (1.72-10.16)	4.86 (1.76-13.4)
Easy bruising	27.5 (16.1-42.8)	98.9 (96.7-99.6)	0.73 (0.61-0.89)	24.11 (7-82.69)	32.87 (8.67-124)
Muscle weakness	2.5 (1-12.88)	99.6 (97.8-99.9)	0.98 (0.93-1.03)	6.56 (0.42-103)	6.72 (0.41-109.6)

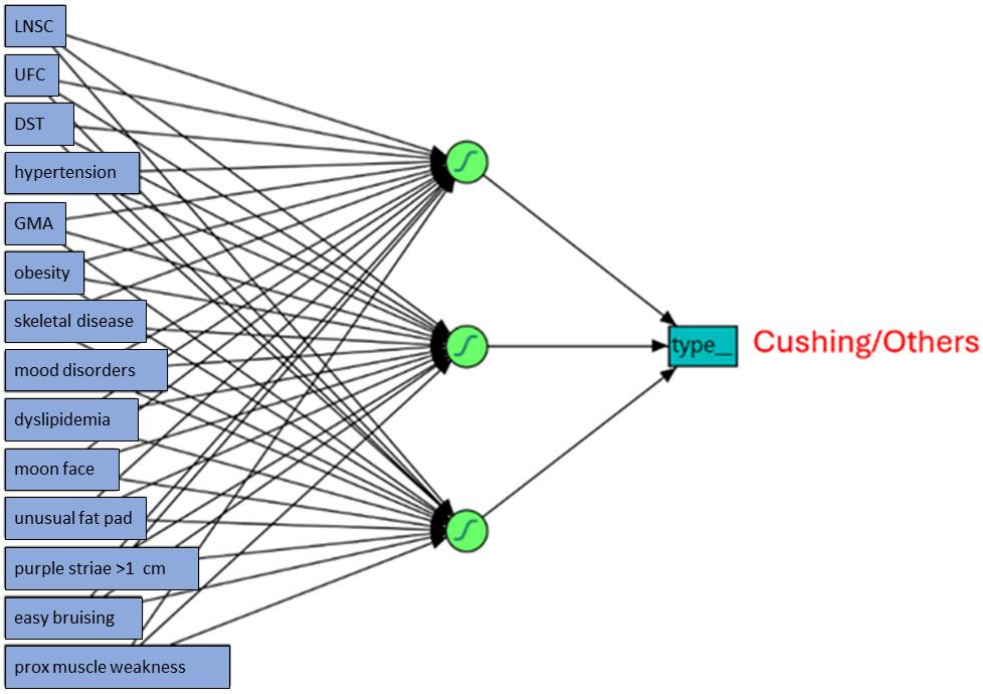
Supplementary Table 3: Clinical picture of patients with adrenal incidentaloma or suspected Cushing's syndrome (CS), considering the severity of the clinical feature. DST: serum cortisol after 1-mg overnight dexamethasone suppression test; UFC: urinary free cortisol; LNSC: late-night salivary cortisol; IFG: impaired fasting glucose; IGT: impaired glucose tolerance; DM: diabetes mellitus; FX: fractures; PCOS: polycystic ovary syndrome. a: $p<0.01$ versus normal; b: $p<0.01$ versus hypertension 1-2 drugs: c: $p<0.05$ vs normal.

		Adrenal incidentaloma			**Suspected CS**		
		DST nmol/L	**UFC nmol/24h**	**LNSC nmol/L**	**DST nmol/L**	**UFC nmol/24h**	**LNSC nmol/L**
Blood Pressure	Normal	56 (5)	103 (8)	3.1 (0.8)	31 (5)	82 (5)	1.4 (0.2)
	Hypertension 1-2 drugs	66 (4)	93 (5)	2 (0.2)	37 (5)	81 (8)	1.8 (0.3)
	Hypertension ≥3 drugs	86 (8) [a,b]	82 (7)	2.5 (0.4)	34 (4)	65 (10)	2.4 (0.7)
Glucose Metabolism Alterations	Normal	65 (5)	92 (6)	2.4 (0.4)	34 (5)	83 (6)	1.6 (0.2)
	IFG-IGT	68 (6)	94 (6)	2.4 (05)	32 (4)	79 (8)	1.4 (0.2)
	DM	76 (7)	98 (9)	2.6 (0.5)	30 (4)	65 (10)	1.9 (0.5)
Weight	Normal	73 (8)	107 (12)	3 (0.9)	48 (11)	104 (19)	1.8 (0.2)
	BMI 25-30 kg/m^2	69 (6)	97 (7)	2.5 (0.5)	25 (2) [c]	67 (6) [c]	1.5 (0.2)
	BMI ≥30 kg/m^2	62 (6)	90 (7)	2.2 (0.3)	25 (2) [a]	74 (5) [c]	1.6 (0.3)
Skeletal disease	Normal	71 (10)	104 (19)	1.6 (0.2)	38 (8)	62 (14)	1.7 (0.5)
	Osteopenia	84 (10)	94 (13)	2.4 (0.7)	27 (3)	79 (11)	2 (0.7)
	Osteoporosis-FX	84 (9)	83 (7)	3 (0.7)	71 (17)	96 (18)	1.8 (1.4)
Mood disorders	Yes	88 (11)	97 (13)	2.6 (1)	44 (10)	90 (19)	1.8 (0.6)
	No	67 (3)	92 (4)	2.3 (0.2)	32 (3)	79 (4)	1.6 (0.1)
PCOS	Yes	n.a.	n.a.	n.a.	19 (1)	82 (10)	1.1 (0.1)
	No	n.a.	n.a.	n.a.	23 (5)	92 (15)	2.2 (0.8)
Dyslipidemia	Yes	69 (4)	87 (5)	2.2 (0.3)	34 (4)	70 (5)	1.8 (0.3)
	No	70 (6)	102 (6)	2.6 (0.5)	32 (5)	88 (8)	1.5 (0.2)

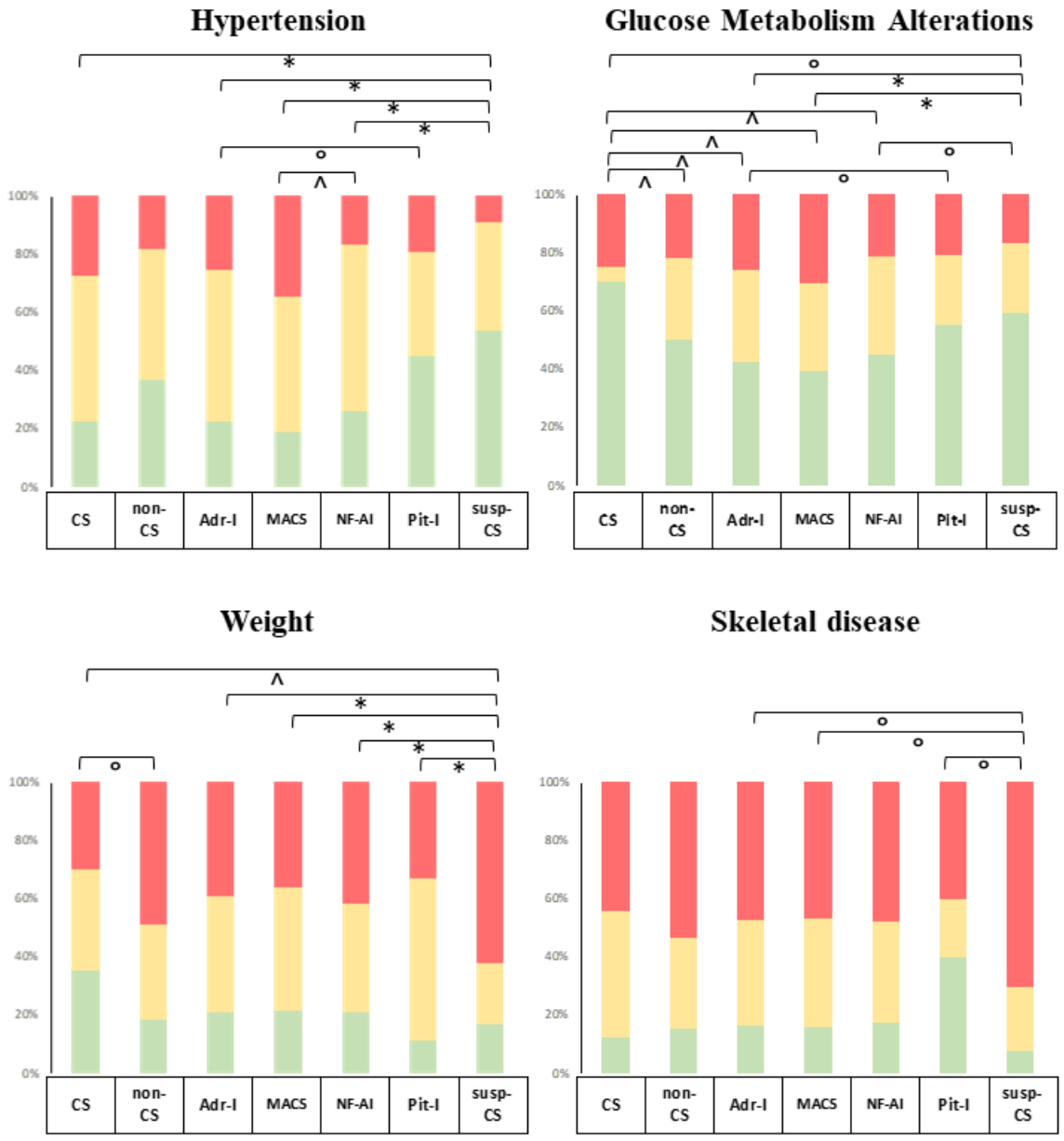
Supplementary Table 4: Diagnostic test accuracy of screening tests used for the diagnosis of Cushing's syndrome (CS) in the high-risk conditions depicted in the first column (hypertension, glucose metabolism alterations, bone disease or weight gain). SE: sensitivity; SP: specificity; LR^{neg}: negative likelihood ratio; LR^{pos}: positive likelihood ratio; AUC: area under the curve and respective 95% of confidence interval (95% CI); DOR: diagnostic odds ratio; LR^{neg}, LR^{pos} and DOR not computable if SE or SP 100%. ROC cut-off: maximum value of Youden's index for the ROC curve; DST: serum cortisol after 1-mg dexamethasone overnight suppression test; UFC: urinary free cortisol; LNSC: late night salivary cortisol.

		Cutoff	SE % (95% CI)	SP % (95% CI)	LR^{neg} (95% CI)	LR^{pos} (95% CI)	DOR (95% CI)	AUC (95% CI)
Patients with hypertension (n=393)	**susp-CS (n=116) vs CS (n=31)**	**DST >50 nmol/L**	100 (89-100)	88.8 (81.8-93.3)	---	8.92 (5.35-14.89)	---	98.5 (97.1-99.9)
		LNSC >2.6 nmol/L	96.8 (83.8-99.4)	85.3 (77.8-90.6)	0.04 (0.01-0.26)	6.6 (4.24-10.29)	174.7 (22.3-1368)	95.1 (89.9-100)
		UFC >170 nmol/24h	90.3 (75.1-96.6)	91.4 (84.9-95.3)	0.11 (0.04-0.31)	10.48 (5.73-19.1)	99 (25.5-384)	96.6 (93.5-99.6)
	Adrenal incidentaloma (n=246) vs CS (n=31)	**DST >50 nmol/L**	100 (89-100)	48.8 (42.6-55)	---	1.95 (1.73-2.21)	---	93.4 (88.8-98.1)
		LNSC >2.6 nmol/L	96.8 (83.8-99.4)	83.7 (78.6-87.8)	0.04 (0.01-0.26)	5.95 (4.45-7.96)	154 (20.5-1166)	95 (89.5-100)
		UFC >170 nmol/24h	90.3 (75.1-96.6)	90.2 (85.9-90.2)	0.11 (0.04-0.31)	9.26 (6.22-13.77)	86 (24.4-305)	95.2 (91.3-99.1)
Patients with GMAs (n=274)	**Susp-CS (n=94) vs CS (n=12)**	**DST >50 nmol/L**	100 (75.8-100)	90.4 (82.8-94.9)	---	10.4 (5.61-19.44)	---	98.5 (96.6-100)
		LNSC >2.6 nmol/L	91.7 (64.6-98.5)	89.4 (81.5-94.1)	0.09 (0.01-0.61)	8.62 (4.68-15.86)	92.4 (10.8-793)	92.2 (80.8-100)
		UFC >170 nmol/24h	75 (46.8-91.1)	93.6 (86.8-97)	0.27 (0.1-0.71)	11.75 (5-27.2)	44 (9.37-206.5)	93.6 (87.2-99.9)
	Adrenal incidentaloma (n=168) vs CS (n=12)	**DST >50 nmol/L**	100 (75.8-100	47.6 (40.2-55.1)	---	1.9 (1.65-2.21)	---	89.5 (76.4-1)
		LNSC >2.6 nmol/L	91.7 (64.6-98.5)	83.9 (77.6-88.7)	0.1 (0.02-0.65)	5.7 (3.88-8.39)	57.4 (7.12-463.6)	95 (91-99.1)
		UFC >170 nmol/24h	75 (46.8-91.1)	86.9 (81-91.2)	0.29 (0.1-0.77)	5.73 (3.44-9.5)	19.9 (5.0-79.25)	89 (78.9-99)
Patients with skeletal disease (n=173)	**Susp- CS (n=50) vs CS (n=22)**	**DST >50 nmol/L**	100 (85.1-100)	84 (71.5-91.7)	---	6.25 (3.31-11.8)	---	94.7 (89.9-99.5)
		LNSC >2.6 nmol/L	100 (85.1-100)	82 (69.2-90.2)	---	5.56 (3.08-10)	---	95 (91-99.1)
		UFC >170 nmol/24h	90.9 (72.2-97.5)	92 (81.2-96.6)	0.1 (0.03-0.37)	11.36 (4.4-29.36)	115 (19.4-679.7)	97.1 (93.0-100)
	Adrenal incidentaloma (n=101) vs CS (n=22)	**DST >50 nmol/L**	100 (85.1-100)	37.6 (28.8-47.4)	---	1.6 (1.38-1.87)	---	94.2 (88.6-99.7)
		LNSC >2.6 nmol/L	100 (85.1-100)	81 (72.2-87.5)	---	5.26 (3.5-7.89)	---	95.5 (92-99.1)
		UFC >170 nmol/24h	90.9 (72.2-97.5)	89.1 (81.5-93.8)	0.1 (0.03-0.38)	8.35 (4.7-14.81)	81.2 (16.8-398.4)	93.6 (88.3-98.8)
Patients with overweight or obesity (n=391)	**Susp-CS (n=174) vs CS (n=26)**	**DST >50 nmol/L**	100 (87.1-100)	94.3 (89.8-96.8)	---	17.4 (9.53-31.76)	---	99.5 (98.9-100)
		LNSC >2.6 nmol/L	96.2 (81.1-99.3)	90.2 (84.2-92.8)	0.04 (0.01-0.29)	9.84 (6.22-15.56)	230.9 (29.4-1812)	95.5 (90.2-100)
		UFC >170 nmol/24h	88.5 (71-96)	93.1 (88.3-96)	0.12 (0.04-0.36)	12.83 (7.3-22.53)	103.5 (27.1-394.6)	96.4 (93.3-99.5)
	Adrenal incidentaloma (n=191) vs CS (n=26)	**DST >50 nmol/L**	100 (87.1-100)	53.9 (46.9-60.8)	---	2.17 (1.86-2.53)	---	94.2 (89.7-98.7)
		LNSC >2.6 nmol/L	96.2 (81.1-99.3)	84.3 (78.5-88.8)	0.05 (0.01-0.31)	6.12 (4.37-8.58)	134.2 (17.5-1028)	93 (86.5-99.6)
		UFC >170 nmol/24h	88.5 (71-96)	86.9 (81.4-91)	0.13 (0.05-0.39)	6.76 (4.57-9.99)	50.9 (14.2-182.1)	93.6 (88.7-98.5)

Supplementary Figure 1: Neural Network Diagram.

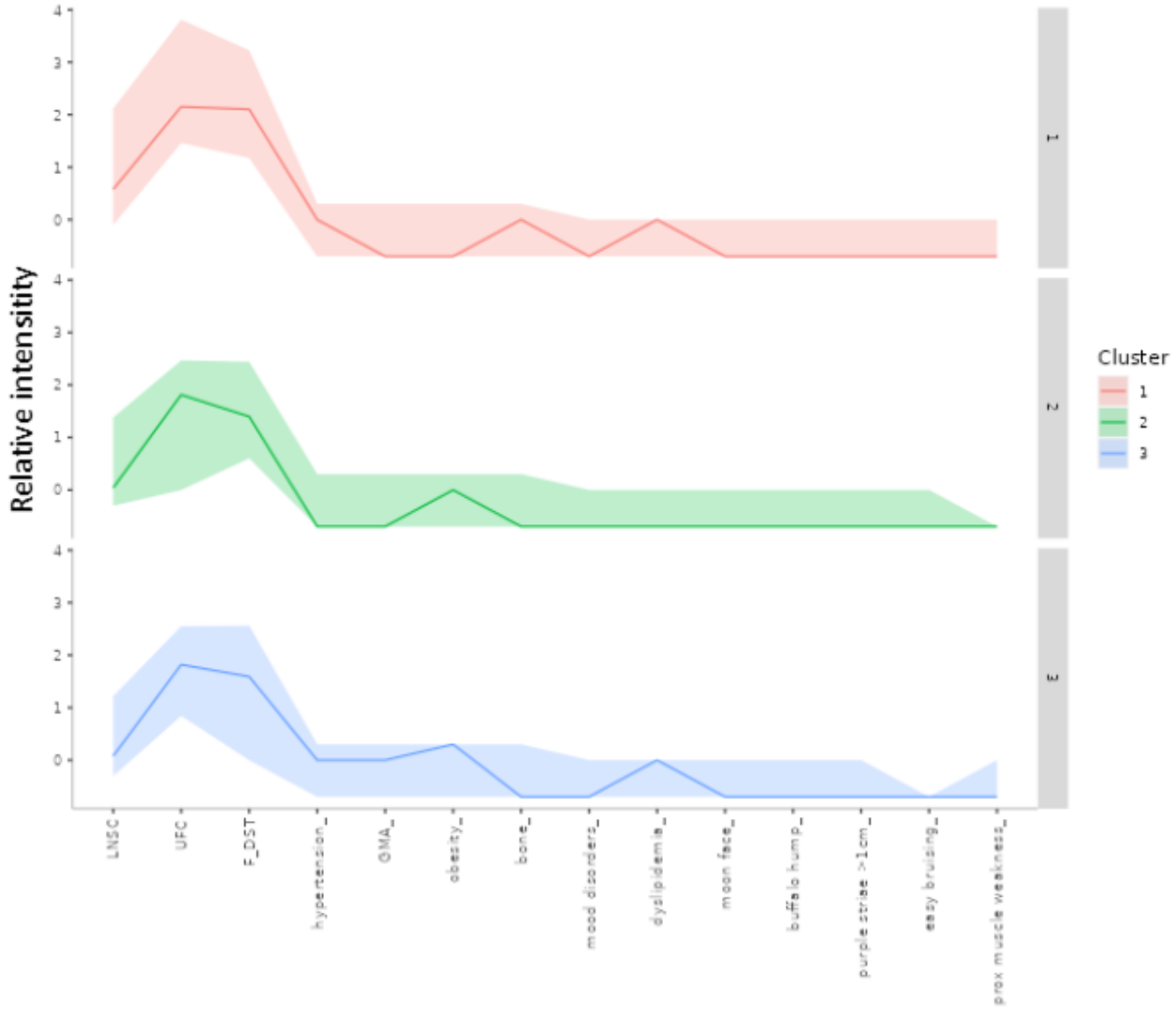


Supplementary Figure 2: Prevalence of chief complaints and cortisol-related comorbidities in the considered groups. CS: Cushing's syndrome; non-CS: the whole number of subjects in whom CS has been ruled out; Adr-I: adrenal incidentaloma; MACS: mild autonomous cortisol secretion; NF-AI: non-functioning adrenal incidentaloma; Pit-I: incidentaloma; susp-CS: suspected CS;*: p <0.001; ^: p <0.01; °: p <0.05.



	Hypertension	Glucose Metabolism Alteration	Weight	Skeletal disease
	Normal	Normal	Normal	Normal
	1-2 drugs	Impaired fasting glucose or tolerance	BMI 25-30 kg/m^2	Osteopenia
	≥3 drugs	Diabetes Mellitus	BMI ≥30 kg/m^2	Osteoporosis or fractures

Supplementary Figure 3: K-means cluster analysis of the whole cohort. The x axes are variable indices and y axes indicate relative intensities. The colored lines represent median intensities of corresponding clusters in Figure 2 (in the manuscript).



Bibliography (managed with Mendeley)

1. Ceccato F. Clinical and biochemical data for the diagnosis of endogenous hypercortisolism: the "Cushingomic" approach. doi:10.25430/researchdata.cab.unipd.it.00001059

2. Grasso M, Boscaro M, Scaroni C, Ceccato F. Secondary Arterial Hypertension: From Routine Clinical Practice to Evidence in Patients with Adrenal Tumor. *High Blood Press Cardiovasc Prev*. 2018;25(4):345-354. doi:10.1007/s40292-018-0288-6

3. Fassnacht M, Tsagarakis S, Terzolo M, et al. European Society of Endocrinology clinical practice guidelines on the management of adrenal incidentalomas, in collaboration with the European Network for the Study of Adrenal Tumors. *Eur J Endocrinol*. 2023;189(1):G1-G42. doi:10.1093/ejendo/lvad066

4. Scaroni C, Mondin A, Ceccato F. How to rule out non-neoplastic hypercortisolemia (previously known as pseudo-cushing). *Pituitary*. 2022;25(5):701-704. doi:10.1007/s11102-022-01222-2

5. Nowak E, Vogel F, Albani A, et al. Diagnostic challenges in cyclic Cushing's syndrome: a systematic review. *lancet Diabetes Endocrinol*. Published online July 7, 2023. doi:10.1016/S2213-8587(23)00150-X

6. Stergiou GS, Palatini P, Parati G, et al. 2021 European Society of Hypertension practice guidelines for office and out-of-office blood pressure measurement. *J Hypertens*. 2021;39(7):1293-1302. doi:10.1097/HJH.0000000000002843

7. ADA standards of care of diabetes 2022. Published 2022. Accessed October 25, 2023. https://www2.diabetes.org/newsroom/press-releases/2022/american-diabetes-association-2023-standards-care-diabetes-guide-for-prevention-diagnosis-treatment-people-living-with-diabetes

8. Ceccato F, Artusi C, Barbot M, et al. Dexamethasone measurement during low-dose suppression test for suspected hypercortisolism: threshold development with and validation. *J Endocrinol Invest*. 2020;43(8):1105-1113. doi:10.1007/s40618-020-01197-6

9. Antonelli G, Ceccato F, Artusi C, Marinova M, Plebani M. Salivary cortisol and cortisone by LC–MS/MS: validation, reference intervals and diagnostic accuracy in Cushing's syndrome. *Clin Chim Acta*. 2015;451:247-251. doi:10.1016/j.cca.2015.10.004

10. Antonelli G, Artusi C, Marinova M, et al. Cortisol and cortisone ratio in urine: LC-MS/MS method validation and preliminary clinical application. *Clin Chem Lab Med*. 2014;52(2):213-220. doi:10.1515/cclm-2013-0471