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Impact of Mild Autonomous Cortisol Secretion on Vertebral Fracture Risk

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1. Introduction

1.1. Adrenal Incidentaloma

Over the past three decades, the widespread use of diagnostic imaging has increased substantially, driven by technological advancements, expanded access to imaging facilities, and a growing emphasis on preventive healthcare (1). This has led to a higher detection rate of incidental pathological findings, among which adrenal incidentalomas (AIs) are among the most frequently identified.

Adrenal incidentalomas are defined as asymptomatic adrenal masses discovered during imaging performed for reasons unrelated to suspected adrenal disease (2,3). According to this strict definition, AIs are detected during investigations for non-adrenal symptoms—such as abdominal or back pain, or to exclude thoracic conditions like pulmonary embolism. This definition excludes adrenal lesions found in the context of hereditary syndromes associated with adrenal neoplasia or during imaging for suspected hormonal excess (e.g., pheochromocytoma, Cushing's syndrome, or primary aldosteronism). Similarly, adrenal masses identified during oncologic staging or surveillance in patients with known extra-adrenal malignancies are not considered true incidentalomas (3).

Although a size threshold of ≥1 cm has traditionally been used to define an adrenal lesion as an incidentaloma (4,5), this criterion may be challenged by the improved resolution of modern imaging modalities such as CT and MRI. Nevertheless, current guidelines continue to adopt this cut-off as the minimum size warranting further diagnostic evaluation, unless clinical signs of hormone excess are present (3).

A 2006 study reported a 4.4% prevalence of adrenal incidentalomas in individuals undergoing CT screening for lung cancer. Subsequent studies have confirmed a consistent prevalence of approximately 5% (6), with rates increasing with age—from around 3% in individuals aged 50 to up to 10% in older adults.

The etiology of adrenal incidentalomas is heterogeneous, encompassing benign and malignant lesions originating from the adrenal cortex or medulla, as well as extra-adrenal masses. The

prevalence of hormone-secreting tumors is notably higher in referral center cohorts compared to unselected populations (3). Despite this variability, malignancy remains relatively uncommon, accounting for 2–12% of cases (7,8). Non-aldosterone-secreting adrenocortical adenomas may be either non-functioning adrenal incidentalomas (NFAIs) or associated with autonomous cortisol secretion (MACS), with NFAIs representing up to 50% of cases (9).

1.3. Cortisol Secreting Adrenal Incidentalomas

Cushing's syndrome (CS), the clinically overt manifestation of cortisol excess, is observed in less than 5% of patients with AIs (9). It presents with distinctive features including purple striae, proximal myopathy, wasting of extremities, increased fat in abdomen, torso and face, facial plethora, easy bruising, and thin skin in the young (10). CS is frequently accompanied by significant metabolic and cardiovascular complications including diabetes mellitus, hypertension, and dyslipidemia (9). Patients commonly present with the associated comorbidities such as hypertension, Cardiovascular events, obesity , and type 2 diabetes (3,11). Additionally, overt Cushing's syndrome is usually associated with catabolic symptoms such as muscle weakness, skin fragility, osteoporosis, and comes with severe morbidity and clearly elevated mortality (3).

Mild autonomous cortisol secretion (MACS), on the other hand, represents a substantial subset of patients with AIs, accounting for 20% to 50% of patients, and is therefore the most common hormonal abnormality. (3,9,12,13)

The European Society of Endocrinology (ESE), in collaboration with the European Network for the Study of Adrenal Tumors (ENSAT), has standardized the definition of MACS in patients with AI as presence of serum cortisol after a fasting 1-mg overnight dexamethasone suppression test (F-1mgDST) exceeding 1.8 µg/dL (50 nmol/L) in the absence of overt clinical signs of cortisol excess (3).

1.4. Systemic effects of MACS

The clinical relevance of MACS extends beyond mere biochemical anomaly. Several studies have identified complications related to cardiovascular, metabolic, neuropsychiatric, infectious, and musculoskeletal in patients with MACS (9,12, 14-18).

In two large population-based studies, patients with adrenal incidentalomas exhibited a higher prevalence and incidence of cardiovascular risk factors—including hypertension, dysglycemia, peripheral vascular disease, chronic kidney disease, cardiac arrhythmias, heart failure, and thromboembolic events—compared to matched controls (19-21). Furthermore, patients with

MACS had a significantly higher burden of cardiometabolic comorbidities than those with NFAIs, including increased prevalence of type 2 diabetes mellitus, hypertension, and dyslipidemia (22).

Notably, prior research has identified a dose-dependent relationship between F-1mgDST cortisol levels and mortality risk. Cortisol levels between 83 and 137 nmol/L have been associated with a twofold increase in mortality, while levels ≥138 nmol/L correspond to a threefold increase (3,23,24).

1.5. MACS and Musculoskeletal System

MACS exerts significant detrimental effects on the musculoskeletal system, primarily through its impact on bone integrity. Chronic exposure to elevated cortisol levels, albeit 'mild', leads to an imbalance in bone remodeling, marked by decreased osteoblastic activity and impaired bone formation, while bone resorption remains unchanged or mildly increased (15), **figure 1**. This disruption results in reduced bone quality and a heightened risk of fragility fractures (25).
Beyond their direct impact on musculoskeletal system, GCs exert several indirect effects that contribute to skeletal fragility **Figure 2**. These effects are mediated through disturbances in systemic regulators of bone metabolism, including alterations in calcium homeostasis, suppression of the hypothalamic-pituitary-gonadal axis, and inhibition of the growth hormone–insulin-like growth factor 1 axis. By disrupting these physiological pathways, GCs amplify bone loss and increase fracture risk, compounding the deleterious consequences of their direct skeletal actions.

Several cross-sectional and longitudinal analyses (25) demonstrate that patients with hypercortisolism, particularly postmenopausal women, exhibit a higher prevalence and incidence of vertebral fractures (VFx) compared to those with nonfunctioning adrenal incidentalomas. Importantly, a significant proportion of these fractures occur even in individuals without osteoporotic bone mineral density (BMD) levels. This suggests that bone fragility in MACS is not fully captured by standard densitometry alone, as bone microarchitecture, rather than density, is impaired (15,25,26). In addition to skeletal effects, MACS has been associated with reduced muscle strength, potentially exacerbating fall risk and contributing to the high incidence of fractures (27).

1.6. Glucocorticoids and fragility fractures

Glucocorticoid (GC) therapy is strongly associated with a marked reduction in bone mass and deterioration of bone quality, resulting in increased skeletal fragility and fracture risk (28). These adverse skeletal effects represent one of the most consistent and clinically relevant complications of long-term GC use. Notably, fracture risk often emerges early—within the first three months of therapy—and increases in a dose- and duration-dependent manner (29,30).

The link between excess glucocorticoids and skeletal fragility was first described by Harvey Cushing in 1932 (31). Since then, extensive clinical and experimental evidence has clarified the profound impact of GCs on bone metabolism and structure (29,30,32,33).

Distinctive Mechanisms and Structural Features

GC-induced bone fragility arises from cellular and molecular mechanisms that differ from those underlying postmenopausal or age-related osteoporosis (27,34). In GC-induced bone loss, trabecular number and connectivity are relatively preserved; however, individual trabeculae become markedly thinned—a phenomenon known as trabecular attenuation. In contrast, postmenopausal osteoporosis is characterized by preserved trabecular width but significant structural disruption due to resorptive perforations, leading to loss of continuity and reduced trabecular surface area (35). These distinct histological patterns contribute to impaired bone strength despite differences in bone mass.

Pathophysiology of GC-Induced Bone Loss

Glucocorticoids exert complex effects on bone metabolism through both direct and indirect mechanisms, ultimately reducing bone strength and increasing fracture risk (34,32,33,36).

- Direct effects: GCs inhibit osteoblastogenesis, promote apoptosis of osteoblasts and osteocytes, and suppress bone matrix protein synthesis, leading to decreased bone formation (34,33,37). Concurrently, they initially enhance osteoclast survival and activity, increasing bone resorption (34) (**Figure 1**).

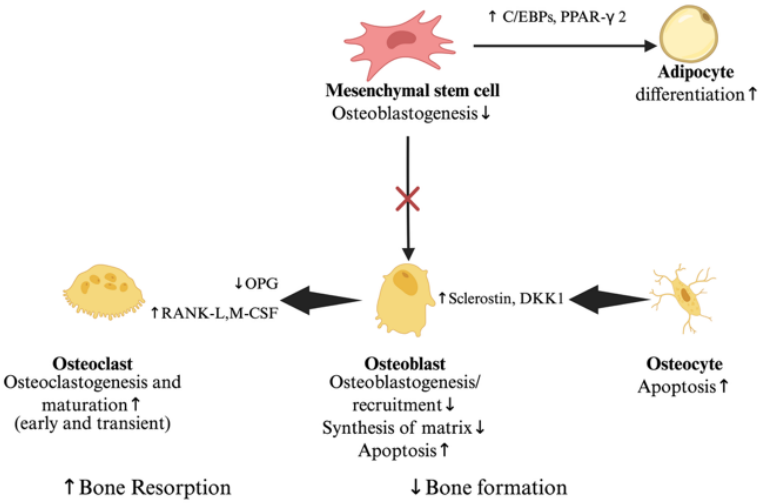

Figure 1 *- Direct action of glucocorticoids on skeletal tissue*

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- Indirect effects: GCs impair calcium homeostasis by reducing intestinal absorption and increasing renal excretion, potentially causing secondary hyperparathyroidism and further bone resorption (34,37). Additionally, they suppress sex hormone and growth factor synthesis, both critical for skeletal integrity. However, fragility fractures have been reported even in eugonadal individuals, suggesting that hypogonadism is not the primary driver of GC-induced bone fragility (34) (**Figure 2**).

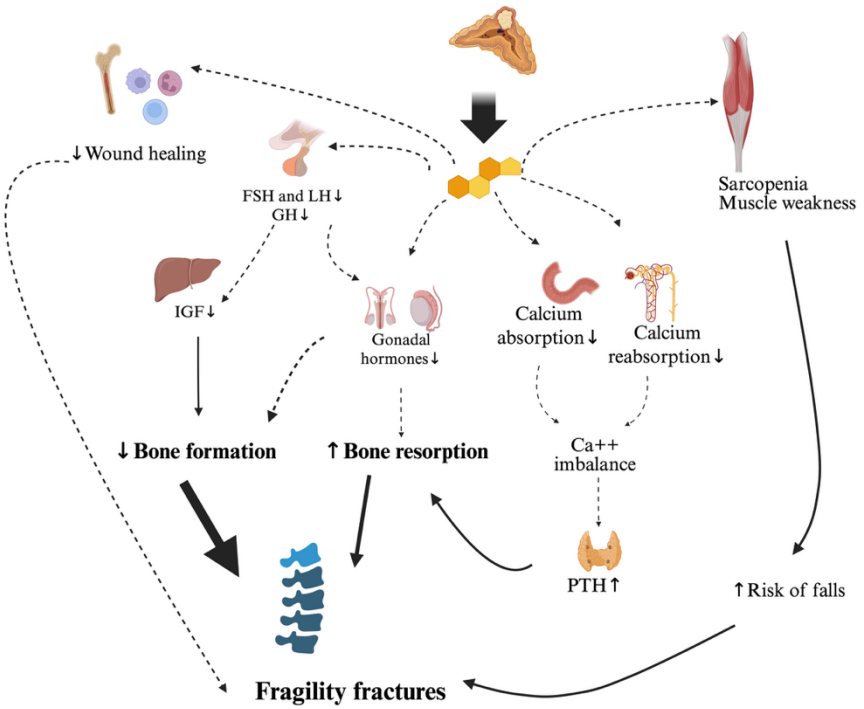

Figure 2 *- Indirect action of glucocorticoids on fragility fractures*

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Clinical Impact in patients with endogenous glucocorticoid secretion

Epidemiological studies estimate that 30–40% of individuals on long-term GC therapy experience at least one fragility fracture, predominantly at trabecular-rich sites such as vertebral bodies (28,36). Similarly, in patients with Cushing's syndrome (CS), osteoporosis prevalence ranges from 30% to 70%. Importantly, fracture risk in CS often exceeds what would be predicted by bone mineral density (BMD) alone: vertebral fractures—frequently asymptomatic—occur in 30–65% of patients and may be present even when BMD is normal in up to 50% of cases (38,39).

Mild autonomous cortisol secretion (MACS) has also been associated with skeletal complications comparable to those seen in overt CS. Among these, osteoporosis and fragility fractures are of particular concern. However, current evidence remains limited, especially in studies applying the most recent diagnostic criteria for MACS. Notably, a fragility fracture may represent the first clinical manifestation of previously unrecognized endogenous cortisol excess (34).

Glucocorticoid (GC) therapy is strongly associated with a significant reduction in bone mass and a deterioration of bone quality, leading to increased skeletal fragility and fracture risk (28). These adverse skeletal effects are among the most consistently observed complications of long-term GC use. Notably, the risk of bone loss and fragility fractures often becomes evident early, within the first three months of therapy (29). This risk escalates in a dose- and duration-dependent manner (30).

The association between excess glucocorticoids and skeletal fragility was first documented by Harvey Cushing in 1932 (31). However, only in recent decades has a robust body of clinical and experimental evidence clarified the full extent of GC-induced bone reduction (28,29,32,33).

Population-based epidemiological studies estimate that 30–40% of individuals on long-term GC therapy will experience at least one fragility fracture. These fractures predominantly occur in trabecular sites, especially the vertebral bodies, although other skeletal sites may also be affected (28,36). Similarly, in patients with CS the prevalence of osteoporosis ranges from 30% to 70%. However, the degree of bone loss does not entirely account for the elevated fracture risk observed in CS (54). Vertebral fragility fractures—often asymptomatic—occur in approximately 30% to 65% of patients and may be present even when BMD is within the normal range in up to 50% and 10% of cases, respectively (38,39).

Furthermore, MACS has been linked to metabolic, cardiovascular, and skeletal complications comparable to those observed in overt CS. Among these, osteoporosis and fragility fractures are of particular concern. However, current evidence remains limited, especially in studies employing the most recent diagnostic criteria for MACS.

1.7. Management of MACS

Current guidelines (2023) recommend that adrenalectomy should be considered in patients with unilateral MACS and comorbidities potentially attributable to cortisol excess—such as hypertension, type 2 diabetes mellitus (T2DM), or osteoporosis—after multidisciplinary discussion and individualized assessment of factors including age, tumor characteristics, surgical expertise, and patient preference (3).

A 2022 randomized study of 62 patients with MACS showed that adrenalectomy led to more improvements in hypertension (68% versus 13%) and glycometabolic control (28% versus 3%) compared with conservative management (40).

Furthermore, another meta-analysis of patients with MACS and NFAT from 2023 reported an improvement in hypertension, glycometabolic control and dyslipidaemia following adrenalectomy (22).

Despite these findings, the impact of adrenalectomy on skeletal health remains largely unexplored. To date, no studies have specifically evaluated whether surgical correction of cortisol excess reduces the risk of osteoporosis or fragility fractures in MACS.

2. Aim of The Study

The primary objective of this research is to investigate the relationship between mild autonomous cortisol secretion (MACS) and skeletal fragility in individuals diagnosed with adrenal incidentalomas (AIs). To address this aim, the study is structured into two complementary parts:

Study 1:

The aim of the first study was to evaluate the prevalence and incidence of vertebral fractures in a cohort of patients with AI, stratified by the presence or absence of MACS.

This study consists of two components:

- Cross-sectional cohort: This part investigates the prevalence of vertebral fractures in patients with adrenal incidentalomas, comparing those with biochemically confirmed MACS to those without biochemical evidence of cortisol autonomy. The goal is to determine whether MACS is independently associated with an increased long-term risk of fragility fractures, regardless of bone mineral density (BMD).
- Longitudinal cohort: This part evaluates the incidence of vertebral fractures over time in patients with adrenal incidentalomas, again stratified by cortisol secretion status. The focus is on patients under conservative follow-up to assess the potential influence of MACS on fracture risk progression.

Study 2:

Building on Study 1, the second part of the research assesses the impact of adrenalectomy on vertebral fracture risk in patients with MACS. This study also includes two components:

- Retrospective arm: This analysis evaluates the effect of adrenalectomy on vertebral fracture incidence in patients with MACS. Outcomes are compared between those who underwent surgical removal of the adrenal adenoma and those managed conservatively, to determine whether adrenalectomy offers a protective effect against fragility fractures.
- Prospective arm: This part analyzes data from a cohort of MACS patients randomly allocated to either surgery or conservative management. The objective is to assess the effect of adrenalectomy on BMD and the incidence of vertebral fractures after 24 months of follow-up.

3. STUDY 1

3.3. Patients and Methods

Design of the Study

Study 1 involved a re-analysis of a previous retrospective dataset, aimed at assessing whether indices of hypothalamic-pituitary-adrenal (HPA) axis activity could help identify patients with adrenal incidentalomas (AI) who are at increased risk of vertebral fractures (VFx) (26).

Participants were stratified into two subgroups based on hormonal evaluation:

- MACS+: Patients with confirmed mild autonomous cortisol secretion (MACS), as defined by the most recent ESE-ENSAT guidelines (3).
- MACS-: Patients with non-functioning adrenal incidentalomas (NFAI), serving as the control group.

In the cross-sectional arm of the study, we analyzed data from 444 patients with AI (271 women, 173 men) to compare the prevalence of VFx between those with MACS (MACS-yes) and those without MACS (MACS-no).

In the longitudinal arm, we assessed the risk of VFx over time in a subgroup of 126 patients with AI who had at least 24 months of follow-up data.

Subjects and Methods

The subjects included in the cross-sectional arm of the study were enrolled from January 1997 to June 2013 in the following referral Italian Hospitals: the “San Giuseppe” Hospital in Milan, the Scientific Institute “Policlinico San Donato” in San Donato Milanese in Milan, the Scientific Institute “Fondazione IRCCS Cà Granda” in Milan, and the Scientific Institute “Casa Sollievo della Sofferenza” in San Giovanni Rotondo. The longitudinal arm included patients with adrenal incidentalomas (AI) enrolled at the same centers between January 2005 and June 2013.

All AI cases were incidentally discovered through imaging—computed tomography (CT),

ultrasonography, or magnetic resonance imaging—performed for unrelated clinical reasons. Adrenal lesions initially detected by ultrasound were subsequently confirmed via unenhanced CT. On CT, all adrenal tumors measured >1 cm and exhibited features consistent with adrenocortical adenomas (i.e., hypodense, homogeneous appearance, and Hounsfield units <10). No patient had evidence of metastatic disease or biochemical signs of aldosterone co-secretion.

Exclusion criteria:

- Clinical signs of overt cortisol excess (e.g., striae rubrae, moon facies, buffalo hump, proximal muscle weakness, skin atrophy);
- History of hypogonadism (defined as fewer than six menstrual cycles per year in premenopausal women or total testosterone <300 ng/dL in men), or the presence of chronic kidney or liver disease, bowel disease, eating disorders, thyrotoxicosis, rheumatologic or hematologic disorders, or alcoholism;
- Use of medications affecting cortisol metabolism/secretion or dexamethasone metabolism;
- Bilateral adrenal masses.

All patients underwent hormonal evaluation, including morning plasma adrenocorticotropin (ACTH) levels (reference: 10–55 pg/mL or 2.2–12 pmol/L), 24-hour urinary free cortisol (UFC) levels (reference: 10–70 µg/24h or 28–193 nmol/24h), and the 1-mg overnight dexamethasone suppression test (F-1mgDST). In patients with F-1mgDST >1.8 µg/dL (50 nmol/L) and ACTH >10 pg/mL (2.2 pmol/L), ACTH-dependent subclinical hypercortisolism was excluded using corticotropin-releasing hormone stimulation testing (15).

Bone assessments included bone mineral density (BMD) measurement at the femoral neck (FN; precision 1.8%) and lumbar spine (LS; precision 1.0%) using dual-energy X-ray absorptiometry (Hologic Discovery, software version 13.3:3). Results were expressed as standard deviation units (Z-scores). Additionally, lateral and anteroposterior radiographs of the thoracolumbar spine (T4–L4) were performed in all patients and interpreted by a radiologist blinded to hormonal and BMD data. Vertebral fractures (VFx) were identified using a semiquantitative visual assessment method (26).

Osteoporosis was defined as a BMD T-score <−2.5 at any skeletal site and/or the presence of

fragility fractures (15). Given the inclusion of both pre- and postmenopausal women and men under 50 years, low BMD was defined as:

- T-score ≤ −2.5 at any site for postmenopausal women and men >50 years, or
- Z-score < −2.0 at any site for premenopausal women and men <50 years

Additional clinical assessments included calculation of body mass index (BMI), diagnosis of type 2 diabetes mellitus (T2D) using World Health Organization (WHO) criteria, and identification of hypertension, defined as systolic blood pressure >140 mmHg.

In accordance with the Italian guidelines (41), all patients with hypovitaminosis D were supplemented with cholecalciferol per os to achieve normal vitamin D levels (ie, above 30 ng/mL, 75 nmol/L). All patients with insufficient dietary calcium intake (ie, less than 1000 mg/d) were supplemented with oral calcium carbonate or calcium citrate, as appropriate.
All subjects signed the informed consent before entering the study. The protocol was approved by the Ethics Committees of the participating centers.

Data Collection

Clinical, radiological, and laboratory data were obtained through a systematic review of electronic medical records. At the time of initial referral, all patients underwent a standardized assessment which included: demographic information (age, sex, BMI); medical history, with particular attention to comorbidities commonly associated with cortisol excess, such as hypertension and type 2 diabetes mellitus, respectively; laboratory evaluation, comprising: Full blood count (FBC), Serum albumin, plasma and urine metanephrines, Serum and 24-hour urinary calcium, serum 25OHVitD, phosphate, PTH, plasma renin and aldosterone concentrations, serum cortisol following the 1-mg overnight dexamethasone suppression test (F-1mgDST), plasma adrenocorticotropic hormone (ACTH), serum dehydroepiandrosterone sulfate (DHEAS); radiological assessment of adrenal incidentalomas, including laterality (right vs. left adrenal gland), maximum tumor diameter (in cm), tumor density, expressed in Hounsfield units (HU), measured on unenhanced computed tomography (CT).

Analytical Methods

Plasma ACTH levels were measured by immunoradiometric assay (BRAHMS Diagnostica GmbH) and reported as the mean of 3 determinations at 20-minute intervals. At baseline, in all

patients we measured serum cortisol and UFC levels (after dichloromethane extraction) immunofluorimetrically by TDxFLx kits (Abbott GmbH Diagnostika). The intra- and interassay coefficients of variation were <15% for ACTH and <10% for all other assays. In both cross-sectional and longitudinal arms, the reported HPA axis parameters were those determined at baseline.

Statistical Analysis

Continuous variables were presented as mean ± standard deviation (SD) for normally distributed data, or as median with interquartile range (IQR) for non-normally distributed data. Prior to analysis, the distribution of each variable was assessed to determine appropriate statistical methods.

The comparison of continuous variables between patients with and without MACS was performed using the Student t test or the Mann-Whitney U test as appropriate. Categorical variables were compared by $\chi 2$ test or Fisher exact test, as appropriate.

The multivariate analysis assessed the independent associations between the presence of prevalent VFx (in the cross sectional arm) or of incident VFx (in the longitudinal arm) after adjusting for the variables that resulted to be different between patients with MACS and those without MACS and for the possible influencing factors, such as age, BMI, gender, BMD, and presence of T2D, which is known to possibly increase VFx risk regardless for BMD (26). In the multivariate analysis, the presence of prevalent VFx was included among the covariates possibly predicting the presence of incident VFx in the longitudinal arm, since it is known that a previous fragility fracture increases the risk of subsequent fractures (26). The results have been expressed as adjusted odds ratio (aOR) and adjusted relative risk (aRR) (26). The adjusted hazard ratio (aHR) has been calculated by Cox regression.

All statistical analyses were conducted using IBM SPSS Statistics for Windows, version 28.0 (IBM Corp., Armonk, NY, USA). A two-tailed p-value < 0.05 was considered statistically significant.

3.4. Results

Cross-Sectional Arm

Demographic, hormonal, clinical, and radiological characteristics of the study population are summarized in **Table 1**. Gender distribution, BMI, prevalence of type 2 diabetes (T2D), and the proportion of premenopausal women were comparable between the two groups.

However, based on clinical and radiological features, patients with mild autonomous cortisol secretion (MACS+) were significantly older and had larger adrenal adenomas compared to those without MACS (MACS-). As expected, patients with MACS+ showed higher F-1mgDST and urinary free cortisol (UFC) levels, lower ACTH levels, and a higher prevalence of suppressed ACTH values (<10 pg/mL; 2.2 pmol/L) compared to patients with MACS-.

Regarding bone health, patients with MACS exhibited a higher prevalence of osteoporosis, along with lower lumbar spine (LS-BMD) and femoral neck (FN-BMD) bone mineral density values. The prevalence of vertebral fractures (VFx)—both symptomatic and asymptomatic—was significantly higher in the MACS group. Specifically, symptomatic VFx occurred in 7.0% of patients with MACS versus 2.3% in those without ($p = 0.022$).

Table 1 *- Clinical and biochemical characteristics of all patients with adrenal incidentalomas and of patients with or without mild autonomous cortisol secretion*

	Total (n=444)	**MACS- (n=214)**	**MACS+ (n=230)**	**p value**
Age (years)	61.8 ± 11.5 (21 – 89)	59.9 ± 13.0 (21 – 89)	63.6 ± 9.5 (24 – 83)	**<0.001**
Females (%)	271 (61.0)	134 (62.6)	137 (59.6)	0.559
BMI (kg/m^2)	29.2 ± 4.8 (19.5 – 40.9)	29.7 ± 5.0 (19.5 – 40.9)	28.7 ± 4.6 (19.5 – 40.9)	0.27
Premenopausal women (%)	31 (11.4)	21 (15.7)	10 (7.3)	**0.03**
F-1mgDST (µg/dL)	2.39 ± 1.89 (0.50 – 12.0)	1.14 ± 0.38 (0.50 –1.8)	3.60 ± 2.00 (1.84 – 12.00)	**<0.001**
UFC (µg/24h)	54.65 ± 32.07 (10.0-175.3)	50.42 ± 27.73 (10.0-169.1)	58.58 ± 35.24 (10.0-175.3)	**0.007**
ACTH (pg/mL)	12.99 ± 9.39 (1.6 – 48.3)	15.89 ± 9.42 (2.8 – 48.3)	10.29 ± 6.21 (1.6 – 48.0)	**<0.001**
Tumor size (cm)	2.58 ± 1.1 (0.8 – 8.0)	2.15 ± 0.87 (0.8 – 6.0)	2.98 ± 1.16 (0.8 – 8.0)	**<0.001**
Type 2 diabetes (%)	73 (16.4)	32 (15)	41 (17.8)	0.246
Osteoporosis (%)	224 (50.5)	77 (36)	147 (63.9)	**<0.001**
Prevalent VFx (%)	193 (43.5)	49 (22.9)	144 (62.6)	**<0.001**
LS BMD (Z-score)	-0.01 ± 1.41 (-4.50 – 3.61)	0.19 ± 1.31 (-3.60 – 3.61)	-0.18 ± 1.48 (-4.50 – 3.61)	**0.005**
FN BMD (Z-score)	-0.02 ± 1.07 (-2.80 – 5.33)	0.13 ± 1.12 (-2.80 – 5.33)	-0.16 ± 1.01 (-2.50 – 2.70)	**0.004**

Categorical variables are reported as absolute number with percentage in parentheses. Continuous variables are reported as mean ± standard deviation with range in parentheses. Statistically significant comparisons are in bold.

F-1mgDST: 1-mg overnight dexamethasone suppression test. ACTH: adrenocorticotrophic hormone: BMI: body mass index. MACS: mild autonomous cortisol secretion. MACS-: patients without mild autonomous cortisol secretion (F-1mgDST ≤1.8 µg/dL µg/dl, 50 nmol/L). MACS+: patients with mild autonomous cortisol secretion (F-1mgDST >1.8 µg/dL µg/dl, 50 nmol/L). UFC: urinary free cortisol. LS: lumbar spine. FN: femoral neck. BMD: bone mineral density. Osteoporosis: BMD T-score < -2.5 at any skeletal site and/or the presence of a fragility fracture

Based on F-1mgDST results, patients with MACS were further stratified according to the European Society of Endocrinology (ESE) guidelines (3):

- Possible Autonomous Cortisol Secretion (PACS): F-1mgDST >1.8 µg/dL and ≤5.0 µg/dL (50–138 nmol/L; n = 199)
- Autonomous Cortisol Secretion (ACS): F-1mgDST >5.0 µg/dL (138 nmol/L; n = 41)

A statistically significant difference in VFx prevalence was found among these subgroups:

- ACS: 70.7%
- PACS: 59.3%
- MACS-: 22.5%

(P < 0.001 for all comparisons)

Additionally, the proportion of patients with normal BMD but prevalent VFx increased with the degree of cortisol secretion:

- MACS-: 15.7%
- PACS: 37.2%
- ACS: 48.4%

(P < 0.001; **Figure 3**)

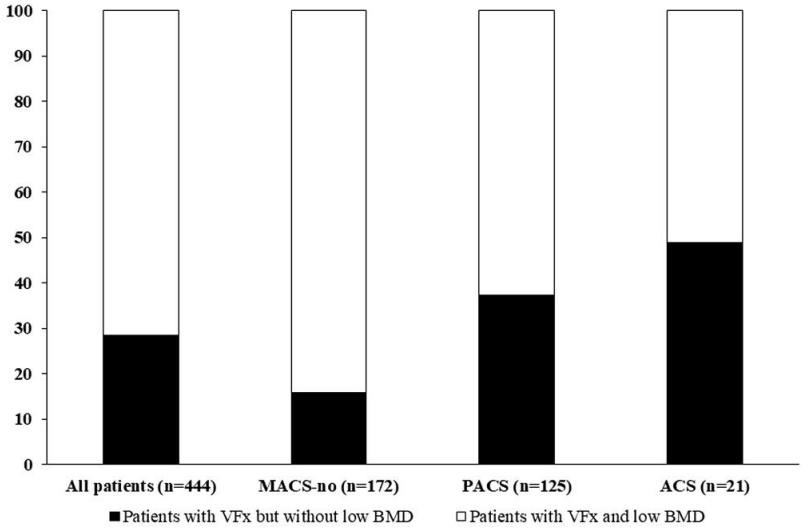

Figure 3 *- Prevalence of vertebral fractures at baseline in relation with the presence of low bone mineral density in all subjects with adrenal incidentalomas (AI), in patients without MACS, in patients with PACS, and in patients with autonomous cortisol secretion*

VFx prevalence is higher in ACS (70.7%) compared with PACS and MACS-no patients (59.3% and 22.5%, respectively) and in PACS patients as compared with MACS-no. patients (P < 0.001 for all comparisons). The percentage of individuals without low BMD but with prevalent VFx significantly increased with increasing. cortisol secretion (MACS-no 15.7%, PACS 37.2%, ACS 48.4%, P < 0.001).

Abbreviations: ACS, patients with autonomous cortisol secretion (F-1mgDST >5.0 µg/dL, 138 nmol/L); MACS-no, patients without mild autonomous cortisol secretion (F-1mgDST ≤1.8 µg/dL µg/dL, 50 nmol/L);

PACS, patients with possible autonomous cortisol secretion (F-1mgDST between 1.8 μg/dL and 5.0 μg/dL, 50-138 nmol/L).

Multivariate analysis showed that the presence of a prevalent VFx was significantly associated with:

- Presence of MACS (aRR 2.07)
- BMD values
- Age

This association remained significant regardless of T2D status, BMI, or gender distribution (**Table 2**).

Table 2 *- Independent associations between the presence of a prevalent vertebral fragility fracture and the presence of MACS, age, body mass index, gender, presence of type 2 diabetes and lumbar spine bone mineral density.*

	OR	**95% CI**	**p value**
MACS (presence vs absence)	5.203	3.361 – 8.055	**<0.001**
Age (1-y increase)	1.049	1.026 - 1.073	**<0.001**
BMI (1 kg/m^2 increase)	1.030	0.984 - 1.079	0.201
Gender (women)	1.114	0.716 - 1.732	0.632
LS-BMD (1 Z-score decrease)	1.395	1.182 – 1.645	**<0.001**
Type 2 diabetes (presence vs absence)	1.391	0.781-2.481	0.263

OR, odds ratio; 95% CI, 95% interval of confidence; MACS, mild autonomous cortisol secretion; BMI: body mass index. MACS+: patients with mild autonomous cortisol secretion. MACS-, patients without mild autonomous cortisol secretion. UFC: urinary free cortisol. LS: lumbar spine. BMD: bone mineral density. Statistically significant associstions are in bold.

Furthermore, patients with low ACTH levels (<10 pg/mL, 2.2 pmol/L) had a significantly higher prevalence of VFx (57.6%) compared to those with normal ACTH levels (30.8%, $p < 0.001$). Importantly, the association between low ACTH levels and prevalent VFx remained significant even after adjustment for age, T2D, BMI, and sex (data not shown).

Longitudinal Arm

As shown in **Table 3**, duration of follow-up, BMI, BMD at both lumbar spine (LS) and femoral neck (FN), and the prevalence of type 2 diabetes (T2D) were similar between patients with MACS

and those without. However, patients with MACS had significantly larger adrenal adenomas, a higher proportion of women, increased prevalence of suppressed ACTH levels, and lower mean ACTH concentrations compared to patients without MACS.

Table 3 *- Clinical and biochemical characteristics of all patients with adrenal incidentalomas and of patients with or without mild autonomous cortisol secretion included in the longitudinal arm*

	Total (n=126)	**MACS- (n=60)**	**MACS+ (n=66)**	**p value**
Age (years)	63.5 ± 9.5 (27 – 83)	61.5 ± 10.6 (27 – 80)	65.5 ± 8.0 (40 – 83)	**0.023**
Women	80 (63.5)	35 (53.0)	45 (75.0)	0.016
Premenopausal women	11 (13.8)	6 (13.3)	5 (14.3)	0.920
BMI (kg/m^2)	27.0 ± 4.3 (19.5 – 40.9)	27.2 ± 4.5 (20.1 – 40.9)	26.7 ± 4.1 (19.5 – 37.0)	0.531
Follow up (months)	24.9 ± 5.3 (24 – 72)	24.0±0.0 (24 – 24)	25.6 ± 7.3 (24 – 72)	0.083
F-1mgDST (µg/dL)	2.20 ±1.20 (0.50 – 7.50)	1.20 ± 0.40 (0.50 – 1.80)	3.00 ± 1.10 (1.84 – 7.50)	**<0.001**
UFC (µg/24h)	49.5 ± 31.1 (10.0 – 175.3)	43.8 ± 24.5 (10.0 – 119.4)	54.7 ± 35.5 (10.0 – 175.3)	**0.050**
ACTH (pg/mL)	13.2 ± 8.2 (3.0 – 35.0)	16.1 ± 8.9 (5.0 – 35.0)	10.5 ± 6.5 (3.0 – 35.0)	**<0.001**
Tumor size (cm)	2.4 ± 0.9 (0.8 – 5.0)	2.0 ± 0.6 (0.8 – 3.7)	2.7 ± 1.0 (0.8 – 5.0)	**<0.001**
Type 2 diabetes	32 (25.4)	12 (20.0)	20 (15.9)	0.221
Osteoporosis	59 (46.8)	19 (31.7)	40 (60.6)	**0.001**
Incident VFx	30 (23.8)	6 (10.0)	24 (36.4)	**<0.001**
LS BMD (Z-score)	-0.07 ± 1.30 (-4.10 – 2.50)	0.03 ± 1.09 (-2.03 – 3.10)	0.11 ± 1.47 (-2.80 – 4.10)	0.739
FN BMD (Z-score)	-0.08 ± 0.86 (-2.4 – 2.7)	0.16 ± 0.76 (-1.6 – 2.1)	-0.01 ± 0.94 (-2.4 – 2.7)	0.292

Categorical variables are reported as absolute number with percentage in parentheses. Continuous variables are reported as mean ± standard deviation with range in parentheses. Statistically significant comparisons are in bold.

F-1mgDST: 1-mg overnight dexamethasone suppression test. ACTH: adrenocorticotrophic hormone: BMI: body mass index. MACS: mild autonomous cortisol secretion. MACS-: patients without mild autonomous cortisol secretion (F-1mgDST ≤1.8 µg/dL µg/dl, 50 nmol/L). MACS+: patients with mild autonomous cortisol secretion (F-1mgDST >1.8 µg/dL µg/dl, 50 nmol/L). UFC: urinary free cortisol. LS: lumbar spine. FN: femoral neck. BMD: bone mineral density. Osteoporosis: BMD T-score < -2.5 at any skeletal site and/or the presence of a fragility fracture

The total number of incident vertebral fractures (VFx) was higher in the MACS group than in the

non-MACS group. The difference in symptomatic VFx incidence approached statistical significance (9.1% vs 1.7%, P = 0.069).

Importantly, these results remained consistent after excluding the four patients with ACS (data not shown). Multivariate analysis identified MACS as an independent predictor of incident VFx. In contrast, other variables—including age, presence of prevalent VFx, T2D, gender, follow-up duration, and LS-BMD—did not independently predict fracture risk (**Table 4**).

Table 4 *- Independent associations between the presence of incident VFx and presence of MACS, age, gender and LS-BMD by Logistic Regression Analysis*

	OR	95% CI	p value
MACS (presence)	4.561	1.600 – 13.003	**0.005**
Age (1-y increase)	1.000	0.944 -1.059	0.991
Gender (women)	1.126	0.413-3.067	0.817
Type 2 diabetes (presence)	1.389	0.475-4.065	0.548
LS-BMD (1 Z-score decrease)	1.279	0.897-1.825	0.175
Patients with prevalent VFx (presence)	1.542	0.602-3.948	0.367

VFx: vertebral fragility fracture; MACS: mild autonomous cortisol secretion; LS-BMD: lumbar spine bone mineral density. Statistically significant associations are in bold.

The adjusted hazard ratio (aHR) for incident VFx associated with MACS was 2.884 (95% CI: 1.108–7.507; P = 0.03), after adjusting for the aforementioned covariates.

Furthermore, the proportion of patients who developed incident VFx despite having normal BMD was significantly higher in the MACS group (25.8%) compared to those without MACS (8.3%, P = 0.017).

Patients with low ACTH levels (<10 pg/mL; 2.2 pmol/L) also had a higher incidence of VFx (31.7%) compared to those with normal ACTH levels (15.9%, P = 0.036). However, this association was not statistically significant after adjusting for age, prevalent VFx, T2D, gender, follow-up duration, and LS-BMD.

3.5. Sex-Specific Analyses in the Cross-Sectional and Longitudinal Arms

The characteristics of men and women with or without MACS included in the cross-sectional arm are presented in **Table 5**, and the corresponding prevalence of vertebral fractures (VFx) is illustrated in **Figure 4**.

Table 5 *- Clinical and biochemical characteristics of men and women patients with adrenal incidentalomas with or without mild autonomous cortisol secretion included in the cross-sectional arm*

	Men			**Women**		
	MACS- (n=80)	**MACS+ (n=93)**	**p**	**MACS- (n=134)**	**MACS+ (n=137)**	**p**
Age (years)	58.4 ± 13.1 (21 – 78)	65.8 ± 9.5 (38 – 83)	**<0.001**	60.7 ± 12.9 (26 – 89)	62.1 ± 9.2 (24 – 83)	0.318
Premenopausal women	-	-	-	21 (15.7)	10 (7.3)	**0.003**
BMI (kg/m^2)	28.7 ± 3.6 (21.3 – 40.4)	28.6 ± 3.7 (20.3 – 39.0)	0.753	30.2 ± 5.6 (19.5 – 40.9)	28.7 ± 521 (19.5 – 40.9)	**0.023**
F-1mgDST (µg/dL)	1.15 ± 0.37 (0.50 – 1.80)	3.58 ± 1.84 (1.84 – 9.15)	**<0.001**	1.10 ± 0.40 (0.50 – 1.8)	3.50 ± 2.10 (1.84 – 12.0)	**<0.001**
UFC (µg/24h)	50.6 ± 24.0 (10.0 – 12.0)	61.6 ± 34.8 (10.0 – 170.6)	**0.019**	50.3 ± 29.8 (10.0 – 169.1)	56.6 ± 35.5 (10.0 – 175.3)	0.118
ACTH (pg/mL)	18.2 ± 9.6 (5.0 – 48.3)	10.9 ± 6.3 (1.6 – 32.2)	**<0.001**	14.5 ± 9.1 (2.8 – 48.3)	9.9 ± 6.2 (1.6 – 48.0)	**<0.001**
Tumor size (cm)	2.2 ± 0.9 (0.8 – 6-.0)	3.0 ± 1.1 (1.0-7.0)	**<0.001**	2.1 ± 0.9 (0.8 – 5.5)	3.0 ± 1.2 (0.8 – 8.0)	**<0.001**
Osteoporosis	27 (33.8)	64 (68.8)	**<0.001**	50 (37.3)	83 (60.6)	**<0.001**
Type 2 diabetes	8 (10.0)	23 (24.7)	**0.012**	24 (17.9)	18 (13.1)	0.278
Prevalent VFx	17 (21.3)	59 (63.4)	**<0.001**	32 (23.9)	85 (62.0)	**<0.001**
LS BMD (Z-score)	0.05 ± 1.43 (-2.80 – 3.61)	-0.24 ± 1.71 (-4.5 – 3.61)	0.239	0.28 ± 1.23 (-3.60 – 3.61)	-0.15 ± 1.32 (-3.07 – 3.61)	**0.006**
FN BMD	-0.05 ±	-0.18 ± 1.03	0.419	-0.24 ± 1.10	-0.14 ± 0.99	**0.003**

(Z-score)	1.13 (-2.8 – 3.0)	(-2.5 – 2.7)		(-2.6 – 5.3)	(-2.5 – 2.5)	

Categorical variables are reported as absolute number with percentage in parentheses. Continuous variables are reported as mean ± standard deviation with range in parentheses. Statistically significant comparisons are in bold.
F-1mgDST: 1-mg overnight dexamethasone suppression test. ACTH: adrenocorticotrophic hormone: BMI: body mass index. MACS: mild autonomous cortisol secretion. MACS-: patients without mild autonomous cortisol secretion (F-1mgDST ≤1.8 µg/dL µg/dl, 50 nmol/L). MACS+: patients with mild autonomous cortisol secretion (F-1mgDST >1.8 µg/dL µg/dl, 50 nmol/L). UFC: urinary free cortisol. LS: lumbar spine. FN: femoral neck. BMD: bone mineral density. Osteoporosis: BMD T-score < -2.5 at any skeletal site and/or the presence of a fragility fracture

Men with MACS had significantly greater tumor size, older age, higher UFC levels, and a higher prevalence of osteoporosis, VFx, and type 2 diabetes mellitus (T2D), along with lower ACTH levels compared to men without MACS. BMI and BMD did not differ between groups.

In men with AI, MACS was independently associated with: prevalent VFx (OR: 5.23, 95% CI: 2.49–11.00, $p < 0.001$), Age (OR: 1.054, 95% CI: 1.01–1.09, $p = 0.05$), and LS-BMD (OR: 1.54, 95% CI: 0.44–2.83, $p < 0.001$), but not with BMI or T2D.

Women with MACS had larger tumor size and higher prevalence of osteoporosis and VFx, along with lower BMI, ACTH levels, BMD at both LS and FN, and lower prevalence of premenopausal status compared to women without MACS. Age, UFC levels, and T2D prevalence were comparable between groups.

In women with AI, MACS was independently associated with: prevalent VFx (OR: 5.36, 95% CI: 3.06–9.38, $p < 0.001$), age (OR: 1.06, 95% CI: 1.02–1.10, $p = 0.002$), and LS-BMD (OR: 1.32, 95% CI: 1.04–1.67, $p = 0.025$), but not with BMI, premenopausal status, or T2D.

The characteristics of men and women in the longitudinal arm are reported in **Table 6**, and the incidence of VFx in these groups is shown in **Figure 4**.

Table 6 *- Clinical and biochemical characteristics of men or women patients with adrenal incidentalomas with or without mild autonomous cortisol secretion included in the longitudinal arm*

	Men			**Women**		
	MACS- (n=15)	**MACS+ (n=31)**	**p**	**MACS- (n=45)**	**MACS+ (n=35)**	**p**
Age (years)	64.1 ± 8.4 (45 – 77)	69.0 ± 6.7 (51 – 83)	**0.037**	60.6 ± 11.2 (27 – 80)	62.1 ± 7.8 (40 – 73)	0.518
Premenopausal femens	-	-	-	6 (13.3)	5 (14.3)	0.920
BMI (kg/m^2)	26.6 ± 2.9 (23.0 – 32.0)	26.6 ± 3.3 (19.0 – 33.1)	0.954	27.4 ± 5.0 (20.0 – 40.0)	26.8 ± 4.8 (20.1 – 37.0)	0.579
Follow up (months)	24.0±0.0 (24 – 24)	24.4±2.2 (24 – 36)	0.493	24.0 ± 0.0 (24 – 24)	26.7 ± 9.7 (24 – 72)	0.061
F-1mgDST (nmol/L)	1.21 ± 0.31 (0.50 – 1.80)	3.16 ± 1.17 (1.84 – 6.20)	**<0.001**	1.26 ± 0.41 (0.50 – 1.80)	2.83 ± 1.10 (1.86 – 7.50)	**<0.001**
UFC (µg/24h)	37.8 ± 16.9 (13.8 – 67.5)	58.6 ± 34.4 (10.0 – 150.7)	**0.033**	45.8 ± 26.5 (10.0 – 111.9)	51.3 ± 36.6 (10.0 – 175.3)	0.446
ACTH (pg/mL)	20.4 ± 10.2 (5.0 – 35.0)	10.0 ± 4.7 (5.0 – 26.7)	**<0.001**	14.7 ± 11.0 (5.0 – 35.0)	11.0 ± 7.7 (3.0 – 35.0)	**0.043**
Tumor size (cm)	1.9 ± 0.8 (0.8 – 3.7)	2.9 ± 1.0 (1.0 – 5.0)	**<0.001**	2.0 ± 0.6 (0.8 – 3.5)	2.6 ± 1.0 (1.0 – 5.0)	**0.02**
Osteoporosis	5 (33.3)	17 (54.8)	0.171	14 (31.1)	23 (65.7)	**0.002**
Type 2 diabetes	3 (20.0)	15 (48.4)	0.107	9 (20.0)	5 (14.3)	0.505
Prevalent VFx	5 (33.3)	19 (61.3)	0.075	8 (17.8)	16 (45.7)	**0.007**
Incident VFx	1 (6.7)	13 (41.9)	**0.018**	5 (11.1)	11 (31.4)	**0.024**
LS BMD (Z-score)	-0.06 ± 1.31 (-2.00 – 1.90)	0.58 ± 1.27 (-2.20 – 3.50)	0.120	0.06 ± 1.01 (-2.03 – 3.10)	-0.31 ± 1.53 (-2.80 – 4.10)	0.198
FN BMD (Z-score)	-0.13± 0.84 (-1.1 – 1.3)	0.23 ± 0.90 (-1.3 – 2.7)	0.208	0.27 ± 0.73 (-1.6 – 2.1)	-0.20 ± 0.94 (-2.4 – 2.0)	**0.016**

Categorical variables are reported as absolute number with percentage in parentheses. Continuous variables

are reported as mean ± standard deviation with range in parentheses. Statistically significant comparisons are in bold. F-1mgDST: 1-mg overnight dexamethasone suppression test. ACTH: adrenocorticotrophic hormone: BMI: body mass index. MACS: mild autonomous cortisol secretion. MACS-: patients without mild autonomous cortisol secretion (F-1mgDST ≤1.8 µg/dL µg/dl, 50 nmol/L). MACS+: patients with mild autonomous cortisol secretion (F-1mgDST >1.8 µg/dL µg/dl, 50 nmol/L). UFC: urinary free cortisol. LS: lumbar spine. FN: femoral neck. BMD: bone mineral density. Osteoporosis: BMD T-score < -2.5 at any skeletal site and/or the presence of a fragility fracture

Men with MACS were older and had greater tumor size, higher UFC levels, and a higher number of incident VFx, along with lower ACTH levels compared to men without MACS.

No significant differences were observed in follow-up duration, BMI, prevalence of osteoporosis, prevalent VFx, or T2D.

In men, MACS was independently associated with: iincident VFx (OR: 15.38, 95% CI: 1.25–189.26, p = 0.03), but not with BMD, age, prevalent VFx, or T2D.

Women with MACS had a higher number of both prevalent and incident VFx, higher prevalence of osteoporosis, greater tumor size, lower ACTH levels, and reduced FN-BMD compared to women without MACS. Age, BMI, follow-up duration, UFC levels, LS-BMD, premenopausal status, and T2D prevalence were similar between groups.

In women, MACS was independently associated with: incident VFx (OR: 3.67, 95% CI: 1.02–13.26, p = 0.047), and T2D (OR: 4.57, 95% CI: 1.01–20.67, p = 0.048), but not with BMD, age, prevalent VFx, or premenopausal status.

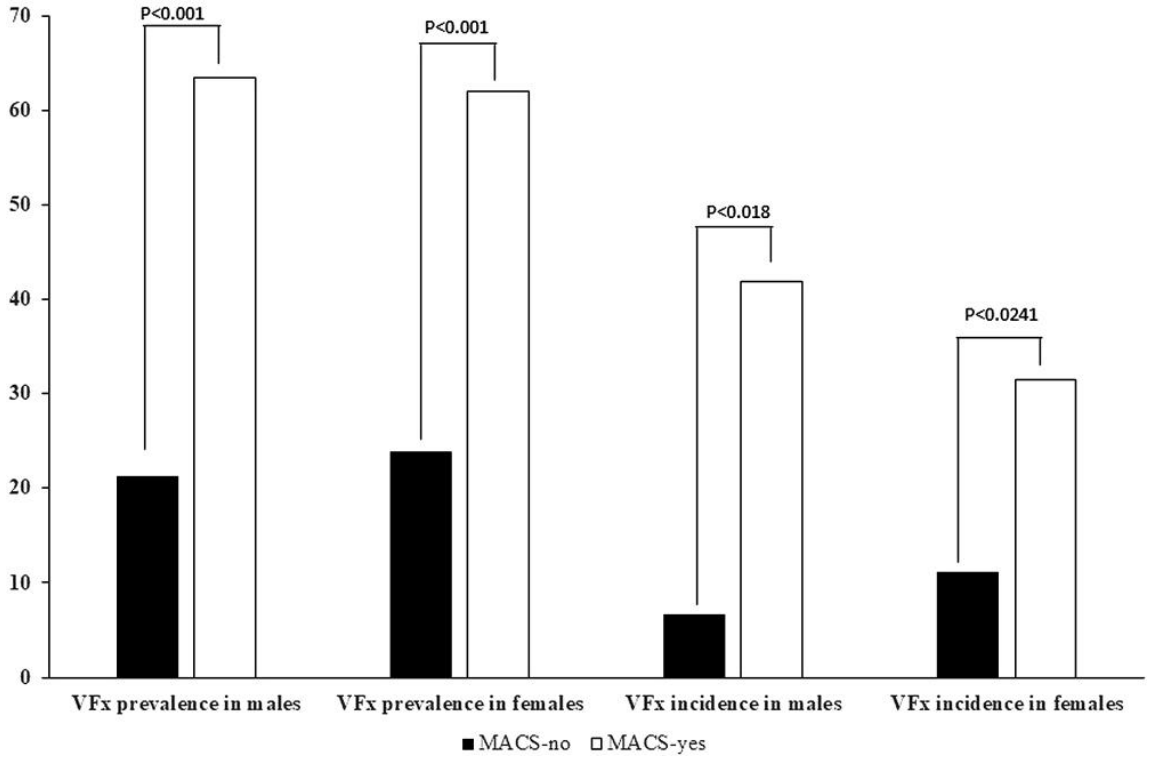

Figure 4 *- Prevalence and incidence of vertebral fractures in relation with gender with (MACS-yes) and without (MACS-no) mild autonomous cortisol secretion*

MACS-: patients without mild autonomous cortisol secretion (F-1mgDST ≤1.8 µg/dL µg/dl, 50 nmol/L). MACS+: patients with mild autonomous cortisol secretion (F-1mgDST >1.8 µg/dL µg/dl, 50 nmol/L). Men and women in the cross-sectional arm: n = 173 and 271, respectively. Men and women in the longitudinal arm: n = 46 and 80, respectively.

4. STUDY 2

4.3. Patients and methods

Design

Study 2 aimed to evaluate the impact of adrenalectomy on vertebral fracture (VFx) risk in patients with mild autonomous cortisol secretion (MACS). This study consisted of two components:

- **Retrospective arm**: The risk of VFx over time was assessed in 53 patients with MACS. Of these, 31 patients (R-Group A) underwent adrenalectomy, while 22 patients who declined surgery were managed conservatively (R-Group B).
- **Prospective arm**: In this component, MACS patients were randomly assigned to either adrenalectomy (P-Group A) or conservative management (P-Group B). The primary objective was to evaluate changes in bone mineral density (BMD) and the incidence of vertebral fractures after 24 months of follow-up. A total of 49 patients (21 in P-Group A and 28 in P-Group B) completed the study protocol.

Subjects and Methods

The retrospective arm included patients with adrenal incidentalomas (AI) enrolled between January 1997 and June 2013 at the following Italian referral centers: San Giuseppe Hospital, Milan; Policlinico San Donato IRCCS, San Donato Milanese (Milan);
Fondazione IRCCS Cà Granda, Milan; Casa Sollievo della Sofferenza, San Giovanni Rotondo.
The prospective randomized arm enrolled patients with mild autonomous cortisol secretion (MACS) between September 2016 and February 2020, with the aim of evaluating the impact of adrenalectomy on outcomes potentially related to hypercortisolism, including changes in: body weight, blood pressure, glucometabolic control, bone mineral density (BMD), risk of incident vertebral fractures (VFx) (42).

Patients in the prospective arm were randomly assigned to either surgical treatment (P-Group A) or conservative management (P-Group B). The inclusion and exclusion criteria were the same as those used in Study 1.

As illustrated in **Figure 5**, out of 71 eligible patients, 9 declined participation, and 62 were randomized: 31 to adrenalectomy (P-Group A) and 31 to conservative treatment (P-Group B). Block randomization was employed to minimize bias and ensure balanced group allocation.
Ultimately, 49 patients (21 in P-Group A and 28 in P-Group B) completed the 24-month study protocol.
In P-Group A:

- 4 patients withdrew consent before surgery
- 1 patient was diagnosed with breast cancer after baseline assessments and discontinued participation
- 1 patient died from COVID-19 prior to undergoing surgery

In P-Group B:

- 1 patient underwent adrenalectomy after 5 months due to rapid tumor growth caused by internal bleeding

All participants were fully informed about the study procedures and provided written informed consent prior to enrollment.

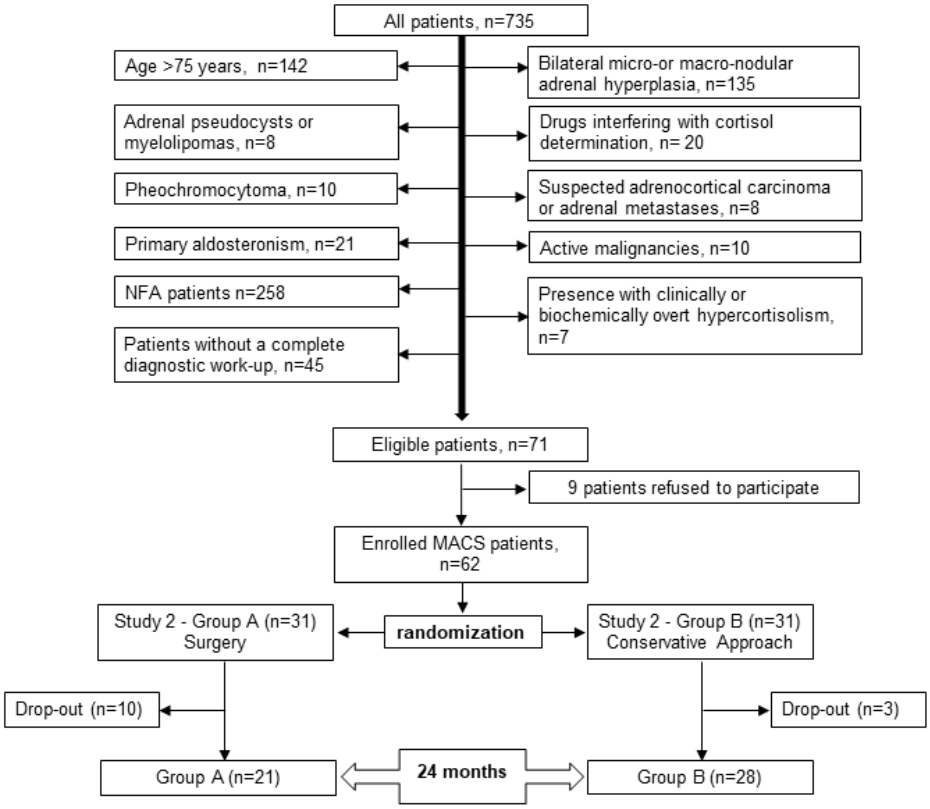

Figure 5 *- Enrolment procedure of prospective randomized arm*

Drop-out causes in Group A: consent withdrawal (n=4), cancer occurrence (n=1), death for COVID-19 (n=1); lost at follow-up (n=10). Drop-out causes in Group B: adrenalectomy for adenoma enlargement (n=2); lost at follow-up (n=1)

According to Italian guidelines, in the presence of hypovitaminosis D patients were supplemented with cholecalciferol in order to achieve 25OHVitD levels above 30 ng/mL (75 nmol/L), and all patients with a dietary calcium intake <1000 mg/day were supplemented with calcium carbonate or calcium citrate.

In all subjects, BMD was measured by dual-energy X-ray absorptiometry (DXA, Hologic Discovery, Bedford MA, USA) at lumbar spine (LS, precision 1.0 %) and femoral neck (FN, precision 1.8 %) and expressed as g/cm2 and SD units (Z-score) in relation to reference population and as the change in Z-scores per year (ΔZ-score/year) between baseline and the end of follow-up. Lateral and antero-posterior spinal radiographs (T4-L4) were performed in all subjects. Two radiologists, who were blinded to hormonal and BMD data, independently reviewed the radiographs and discussed the questionable cases. Prevalent and incident VFx were diagnosed using the semiquantitative visual assessment (30): fractures assessed on lateral T4-L4 spine

radiographs were defined as reductions of more than 20% in anterior, middle, or posterior vertebral height. In order to increase specificity, we considered as VFx only vertebrae with at least moderate (>25% compression) deformity. Fractured vertebrae were excluded from BMD measurement. Type 2 diabetes was diagnosed using World Health Organization criteria (42).

In patients enrolled in retrospective arm, as per our protocols, calcium-phosphate parameters are measured every 12 months. The prospective arm contemplated to measure calcium-phosphate parameters every 6 months until the end of follow-up.

In all patients from P-Group B on the basis of our national guidelines alendronate was offered to 11 patients with a FRAX® 10-year probability of a major osteoporotic fracture ≥10% (41, 43). Out of the 7 patients who accepted the weekly bisphosphonate therapy, only 5 patients had an adherence ≥80%.

On the basis of our national guidelines an antiresorptive therapy (alendronate or risedronate) was prescribed in 2 and 5 patients from R-Group B and Study 2-Group B, respectively. No patient from R-Group A and from Study 2-Group A was treated with bone active drugs as per our protocol.

Analytical Methods

Serum and 24-hour urinary calcium, serum 25-hydroxyvitamin D (25OHVitD), phosphate, PTH and albumin levels were assessed. Calcium, albumin and phosphate were measured by standard colorimetric techniques. Serum intact PTH and 25OHVitD concentration was measured by electrochemiluminescence immunoassay (ECLIA) (reference interval 15–65 pg/mL and 30–100 ng/mL, respectively).

Statistical analysis

In both retrospective and prospective arm, the results are expressed as mean±SD or median (interquartile range, IQR) as appropriate. Categorical variables were compared by $\chi 2$ test or Fisher Exact test as appropriate. Comparison of continuous variables among the different groups was performed using Student t test for paired or unpaired data, as appropriate, for normally distributed variables and using the Mann-Whitney U test or the Wilcoxon signed-rank test, as appropriate, for not normally distributed variables.

The logistic regression analysis was used in retrospective arm to assess the association between

the occurrence of incident VFx after adjusting for adrenalectomy (yes/no) and for the independent variables known to be associated with increased fracture risk (i.e. age, gender, F-1mgDST levels, LS BMD and VFx at baseline) as well as other variables that were found to be different between surgically and not surgically treated patients at baseline. This analysis was repeated also including in the model the use of bone active drugs (as explanatory variable).

Statistical analysis was performed by SPSS version 28.0 statistical package (IBM Corporation, Armonk, New York, United States). P-values of less than 0.05 were considered significant.

4.4. Results

Retrospective arm

Clinical characteristics of patients surgically (R-Group A) and conservatively treated (R-Group B) at the beginning and at the end of follow-up are reported in **Table 7**. In both groups, serum calcium, creatinine, phosphorous and PTH levels and 24-hour urinary calcium levels were within the normal range and did not change between baseline and the end of follow-up (data not shown). The number of patients with prevalent VFx was not different between the two groups.

At baseline, age, BMI, adenoma size, 25OHD, ACTH, 1mg-DST and UFC levels and prevalence of hypertension were not different between the two groups, while LS and FN BMD and prevalence of type 2 diabetes was lower in R- Group A than in R- Grop B group.

Table 7 *- Retrospective Arm: comparison of clinical and biochemical characteristics between surgically treated (R-Group A) and conservatively treated (R-Group B) MACS patients at baseline and at the end of follow-up.*

	R-Group A (n=31)			**R-Group B (n=22)**		
	Baseline	**End of FU**	**p**	**Baseline**	**End of FU**	**p**
Age (yrs)	63 (57–67)	65.7 (60.5–70.6)	0.10	64 (61–72)	66 (64–75)	0.20
Gender (Females)	23[a] (74.2)	-	-	10 (45.5)	-	-
BMI (kg/m^2)	26.0 (24.5–29.1)	26.4 (23.7–29.6)	0.93	25.2 (23.3–29.6)	25.6 (23.9–31.1)	0.41
Duration of FU (months)	-	36[c] (24–48)	-	-	27 (24–44)	-
Diameter of adenoma (cm)	3.2 (2.2–4.0)	-	-	2.5 (2.1–3.5)	-	-
25hydroxyvitamin D (ng/mL)	20.7 (14.7–26)	38 (34.4–42)	0.001	20.5 (16.3–28.8)	36.4 (33.8–43.2)	0.001
ACTH (pg/mL)	7.2 (5.0–9.1)	20.6[d] (12.8–46.6)	0.001	8.4 (5.9–9.0)	8.9 (7.7–9.4)	0.26
F-1mgDST (µg/dL)	3.6 (2.4–5.9)	0.9[d] (0.7–1.0)	0.001	3.4 (3.2–3.6)	3.1 (2.9–3.3)	0.02
UFC (µg/24 hour)	68 (41–96)	25[d] (20–50)	0.001	56.1 (33.2–81.4)	56.3 (30.1–56.3)	0.72
L1-L4 BMD (Z-score)	-0.81[a] (-2.0–0.1)	-0.54 (-1.3–0.4)	0.29	0.2 (-1.4–1.2)	0.4 (-1.5–1.4)	0.96
L1-L4 ΔZ-score/year	-	0.10 (-0.05–0.2)	-	-	-0.01 (-0.01–0.1)	-
Femoral Neck BMD (Z-score)	-0.7[b] (-1.2–0.1)	-0.4 (-1.0–0.0)	0.44	0.0 (-0.6–0.5)	-0.2 (-0.4–0.7)	0.89
Femoral Neck ΔZ-score/year		0.05 (-1.05–0.74)			-0.5 (-1.7–1.2)	
Total Hip BMD (Z-score)	-0.8[b] (-1.4–0.1)	-0.5 (-0.8–0.4)	0.21	0.05 (-0.8–0.5)	0.05 (-0.6–0.7)	0.69
Total Hip ΔZ-score/year		0.05 (-0.05–0.15)			-0.01 (-0.05–0.2)	
Patients with prevalent VFx (%)	14 (45.2)	-	-	14 (63.6)	-	-
Patients with incident VFx (%)	-	3[e] (9.7)	-	-	11 (50.0)	-
Patients with type 2 diabetes (%)	5[a] (16.1)	5[c] (16.1)	1.00	9 (40.9)	9 (40.9)	1.00

Data are median (interquartile range, IQR) or absolute number with percentage in parenthesis. [a]$p<0.005$ and [b]$p<0.05$ vs conservatively treated patients at baseline; [c]$p<0.05$, [d]$p<0.001$ and [e]$p<0.005$ vs conservatively treated patients at the end of FU. FU: follow-up. BMI: body mass index; F-1mgDST: serum cortisol levels after 1–mg dexamethasone suppression test; UFC: urinary free cortisol; ACTH: adrenocorticotroph hormone.
MACS: mild autonomous cortisol secretion was diagnosed in presence of F-1mgDST >1.8 µg/dL (50 nmoL/L)
LS: lumbar spne, FN, femoral neck, TH: total hip. BMD: bone mineral density. ΔZ-score/year: change of Z-score

per year between baseline and end of FU**.**

Compared to R – Group A, R – Group B showed a higher incidence of VFx at the end of follow-up. In Group B, F-1mgDST levels increased over time, while BMD at both the LS and TH remained unchanged from baseline to follow-up.

A new vertebral fracture occurred in 11 patients (50%) in Group B. Of these, 2 out of 5 patients receiving bisphosphonates still developed a new VFx, and 5 patients already had a prevalent VFx at baseline.

In Study 1 – Group A, BMD at both LS and femoral neck (FN) remained stable during follow-up. Only 3 patients (9.4%) experienced incident VFx, all of whom had a prevalent VFx at baseline.

Baseline F-1mgDST levels were not significantly different between patients who developed incident VFx and those who did not (data not shown).

In the entire cohort, logistic regression analysis revealed that surgical treatment of MACS was independently associated with a 6.8-fold lower risk of developing new vertebral fractures (OR: 0.147; 95% CI: 0.023–0.924; p = 0.041). This association remained significant after adjusting for potential confounders, including: age, gender, duration of follow-up, degree of hypercortisolism (F-1mgDST levels), LS-BMD (Z-score), presence of baseline VFx, presence of type 2 diabetes at baseline (**Table 8**). The results were confirmed even after adjusting for the use of bone-active medications (data not shown).

Table 8 *- Results of a multivariable logistic regression analysis for retrospective arm*

Multivariate logistic regression analysis showing independent associations between the risk of incident VFx and adrenalectomy (yes vs. no), age, gender, duration of follow-up, severity of hypercortisolism (F-1mgDST), LS-BMD, presence VFx at baseline, and type 2 diabetes mellitus at baseline in patients with MACS.

	aOR	**95% CI**	**p value**
Adrenalectomy (yes vs no)	0.147	0.023 – 0.924	**<0.041**
Age (1-y increase)	1.061	0.919 – 1.225	0.417
Gender (women)	0.971	0.167 – 5.659	0.818
Duration of follow up (1 month increase)	0.994	0.943 – 1.048	0.632
F-1mgDST (1 µg/dL increase)	0.649	0.280 – 1.508	0.315
LS-BMD (1 Z-score increase)	1.139	0.579 – 2.240	0.707
Prevalent vertebral fracture at baseline (yes vs no)	0.551	0.119 – 2.538	0.444
Type 2 diabetes mellitus (yes vs no)	0.547	0.091 – 3.274	0.251

aOR: odds ratio adjusted for the variables included in the model. 95% CI: 95% interval of confidence. MACS: mild autonomous cortisol secretion. LS: lumbar spine. BMD: bone mineral density.

4.5. Prospective arm

The baseline clinical characteristics of patients in P—Group A and P—Group B are presented in **Table 9**. No significant differences were observed between the two groups in terms of demographic features (age, sex, and BMI), cortisol secretion parameters (F-1mgDST, UFC, and ACTH), or bone health indicators, including bone mineral density (BMD) at the lumbar spine, femoral neck, and total hip. Similarly, the prevalence of vertebral fractures at baseline was comparable between the groups. Two patients and no patients had severe VFx in P-Group B and P-Group A, respectively.

Table 9 *- Randomized Prospective Study: comparison of clinical and biochemical characteristics between surgically treated (P-Group A) and conservatively treated (P-Group B) MACS patients at baseline*

	P-Group A (n=21)	**P-Group B (n=28)**	
Age (yrs)	63 (56.5–72.5)	69 (61–73)	0.19
Women, n (%)	14 (67)	22 (78)	0.35
BMI (kg/m^2)	27.7 (24.4–31.1)	26 (23.3–30.6)	0.37
Diameter of adenoma (cm)	3.2 (2.7–3.8)	2.9 (2.3–3.4)	0.1
ACTH (pg/mL)	8.8 (5.9–11.5)	9 (5.7–12.3)	0.74
F-1mgDST (μg/dL)	3.5 (2.3–4.5)	2.5 (2.2–3.6)	0.10
UFC (μg/24 hour)	27.1 (16.5–40.9)	22 (12.6–29.1)	0.11
Patients with type 2 diabetes (%)	5 (23.8)	5 (17.9)	0.37
L1-L4 BMD (Z-score)	0.15 (-0.57–0.75)	0.1 (-0.7–0.9)	0.99
Femoral neck BMD (Z-score)	-0.55 (-0.30–0.37)	-0.1 (-1.1–0.4)	0.72
Total hip BMD (Z-score)	0.28 (-0.6–0.9)	-0.05 (-0.57–0.82)	0.67
Patients with prevalent VFx (%)	4 (21)	6 (21.4)	0.97

MACS: mild autonomous cortisol secretion was diagnosed in presence of F-1mgDST >1.8 μg/dL (50 nmoL/L) Data are median (interquartile range, IQR) or absolute number with percentage in parenthesis. BMI: body mass index; ACTH: adrenocorticotroph hormone; F-1mgDST: serum cortisol levels after 1–mg dexamethasone suppression test; UFC: urinary free cortisol. BMD: bone mineral density. ΔZ-score/year: change of Z-score per year between baseline and end of FU**.**

The patients were followed over time (**Table 10**). Over the 24-month follow-up period, patients in the surgical group (Group A) showed a normalization of F-1mgDST levels and, as expected, a significant increase in ACTH concentrations ($p < 0.001$). Additionally, a significant rise in serum calcium and phosphorus levels was observed in this group between baseline and the end of follow-up ($p = 0.03$ and $p = 0.04$, respectively), as illustrated in **Figure 6**.

Table 10 *- Randomized Prospective Study (Study 2): comparison of clinical and biochemical characteristics between surgically treated (Study 2-Group A) and conservatively treated (Study 2-Group B) MACS patients at baseline and at the end of follow-up*

	Study 2-Group A (n=21)			**Study 2-Group B (n=28)**		
	Baseline	24 months of follow up		Baseline	24 months of follow up	
BMI (kg/m^2)	27.7 (24.4–31.1)	27.7 (23.1–31.8)	0.88	26 (23.3–30.6)	25.2 (22.9–27.5)	0.52
ACTH (pg/mL)	8.8 (5.9–11.5)	25.9 (17.3–44.3)	<0.001	9 (5.7–12.3)	10.6 (8.3–14.4)	0.13
F-1mgDST (µg/dL)	3.5 (2.5–4.5)	0.8 (0.59–1.45)	<0.001	2.5 (2.2–3.6)	2.8 (2.1–4.2)	0.57
UFC (µg/24 hour)	27.1 (16.5–40.9)	26.5 (16.6–41.6)	0.78	22 (12.6–29.1)	22.2 (14.3–32.8)	0.35
Calcium (mg/dL)	9.2 (9.1–9.5)	9.5 (9.2–9.8)	0.03	9.3 (8.9–9.5)	9.4 (9.2–9.6)	0.56
Phosphate (mg/dL)	3.0 (2.8–3.4)	3.4 (3.0–3.7)	0.04	3.5 (3.3–3.7)	3.6 (3.5–3.8)	0.17
25hydroxyvitamin D (ng/mL)	27.8 (19–36)	32.9 (29.1–37.7)	0.15	33.2 (26.7–43.5)	35.9 (32.2–43.0)	0.40
24 hrs urinary Calcium (mg/kg/die)	1.35 (1.01–2.53)	1.51 (0.95–2.08)	0.71	2.10 (1.30–2.97)	1.43 (0.79–2.92)	0.29
Alkaline Phosphatase (U/L)	62.5 (52.0–77.5)	66 (53–86)	0.53	69 (60.5–74.5)	69 (57–81)	0.78
L1-L4 BMD (Z-score)	0.2 (-0.6–0.8)	0.2 (-0.3 – 1.0)	0.55	0.1 (-0.7–0.9)	0.3 (-0.6–1.3)	0.38
Femoral Neck BMD (Z-score)	-0.5 (-0.3–0.4)	-0.2 (-0.4–0.3)	0.72	-0.1 (-1.1–0.4)	-0.3 (-0.9–0.5)	0.92
Total Hip BMD (Z-score)	0.2 (-0.6–0.9)	0.3 (-0.3–0.7)	0.78	-0.1 (-0.6–0.8)	0 (-0.7–1.1)	0.80
Patients with incident VFx (%)	-	1 (4.8)[a]		-	7 (25)	

Data are median (interquartile range, IQR) or absolute number with percentage in parenthesis. Data are mean ± SD with range in parenthesis or absolute number with percentage in parenthesis. ap<0.05 vs conservatively treated patients at the end of FU. FU: follow-up. BMI: body mass index; F-1mgDST: serum cortisol levels after 1–mg dexamethasone suppression test; UFC: 24hrs urinary free cortisol; ACTH: adrenocorticotroph hormone.
MACS: mild autonomous cortisol secretion was diagnosed in presence of F-1mgDST >1.8 µg/dL.

LS: lumbar spine, FN, femoral neck, TH: total hip. BMD: bone mineral density. ΔZ-score/year: change of Z-score per year between baseline and end of FU.

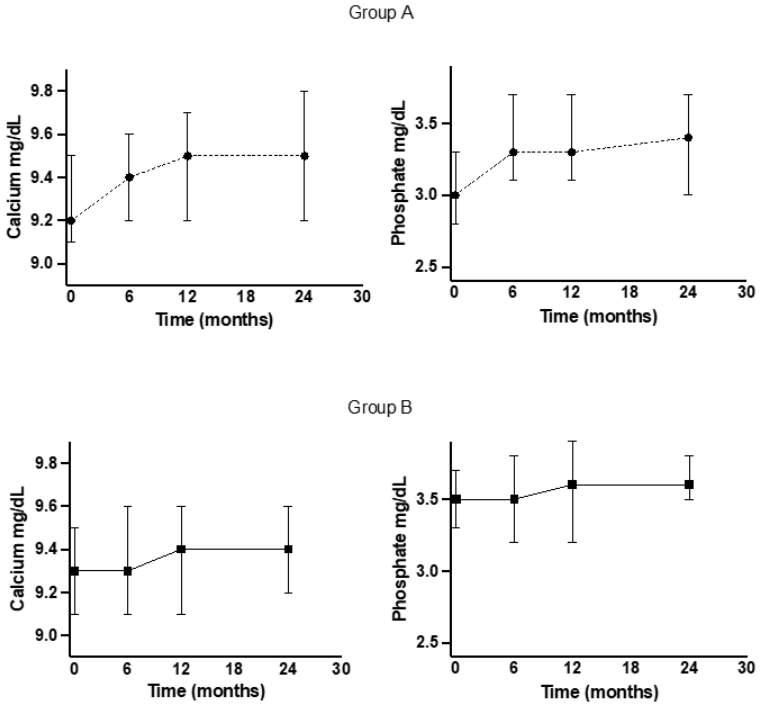

Figure 6 *- Prospective randomized arm: changes in calcium and phosphate levels from baseline to end of follow-up in surgically treated subjects (Group A) and in conservatively treated ones (Group B)*

In surgically treated patients' calcium levels significantly increase from baseline to 6 months (p=0.01) and 12 months (p=0.001) and from 6 to 12 months (p=0.05) and phosphate levels increased from baseline to 6 months (p=0.01). No statistically significant changes in calcium and phosphate levels were found from baseline to 6, 12 and 24 months

In contrast, patients in the conservative group (Group B) did not experience significant changes in cortisol or ACTH levels, nor in serum calcium or phosphorus levels over the same period. BMD remained stable in both groups throughout the 24-month follow-up.

The incidence of vertebral fractures was assessed in all patients. At the end of the 24 months, a total of eight incident fragility fractures were recorded. All of these were morphometric vertebral fractures, with only one occurring in the surgical group and seven in the conservatively treated group ($p = 0.04$). Notably, two of the patients in the conservative group who developed new fractures were receiving bisphosphonate therapy. Among the eight patients with incident vertebral fractures, five had a vertebral fracture at baseline—one in the surgical group and four in the conservative group.

Baseline F-1mgDST levels were not significantly different between patients who developed a new fracture and those who did not (data not shown). However, logistic regression analysis revealed

that surgical treatment of MACS was significantly associated with a reduced risk of incident vertebral fracture, corresponding to a 4.5-fold decrease in risk (odds ratio 0.22, 95% confidence interval 0.07–0.71), even after adjusting for age (OR 0.65, 95% CI 0.05-0.84).

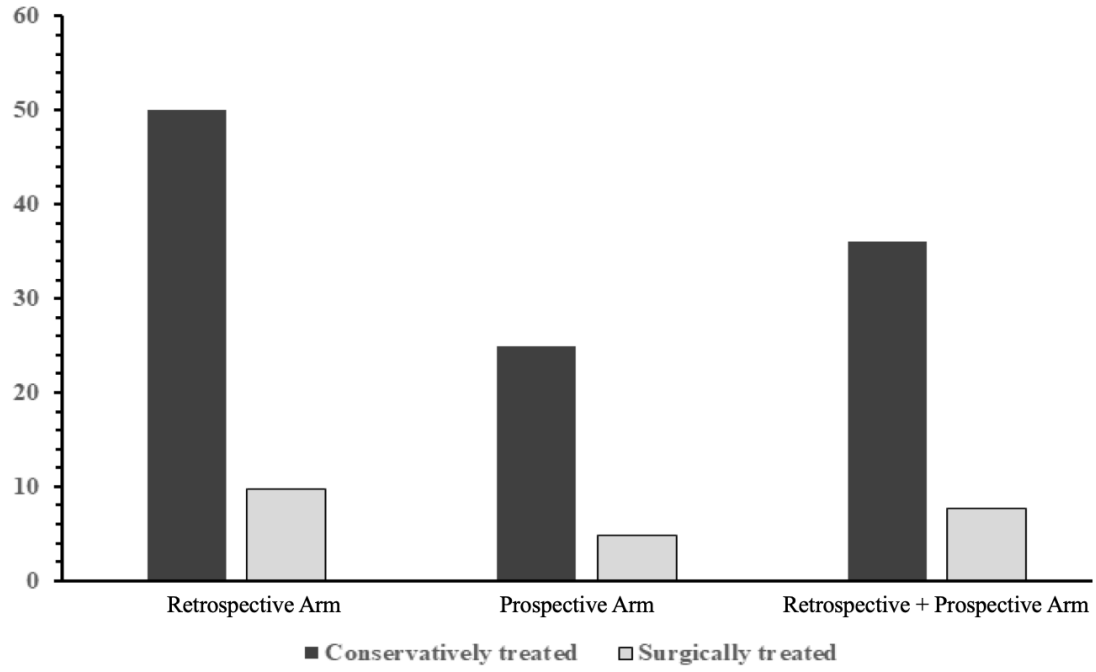

Figure 7 *- Incidence of vertebral fractures in conservatively treated patients and surgically treated patients from Retrospective Arm, Prospective Arm and form both arm taken together*

For each comparison the difference between conservatively treated subjects and surgically treated subjects is statistically significant. As compared with conservatively treated patients, in retrospective arm, prospective arm and both arms surgically treated subjects have a 6.8fold, 4.5fold and 6.5fold reduced risk of fragility fractures, respectively.

5. Discussion

This study (study 1) demonstrates that patients with MACS have a significantly higher prevalence and incidence of vertebral fractures compared to those with non-functioning adrenal incidentalomas. In the second part of the study (study 2), we evaluated the impact of adrenalectomy on fracture risk. Patients who underwent surgical removal of the adrenal adenoma responsible for MACS experienced a marked reduction in VFx incidence. In contrast, those

managed conservatively continued to show a high rate of new vertebral fractures during follow-up, underscoring the potential benefit of surgical intervention in mitigating bone fragility in this population.

Study 1 indeed demonstrates that patients with MACS exhibit a significantly higher prevalence and incidence of VFx compared to patients without MACS. These associations persist independently of age, gender, BMI, presence of type 2 diabetes mellitus, and—regarding incident fractures—even of baseline VFx status. Importantly, these findings were consistent across both sexes and remained significant after adjusting for premenopausal status in women.

A notable observation is the increased number of patients with prevalent VFx despite preserved BMD, which correlated with the degree of cortisol secretion. Consistently, BMD did not predict incident VFx, underscoring the limited utility of BMD alone in assessing fracture risk in MACS. Furthermore, over one-third of conservatively managed MACS patients experienced incident VFx during follow-up, highlighting the clinical relevance of this condition.

The diagnostic criteria for mild hypercortisolism have evolved substantially in recent years. Historically, heterogeneous definitions hindered comparability across studies (3). The 2016 ESE-ENSAT guidelines introduced a stratification based on post-dexamethasone cortisol levels (F-1mgDST), distinguishing between ACS, PACS, and non-functioning AI (44). The updated guidelines now define MACS as F-1mgDST >1.8 µg/dL, without further subclassification. This shift renders earlier studies on bone health in AI patients less applicable to current clinical practice (45-47).

Previous research has linked mild hypercortisolism to increased bone fragility and suggested that its resolution may reduce fracture risk. However, these studies used varying diagnostic thresholds, limiting their relevance under current guidelines. Consequently, these latter still consider the association between MACS and osteoporosis not yet well established and suggest that prospective cohorts should evaluate the fracture risk in AI with MACS (3).

Our study addresses this gap by demonstrating, in a large cohort, that MACS is associated with increased VFx prevalence, corroborating earlier, less uniform findings. These results align partially with a recent cross-sectional study by Zavatta et al., which reported an association between MACS and prevalent fragility fractures in postmenopausal women (25). Unlike Zavatta's

study, we observed increased VFx prevalence in men as well. This discrepancy may reflect differences in disease severity, as our cohort exhibited higher cortisol levels (mean F-1mgDST: 3.6 µg/dL vs. 3.1 µg/dL in Zavatta's study).

Both studies found a positive correlation between cortisol levels and VFx prevalence, reinforcing the role of cortisol excess in bone fragility. Notably, our data suggest that patients previously classified as PACS also have elevated fracture risk, indicating that these individuals should not be overlooked in clinical assessments of bone health.

A key novel finding is the comparable incidence of VFx in MACS men and women. Clinically, this implies that both sexes are at risk for asymptomatic VFx, which are strong predictors of future symptomatic fractures and hip fractures (26,48). This is in line with the fact that the last ESE-ENSAT guidelines have anyway decided to suggest to screen patients with MACS for VFx, though, at the time of drafting the guidelines, they considered the relation between MACS and VFx not yet well established (3). Secondly, if a conservative therapeutical approach is chosen in patients with MACS, a very careful evaluation of the bone fragility risk should be carried on, since a third of patients may experience a VFx during the follow-up.

This latter point deserves interest since, to date, a reliable evaluation of the fracture risk in patients with hypercortisolism remains a challenge even for bone experts. Indeed, the fracture risk in patients with hypercortisolism largely depends on the reduction of bone quality rather than on the bone density decrease (32,36,49,50). Patients with MACS do not represent an exception. In the present study, indeed, we found that in MACS patients, the VFx prevalence and incidence are increased independently of BMD, with no gender related differences. Interestingly, we found that the number of fractured patients without low BMD increases with the increase of cortisol secretion, suggesting that the higher the cortisol secretion, the more damaged the bone quality is. Unfortunately, nowadays, a reliable assessment of bone quality, particularly in patients with hypercortisolism, is still not obtainable in the clinical practice (51). Given the limited role of BMD in evaluating fracture risk in AI patients with MACS, the evaluation of other possible risk factors for fracture becomes even more important in MACS patients. In the present study, for example, at least in women, the presence of type 2 diabetes mellitus was associated with the VFx incidence independently of BMD and the presence of MACS itself. This finding, which was somewhat expected on the basis of literature data (78), suggests the bone fragility should be even more carefully evaluated in women with MACS and type 2 diabetes mellitus.

This study has some limitations. Firstly, given the retrospective design, we cannot exclude that some possible unknown factors could have biased the results. Secondly, the relatively small sample size of men patients included in the longitudinal arm could have prevented us to find other possible associations. Thirdly, in AI patients the cortisol secretion is known to possibly fluctuating and, thus, measuring the degree of cortisol secretion at the beginning of a follow-up may be not representative of the disease activity over time.

Notwithstanding these limitations, the study 1 is of interest since it shows that in both women and men with MACS both the prevalence and incidence of VFx are increased as compared with patients without MACS independently of BMD and of other possible confounding factors. These results give the scientific support to the last ESE-ENSAT decision to suggest to screen patients with MACS for the presence of VFx.

Building on these findings (Study 1), the second part of our research (Study 2) evaluates the effect of adrenalectomy on fracture risk in MACS patients. This is the first study to assess the impact of resolving MACS on bone health. Both retrospective and prospective analyses revealed a significant reduction in VFx risk following adrenalectomy, whereas conservatively treated patients continued to experience high fracture rates. Notably, BMD remained unchanged during follow-up in both surgically and conservatively treated groups, reinforcing the notion that fracture risk in MACS is driven more by impaired bone quality than by changes in BMD.

The observation that resolution of mild cortisol excess leads to a substantial reduction in fracture risk aligns with previous studies on subclinical hypercortisolism, a condition historically diagnosed using different criteria than those currently used for MACS (25,26,45).

However, as seen in subclinical hypercortisolism, fracture risk in MACS is not entirely eliminated by surgery. Several factors may contribute to this residual risk. Firstly, even though in surgically treated patients the follow-up period started after the withdrawal of the steroid substitutive therapy, we cannot exclude that this therapy could have been excessive for bone health at least in some patients. Moreover, the length of the recovery from sarcopenia may have played a role in slowing down the fracture risk reduction after surgery Importantly, in both studies all surgically treated MACS patients who experienced an incident VFx had a prevalent VFx at baseline and, therefore, might themselves be at increased risk of subsequent VFx (53). Finally, although all patients with a dietary calcium intake below 1000 mg/day were supplemented with calcium carbonate or

calcium citrate, it is not possible to exclude that the adherence to calcium supplements has been inadequate in some patients. This is important because after the cure of glucocorticoid excess an adequate calcium intake is crucial for the bone health recovery (32), which, in patients withdrawing glucocorticoids, is partially compensated by the increase of calcium and phosphate absorption by the gut (53,54). At this regard, it is of note that in the prospective randomized study, calcium and phosphate levels increased in surgically treated patients but not in conservatively treated ones, thus confirming the beneficial effect on bone of the recovery from even mild hypercortisolism.

The randomized study also confirmed that conservatively treated MACS patients remain at elevated risk for VFx, consistent with retrospective data reported in study 1. Although our studies were not designed to identify predictors of incident fractures in this group, pooled data revealed that 11 of 18 conservatively managed patients with incident VFx had prevalent fractures at baseline. This underscores the importance of baseline VFx as a strong predictor of future fractures (53). While F-1mgDST levels did not emerge as a statistically significant predictor in our cohort, a trend was observed across studies: higher mean F-1mgDST levels were associated with greater VFx incidence (2.5 μg/dL and 25% in the present randomized study; 3.0 μg/dL and 36.4% in a previous retrospective study; 3.5 μg/dL and 50.0% in our retrospective cohort). These findings suggest a possible dose-response relationship between cortisol excess and fracture risk, consistent with study 1 cross-sectional data.

Some findings of the present studies were somewhat expected. The fact that fragility fractures were only at vertebral sites is in line with previous studies on both subclinical hypercortisolism and MACS (25,26) and it could be explained by the fact that trabecular bone is highly sensitive to glucocorticoid excess (43). A further expected finding is that the prevalence of patients with an inadequate response to bisphosphonates (4 out of 7 patients, 57.1% considering the two arms together) is higher than that expected in post-menopausal osteoporosis (51,55), confirming that the MACS condition confers an additional risk of fragility fractures. Finally, even the present studies confirm that the absence of a low BMD at baseline and/or of a BMD reduction during the follow-up could not be considered a safe parameter in evaluating the fracture risk in MACS patients, as already described in patients with subclinical hypercortisolism (56). Indeed, it is widely accepted that BMD evaluation is not entirely reliable for predicting the fracture risk in glucocorticoid induced osteoporosis since, bone quality, which is not captured by BMD

assessment, plays an important role (52). Therefore, in the clinical evaluation of patients with MACS, other tools, beside BMD (trabecular bone score for example), should be studied in order to identify patients at higher risk of incident fractures (56-58).

Study 2 have several limitations. First, due to its retrospective design, we cannot exclude that in the retrospective arm some confounding factors could have exerted a role. For example, the higher disease activity in conservatively managed patients (as mirrored by F-1mgDST levels) may have impacted on the fracture risk. However, the fact that the association between the surgical treatment and the fracture risk reduction was independent of F-1mgDST levels renders this hypothesis less likely. Secondly, due to the need of steroid substitutive therapy, the observation period in the surgically treated patients lasted for a mean of 12 months more than that in the conservatively managed subjects. However, the shorter follow-up in conservatively treated subjects should have decreased rather than increased the rate of VFx. Finally, the use of bone turnover markers, which were not included in both retrospective and prospective arm determinations, could have been informative, particularly for the prediction of MACS patients at higher risk of incident VFx (59).

Notwithstanding these limitations, this study is important since it shows for the first time with both retrospective and randomized prospective approaches that subjects undergoing the removal of the adrenal adenoma causing MACS have an important VFx risk reduction, while conservatively treated subjects maintain a high rate of VFx incidence. Further larger studies should be designed in order to identify MACS patients at higher risk of incident VFx. In the meanwhile, while approaching patients with MACS, physicians should take into consideration the risk of VFx in patients who do not undergo surgery.

6. Future perspectives

While further large-scale studies with extended follow-up are warranted, the present findings offer valuable insights for future research.

First, it is essential to recognize that the prevalence of MACS is not negligible, and a substantial number of patients may benefit from targeted treatment for cortisol excess. Currently, surgery is the primary therapeutic option; however, it may not be feasible in elderly patients or those with several comorbidities. Pharmacological medications that act on cortisol secretion and cortisol peripheral activity —already used in other clinical settings such as Cushing's syndrome—could

potentially be repurposed for MACS patients, possibly at lower doses. Future studies should explore whether such medical therapies can also reduce fracture risk.

Second, given that MACS patients frequently present with vertebral fractures and often show poor response to standard osteoporosis treatments, it would be valuable to investigate the prevalence of MACS among individuals with osteoporosis. Furthermore, clinical criteria should be developed to identify which osteoporotic patients should undergo screening for MACS.

Finally, cortisol secretion should be viewed as a continuum rather than a binary phenomenon. Even patients with non-functioning adrenal tumors may exhibit minimal cortisol secretion, which could negatively impact bone quality and increase fracture risk.

7. Conclusions

This study provides robust evidence that MACS is independently associated with an elevated risk of vertebral fractures, irrespective of bone mineral density (BMD) or other conventional risk factors. Both retrospective and prospective analyses demonstrate that adrenalectomy significantly lowers fracture incidence, emphasizing the clinical importance of treating even mild cortisol excess. Notably, the occurrence of vertebral fractures in patients with normal BMD highlights the limitations of densitometry and the need for diagnostic tools that better capture bone quality. These findings underscore the necessity of incorporating fracture risk into the clinical management of MACS and support considering bone fragility as a key factor in therapeutic decision-making. Future research should aim to identify reliable predictors of fracture risk and evaluate non-surgical treatment strategies to expand management options for MACS.

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