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Dissertation

**A MURINE MODEL OF GLUCOCORTICOID MYOPATHY**

**ALLEVIATION USING ANDROGEN THERAPY**

by

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*”Se questa non piace, non voglio più scrivere di musica.”*

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**A MURINE MODEL OF GLUCOCORTICOID MYOPATHY**

**ALLEVIATION USING ANDROGEN THERAPY**

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Boston University School of Medicine, 2015

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# ABSTRACT

Glucocorticoids (GC) are used widely for the treatment of a large number of inflammatory conditions. A loss in muscle mass and increases in muscle weakness are common complications of GC therapy. Androgen therapy has been suggested to reverse GC-associated muscle loss (GAML), but evidence of its effectiveness is inconsistent. Herein, I established a mouse model of GAML. Young adult male mice receiving 0.25 mg/kg/day of the GC dexamethasone (D) s.c. daily, for a week, lost 3% of their total body weight. Based on NMR lean body mass quantification and muscle dissection, more than 10% of their muscle mass was lost. More than half of the D-induced muscle loss could be reversed by co-administration of 0.7 mg/kg/day of testosterone (T). To my knowledge, this is the first mouse model of GAML demonstrating alleviation by T.

D-upregulated intramuscular atrogene expression and proteasome catalytic activity were suppressed by T co-administration. D downregulated cathepsin L enzymatic activity and beclin expression, indicating that lysosome was not a major effector of GAML. Changes in calpain 1 and in translation factors 4E-BP, eIF3f and eIF2, following T treatment, were inconclusive. The changes in proteasome activity and atrogene expression were correlated with changes in expression of Foxo 1, 3a, and 4. Pro-catabolic factors REDD1 and Klf15 were repressed by T co-administration.

C2C12 differentiated myotubes were used to model GAML in vitro. Myotube diameter and total protein were reduced by D, and restored by T co-administration. Changes in C2C12 total protein were correlated with changes in protein degradation. D-induced proteolysis was inhibited by the proteasome inhibitor MG132.

In vivo, D reduced intramuscular IGF-I expression, an effect reversed by T co-administration. In C2C12, inhibition of IGF-1R signaling with picropodophyllin did not modify T protective effect. Mechanisms potentially explaining these observations are discussed.

In summary, my model demonstrates that T protective effect in GAML is mainly anti-catabolic, through the reversal of proteasome upregulation induced by D. In vivo, T stimulates a potentially protective intramuscular IGF-I response. The roles of protein synthesis and IGF-I in anabolic myoprotection could not be addressed in these models, and require further investigations.

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# LIST OF ABBREVIATIONS

24HUC 24-hour urine cortisol

3MH 3-methylhistidine

4E-BP eIF4E binding protein

AAP adrenal androgen precursor

AAS anabolic androgenic steroids

ACTH adrenocorticotropic hormone

ActRIIB activin A receptor, type IIB

AMPK adenosine monophosphate kinase

ANOVA analysis of variance

AP-1 activator protein 1

AR androgen receptor

ATF4 activating transcription factor 4

Atg12 autophagy-related 12

BCA bicinchoninic acid

BCAA branched-chain amino acids

Bcl3 B-cell leukemia/lymphoma 3

bFGF basic fibroblast growth factor

BSA bovine serum albumin

C/EBP CCAAT-enhancer-binding protein

Comb combination of Dexa and Testo

COPD chronic obstructive pulmonary disease

CS Cushing’s syndrome

CSA cross-sectional area

CT computed tomography

Ct cycle threshold

D dexamethasone-only group

Dexa dexamethasone

DHEA dehydroepiandrosterone

DHEAS dehydroepiandrosterone sulfate

DHT dihydrotestosterone

DMEM Dulbecco’s Modified Eagle Medium

DMSO dimethyl sulfoxide

DT dexamethasone and testosterone group

DTT dithiothreitol

EC50 half maximal effective concentration

EDTA ethylenediaminetetraacetate

eIF2 eukaryotic initiation factor 2

eIF2B eukaryotic initiation factor 2B

eIF3-f eukaryotic initiation factor 3f

eIF4E eukaryotic translation initiation factor 4E

eNOS endothelial nitric oxide synthase

FBS fetal bovine serum

FDA Food and Drug Administration

FSH follicle stimulating hormone

FSR fractional synthesis rate

Gadd45 Growth Arrest and DNA Damage 45

GAML GC-associated muscle loss

GC glucocorticoid

GCN2 General Control Nonderepressible 2

GFP green fluorescent protein

GH growth hormone

GLUT glucose transporter

GnRH gonadotropin-releasing hormone

GR glucocorticoid receptor

Grb2 Growth factor receptor-bound protein 2

GRE glucocorticoid responsive-elements

GSK3 glycogen synthase kinase 3

HAT histone acetyltransferase

HDAC histone deacetylase

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonate

HIF 1a hypoxia-induced factor 1a

HOMA-IR homeostatic model assessment - insulin resistance

HPA hypothalamic - pituitary - adrenal

HS horse serum

HSD honest significant difference

IBMX 3-isobutyl-1-methylxanthine

IGF-1R IGF-I receptor

IGF-I insulin-like growth factor I

IGF2 insulin-like growth factor 2

IGFBP IGF-I binding protein

IL-1 interleukin 1

IL-1RII interleukin 1 receptor type II

IL-2 interleukin 2

IRS insulin receptor substrate

IκBα inhibitory κBα

Kd dissociation constant

Ki inhibitory constant

Klf15 Krüppel-like factor-15

LC3 microtubule-associated protein 1 light chain 3

LH luteinizing hormone

LKB1 Liver Kinase B1

MAFbx muscle atrophy F-box

MAPK mitogen-activated protein kinases

MEF2A myocyte enhancer factor 2A

MNK2 mitogen-activated protein kinase–interacting kinase

MR mineralocorticoid receptor

MRF muscle regulatory factor

Mrf4 myogenic regulatory factor 4

mTOR Mechanistic Target of Rapamycin

mTORC1 mTOR complex 1

MuRF1 muscle RING finger 1

Myf myogenic factor

MyHC myosin heavy chain

NEFA non-esterified fatty acids

NF-κB nuclear factor kappa - light-chain enhancer of activated B cells

NFAT nuclear factor of activated T-cells

NMJ neuro-muscular junction

NMR nuclear magnetic resonance

OMIM Online Mendelian Inheritance in Man

ORF open reading frame

p70-S6K ribosomal protein S6 kinase, 70 kDa

p90-RSK ribosomal protein S6 kinase, 90 kDa

PAGE polyacrylamide gel electrophoresis

PBS phosphate buffered saline

PDK1 3-phosphoinositide-dependent protein kinase 1

PGC-1 PPARγ coactivator 1

PI3K phosphatidylinositol 3-kinase

Pitx paired-like homeodomain

PLC phospholipase C

POMC pro-opiomelanocortin

PPAR peroxisome proliferator-activated receptor

PPP picropodophyllin

PRAS40 proline-rich Akt substrate of 40-kDa

qRT-PCR quantitative real-time polymerase chain reaction

REDD1 regulated in development and DNA damage responses-1

RIPA radioimmunoprecipitation assay

SDS sodium dodecyl sulfate

SGKL serum and glucocorticoid-inducible kinase-like kinase

Shc Src homology 2 domain containing

Sos Son of sevenless homolog

Sp1 SV40 promoter-specific 1

STAT Signal Transducer and Activator of Transcription

T testosterone-only group

Tbx T-box

TCA trichloroacetic acid

TCF4 transcription factor 4

Testo testosterone

TGF-β transforming growth factor-beta

Tp testosterone propionate

TSC1 Tuberous Sclerosis Complex 1

TSC2 Tuberous Sclerosis Complex 2

ULK1 UNC-51-like kinase 1

V Vehicle-only

VEGFR VEGF receptor

# CLINICAL QUESTIONS AND EVIDENCE

## Cushing’s syndrome and hints of an atrophy mechanism

Maintenance of muscle mass and force is dependent on the well-adjusted endocrine system. The first evidence for this muscle-hormones interaction came from diseases, interpreted as natural experiments. Interestingly, the role of adrenal hormones in muscle homeostasis was deduced from perturbations of another gland, the pituitary.

Through the detailed case series written by Harvey Cushing[3], the scientific and medical community became aware of an otherwise rare disease, which bears his name. Unlike the earlier and better-studied deficiencies of the thyroid and pancreas, pituitary defects are more variable in manifestation and therefore harder to unify in a single clinical entity. Even when macroscopic hypertrophy of pituitary was localized to a gland subdomain, pathological mechanisms were ambiguous. Symptoms could have been attributed to a hypersecretion from the hypertrophied sector, or to a deficiency in the neighboring compressed structures. Pituitary extracts caused multiple, and even opposite effects, in animal models[4], further proving the heterogeneous nature of pituitary secretion.

Among 50 cases described by Cushing, about five stood out due to the involvement of other glands. In each of them, and, to a lesser extent, in a few more cases, “hyperadrenalism” was blamed for asthenia, hyperpigmentation of skin, low blood pressure, and hypoglycemia. Histopathology tests localized the adrenal abnormalities to the zona fasciculata of the cortex. Cushing wrote that some of these abnormalities reflect current adrenal hypoactivity, caused by exhaustion after preceding intense stimulation and hyperactivity.

Twenty years later, Cushing narrowed the focus in an updated case series of combined pituitary-adrenal pathology[5]. Cushing noted that basophile adenomata of the pituitary and hypertrophy of the adrenal glands often coexisted. Based on the curative effect of pituitary surgery, he hypothesized that the adrenal defect is secondary to the pituitary abnormality. In turn, he inferred that the adrenal changes mediate the disease phenotype, which includes obesity with ectopic adipose deposits, kyphosis, amenorrhea / impotence, hypertrichosis, lineae atrophicae, fatigability and weakness. Among these disease manifestations, muscle impairment was a serious, if variable, component. Cushing considered intense muscle loss the cause of death for one of these cases.

Cushing’s work did little to elucidate mechanisms leading to the phenotype. The variability in pituitary changes between the cases he described meant that many scientists rejected his hypothesis of pituitary primacy. A group at the Mayo Clinic was actively pursuing the opposite hypothesis, with the adrenal as the primary site of impairment in adrenal-pituitary combined afflictions[6]. On the clinical side, it was noted that some of Cushing’s patients lacked observable pituitary changes. Moreover, some of the Mayo patients were cured by adrenal surgery. From a theoretical perspective, the adrenal hypothesis was more tempting because the adrenal deficiency (termed Addison’s disease) and its reversal by administration of adrenal cortex extracts were better known than pituitary pathology[7].

Today, we know that the truth was more nuanced. Hypersecretion of the adrenal cortex hormones cortisol and / or corticosterone is termed hypercortisolism. One or more clinical signs listed by Cushing (see above) suggest to the practitioner the activation of the hypothalamic - pituitary - adrenal (HPA) axis. If concomitant hypercortisolism is confirmed by an increase of urine free cortisol measurements, or by the effacement of the evening trough in circulating cortisol, there is suspicion for Cushing’s syndrome (CS)[8]. Some hypercortisolism cases, termed pseudo-Cushing’s syndrome, are ascribed to causes outside the HPA axis, such as in depression, morbid obesity, uncontrolled diabetes mellitus, and sleep apnea (reviewed in [9]).

True CS cases are further classified based on the role of the adrenal-stimulating pituitary hormone corticotropin (adrenocorticotropic hormone; ACTH). In some CS patients, hypercortisolism is paralleled by an increase in ACTH. Their adrenals are usually responsive to further ACTH stimulation tests, indicating that previously intact adrenals underwent hyperplasia in response to a pathological overstimulation with ACTH. When attributed to the pituitary, such ACTH hypersecretion, followed by secondary hypercortisolism, is termed Cushing’s disease (reviewed in [10]). Cushing’s disease remains a staple of physiology textbooks, because it provides an excellent didactic example of a hormone hierarchy.

The remainder of CS cases consists of hypercortisolism despite low ACTH. In primary hypercortisolism, ACTH is typically suppressed by negative feedback. Adrenal neoplasms are the most frequent cause of primary hypercortisolism. Ectopic or diffuse unregulated sources of ACTH or cortisol may cause hypercortisolism. In recent decades, overdose with synthetic derivatives of cortisol became the most important cause of low-intensity CS (discussed in the next section).

Although CS may originate in various HPA pathologies, muscle impairment is one of its most common, unifying features.

## Glucocorticoid therapy

A series of serendipitous decisions brought impressive knowledge about CS of non-pituitary etiology (reviewed in [11]). First, during World War II, US intelligence learned that Germans were importing large quantities of adrenal glands from neutral Argentina. This reignited US government interest in corticoadrenal research, despite the lackluster results with earlier adrenal extracts. At the end of the war, only a few grams of pure adrenal steroids were manufactured, from endogenous sources and at a high cost. The second opportunity was in the allocation of those scarce steroids. One of them, cortisone, made by Merck, was shared by a few clinical researchers, including Phillip Hench. Hench’s request was based on his previous work on rheumatoid arthritis. He observed that rheumatoid arthritis was alleviated in jaundice, and hypothesized the existence of a steroidal “anti-rheumatoid factor.” Third, Hench’s choice of dose and route elicited an extraordinary reversal in arthritic pain and dysfunction. In 1949, after treating only five patients[12], impressive improvements in those cases reordered priorities in corticosteroid research.

Previous work described multiple effects for adrenal extracts. In fact, adrenal research was considered a dead end prior to cortisone purification, because less pure extracts combined antagonistic hormones in variable doses, seemingly lacking defined pharmacological or endocrine relevance. Even with purified cortisone, Hench saw a very diverse set of consequences for cortisone administration[13]. However, Hench’s observations were replicable, demonstrating the complex and vital role of the adrenal.

First, cortisone’s action on metabolism was accessible even to the less sophisticated clinical measurements used 60 years ago. Patients receiving cortisone gain weight. Chronic cortisone therapy leads to accumulation of adipose tissue, often in ectopic locations, such as the interscapular “buffalo hump.” Cortisone also induces hyperglycemia, and subsequent glycosuria. For this reason, cortisone and its endogenous and synthetic analogs are grouped in the glucocorticoid (GC) family.

Hench and collaborators hypothesized that cortisone’s protective action is not limited to rheumatoid arthritis. In his 1950 Nobel lecture, Hench envisaged a role for alleviation of most inflammatory diseases. GCs share the ability to reduce inflammation (reviewed in [14, 15]). Some of these anti-inflammatory effects, such as reduction in the number of circulating white blood cells, are ample and robust. Other aspects of GC action remain under active study, facilitated by the rapid progress of immunology. The questions still open illustrate the convoluted ways in which GC signals are relayed in the cell. For example, GCs are often acting in a manner shared with all steroid hormones, by binding and activating the glucocorticoid receptor (GR). Activated GR translocates from cytosol to the nucleus, where it dimerizes on specific DNA sequences, termed glucocorticoid responsive-elements (GRE)[16]. The classical effect of the GRE-GR interaction is increased transcription for neighboring target genes (transactivation), as it is the case in polymorphonucleate cells for interleukin 1 (IL-1) receptor type II (IL-1RII)[17], a decoy inhibitor for the pro-inflammatory IL-1. In other circumstances, the activated receptor inhibits transcription directly (transrepression), or by interfering with transcription factors. For example, in human T lymphocytes, GCs inhibit the transcription factor activator protein 1 (AP-1), thus causing a reduction in their ability to synthesize pro-inflammatory interleukin 2 (IL-2)[18]. GCs employ nongenomic mechanisms, such as mRNA stability and enzymatic activity modulations. In airway epithelia, GCs reduce the half-life of the mRNA for interleukin 8 (IL-8), the major chemoattractant for neutrophils[19]. Within minutes, GC administration induces vasodilation, through direct, nongenomic activation of endothelial phosphatidylinositol 3-kinase (PI3K) leading to activation of endothelial nitric oxide synthase (eNOS)[20].

Some GC effects may be limited to a range of doses, durations, and frequencies of administration. Moreover, the example of adrenalectomized rats re-supplemented with corticosterone, their most abundant endogenous GC, illustrates how, at times, the same GC can induce or repress the same cellular response, depending on the dose. A lower 5 mg/kg dose enhanced the immune skin delayed-type hypersensitivity, while a 40 mg/kg dose yielded the opposite, expected immunosuppressive response[21]. This biphasic behavior, suggestive of a U- (or inverted-U) shaped curve, poses great challenges, both to the investigative scientist, and to the clinician attempting to establish a therapeutic regimen.

In 1950, endogenous GCs corticosterone and cortisol, were synthesized at Merck[22], thus lowering the price and creating the opportunity for large-scale trials. The Empire Rheumatism Council organized a randomized trial comparing cortisone with acetylsalicylate, and concluded that there is no benefit in cortisone[23]. While participants receiving cortisone claimed an improvement in subjective well-being, they were afflicted more often with deleterious side effects, including edema and hypertension. In retrospect, a comparison between two palliative symptomatic therapies using cure-indicating outcomes was likely misleading. Nevertheless, cortisone was deemed unfit for therapeutic purposes, at least in Britain. This failure initiated a race for improving endogenous GCs with chemical modifications (reviewed in [24]). While synthesizing esters with a better half-life, Schering chemists introduced a double bond in the A ring of cortisone, thus discovering prednisone, the first widely used oral GC[25]. Prednisone was a better anti-inflammatory than cortisone, but had a lower ability to cause edema. This was the first suggestion that the many GC effects could be separated by chemical modifications. In 1955, NIH researchers synthesized and characterized prednisone’s active metabolite, prednisolone[26]. In a trial of prednisolone versus acetylsalicylate in rheumatoid arthritis, the GC provided better functional protection to the articulations[27], thus establishing prednisolone as a standard of care and making GCs even more interesting for chemists.

Further improvements were made at Squibb, where it was found that insertion of a halogen atom improves GCs anti-inflammatory effect[28, 29]. In 1958, Merck chemists led by Arth modified cortisol with the unsaturated A ring (Δ1 ), the fluoride addition at position 9α, and with a methyl group on the 16α position to obtain dexamethasone (Dexa)[30, 31]. Dexa is the most effective and specific therapeutic synthetic GC to date, with 170 times higher ability to inhibit the immune reaction to subcutaneous foreign bodies (granuloma) compared to cortisol[32]. The other benefit of Dexa is its virtual inability to cause edema and electrolyte imbalance.

In addition to being a strong anti-inflammatory, Dexa is 52 times more potent in suppressing endogenous GC secretion, and 35 times more potent in causing hyperglycemia compared to cortisone[33, 34]. Efforts to synthesize steroids with anti-inflammatory action that do not interfere with metabolism have failed. Compounds such as A276575[35] and RU 24858[36] failed in preclinical studies. Mapracorat[37] did not progress beyond phase II clinical trials. These facts demonstrate that the anti-inflammatory and hyperglycemic actions are intermediated by the same specific, Dexa-sensitive receptor, whereas electrolyte changes are caused by cortisol through a different pathway. Clinicians prescribing GCs in these five decades had to balance therapeutic benefit with metabolic side effects, and, in the case of less specific GCs, with the water retention.

Edema is an example of non-specific GC effect, caused by a less typical interaction of the hormone with the mineralocorticoid receptor (MR). The GC family spans tens of active principles and thousands of formulations, from weak GCs with lower specificity, such as cortisone, to strong, specific GCs, such as Dexa. When strong GR activation is desired, clinicians have to use Dexa in order to avoid MR activation. When safety is desired, such as in over-the-counter products, low-activity, low-specificity compounds are preferred.

Chronic GC therapy causes glucose metabolism disturbance, osteoporosis, and muscle loss, suggesting that their therapeutic use is limited. However, their efficacy makes them some of the most commonly used drugs. The trivial case for using GC therapy is in hormone replacement, such as in adrenocortical insufficiency (reviewed in [38]). In addition, many other diseases are alleviated by GCs to a degree that deleterious effects are outweighed. Based on their ability to lower white blood cell count, GC are an important adjuvant in the palliative and even etiologic treatment of leukemias and lymphomas[39, 40, 41]. On the balance of benefits and drawbacks, GCs are recommended for many life threatening or impairing immune reactions, such as polymyositis (reviewed in [42]), severe sarcoidosis (reviewed in [43]), and disseminated pulmonary tuberculosis. GCs are relatively safe in topical applications in dermatological conditions (pemphigus, psoriasis, most types of dermatitis; reviewed in [44]). Similarly, GCs are commonly used in eye inflammatory conditions[45, 46], such as diffuse posterior uveitis and optical neuritis. GC therapy is suitable for brief administration in acute immune or allergic conditions, such as seasonal rhinitis (reviewed in [38]). In chronic diseases, GCs are recommended for short-term alleviation of exacerbations. Short-term GC therapy is recommended for rheumatoid arthritis, gouty arthritis, psoriatic arthritis, ankylosing spondylitis, asthma[47, 48], ulcerative colitis[49, 50], and idiopathic nephrotic syndrome[51].

As envisaged by Hench in his Nobel Lecture, GCs do not address disease causes, and are recommended for temporary respite. For many immune diseases, more specific therapeutic alternatives have been developed. The list of Food and Drug Administration (FDA)-approved indications for cortisone, Dexa, and prednisone is often narrowed by additional precautions, and by newly discovered drugs[52, 53, 54]. Nevertheless, off-label use of GCs is very frequent. For example, GCs are perceived by physicians as a fallback therapeutic alternative for cerebral hypertensive conditions, despite scarce evidence for efficacy in any specific conditions. Small trials suggest GCs reduce vasogenic cerebral edema[55] and prevent acute mountain sickness[56]. These studies have been carried although earlier systematic reviews showed that, in fact, GCs worsen outcomes for acute brain trauma victims[57]. This example illustrates how strongly rooted are off-label uses of GCs.

A similar, paradoxical situation is seen in ongoing clinical research. As of 2015, the patent-free status of the GCs discourages trials for new indications, while their de facto standard-of-care status makes them a common comparator in clinical trials. The National Cancer Institute sponsors 311 ongoing clinical studies employing Dexa, mainly in the standard-of-care arm, thus providing a plethora of data that have been, and may still be, misconstrued as support for the use of GCs. Everyday practice may drift further apart for the officially sanctioned label, thus providing new opportunities for unjustifiable overdose.

This wide array of uses make GCs some of the most prescribed and used drugs. Despite the low prevalence of the conditions proven to benefit from GC therapy, every year, about 1% of the Americans and British receive some form of GC[58, 59]. GCs are likely even more prescribed in the developing world, due to affordability and lack of alternatives, poor access to health care notwithstanding. Dexa and cortisol are the only drugs listed five times in the World Health Organization’s List of Essential Medicines[60]. Due to their widespread use, GCs are likely to cause covert iatrogenic CS in a large population, impairing muscle mass and quality of life to a certain and understudied degree.

## Hypercortisolism-induced muscle loss

Primary and secondary endogenous hypercortisolism are rare diseases (1-2 cases per million and year each[61]), despite a recent boost from incidental imaging diagnoses. The symptomatology is non-specific, meaning that, even today in the developed world, an average of 6 years pass from signs onset until diagnosis is made and treatment is initiated[62]. Endogenous hypercortisolism is a life-threatening disease, with untreated patients having a median survival rate of 5 years after diagnosis[63]. Some of the changes occurring in Cushing’s disease are irreversible, especially at the level of brain, bone, adipose tissue, and liver levels (reviewed in [64]). Even after surgical adjustments of the hyperactive pituitary, the quality of life for CS patients lags behind that of the unaffected population.

About two thirds of patients with Cushing’s syndrome acknowledge muscular problems at presentation, with similar incidence among pituitary and adrenal conditions[65]. Among patients diagnosed with endogenous CS, one fifth are referred to the endocrinologist due to muscle weakness[66]. Two fifths of those whose endogenous hypercortisolism is successfully corrected by surgery still complain of fatigue[67].

On the other hand, therapy-induced (iatrogenic) CS is common. The glut of GC indications and off-label uses makes them some of the most used drugs in the developed countries, as described earlier. In most cases, the cause of iatrogenic CS can be identified by careful history taking and medication reviews. However, an increasing number of cases are not as easily diagnosed, because the excess GC is not from prescription medicine. In United States, FDA approved in 1979 over-the-counter sale of 0.5% hydrocortisone cream for itching and minor skin inflammation. In 1990, 1% hydrocortisone creams were also permitted[68]. In 1987, hydrocortisone creams became over-the-counter in Great Britain. Regulated over-the-counter GC creams rarely cause CS on their own, but have been frequently suspected to lower the threshold for CS in patients who are also prescribed oral GC. Unregulated, mislabeled, overdosing GC creams sold as skin-bleaching products pose a great CS risk to patients from ethnic groups with darker skin. Half of the respondents in a Nigeria poll admitted using GC-based skin bleaching products[69]. In 2015, the Ivory Coast government made illegal skin bleaching products, due to worries about GC overdose side effects[70]. The side effects of skin bleaching are well recognized by the sub-Saharan medical community. Paradoxically, CS caused by bleaching products may be less identifiable to practitioners who care for the African diaspora in the developed world, where bleaching is more frequent, due to improved financial access and social pressures[71, 72].

Other, less frequent causes of iatrogenic CS include the interaction between low dose GC therapy and cytochrome P450 3A4 inhibitors, such as the antiretroviral ritonavir[73]. Other steroid drugs may interact with GR and cause CS when overdosed, as it is the case with the synthetic progestin megestrol acetate[74].

Due to its insidious and erratic symptomatology, iatrogenic CS is often diagnosed years after onset or completely unrecognized[62]. The incidence of iatrogenic CS is difficult to estimate, because there is no reporting requirement. In the developed world, iatrogenic CS could be as frequent as one case per thousand and year[75].

Signs of iatrogenic CS are as varied as those of Cushing’s disease. In a cohort of patients receiving for three months more than 0.4 mg/(kg d) prednisone, the most common signs were development of ectopic adipose deposits (50%), hyperphagia (47%), and muscle cramps (32%)[76]. In the same cohort, 15% complained of muscle weakness. Patients stated that the most distressing signs of hypercortisolism were, in order, body shape changes, neuropsychiatric disorders, muscle cramps, and hand tremor. In 1982, the most common cause of iatrogenic muscle weakness was GC therapy[77].

There are differences between GC-induced cardiovascular changes, depending on the nature of the GC. Endogenous GCs, such as cortisol, have hypertensive effects, while some synthetic GCs, Dexa included, lack such non-specific MR-dependent action. Nevertheless, excess exogenous and endogenous GC causes the same disabling effects on muscle[78], indicating that muscle damage is mediated by GR. GCs differ quantitatively in their ability to cause myopathy. Myopathy is invariably induced in two weeks by either 0.2 mg/(kg d) Dexa[79], or by 0.5 mg/(kg d) prednisone[80]. Based on animal studies, it is likely that the catabolic potency ratio is even more tilted towards Dexa than the referenced studies indicate. Modern human pharmacodynamics and epidemiological studies are needed, in order to establish actual safety thresholds.

In their 1958 case series, Muller and Kugelberg were the first to describe muscle changes associated with long-term Cushing’s disease[81]. In their mixed, primary and secondary, endogenous hypercortisolic cohort, they found that complaints of muscle weakness were primarily localized on the thigh. Objective loss of muscle force was correlated with histopathological changes indicative of a muscle fiber defect, such as degenerated fibers, at times hyalinized or with loss of striation, muscle replacement with fat and connective tissue, and rare hypertrophic fibers. Through electromyography, they established that the number of motor units is unaffected. Together with lack of changes in reflexes, their work negated a neurological component of CS. Muller and Kugelberg noted that hypercortisolism is correlated with faster extinction of the action potential, which is typically caused by a reduction in the number of muscle fibers, or by fiber atrophy[82]. Based on the evidence that CS is a muscle fiber disease, they coined the phrase “steroid myopathy” (in opposition to a hypothetical “neuropathy”). Similar electromyography changes are induced by long-term GC therapy[83], making some authors reserve the term “steroid myopathy” to muscle complaints of iatrogenic etiology. In 1966, D’Agostino and Chiga, confirming histological fiber changes in a rabbit model of iatrogenic CS, formulated the more precise, yet less commonly used “glucocorticoid myopathy”[84]. Owing to the fact that glucocorticoid myopathy is not a standalone disease or syndrome, terminology has never been standardized. In the present work, the human condition will be designated glucocorticoid myopathy, while its animal models will be termed GC-associated muscle loss (GAML).

In exogenous CS, GC excess can be better quantified. In a population with neurological maladies receiving long-term Dexa, the threshold for manifest glucocorticoid myopathy appears to be 50 µg/(kg d)[85]. However, the most significant predictor of clinical GAML is total dose[79, 86]. When GAML develops, the amplitude of electromyography changes (that is, the reduction in action potential duration) is proportional with the total GC dose[87]. These findings imply that glucocorticoid myopathy can be induced in shorter periods, if the GC dose is extremely high. Foye and colleagues drew a distinction between “classic” or “chronic” glucocorticoid myopathy, induced “within weeks to years,” and “acute” glucocorticoid myopathy, induced in 5-7 days of high-dose GC[88]. However, their description of the two forms of GAML is almost identical, suggesting that the two clinical entities are largely overlapping.

In a comparative study of patients receiving GC therapy for asthma, half of the patients receiving more than 0.2 mg/(kg d) prednisone exhibited a reduction in hip flexor strength of 2 SD or more, compared with healthy age- and sex-matched controls[80]. In a study of adults with brain or spine cancer, 60% of the participants experienced loss of iliopsoas muscle force in response to GC therapy for cerebral edema[79]. In a small cohort, 6 months of 0.16 mg/(kg d) prednisone treatment was associated with a 20% reduction in thigh muscle force, compared to healthy controls[89]. Such findings suggest that GC-induced weakness has functional consequences.

In a post-hoc analysis of a chronic obstructive pulmonary disease (COPD) trial, the placebo arm was stratified in GC-treated and GC-naive groups[90]. The maximal inspiratory mouth pressure, a proxy measurement for respiratory muscle strength, was significantly better maintained over the 8 weeks of the trial in the GC-naive, compared to GC-treated participants. Involvement of partly-involuntary muscles further proves that glucocorticoid myopathy is caused by an objective muscle disorder, and negate the alternative, neuropsychiatric etiology.

Another investigative direction in the study of GC-induced muscle weakness focused on muscle mass and volume. Although correlated, muscle force, mass, and volume are not completely reflecting each other. The most accessible proxy measurements of muscle mass, such as mid upper-arm or thigh circumference, are not sensitive enough in monitoring GC-induced muscle loss, even after subtracting skin fold, because GC stimulate intramuscular adipose deposits[91]. The advent of modern imaging allowed non-invasive muscle measurements. Chronic prednisone administration causes a 20% reduction in mid-thigh muscle area measured by computed tomography, and a 36% increase in the ratio of fat-to-muscle areas (CT)[92]. Psoas muscle area and density, measured by computed tomography, are inversely correlated with GC levels indicated by 24-hour urine cortisol (24HUC)[93].

Muscle fibers are classified in types, based on their adaptation to either endurance or brief strong bursts. Fast-twitch fibers are further classified based on their propensity for aerobic or anaerobic (glycolytic) metabolism. Differential effects on fiber types and inter-type conversions have been observed in many muscle-afflicting maladies. For example, gains in the ratio fast-to-slow twitch fibers are associated with insulin resistance[94]. In contrast, aging is correlated with preferential loss of fast fibers[95]. Reports of type-specific effects of GC are inconclusive. In one study, women with CS had an increased proportion of type IIx (fast twitch, glycolytic) and a lower proportion of type I (slow twitch, oxidative) fibers in their vastus lateralis muscles[96]. Renal transplant patients receiving 25 mg/(kg d) prednisone over three months had lower cross-sectional area (CSA) in type IIa (slow twitch, oxidative / glycolytic) and I fibers[97]. Others found that all types of fibers are uniformly affected by GC[98]. This hypothesis was further followed in animal studies.

A set of muscle mononucleate cells, expressing the paired-box transcription factor Pax7, are presumed to support muscle development and regeneration, and are termed satellite cells (reviewed in [99]). There are no definitive studies describing the effect of GC in human satellite cells. Some or all satellite cells may be activated to proliferate, thus becoming myoblasts. Many in vitro assays use proliferating cells from human muscle, at times assumed to be myoblasts. These human “myoblasts” do not proliferate in the absence of at least 1 µM Dexa([100], and personal observation; data not shown). For comparison, maximum normal concentration of endogenous cortisol in humans is 0.78 µM[101], that is, tens of times less potent. Therefore, it is impossible to conceive an experiment where human myoblasts in culture are subjected to meaningful manipulations of GC concentration. The fact that GCs are vital for in vitro human muscle development and maintenance suggests that cell lines that do not require GC may be less accurate models of human muscle.

There are no published cases of increase in circulating myoglobin or creatine kinase in response to GC monotherapy, or as a consequence of Cushing’s disease. The absence of such intramuscular protein from the blood flow suggests GC do not cause rhabdomyolysis, that is, loss of muscle through uncontrolled rapid membrane leakage.

GC therapy induces a massive loss of nitrogen, a side effect seen from its first trial[102]. The ample increase in urinary creatine and creatinine is evidence for upregulated tissue protein breakdown. As little as 20 µg/kg cortisol infused over 8 hours increases by a quarter the rate of appearance of leucine into the bloodstream, suggestive of acute proteolysis upregulation[103]. Leucine’s rate of appearance is even higher when the GC-induced hyperinsulinemia is prevented, indicating that whole-body experiments do not capture the amplitude of the GC-induced proteolysis[104]. More modern mass spectrometric methods revealed that a single dose of 1 mg/kg prednisolone cause increases in all blood amino acids, presumably due to mobilization from muscle sources[105]. The same acute treatment causes an increase in 3-methylhistidine (3MH), a non-recyclable degradation product specific to muscle actin and myosin[106]. Similar increases in 3MH are seen with control diet in chronic GC excess of endogenous or exogenous nature[98]. These findings demonstrate that GC-induced loss of muscle mass is mediated by stimulation of protein degradation.

The last three decades brought a better understanding of protein degradative pathways and of muscle atrophy. Two distinct proteolytic systems, the proteasome - ubiquitin system and the autophagosome (discussed in later sections), have been discovered. Unfortunately, only one published trial investigated the action of GC in human muscle biopsies, at a molecular level. It failed to find a significant change in mRNA of ubiquitin and the C3 subunit of the proteasome[107]. The result is unsurprising, given that the control of the proteasome system may be exercised in other, unexplored ways. In animal models, the genes most correlated with muscle loss, including GAML, are two E3 ubiquitin ligases, muscle atrophy F-box (MAFbx; gene known as Fbxo32) and muscle RING finger 1 (MuRF1; gene known as Trim63), but no published studies confirm or refute their modulation in humans (reviewed in [108]).

Recently, pharmacological inhibitors of the proteasome became widely available. The first proteasome inhibitor, bortezomib, is recommended by the FDA for multiple myeloma and mantle cell lymphoma[109]. The second generation, irreversible proteasome blocker carfilzomib is also approved for advanced myeloma therapy[110]. In the light of data from the animal models of muscle loss, these drugs should have been useful in cachexia, but, to date, no human trials investigated their ability to prevent muscle atrophy.

There are no trials comparing GC with the combination (GC + bortezomib). However, an indirect comparison can be made. In a trial for multiple myeloma, fatigue was a complaint of 32% of the participants receiving 40 mg Dexa, compared to 42% for bortezomib[111]. In another trial, addition of 20 mg Dexa to bortezomib lowered the rate of fatigue from 57% to 25%[112]. Neither finding is suggestive for superiority of that the combination (Dexa + bortezomib) to Dexa alone. Clinical studies directly addressing this comparison are recommended, given that the most commonly accepted hypothesis centers on the proteasome as main effector of GC-induced muscle loss. Proving a beneficial action of bortezomib in co-administration with GC will have major practice-changing implications. Even proving the opposite, that bortezomib has no protective action, will be very valuable in better understanding and eventually preventing GC-induced muscle loss.

The inhibition of the other proteolytic system, the autophagosome, is also the focus of clinical studies. Starting with the inexpensive antimalarials chloroquine and hydroxychloroquine, autophagosome inhibitors are now the focus of phase II clinical studies in many cancers[113]. Interestingly, hydroxychloroquine is also recommended for rheumatoid arthritis, where it may be prescribed for up to six months[114]. However, to my knowledge, no clinical trial compared hydroxychloroquine with GC. Chronic hydroxychloroquine therapy is known to induce muscle weakness and sporadic myopathy, through a distinct, vacuolar mechanism. The hydroxychloroquine-induced myopathy is associated with an increase in autophagosome markers in muscle, demonstrating the importance of autophagosome in muscle regulation[115]. In two separate case reports, co-administration of prednisone and hydroxychloroquine led to vacuolar myopathy, which could be caused by the choice of doses, or could be indicative of true epistasis[116, 117]. Potential benefits of anti-lysosome co-therapy in glucocorticoid myopathy remain the subject of speculation.

Another putative parallel mechanism for GC-induced loss of muscle is downregulation of protein synthesis. Few human trials measured directly the effect of GC on protein synthesis in healthy volunteers. Brillion and colleagues[104] found that an 80 mg cortisol infusion over 13 hours led to 8% increase in non-oxidative leucine uptake, indicating an upregulation of protein synthesis. However, using a 200 mg cortisol infusion in the same protocol failed to cause a detectable change in protein synthesis compared to placebo, suggesting a biphasic response. Löfberg and colleagues[107] found that three days of 65 mg / day prednisolone caused a non-significant 21% increase in protein synthesis rate and a statistically significant 52% increase in the rate of protein degradation, based on the difference between arterial and venous levels of tritiated phenylalanine at leg level. Short and colleagues employed fractional synthesis rate (FSR), which describes the time rate of enrichment in muscle tracer, normalized to the circulating tracer concentration. They concluded repeatedly that, in leg muscles, 35 mg / day prednisone for 6 days “has no effect on [...] muscle protein metabolism or muscle function”[118, 119]. Some of these studies may have been underpowered (sample size n = 6-7) or may be troubled by the use of a small dose. Nevertheless, their validity is confirmed by the fact that, in each case, the expected hyperglycemic response to GC was observed.

The hypothesis that GC cause muscle loss by inhibition of protein synthesis is still debated, due to a plethora of indirect evidence. In Löfberg’s study, biopsies revealed a prednisolone-induced loss of muscle polyribosomes, interpreted as evidence for decrease in protein synthesis rate. Even in studies where GC failed to elicit reductions in protein synthesis, they inhibited translation-stimulating signals in muscle from anabolic factors such as insulin[120], branched chain amino acids[121], and exercise[122].

At a molecular level, it appears that Dexa inhibits anabolic signals centered on the Akt / mechanistic target of rapamycin (mTOR) axis. Rather than directly repressing this axis, GCs appear to reduce sensitivity of this axis to upstream stimuli. One study on humans described how Dexa inhibits branched chain amino acids’ ability to induce phosphorylation of mTOR substrates eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP) and p70-S6K[123]. In the same study, Dexa had no effect on another translation regulator, the α subunit of the eukaryotic initiation factor 2 (eIF2). More evidence has been obtained from animal models (discussed in a dedicated section).

In addition to GC excess, muscle weakness is observed with GC withdrawal[124], and by GC deficiency, illustrated by the Addisonian crisis[125]. In both hypercortisolism and hypocortisolism, effects on human muscle remain understudied. Animal models have been essential for the study of GC-induced muscle loss (discussed in the dedicated section). Human studies agree that GC-induced loss of muscle force is an objective finding caused by an increased proteolytic activity. Indirect evidence indicates that human GAML is associated with changes in protein synthesis. Current guidelines suggest GC discontinuation if myopathy develops, because proven mitigating interventions have not been developed.

## Muscle protection with androgen therapy

A series of historical circumstances brought anabolic androgenic steroids (AAS) in the attention of clinicians treating hypercortisolism in muscle. The same circumstances meant that utility of AAS therapy in glucocorticoid myopathy has never been fully explored.

Male hormones have been considered an efficacious anabolic therapy long before they were purified and tested. The effects of male castration, such as reductions in aggressiveness and muscle force, were discovered independently by many human civilizations, starting more than three thousand years ago. Castration is omnipresent in ancient mythology, and, more mundanely, in primitive farming. For almost as long, people perceived testis ingestion as a reversal of castration, thought to improve muscle force. Such perceptions were caused by the placebo effect alone, given that this testis active principle is almost completely degraded by liver.

Testis extract benefits received more attention starting around 1889, when Brown-Séquard published his theory about rejuvenating abilities of sperm. He thought that loss of sperm during aging or masturbation causes degradation in muscle and brain performance, and hypothesized that chemicals from sperm may pass into blood where they have “a most-essential use in giving strength to the nervous system and to other parts.” Consequently, he injected himself with a combination of sperm and testis extracts, which led to self-reported improvements in physical and intellectual abilities[126]. He describes how, at the age of 72, a single injection enables him stand for hours, or write longer scientific papers. Later on, he describes how testis extracts appeared to alleviate “serious affections of any kind,” including cachexia, pulmonary tuberculosis, cancer and leprosy ulcers[127]. Because the active principle in testis is made as needed, rather than stored in high-concentration depots, Brown-Séquard’s injections must have contained very little male hormones. His observations were likely caused by the placebo effect.

The cultural context in which Brown-Séquard worked introduced multiple biases in his experiments and conclusions. His mistaken theses were constrained into rather low-quality experiments, which luckily provided useful, testable, and eventually proven scientific hypotheses. First, the logical conclusion for Brown-Séquard’s theory would have been endorsement for semen therapy. Instead, due to the semen taboo, Brown-Séquard and his disciples resorted to surrogate interventions, such as vasectomy, believed to preserve sperm in the body, and injections with testis extracts. The introduction of injections gave a new lease of life to the therapeutic use of organ extracts, called “organotherapy,” which had been banished from the British Pharmacopoeia in 1788 after failing the test of oral administration. Some organotherapies were shams or even harmful. Yet a few of them provided evidence that specific parts of the body store or release into the blood stream chemicals, which subsequently induce changes in other specific parts of the body. This conjecture led the discovery of endocrine glands and the establishment of endocrinology as a science. In fact, androgen organotherapy provided the blueprint for GC discovery.

Second, the Victorian era was an age of body rediscovery. Georgian pastimes, such as cock fighting, horse racing, or cricket, were replaced by more muscular sports, such as football, rugby, gymnastics, and swimming. Bodybuilding became fashionable, with the first professional competition selling out Royal Albert Hall in 1901. Brown-Séquard’s promise of muscle without effort made testis organotherapy a widespread, well-earning business. When Voronoff was barred from practicing in Paris and judged as fraudulent by the Royal Society of Medicine, he took his testis transplant business to Algiers, where he received patients from all over the world (reviewed in [128]). Private sponsorship led to investment in androgen research, but with a focus on commercial rather than clinical efficacy.

Finally, Brown-Séquard’s era tolerated unscientific theories, which ignored the physical and intellectual ability of women. Brown-Séquard claimed that ovary extracts provide some benefits, but with “less power” than testis extracts[127]. Such conclusions stemmed from cultural biases rather than comparative experiments. In 1849, Berthold showed that, through testis implants, roosters regain male characteristics they lost through castration, such as aggressiveness, libido, and larger combs[129]. With maintenance of secondary sex characteristics as its sole ability, Berthold’s secreted agent was therefore androgenic. In contrast, Brown-Séquard claimed that his extract increases muscle force, without mentioning any virilizing side effects. Moreover, in 1935, Kochakian proved that urine-extracted “male hormone” stimulates muscle accretion in castrated dogs, that is, that it is anabolic[130]. While ultimately proven correct, the idea that “male hormones” were simultaneously androgenic, anabolic, and ergogenic was based on a cultural construct that confounded manliness and physical force, rather than the product of direct scientific evidence.

The belief in a male-secreted ergogenic substance inspired many commercial enterprises to sponsor research in male endocrinology, through the decades where the evidence was confined to changes in the combs of roosters. These dark ages ended in 1927, when McGee and Koch extracted a lipophilic virilizing mixture from rooster testis[131, 132]. A pure and even more androgenic chemical was extracted in 1935 from bull testis by Laqueur, working for Organon[133]. Laqueur named his discovery testosterone (Testo). Three months later, Butenandt and Ruzicka, sponsored by Schering and Ciba respectively, announced the development of manufacturing methods for synthetic testosterone, an achievement that brought them the 1939 Nobel Chemistry Prize (reviewed in [134]). The first beneficiaries of the new drug were hypogonadal men, that is, adult males with pathological decreases in circulating Testo. At the University of Chicago, Kenyon tested Testo on four eunuchoid patients of testicular and pituitary etiology. Daily injections of 25 mg testosterone propionate (Tp) caused a doubling in prostate and penis size[135] after less than two weeks, thus establishing the efficacy of Testo replacement therapy in hypogonadal men. Except for a few, narrow exceptions, this population was and remains the sole generally accepted, FDA-approved indication for Testo therapy[136, 137, 138]. Recent Testo preparations are also recommended for some breast cancers, but this indication is describes as having small, unpredictable efficacy.

Due to manufacturing costs, limited commercial target, and governments’ lack of interest, Testo therapy traversed a very long experimental stage, which could easily be called “the second dark age of androgens.” Only in 1953, did the FDA give its first approval for an androgenic therapy, a Testo enanthate injection. Then, as now, FDA’s approval was based on Testo ability to restore normal levels of androgens, rather than other, more functional or curative, outcomes[137]. However, in 18 years of life as experimental drugs, androgenic steroids have been trialed in diverse diseases, including male functional impotence[139], unwanted lactation[140], uterine bleeding and dysmenorrhea[141], or osteoporosis[142]. These early studies share the extremely small sample size, and the scarcity of controls, blinding, and objective outcomes. For example, a study found that 14-35 injections of Tp (cumulative dose 255-455 mg) caused an improvement of acne in half of the male participants[143]. Such findings are at odds with more modern trials, where weekly i.m. androgen injection lead to an increase in absolute risk of acne by 15%, in healthy males[144], and are possibly explained by the variability in the androgen arm, small sample size (n = 12), lack of blinding, and early stopping in the placebo arm. Nevertheless, these trials are, in many cases, the only source of information about the action of Testo in the normogonadal population. For example, early trials of oral methyltestosterone revealed its hepatic toxicity. Fifty years later, those limited trials are still the main factor discouraging the development of oral synthetic androgenic therapies.

The second dark age of Testo was a time of poor knowledge and poor clinical study design. Yet in these years, androgenic steroids first gained their reputation as ergogenics. Kenyon noted in his studies on eunuchoid men that Testo injections helped them gain weight through protein accretion, as demonstrated by a reduction in urinary nitrogen despite fixed dietary intake. Other trials evidenced benefits from androgenic therapy in muscle-depleting conditions, including thyrotoxic myopathy[145] and muscular dystrophy[146]. By 1940, Kenyon confirmed that Tp caused nitrogen retention, caused by increased protein accretion, even in healthy men and women[147]. In 1942, Samuels and colleagues concluded that Testo does not change grip strength in healthy males[148]. According to a meta-analysis[149] and my literature search, no other test of androgens’ effect on muscle strength was published until 1968. Despite the lack of evidence, androgens were used as ergogenics in healthy people, starting with Olympic athletes around 1954[150].

As exemplified by the ergogenic hypothesis, benefits of androgen therapy on men with Testo deficiency have been extrapolated by clinicians and theoreticians to other muscle-depleting conditions, and even to healthy humans. One of the conditions associated with loss of muscle mass that clinicians hoped to improve was hypercortisolism. In 1941, Albright showed that the newly discovered Tp, in 25 mg daily injections, was better than estradiol benzoate, progesterone, or vitamin D in restoring nitrogen balance in three cases of Cushing’s disease[151]. Similarly, in 1950, the Mayo Clinic team who discovered cortisone remarked that, in one case, 25 mg Tp daily injections reduced urinary nitrogen losses caused by 200 mg cortisone administration[13]. Some of the aforementioned researchers publish similar case reports, sharing the small sample size and the use of surrogate outcomes[152]. These shortcomings do not prevent each investigator from subjective claims of improvements in physical function.

During the 1950’s, AAS became part of the standard of care for endogenous hypercortisolism during the gap between diagnosis and curative surgery. However, this gap narrowed to a few weeks, due to improvements in differential diagnosis. Today, development of accurate cortisol assays allowed the measurement of its changes in response to Dexa, thus discriminating conditions where feedback mechanisms fail (mainly endocrine neoplasms) from cortisol-stimulating non-endocrine conditions. ACTH assays differentiate ACTH-independent cases (typically adrenal tumors) from the ACTH-dependent ones (usually localized in the pituitary). Modern imaging, including computed tomography of the adrenal and magnetic resonance imaging of the pituitary, rapidly identify the target for surgery. AAS therapy is now confined to inoperable cases, including unidentified ectopic sources of ACTH or cortisone. Even these cases benefit from more targeted interventions (see Table 1).

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| Class | Medications |
| ACTH inhibitors | * Subtype 5 somatostatin receptor agonists: pasireotide (FDA-approved)[153] * Dopamine D2 receptor blockers: cabergoline |
| 11-β hydroxylase inhibitors | Metyrapone, mitotane, ketoconazole. |
| Inhibitor of 3 β-  hydroxysteroid dehydrogenase | Trilostane (EMA-approved, FDA-withdrawn)[154] |
| Inhibitor of the  cholesterol side-chain cleavage enzyme | Aminoglutethimide |
| GR antagonist | Mifepristone (FDA-approved)[155] |

Table 1. Pharmacological agents used in Cushing’s disease of unidentified ectopic, or diffuse localization (reviewed in [1, 2])

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## Section Two

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### Subsection One

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Table 2. My Other Table.

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# CHAPTER TWO

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# CHAPTER THREE

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# BIBLIOGRAPHY

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