A murine model of glucocorticoid myopathy alleviation using androgen therapy

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Keep calm and carry on.

Acknowledgments

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Abstract

Glucocorticoids (GC) are used widely for the treatment of a large number of inflammatory conditions. Loss of muscle mass and 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 lacking.

Here, I established a mouse model of GAML. Young adult male mice receiving 0.25 mg/kg/day dexamethasone (D) s.c. daily, for a week, lost 3% of their body weight. Through NMR lean body mass quantification and muscle dissection, a loss of more than 10% of their muscle mass was lost. More than half of the muscle loss was reversed by co-administration of 0.7 mg/kg/day testosterone (T). This is the first mouse model of GAML alleviation by T.

Intramuscular atrogene expression and proteasome catalytic activity were upregulated by D and suppressed by T co-administration. T co-administration caused intramuscular downregulation of atrogene-activating Foxo transcription factors. Intramuscular pro-autophagic REDD1 and Klf15 were repressed by T co-administration. T co-administration reduced the autophagosome-characteristic lipidated form of LC3B. Translation regulators 4E-BP, eIF3f and eIF2 did not change significantly as a result of androgen co-administration. Calpains activity and levels were unchanged by D and T.

C2C12 differentiated myotubes were used to determine the effects of T and D on protein synthesis and degradation. Myotube diameters were reduced by D, while T co-administration suppressed D effect. Protein degradation was increased

by 24 hour D treatment. D-stimulated protein degradation was inhibited by proteasomal inhibitor MG132, and, to a lesser degree, by lysosome inhibitor chloroquine. T co-administration returned protein degradation to basal levels. Protein synthesis response to D and T did not correlate with the observed phenotypes.

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 effects.

In conclusion, T protective action in GAML is mainly anti-catabolic, through reversal of proteasome and autophagosome upregulation induced by D. T stimulates a potentially protective intramuscular IGF-I response. Different models are needed to determine the role of protein synthesis and of IGF-I in GAML.

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Abbreviations

24HUC 24-hour urine cortisol

3MH 3-methylhistidine

4E-BP eIF4E binding protein

4E-BP1 eIF4E binding protein 1

AAP adrenal androgen precursor

AAS anabolic androgenic steroids

ACTH adrenocorticotropic hormone;

ActRIIB activin A receptor, type IIB

AMPK adenosine monophosphate kinase

AP-1 activator protein 1

AR androgen receptor

BCAA branched-chain amino acids

bFGF basic fibroblast growth factor

COPD chronic obstructive pulmonary disease

CS Cushing's syndrome

CSA cross-sectional area

CT computed tomography

Dexa dexamethasone

DHEA dehydroepiandrosterone

DHEAS dehydroepiandrosterone sulfate

DHT dihydrotestosterone

EC50 half maximal effective concentration

eIF2B eukaryotic initiation factor 2B

eIF4B eukaryotic initiation factor 4B

eIF4E eukaryotic translation initiation factor 4E

eNOS endothelial nitric oxide synthase

FDA Food and Drug Administration

FSH follicle stimulating hormone

FSR fractional synthesis rate

GAML GC-associated muscle loss

GC glucocorticoid

GH growth hormone

GLUT glucose transporter

GnRH gonadotropin-releasing hormone

GR glucocorticoid receptor

GRE glucocorticoid responsive-elements

GSK3 glycogen synthase kinase 3

HDAC histone deacetylase

HIF 1a hypoxia-induced factor 1a

HOMA-IR homeostatic model assessment - insulin resistance

HPA hypothalamic - pituitary - adrenal

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

LH luteinizing hormone

MAFbx muscle atrophy F-box

MAPK mitogen-activated protein kinases

MEF2A myocyte enhancer factor 2A

MR mineralocorticoid receptor

MRF muscle regulatory factor

Mrf myogenic regulatory factor

mTOR Mechanistic Target of Rapamycin

MuRF1 muscle RING-finger protein-1

Myf myogenic factor

MyHC myosin heavy chain

NEFA non-esterified fatty acids

NFAT nuclear factor of activated T-cells

NMJ neuro-muscular junction

OMIM Online Mendelian Inheritance in Man

p70-S6K ribosomal protein S6 kinase, 70 kDa

p90-RSK ribosomal protein S6 kinase, 90kDa

PDK1 3-phosphoinositide-dependent protein kinase 1

PGC-1 PPARg coactivator 1

PI3K phosphatidylinositol 3-kinase

PI3K phosphatidylinositol 3-kinase

Pitx paired-like homeodomain

PLC phospholipase C

POMC pro-opiomelanocortin

PPAR peroxisome proliferator-activated receptor

REDD1 regulated in development and DNA damage responses-1

SGKL serum and glucocorticoid-inducible kinase-like kinase

SOS Son of Sevenless

STAT Signal Transducer and Activator of Transcription

T testosterone

Tbx T-box

TCF4 transcription factor 4

TGF-β transforming growth factor-beta

Tp testosterone propionate

TSC2 Tuberous Sclerosis Complex 2

VEGFR VEGF receptor

Chapter 1

Clinical questions and evidence

Cushing's syndrome

Through the detailed case series written by Harvey Cushing[4], 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 were more variable in manifestation and therefore harder to unify in a single clinical entity. Even when macroscopic hypertrophy of pituitary were localized to a gland subdomain, it was unclear whether observed pathology could be attributed to a hypersecretion from the hypertrophied sector, or to a deficiency in the neighboring compressed structures. Similarly, pituitary extracts caused multiple, and even opposite effects, in animal models[5], suggestive of a mixture of hormones.

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[6]. 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, amenorrhoea / 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[7]. 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[8].

Today, we know that the truth was more nuanced. Oversecretion 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)[9]. 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 [10]).In theory, pseudo-Cushing responds to suppression tests by low dose Dexa, while true CS does not. In practice, specificity is very low and sensitivity is 95%, so latest guidelines are recommending against suppression tests. Tumors may be less responsive in Dexa suppression and in ACTH stimulation tests than hyperplasias per PMID: 4342889

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 oversecretion, followed by secondary hypercortisolism, is termed Cushing's disease (reviewed in [11]). 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 in spite of low ACTH. In primary hypercortisolism, ACTH is typically suppressed by negative feedback. Adrenal neoplasms are 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).

Despite being caused by a diverse set of HPA pathologies, CS is quite invaiable in its ability to cause muscle impairment.

Glucocorticoid therapy

A series of serendipitous decisions brought impressive knowledge about CS of non-pituitary etiology (reviewed in[12]). 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[13], impressive improvements in those cases triggered redirected corticosteroid research.

Previous work did describe multiple effects for adrenal extracts, but little was known about purer preparations, such as cortisone. In fact, adrenal research was discouraged prior to cortisone purification, because less pure extracts combined antagonistic hormones in variable doses, leading to the impression that they lack a defined pharmacological effect. However, even with purified cortisone, Hench saw a very diverse set of consequences for cortisone administration[14].

First, cortisone’s action on metabolism was accessible even to the less sophisticated clinical measurements used 60 years ago. Patients receiving cortisone gained weight. Chronic cortisone therapy led to accumulation of adipose tissue, often in ectopic locations, such as the interscapular “buffalo hump”. Cortisone also induced hyperglycemia which can induce 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 [15, 16]). Some of these anti-inflammatory effects, such as reduction in the number of circulating white blood cells, are ample and robust. The cellular and molecular anti-inflammatory mechanisms are still subject of active research.

The knowledge gap around the anti-inflammatory actions of GCs is in part caused by the immunology progress. Still, some questions remain open, and 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 steroids, 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) [17]. The classical effect of the GRE-GR interaction is increased transcription for neighboring target genes (transactivation), as it is the case in polymorphonucleate cells with interleukin 1 (IL-1) receptor type II (IL-1RII) [18], a decoy inhibitor of 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)[19]. 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[20]. Within minutes, GC administration induces vasodilation, through direct, non-genomic activation of phosphatidylinositol 3-kinase (PI3K) leading to activation of endothelial nitric oxide synthase (eNOS)[21].

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 5 mg / kg physiological dose enhances the immune skin delayed-type hypersensitivity, while a pharmacological 40 mg / kg dose yields the more typical GC immunosuppressive behavior [22]. This behavior, suggestive of a U (or inverted U) shaped response curve, is called hormesis or biphasic response, and 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[23], thus lowering the price and creating the opportunity for large-scale trials. The Empire Rheumatism Council organized a randomized trial comparing cortisone with acetylsalycilate, and concluded that there is no benefit in

cortisone[24]. 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 misinformative,

By the time this trial was reported, the chemists were providing more effective and safe synthetic GCs (reviewed in [25]). While synthesizing esters with a longer half-life, Scherring chemists introduced a double bond in the A ring of cortisone, thus discovering prednisone, the first widely used oral GC[26]. In addition to an improved ability to induce hyperglycemia, prednisone lost some of the cortisone's ability to cause edema. NIH researchers synthesized and characterized prednisone's active metabolite, prednisolone[27]. In a trial of prednisolone versus acetylsalycilate in rheumatoid arthritis, the GC provided better functional protection to the articulations[28].

At Squibb, insertion of a halogen atom was found to abolish GCs ability to induce hyperglycemia[29, 30]. In 1958, Merck chemists led by Arth modified cortisol with the unsaturated A ring (Δ^1), the fluoride addition at position 9α, and with a methyl group on the 16α position to obtain dexamethasone (Dexa) [31, 32]. 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.

Dexa is completely unable to cause edema and electrolyte imbalance. Nevertheless, Dexa is 52 times more potent in suppressing endogenous GC secretion, and 35 times more potent in causing hyperglycemia[33, 34]. In animals, Dexa is 160 times more potent than cortisol in the inhibition of granuloma test of immune function[35]. These findings suggest that 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. Further supporting the idea of a unique

Dexa receptor, efforts to synthesize steroids with anti-inflammatory action that do not interfere with metabolism have failed. Compounds such as A276575[36] and RU 24858[37] did not reach the human studies stage. Mapracorat[38] did nor progress beyond phase II clinical trials. Therefore, clinicians prescribing GCs in these five decades had to balance therapeutic benefit with metabolic side-effects.

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. The list of Food and Drug Administration (FDA)-approved indications for cortisone, Dexa, and prednisone is often changed, as better, more specific drugs are developed, and more precautions are added[39, 40, 41].

The trivial case for using GC therapy is in hormone replacement, such as in adrenocortical insufficiency (reviewed in [42]). GC therapy is suitable for acute immune or allergic conditions, such as seasonal rhinitis (reviewed in [42]). GCs are relatively safe in topical applications in dermatological conditions (pemphigus, psoriasis, most types of dermatitis; reviewed in [43]). Similarly, GCs are commonly used in eye inflammatory conditions[44, 45], such as diffuse posterior uveitis and optical neuritis.

As envisaged by Hench in his Nobel Lecture, GCs do not address disease causes and are recommended for temporary respite. For many autoimmune diseases, there are more specific therapeutic alternatives, often addressing the cause of disease. In addition to the glucose metabolism disturbance, long term GC administration causes osteoporosis and muscle loss. On the balance of benefits and drawbacks, GCs are recommended for life-threatening or impairing immune reactions, such as in polymyositis (reviewed in [46]), severe sarcoidosis (reviewed in [47]), and disseminated pulmonary tuberculosis. 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[48, 49, 50].

Even 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[51, 52], ulcerative colitis[53, 54], and idiopathic nephrotic syndrome[55].

GCs have been used off-label in many other diseases. Most commonly, GCs are perceived by physicians as a fall-back therapeutic alternative for cerebral hypertensive conditions, despite sparse evidence for efficacy in specific conditions. Small trials suggest GCs reduce vasogenic cerebral edema[56] and prevent acute mountain sickness[57], while systematic reviews suggest they might in fact worsen outcomes for acute brain trauma victims[58]. 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 which have been, and may still be, misconstrued as support for the use of GCs. Every day practice may drift further apart for the officially sanctioned label, thus providing new opportunities for unjustifiable overdose.

Due their widespread use, GCs are likely to cause covert iatrogenic CS in a large population, impairing muscle 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[59]), despite a recent boost from incidental findings during imaging tests for other needs. 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[60].

It is a life-threatening disease, with untreated patients having a median survival rate of 5 years after diagnosis[61]. 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 [62]). Even after surgical adjustments of the hyperactive pituitary, the quality of life for CS patients lags behind that of the unaffected population.

At presentation, about two thirds of Cushing's syndrome cases present with muscular complaints, with similar incidence among pituitary and adrenal conditions[63]. Among patients suspected of endogenous CS, one fifth are referred to the endocrinologist due to muscle weakness[64]. Two-fifths of those whose endogenous hypercortisolism is successfully corrected by surgery still complain of fatigue[65].

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. Every year, about 1% of the Americans and British receive some form of GC[66, 67]. GCs are likely even more often prescribed in the developing world, due to affordability and lack of alternatives, poor access to health care not withstanding. Dexa and cortisol are the only drugs listed five times in the World Health Organization's List of Essential Medicines[68].

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 prescribed 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[69]. Where regulated, over-the-counter GC creams rarely cause CS on their own, but may 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. In a study in Togo, one fifth

of the skin-bleaching creams listed GC as an ingredient[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 likely 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[60]. 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 1 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 $0.4\ \mathrm{mg/(kg\,d)}$ 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. Mastaglia estimated that, in 1982, the most common cause of iatrogenic muscle weakness was caused by GC[77].

There are differences between GC-induced cardiovascular changes, depending on the nature of the GC. Endogenous GC, 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 cause essentially the same disabling effects on muscle[78], indicating that muscle

damage is mediated by GR. GCs do differ quantitatively in their ability to cause myopathy. Myopathy is invariably induced in two weeks by either $0.2\,\mathrm{mg/(kg\,d)}$ Dexa[79], or by $0.5\,\mathrm{mg/(kg\,d)}$ prednisone[80]. Based on other metabolic effects, it is likely that the catabolic potency ratio is even more tilted towards Dexa than the referenced studies indicate, but more detailed human pharmacodynamic studies are lacking.

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 focused 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 faster extinction of the action potential, which may be caused by a reduction in the number of fibers, or by fiber atrophy[82]. Based on the evidence that GC is a muscle fiber disease, they coined the phrase "steroid myopathy". Similar electromyographic 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 steroid myopathy is not a standalone disease or syndrome, terminology has never been standardized. In the present work, where an experimental and objective angle is taken, through the use of an animal model, the condition of interest 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 steroid 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 electromyographic changes (that is, the reduction in action potential duration) is proportional with the total GC dose[87]. These findings imply that steroid myopathy can be induced in shorter periods, if the GC dose is extremely high. Foye and colleagues drew a distinction between "classical" chronic steroid myopathy, induced "within weeks to years", and acute steroid 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 overlapping to a great extend.

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 health 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]. 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. Such findings suggest that GC-induced weakness 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 and muscle mass 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].

In an early study of chronic hypercortisolism, it was found that all types of fibers are affected by GC[94]. In more recent ones, a type-specific effect was found. Women with CS have 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[95]. Similar histological findings were made in renal transplant patients receiving 25 $\mathrm{mg}/(\mathrm{kg\,d})$ prednisone over three months[96]. In the latter, GC caused an increase in the cross-sectional area (CSA) of the type I and IIa (slow twitch, oxidative / glycolytic) fibers. Gains in the ratio fast-to-slow twitch fibers are associated with insulin resistance[97]. Diameter increases in spite of loss of function and protein content have been explained by a disorganized intracellular structure. A more practical consequence of these findings is the dereliction of CSA measurements in GAML.

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 [98]). 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 dividing cells from human muscle, at times assumed to be myoblasts. These human “myoblasts” do not proliferate in the absence of at least 1 μM ([99], and personal observation; data not shown). For comparison, maximum normal concentrations of endogenous cortisol in humans is 0.78 μM[100], that is, tens of

times less potent. Therefore, it is impossible to conceive an experiment where these human myoblasts are subjected to meaningful manipulations of GC concentration. Moreover, it appears that GCs are vital for human muscle development and maintenance. This implies that cell lines which do not require GC, such as those surviving in serum-free media, may be less accurate models of muscle.

There are no published cases of increase in circulating myoglobin or creatin kinase in response to GC monotherapy, or as a consequence of Cushing's disease.There are some mentions of the opposite in Uptodate, but the rumor has no literature to support it. Not even anecdotal. 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.

From its first trial, GC therapy ability to induce a negative nitrogen balance, through an ample increase in urinary creatine and creatinine, was interpreted as evidence for stimulation of tissue protein breakdown[101]. 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 proteolysis upregulation[102]. 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[103]. 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[104]. The same acute treatment causes an increase in 3-methylhistidine (3MH), a degradation product specific to muscle actin and myosin[105]. Similar increases in 3MH are seen with control diet in chronic GC excess of endogenous or exogenous nature[94]. 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 major proteolytic systems, the

proteasome-ubiquitin system and the autophagosome (discussed in later sections), have been discovered and dissected. But 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[106]. The result is unsurprising, given that the control of the proteasome system may be exercised in other, unprobed ways, such as E3 ligases.

Recently, pharmacological inhibition of the proteasome become widely available. The first proteasome inhibitor, bortezomib, is recommended by the FDA for multiple myeloma and mantle cell lymphoma[107]. The second generation, irreversible proteasome blocker carfilzomib is also approved for advanced myeloma therapy[108]. 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[109]. In another trial, addition of 20 mg Dexa to bortezomib lowered the rate of fatigue from 57% to 25%[110]. Taking into account the large variability between trials and the use of a atypical population, the comparison is of marginal use, but it does not appear that the combination (Dexa + bortezomib) is more muscle protective than Dexa alone. Clinical studies directly addressing this comparison in patients prescribed high-dose GC are recommended, given that the most commonly accepted hypothesis centers on the proteasome as main effector of GC-induced muscle loss. It is still more likely to find that bortezomib provides muscle protection. Proving a beneficial action of bortezomib in co-administration with GC will have major practice 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[111]. Interestingly, hydroxychloroquine is also recommended for rheumatoid arthritis, where it may be prescribed for up to six months[112]. 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 autophagosomal markers in muscle, demonstrating autophagosome's importance in muscle regulation[113]. 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[114, 115]. Potential benefits of anti-lysosomal co-therapy for the atrophying muscle 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[103] found that a 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 hormesis may confound experiments. Moreover, this finding was never replicated. Löfberg and colleagues[106] 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 phenyl alanine 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”[116, 117] . Some of these studies may have been underpowered (sample size n = 6-7) or may be troubled by the use of a small dose, but 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[118], branched chain amino acids[119], and exercise[120]. In the case of branched chain amino acids, Dexa inhibit their ability to induce phosphorylation of eIF4E binding protein 1 (4E-BP1), despite lacking any independent action on 4E-BP1[121]. Dexa lacked similar action on other translation regulators, p70S6K and eIF2α.

In addition to GC excess, muscle weakness is caused by GC withdrawal[122], and by GC deficiency, illustrated by the Addisonian crisis[123]. In both hyercortisolism and hypocortisolism, effects on human muscle remain understudied. Human studies concord that GC-induced loss of muscle force is an objective finding caused by an increased proteolytic activity. Indirect evidence indicate human GAML is associated with changes in protein synthesis. In the absence of other proven mitigating interventions, current guidelines suggest GC discontinuation if myopathy develops. Animal models have been essential for the study of GC-induced muscle loss, although they have been confounded by hormesis (discussed in future sections).

In conclusion, CS of various etiologies leads to an increase in muscle protein catabolism, and, less certainly, to a reduction in muscle protein synthesis.

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 steroid 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[124]. 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[125]. Because the active principle in testis is made as needed, rather than stored in high-concentration depots, it is now obvious that these observations were the product of preconception.

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 pattern to GC discovery.

Second, the Victorian era is an age of body rediscovery. Georgian pastimes, such as cock fighting, horse racing, or cricket, are replaced by more muscular sports, such as football, rugby, gymnastics, and swimming. Body building becomes fashionable, with the first professional competition selling out Royal Albert Hall in 1901. Brown-Séquard's promise of muscle without effort makes testis organotherapy a widespread, well-earning business. When Voronoff is barred from practicing in Paris and judged as fraudulent by the Royal Society of Medicine, he takes his testis transplant business to Algiers, where he receives patients from all over the world (reviewed in [126]). 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[125]. 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[127]. 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[128]. 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 evidence.

The belief in an 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 end in 1927, McGee and Koch extract a lipophilic virilizing mixture from rooster testis[129, 130]. A pure and even more androgenic chemical is extracted in 1935 from bull testis by Laqueur, working for Organon[131]. Laqueur names his discovery testosterone (T). 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 [132]). At the University of Chicago, Kenyon tests T on four eunuchoid patients of testicular and pituitary etiology. Daily injections of 25 mg testosterone propionate (Tp) cause an doubling in prostate and penis size[133] after less than two weeks, thus establishing the efficacy of T replacement therapy in men with pathological decreases in circulating T. With few, narrow exceptions, this population was and remains the only generally accepted, FDA-approved indication for T therapy[134, 135, 136].

Recent T preparations are still recommended for some breast cancers, but this indication is limited to a few, unpredictable, cases.

Due to manufacturing costs, limited commercial target, and governments' lack of interest, T therapy traversed a very long experimental stage, which could easily be called "the second dark age of androgens". Only in 1953, FDA gives its first approval for an androgenic therapy, a T enanthate injection. Then, as now, FDA's approval was based on T ability to restore normal levels of androgens, rather than other, more functional or curative, outcome[135]. But in 18 years of life as experimental drugs, androgenic steroids have been trialled in diverse diseases, including male functional impotence[137], unwanted lactation[138], uterine bleeding and dysmenorrhea[139], or osteoporosis[140]. 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[141]. 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[142], 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 T in the normogonadal population. For example, early trials of oral methyltestosterone revealed its hepatic toxicity, with the effect that, 50 years later, the development of oral androgenic therapies is still discouraged.

The second dark age of T were times of limited knowledge, and even more limited adherence to the principles of clinical research. Yet in these years, androgenic steroids first gained their reputation as ergogens. Kenyon noted in his studies on eunuchoid men that T injections helped them gain weight through protein accretion, as demonstrated by a reduction in urinary nitrogen. Other trials evidenced benefits from androgenic therapy in muscle-depleting conditions,

including thyrotoxic myopathy[143] and muscular dystrophy[144]. By 1940, Kenyon confirmed that Tp caused nitrogen retention, caused by increased protein accretion, even in healthy men and women[145]. Interestingly, in 1942, Samuels and colleagues state that T does not change grip strength in healthy males[146]. According to a meta-analysis[147] and my literature search, no other test of androgens' effect on muscle strength is published until 1968. Despite the lack of evidence, androgens are used as ergogens in healthy people, starting with Olympic athletes around 1954[148].

As exemplified by the ergogenic hypothesis, benefits of androgen therapy on men with T deficiency have been extrapolated by clinicians and theoreticians to other muscle-depleting conditions, and even to healthy humans. Naturally, one of the conditions associated with loss of muscle mass that clinicians hoped to improve was hypercortisolism. In 1941, Albright shows 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[149]. 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[14]. Some of the aforementioned researchers publish similar case reports, sharing the small sample size and the use of surrogate outcomes. 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. 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, identify the target of 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 (see1.1).

Similarly, the opportunities for AAS as adjuvant to GC therapy are very limited. Many of the diseases previously treated by high-dose GC are now treated with more specific drugs. As practitioners became more accustomed with the risks of GC therapy, doses and durations were reduced. With the exception of life-threatening conditions, typical GC prescriptions switched to lower-potency compounds, such as prednisone or even cortisol. In particular, practitioners became well aware of the issues of GC withdrawal syndrome, where adrenal atrophy is aggravated by some other, still undiscovered, component[122]. By mid-1970's, it became common advice that "prescriptions for [glucocorticoid] steroids should not be refillable"[153]. By the time modern trials with AAS began, the incidence of overt hypercortisolism have been greatly reduced. Despite a potential epidemic of covert hypercortisolism, with deleterious effects of life quality and expectancy, the interest for studies on hypercortisolism has largely waned. Clinical studies investigating the benefits of AAS in hyepercortisolism are scarce and small-scale. For example, there are no significant-size clinical studies analyzing the effect of AAS on the muscle strength of the endogenous CS patient.

An unblinded trial observed AAS-induced increases in lean body mass and apendicular muscle mass, in men already receiving an average of 6 mg prednisone a day over 9 years[154]. A randomized, blinded, placebo-controlled trial by Crawford and colleagues tested the benefits of testosterone or nandrolone decanoate as an adjuvant to chronic GC therapy for diverse pathologies[155]. The exposure to GC was an average of 12 mg prednisone a day, over more than 8 years, and was already causing osteopenia, hyperlipidemia, hypercholesterolemia, and a reduction in quality of life compared to historical controls[156]. Such findings

Class	Medications
ACTH inhibitors	• Subtype 5 somatostatin receptor agonists: pasireotide (FDA-approved)[150] • Dopamine D2 receptor blockers: cabergoline
11-β hydroxylase inhibitors	Metyrapone, mitotane, ketoconazole.
Inhibitor of 3 β-hydroxysteroid dehydrogenase	Trilostane (EMA-approved, FDA-withdrawn)[151]
Inhibitor of the cholesterol side-chain cleavage enzyme	Aminoglutethimide
GR antagonist	Mifepristone (FDA-approved)[152]

Table 1.1: Pharmacological agents used in Cushing's disease of unidentified ectopic, or diffuse localization (reviewed in [1, 2])

could arguably be considered evidence for mild iatrogenic CS in this study sample. After six months of 200 mg testosterone injections every other week, the AAS group had higher bone density, muscle mass an strength, and a better quality of life, compared to the placebo group. To date, Crawford's study is the best evidence for effectiveness of AAS as adjuvant in GC therapy.

In a subset of CS patients, androgen administration improves muscle mass, and, presumably, quality of life.

Hypercortisolism-induced changes in endogenous androgens levels

The period when AAS were frequently used as therapy of CS or as adjuvant to high-dose GC pre-dates modern molecular biology and genomics. Therefore, there are no published clinical trials to describe in molecular terms the interaction of GCs and AAS at muscle level. Most of our knowledge is derived from animal models (discussed later). On the other hand, clinical observational studies of circulating biomarkers remain common, and reveal an interesting interaction between the two classes of steroids. More specifically, in many cases. hypercortisolism suppresses endogenous AAS. Because loss of endogenous AAS, also termed hypoandrogenism, is associated with loss of muscle mass and strength[157, 158], an AAS replacement strategy in hypoandrogenic hypercortisolism may be beneficial for the muscle.

A series of trials observed the effect of short-term (hours or days) hypercortisolism in healthy volunteers. Experimental acute hypercortisolism represses circulating levels of T, in a reversible manner, in males and, to a lesser degree, in females [159, 160]. The mechanisms through which hypercortisolism causes hypoandrogenism are still to be elucidated. Some studies suggest that acute hypercortisolism downregulates the pituitary-secreted, T-upregulating,

luteinizing hormone (LH)[161, 162, 163]. Others counter that GC induce hypoandrogenism even when LH is unchanged[164]. Another hypothesis is that the negative feedback loop repressing ACTH in hypercortisolism has a side effect of androgen suppression[165]. A few groups have even hypothesized the existence of another, still unknown hormone, synthesized from ACTH precursor, pro-opiomelanocortin (POMC), with the ability to stimulate androstenedione synthesis and secretion[166, 167]. Certainly, GC-induced repression of POMC should also repress this unknown androgen-stimulating hormone, but its existence was never proven.

In males, chronic hypercortisolism is also associated with hypoandrogenism. Long term prednisone therapy reduces circulating T levels[168]. Similar observations have been made in endogenous CS, where exposure is longer and, depending on etiology, ACTH is increased or decreased compared to normal. Some studies found that, in CS, LH and another gonad-stimulating pituitary hormone, follicle stimulating hormone (FSH) are lower than normal[169]. This has been explained as a CS-associated pituitary defect, with loss of LH response to stimulation by its hypothalamic regulator, gonadotropin-releasing hormone (GnRH)[169, 170]. Alternatively, others concluded that hypercortisolism impairs hypothalamic GnRH secretion[171]. Finally, a small study found that male asthma patients receiving long-term prednisone have lower circulating T levels despite increases in LH and FSH, and concluded that prednisone has an direct inhibitory action on the testes[172]. Despite disagreeing on the mechanism, all these studies agree that chronic hypercortisolism represses testicular androgen secretion.

AAS therapy does not change circulating cortisol levels[173], suggesting that a reverse effect probably does not exist. Similarly, it is possible that the direct effect of GC on AAS is only an artifact caused by pathological and pharmacological doses.

In both sexes, the most concentrated circulating steroids are dehydroepiandrosterone (DHEA) and its ester, DHEA sulfate (DHEAS), which

originate from the adrenal and, to a lesser degree, from gonads. Their most important role appears to be that of precursors for synthesis, in glands and peripheral tissue, of androgens and estrogens. DHEA has some affinity for the AR, which suggested it may be an AAS. Recent studies indicate that, in human female tissue, DHEA may in fact be a partial agonist, hindering the action of T[174]. DHEA and DHEAS, now termed adrenal androgen precursors (AAP), are upregulated by ACTH, through increased synthesis of DHEA in the adrenal and rapid bidirectional interconversion[175, 176]. Therefore, Cushing's disease and other conditions associated with increases in ACTH will present with increases in AAPs, while primary hypercortisolism will be associated with ACTH repression and consequent AAP decrease[177, 178, 166, 179]. Both types of hypercortisolism manifest GAML, despite opposite effects on AAPs, suggesting that AAPs changes are not mediating GAML.

In adult women, the regulation of AAS is more complex. During reproductive age and a few years afterwards, the main source of androgenic stimulation is the ovary[180], where T is an intermediate product in the synthesis of estrogens (reviewed in [181]). A feedback loop links LH and estrogens levels, with LH stimulating synthesis and secretion of estrogens from the developing and atretic follicles[182]. The reverse link is more complex, with estrogens inhibiting LH for most of the menstrual cycle[183], with the possible exception of ovulation. In the direct link, LH must stimulate ovarian T synthesis, but a reverse link, where T directly inhibits LH, is absent in women[184]. Although measurement methods and normal ranges are still to be perfected, it appears that circulating T level in women are reflecting the menstruation-related cyclical interplay of estrogen and LH, rather than being independently controlled[185, 186].

This sexual dimorphism differentiates male and female AAS response to chronic hypercortisolism. Women with CS have lower muscle mass compared to general population [187]. Decreased libido, a sign of hypoandrogenism in both genders, is reported by 40% of female CS patients[63]. But, in contrast to males,

females with CS have normal or even increased AAS synthesis and levels, compared to healthy controls [188, 189]. Four fifths of women with CS have menstrual irregularities, which has been attributed to hyperandrogenism, direct cortisol action, or depletion of LH or estradiol[171]. More than 75% of CS cases present with hirsutism, that is, male-patterned body and face hair growth in female patients, and a clear sign of hyperandrogenism[1, 63]. Women with CS-related hirsutism have androgen levels higher than healthy controls[190]. Per PMID 5922193, urinary androgens in female CS are repressed by Dexa. That could be a biphasic response. Other signs of hyperandrogenism, such as voice changes or acne, are rare in female CS.

In infants, tumors causing CS are exceedingly rare. In pediatric Cushing's disease and adrenocortical carcinoma, AAP circulating levels are usually normal for the age[191, 192]. Virilization signs such as change in voice, penile or clitoridian overgrowth, and hirsutism are common [193]. Published studies do not describe muscle changes in these children, possibly due to difficulties in assessment.

In adult female and in pediatric CS, virilization, muscle catabolism, and circulating androgens changes are not correlated. These examples suggest that relative hyperandrogenism in some tissues may be paralleled by relative hypoandrogenism in others. PMID 14329633 implies Cushing causes polycytemia, which would be another sign of hyperandrogenism. Some old textbooks also clam the same. There is no evidence in that paper nor anywhere else. PMID 10409572 shows acute Dexa in healthy men has no effect on hematocrit. Nieman reviews do not mention any RBC effect in CS either. For example, it may be possible that, in some tissues, excess GC activates the androgen receptor (AR)[194], the nuclear receptor specific for AAS at physiological concentrations. Because short-term Dexa inhibits AR expression in women's muscle[195], it may be possible that GCs interfere with T signals in a tissue-specific manner.

Understanding causality in the case of simultaneous muscle loss and

hirsutism is complicated by dose- and compound-dependent crossconversion of GCs to AAS and interference of GCs in AAS synthesis and degradation. It is unclear to what degree muscle loss in CS is influenced by the changes in endogenous AAP and AAS. Based on endogenous levels, it appears that AAS therapy may benefit men, but not women and children, with CS.

Interestingly, the Crawford and colleagues trial observed muscle protection by AAS as adjuvant to GC therapy although, at enrollment, these men had circulating T levels in the lower normal range[155]. This confirms that GC deleterious effects are not solely caused by hypoandrogenism.

Hypercortisolism is associated with hypoandrogenism solely in adult males. Androgen therapy for muscle protection in CS is predicted to benefit them more than other populations.

Molecular mechanisms of androgenic myoprotection in humans

GAML is a phenomenon well-studied, with its molecular mechanisms dissected in human studies. In contrast, the effect of AAS in GAML was studied in a few case reports, marred by the absence of objective physical outcomes and of molecular analysis. More information can be gleaned from the effect of AAS in other muscle-depleting conditions.

Most commonly, studies of AAS on muscle are carried on men with lower than normal circulating T and / or associated symptoms, also called hypogonadal. In male primary hypogonadism, rates of cortisol synthesis and degradation are typically normal[196]. In this population, AAS therapy, even with low, “replacement” doses, causes an increase in muscle mass and force[197, 198]. The gain in muscle mass is caused mainly by an increase in protein synthesis, as evidenced by increased nonoxidative uptake of labeled leucine[198]. Moreover, T

causes an increase in FSR of myosin heavy chain (MyHC), indicating that protein accretion is localized in the myotubes.

The referenced studies also measured leucine flux, a proxy for protein degradation, but failed to detect significant changes as a result to T therapy. The absence of a detectable change in leucine flux may be attributed to a true lack of effect on catabolism, or may be an artifact caused by the use of whole-body, rather than isolated muscle, methods.

Typical naturally-occurring male hypogonadism is usually associated with pleiotropic pathology, such as Klinefelter's syndrome, where deficient androgen synthesis may be complicated by other peripheral defects. For this reason, some studies were conducted in males with iatrogenic hypogonadism, induced by administration of GnRH agonists, such as goserelin or leuprolide, which disrupts and eventually abolishes LH secretion. Leuprolide-induced hypoandrogenism causes loss of muscle mass in healthy volunteers and in prostate cancer patients[199, 200]. In the former, most of the muscle losses are reversed if exogenous T is co-administered. Chemical castration causes decreases in both protein synthesis and degradation[201], suggesting that, in some cases, such as restoration of physiological levels, T supplementation may be followed by a paradoxical increase in protein degradation.

The protective action of AAS therapy in iatrogenic hypoandrogenism is not affected by co-administration of an aromatase inhibitor such as anastrozole[202]. Aromatase converts T in estradiol. The continuing muscle protection when T cannot be converted to estrogens demonstrates that muscle protection is an intrinsic ability of T. A more plausible mediator is the anabolic hormone insulin-like growth factor I (IGF-I), whose muscle expression is decreased by iatrogenic hypogonadism[201], and by short-term, high-dose Dexa.

Another well-studies group comprises older men, whose T levels and muscle mass are naturally declining[203, 204]. An argument has been made about benefits of T replacement therapy in this population. Multiple clinical studies

tested this hypothesis. In older men with low bioavailable T, muscle mass and strength is improved by 200 mg T every other week[205, 206]. As in hypogonadal men, muscle recovery can be localized to the contractile cells, as indicated by increases in the CSA of fast- and slow-twitching fibers[207]. No evidence of fiber type switching or fiber type-specific effects in response to AAS therapy has been seen. Instead, histological studies reveal that elderly treated with AAS have significantly more satellite cells[207].

T causes improvement in the net balance between protein synthesis and degradation at muscle level[208]. The cause of protein accretion is an increase in protein synthesis, as shown by an augmentation of mixed-muscle FSR[209]. Interestingly, some of this newly accrued protein is extracellular matrix, as indicated by the upregulation of circulating N-terminal propeptide of type III procollagen[210].

Ferrando and colleagues made the case for an anti-catabolic action of AAS in older men[3]. However, their study differs in key aspects from the other studies and the medical practice. They tested a variable, moderate dose of T on normogonadal older men, with the goal of maintaining a physiological T level. Moderate T therapy caused an improvement in muscle mass, strength, and net protein balance. However, they failed to observe an improvement in protein FSR, and concluded that the net protein balance improvement must be caused by T-induced inhibition of protein degradation. The failure to detect protein synthesis rate changes indicates that, perhaps due to unusual treatments, this study yielded unusual outcomes, which cannot be extrapolated to other studies or populations.

In support of their hypothesis, Ferrando and colleagues showed a significant decrease in the rate of phenylalanine disappearance at muscle level and in the proteasomal enzymatic activity. However, the data they provide show that, on the contrary, proteasomal activity is not reduced by T therapy. Digitizing their plot indicates that six-months of placebo changed the normalized lactastatin-sensitive

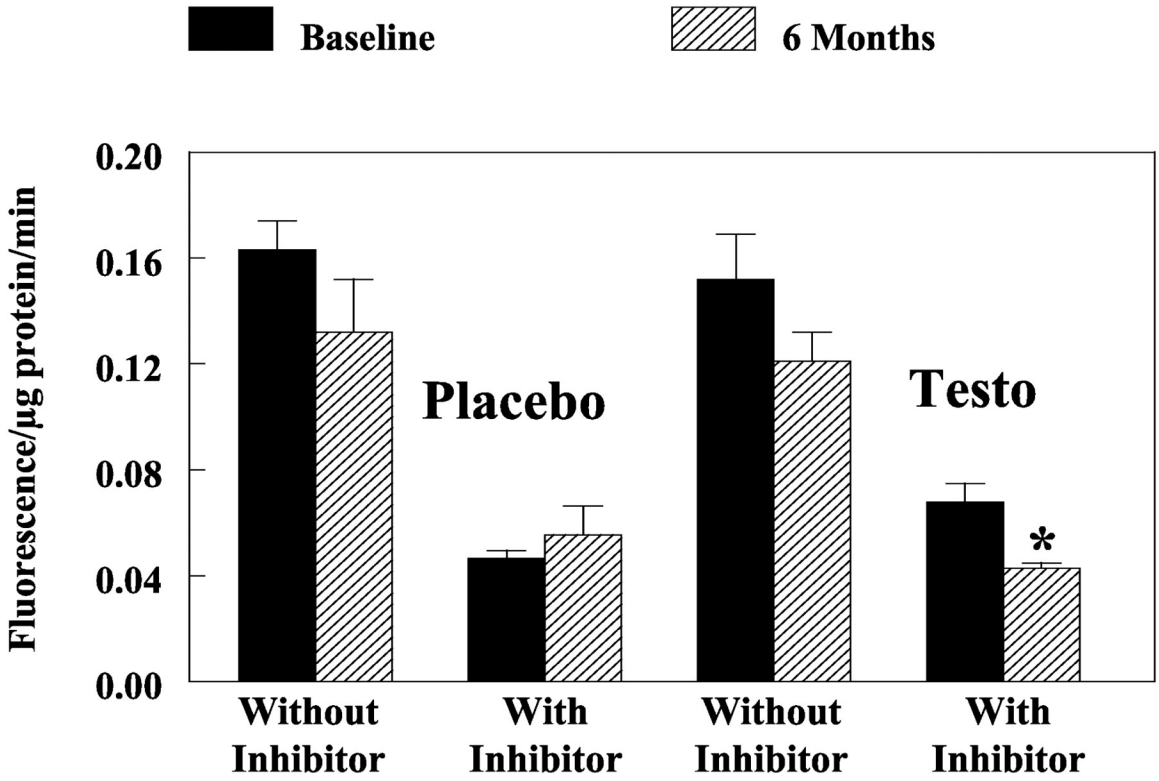

Figure 1.1: Changes in proteasome enzymatic activity following 6 months of T therapy (reproduced from [3] with permission).

proteasome activity from 0.12 to 0.076 relative units, whereas six months of T changed it from 0.084 to 0.078 relative units, a likely non-significant set of changes. The same group found a similar pattern of anticatabolic action, in a short-term trial of T on men with severe burns, once again doubled by an apparent absence of the pro-anabolic component[211]. It may be possible that the protective action of T changes qualitatively, depending on the cumulative dose. Alternatively, the anticatabolic action may be more salient when T supplementation is given to the normogonadal. The hypothesis that T inhibits protein degradation remains tempting, but better studies are needed.

In older men, T upregulated intramuscular and circulating levels of IGF-I[208, 212]. The protection of muscle force provided by T to the older

hypogonadal men is not hindered by co-administration of finasteride, an inhibitor of 5α-reductase which causes the transformation of T to 5α-dihydrotestosterone (DHT)[213]. Similar lack of effect was seen with dutasteride, a less specific inhibitor of 5α-reductase, added to exogenous T, in a younger, possibly less hypoandrogenic cohort[214]. In human males, conversion to DHT is not required or T's regulation of muscle mass. Possibly more relevant, T upregulates IGF-I in the muscle and in the serum of the older men[208, 212].

While women's endogenous AAS levels are lower than men's, it is unclear if benefits of T therapy outweigh the deleterious effects[215, 216]. There is no FDA-approved T preparation for women. Therefore the action of T in women losing muscle is yet to be investigated.

The best molecular observations on the action of T on muscle loss have been obtained from studies of HIV-positive men, who have significantly lower circulating T levels[217]. Some of the drugs used in HIV-AIDS, the anticachectic megestrol and the protease inhibitor ritonavir, may cause hypercortisolism, making it even more informative about the action of AAS in CS. AAS delays loss of muscle mass in AIDS wasting syndrome, leading to better quality of life[218]. Microarray analysis indicated that T-treated muscle upregulated, as expected, expression of genes from the IGF-I- and AR-stimulated signaling pathways[219]. Immunoblot confirmatory studies indicated that T caused the upregulation of a key component of the IGF-I signaling pathway, the protein kinase B, also known as Akt, in its Serine-473 phosphorylated, that is, activated form. Other genes upregulated by T are muscle development regulators, such as the myocyte enhancer factor 2A (MEF2A) and a host of macrophage-associated markers. In addition, T stimulated expression of genes from other pathways, including transcription factor 4 (TCF4) from the Wnt / β-catenin pathway, AMP kinase (AMPK), and the guanine nucleotide exchange factor Son of Sevenless (SOS), involved in the mitogen-activated protein kinase (MAPK) pathway. However, in the same study, at protein level, MAPK did not appear to be modulated by AAS

therapy. The referenced microarray study failed to find a change in expression of the major muscle regulator myostatin[220, 221], or of the two E3 ligases typically associated with muscle loss, muscle atrophy F-box (MAFbx) and muscle RING-finger protein-1 (MuRF1)[222, 223].

The histological and molecular findings from hypogonadal and HIV-positive males receiving AAS have been confirmed in many other pathologies that cause loss of muscle. AAS therapy improves muscle mass and strength in males with chronic kidney disease and liver cirrhosis[224, 225]. In men with COPD, 100 mg T enanthate injected weekly led to improvements in muscle mass and strength, potentially augmenting quality of life[226]. These improvements are caused by an increase in fiber CSA, regardless of fiber type, and by an upregulation of the IGF-I mRNA isoform known as mechanogrowth factor or IGF-IEc[227]. In these men, MyHC upregulated by T in these men was of isoform 3, also known as embryonic MyHC. This is also one of the two MyHC isoforms upregulated by T in the HIV-positive men. In the COPD cohort, embryonic MyHC was found in thinnest fibers, possibly marking them as newly formed.

A cross-sectional study split a cohort of males with heart failure, without cachexia and with normal circulating cortisol, T and ACTH levels in two halves based on their cortisol level. I am rebranding CHF as HF, following the recent guidelines from PMID 16160202. The subgroup with lower circulating cortisol achieved a higher peak work rate, suggestive of GC-induced muscle damage[228]. Some randomized trial showed that heart failure patients improve their muscle force with AAS therapy[229, 230]. However, a series of recent studies found deleterious cardiovascular effects of AAS[231, 232], which will discourage the use of T in heart failure. In fact, many of the aforementioned conditions where AAS was used are marred by higher cardiovascular risks and frailty, pressing the need for alternative, equally efficient, anabolic adjuvants. To this end, a deeper understanding of AAS therapy at molecular level is required.

In various conditions that cause muscle loss, AAS benefits share a pattern

including improved muscle mass and strength, fiber hypertrophy, tissue remodeling, and increased protein synthesis. In some conditions, AAS-driven muscle rehabilitation is associated with an increase in satellite cells and / or an inhibition of protein degradation. Putative molecular mediators known from animal models have not been confirmed in humans, with the invariant exception of the upregulation of IGF-I. Better clinical studies are required. Animal models and in vitro studies sketch the road ahead.

Chapter 2

Biological premises

Skeletal muscle histology

Muscles are specialized for their main ability, contractility. For mammalians, ability to move is vital for survival, meaning that a large portion of their bodies is muscle. In a cohort of 300 borderline overweight US Americans, skeletal muscle as a proportion of body weight was on average 41% for men and 31% for women[233]. Owing to the ability to measure individual muscles in any experiment, the scientific community has not been under pressure to develop accurate techniques that measure total skeletal muscle mass in mice. A proxy measure of murine skeletal muscle mass, lean body mass averaged 81% of total body weight in adult males (detailed in results section). Three fifths of the human body's protein is confined to the muscle contractile and support structures[234].

For the most part, the skeletal muscles confers the three-dimensional intricate conformation of the body, suggesting a complex, detailed organization, at least at macroscopic level. In contrast, at cell level, the relatively high specialization of the skeletal muscle leaves little space for diversity or inhomogeneity. Skeletal muscles are organized in anatomical units that may impose a force on two moving body segments (typically, bones), determining them to come closer to each other. This specificity of action is ensured by the presence of distinct, well-determined insertion points, to which skeletal muscles are attached by the means of tendons and aponeuroses (reviewed in [235]).

The tendons are dense connective tissue structure, which extend into the epimysium, a connective tissue sheath surrounding the muscle. In turn, epimysium emits connective septing structures termed perimysium, splitting muscles into subunits termed fascicles. At an even lower level, a thin, sparse connective structure called endomysium coats each polynucleate, elongated cell (termed myofiber). Connective tissue supports the terminal branches of the nervous, circulatory and lymphatic systems. In addition, connective tissue inside the muscle provides mechanical anchoring between fibers, longitudinally, laterally

and with the tendons. This is particularly true of perimysium, whose collagen content is 95%[236]. The collagen in muscle-associated connective tissue is mainly of types I and III (reticulin), with traces of type V collagen and fibronectin, while the most frequent proteoglycan is collagen I-binding decorin[237]. The external lamina is the equivalent of basement membranes in other tissues, an proteic structure surrounding each multinucleate, roughly tubular cell. The external lamina contains collagen IV, laminin, and heparan sulfates [238, 239]. Muscle mass changes should require remodeling of all these connective structures, with novel collagen synthesis possibly misleading measurements of protein synthesis in atrophying muscle. In addition to the contractile and connective components, muscles include vascular, nervous, adipose, blood and immune cells.

The myofiber is the histological base unit of contractile tissue. Another muscle-specific population of cells comprises the mononuclear, proliferating cells, called satellite cells. Satellite cells are nearly devoid of cytosol, sitting in close proximity to the fiber, under the external lamina. They can be identified by markers such as Pax7 and, on the membrane, CD34 (reviewed in [240]).

Each myofiber is a syncytium, that forms and grows by fusion with neighboring cells. The only way to acquire nuclei to the myofibers is fusion with surrounding proliferative mononucleate cells, or with neighboring myofibers. Cells that have the ability to undergo mitosis and to fuse with myofibers are typically designated myoblasts. In vivo, myoblasts are derived from a subset of satellite cells. Experimentally, α7 integrin is an effective marker for selecting proliferative precursors from muscle[241], although its sensitivity and specificity are yet to be established. Alternate sources of nuclei in the myofiber are subject of ongoing research, but their relative importance is expected to be minor at best (reviewed in [242]). A transplant of seven satellite cells from an adult mouse is capable of yielding more than a hundred multinucleate myofibers, thus demonstrating former's ability to regenerate muscle[243]. The transformation of quiescent satellite cells to proliferating myoblast is regulated by the interplay of

growth factors, external lamina, and contact with myofibers[244]. In vivo, myofiber nuclei are typically peripheral, while in vitro incomplete models often yield elongated, contractile cells, with central nuclei, akin to regenerating fibers. The latter are typically termed myotubes.

The proliferative niche can play an important role in muscle atrophy and recovery. However, muscle hypertrophy may occur without cell divisions. For example, the muscles of mice receiving clenbuterol and of rats undergoing eccentric training gain 20-30% muscle mass without apparent DNA changes[245, 246]. Quail muscles depleted of proliferating cells by irradiation still undergo hypertrophy in response to stretch-overload[247]. These example of amitotic hypertrophy demonstrate that, in some circumstances, the number of nuclei and the pace of transcription are not limiting factors in muscle growth.

A large majority of the myofiber cytosol is the contractile apparatus, in the shape of bundles of protein filaments termed myofibrils. Within myofibrils, myosin and actin filaments alternate, held together by multi-protein complexes containing titin. Myofibril proteins are about two thirds of the total myofiber protein[248]. Therefore, any myofiber size change with functional relevance should correlate with changes in myosin II and actin protein content. In rat muscles, three days of streptozotocin-induced acute diabetes causes intramuscular formation of a actin degradation fragment[249]. The apparent sensitivity of actin to muscle mass regulators poses a technical challenge to protein and even mRNA measurements, because traditionally actin is considered a housekeeping, unregulated, and rather constant protein, and is used in level normalization. If, in an atrophying muscle, some protein of interest is depleted faster than actin, immunoblots will convey the certainty that the former is downregulated. But if the protein of interest is lost in a less preferential manner, at the same rate with actin, immunoblots normalized to actin will convey the mistaken appearance of constancy.

Similar issues govern the use of 3MH as a marker for myofibril protein

catabolism. Given that the main correlate of urinary 3MH is muscle mass[250], 3MH measurement may lack sensitivity when used as a indicator for muscle catabolic rate. On one hand, increased catabolism is expected to cause increased 3MH output, but on the other, an atrophic muscle has less 3MH to release.

During experiments that perturb muscle mass equilibrium, the level of the regulators, of typical housekeeping proteins, and of non-myofiber proteins may fluctuate in manners that convolve their specific modulation with overall muscle protein kinetics. Investigation of recovery from muscle loss is burdened by the fact that it aims to dissect protein regulation, when the regulators are proteins themselves.

Preadult muscle development

Around puberty, muscle growth is associated with a massive shift of nuclei from the satellite cells to the myofibers[251]. Even earlier, de novo muscle development is remarkable for its accretion of new nuclei to the myofiber. Pre-adulthood muscle growth appears reliant on hyperplasia, and cell proliferation regulators are likely crucial in determination of muscle mass.

In utero, the mesoderm, which is the source of muscle progenitor cells, undergoes segmentation and differentiation to form somites, dermomyotomes, and eventually myotomes (reviewed in [252]). The latter contain the earliest cells expressing muscle regulatory factors (MRF). In animal models, the cells in epaxial and hypaxial muscle initiate the formation of trunk and limb muscles. The limb muscle early progenitors express myogenic factor (Myf) 5, due to stimulation from the transcription factor Pax3[253, 254]. Myf5 is a strong inductor of the muscle transcriptional program and phenotype, with the ability to convert embryonic fibroblasts to myosin-containing syncitia[255]. Cranial muscle formation is coordinated in a partly different manner, through the transcription factors T-box (Tbx) 1 and paired-like homeodomain (Pitx) 2 (reviewed in [256]). Once this early

stage is completed, later fetal muscle progenitors converge to a phenotype remarkable for the expression of the MRF MyoD, due to stimulation by the transcription factor Pax7[257]. MyoD knockout mice are normal, with Myf5 supplanting its absence[258]. In the Online Mendelian Inheritance in Man (OMIM) database, there is no reported case of human mutation of Myf5 or MyoD, further supporting the idea of duplicate function. Neither Myf5, nor MyoD induce muscle attributes. However, the expression of either will promote expression of another MRF, myogenin, which marks the transition form specification to differentiation. MRFs share structural features, and some are juxtaposed in the genome, making their study difficult.

Initiation of myogenin expression marks the transition from specification to differentiation stage. In cultured cells, myogenin expression is followed by p21 expression, which removes the muscle precursor from the cell cycle[259]. Subsequent changes include expression of muscle-specific enzymes and contractile proteins[260], of a fourth MRF, the myogenic regulatory factor (Mrf) 4[261], and, finally, acquisition of fusogenic abilities. In humans, by the seventh week of gestation, the initial wave of myoblast fusion slows down, and a second proliferative stage starts. The latter tapers off, leading to formation of secondary myotubes, within the same laminar sheath with a primary myotube. Around the seventeenth week, some secondary myotubes migrate to form independent centers of coalescence for a third set of myotubes[262].

In chick embryos, overexpression of IGF-I induces a rapid increase in the ratio of myoblast to myofiber nuclei, while fiber density is unchanged[263]. In addition to the pro-proliferative effect, IGF-I stimulates protein anabolism in prenatal muscle[264]. Defects in IGF-1R signaling determine low birth weight and subsequent growth retardation in humans and transgenic mice, although it is unclear this is due to the mitotic or to the anabolic deficiency[265, 266]. Moreover, the murine model may be marred by the formation of hybrid dimers between IGF-1R and insulin receptor, which is likely to impair insulin signaling as

well.

In mice, phenotypic differences between myostatin-null and wild type develop by the second week of embryogenesis[267]. Experimental myostatin perturbations in chicken embryos alter the population of muscle precursor cells[268]. The few reports of viable human mutations in the myostatin gene concern newborns with unusual unusually large muscles, due to loss of function[269]. Nevertheless, myostatin defects appear to cause ampler changes in muscle mass after birth[270, 269]. The relative importance of myostatin in embryo muscle development is to be determined.

Immediately after birth, the number of satellite cells is much higher than in the adult, with a one magnitude order drops between birth and 10 years[271]. This decay carries on throughout the life time at slower rate. Perinatal Pax7 knockout reduces muscle ability to regenerate, while its genetic depletion in utero or at adulthood does not exhibit pathological traits[272], suggesting that juvenile muscle growth is distinct from muscle development at other ages. Overall, muscle formation in children appears qualitatively different from adult muscle hypertrophy and regeneration, and should be more susceptible to modulation via mitotic mechanisms. This may be underlie a difference between juvenile and adult in the atrophying effect of GC on muscle.

The most likely source of new myofiber nuclei are the adult satellite cells, derived from a subset of fetal $Pax3^{+}Pax7^{+}$ cells. Some cells, such as bone marrow stem cells and pericytes, have the ability to fuse with myotubes, and possibly contribute to the muscle stem cell population[273, 274]. In adulthood, most limb and trunk satellite cells are $Pax7^{+}$, while head satellite cells are often $Pax3^{+}Pax7^{-}$[275]. However, in longitudinal studies, human muscle mass, as estimated by body potassium, decreases in men and and stagnates in women over the age of 30[276]. As evidenced by studies such as the New Mexico Elder Health Survey, 1993-1995, with aging, loss of muscle mass accelerates [277]. Any muscle growth mechanism, including that dependent on satellite cells, is likely less

powerful in adulthood than in childhood. Indeed, after the age of 20, nuclei in human muscle maintain an almost constant length of telomeres, indicating that mitosis is a rare phenomenon in the adult muscle[278]. Moreover, the proportion of satellite cells with proliferating abilities decreases with age, as more of them approach the Hayflick limit[279].

Physiological muscle metabolism

Muscle is a major energy user in the body, using fat during rest and glucose during exercise (reviewed in [280, 281]). Because its ability to fatty acid is negligible, muscle is a consumer and a minor store, but not a generator, of fatty acids. During fast, more than half of infused non-esterified fatty acids (NEFA) are taken up by muscle, with a higher rate of incorporation in type I oxidative muscle[282]. In contrast, after feeding, oversupply of NEFA is compensated mainly by increased uptake, in absolute terms, in the visceral adipose tissue, with minor contributions from other adipose tissue, liver, and muscle. After repeated exercise, muscle lipoprotein lipase expression is increased, indicative of an adaptive improvement in muscle ability to extract NEFA from circulating triglycerides[283]. Ongoing studies suggest that muscle oxidative (catabolic) uptake of NEFA is upregulated by peroxisome proliferator-activated receptor (PPAR) β/δ, which stimulates expression of the lipolysis rate-limiting enzyme, carnitine palmitoyltransferase I[284, 285]. This hypothesis is supported by fast- and exercise-induced upregulation of muscle PPAR δ[286, 287]. Moreover, PPAR δ overexpression leads to increase in type I fibers and subsequent resistance to high-fat diet[284]. Organ-level studies are impaired by the existence of nontrivial intramuscular adipose tissue.

In humans, up to 90% of the glucose absorbed after a meal is removed from circulation by the skeletal muscles[288, 289], meaning that muscles should have a paramount role in the development of insulin resistance and eventually diabetes

mellitus. After normal feeding, muscle builds polysaccharides reserves, in part because it can synthesize and deposit the largest glycogen stores in the body, but also because it cannot release glucose.

Muscle work is generated at such high rates, that most glucose is processed solely through glycolysis, in the cytosol, to the three-carbon pyruvate. Some of the pyruvate is further oxidized in the muscle, through the tricarboxylic acid cycle, but a significant amount is converted to lactate and released in the blood stream. As part of the Cori cycle, circulating lactate is reassembled into glucose by the liver, and re-released into the blood stream, for muscle use. A similar shuttling mechanism further enables muscle to rely on glycolysis, by transaminating excess pyruvate to the amino acid alanine, similarly released in the blood. Circulating alanine is converted by liver to glucose. Pyruvate transamination requires the amino acid glutamate. Muscle uses glutamate for other metabolic processes, including the synthesis of non-essential amino acids, including proline and arginine. Therefore, at rest, human muscle uptakes significant amounts of glutamate, less serine, while releasing alanine, glutamine, and smaller amounts of the other amino acids[290]. Glutamine is synthesized in muscle in order to carry to the liver, through the blood, the excess nitrogen yielded by amino acid release during protein degradation.

The glutamine secretion is mainly a vehicle for shuttling excess nitrogen to the liver, where it is further converted to urea.

Among the amino acids with a trend for release between meals, isoleucine, leucine, methionine, phenylalanine, threonine, and valine cannot be synthesized by humans. Their net release indicates that, at rest, basal level of protein degradation slightly surpass protein synthesis. Conversely, for a steady muscle mass, there must be net protein synthesis in the fed state. Feeding status is relayed to the muscle by a surge in insulin and, independently, by an increase in circulating levels of essential branched-chain amino acids (BCAA), that is, valine, isoleucine, and leucine. The signaling effects of BCAA are not completely

understood. Some studies show that BCAA ingestion or infusion increase protein synthesis rate[291], while others claim that BCAA solely reduce protein degradation[292]. However, staying true to their energetic value, BCAA supplementation caused increases in insulin in all the referenced studies. It becomes difficult to extricate BCAA intrinsic effect from that of insulin. Moreover, the combination of insulin and BCAA is a potent synergistic anabolic stimulus in healthy human muscle[293]. One study investigated the action of BCAA at clamped normal insulin levels[294]. In young, healthy controls, BCAA alone were able to increase the fractional synthesis rate for myofibrilar protein. At the same time, BCAA caused an increase in phosphorylation of mechanistic target of rapamycin (mTOR) at Serine 2448. This posttranslational modification is caused by the ribosomal protein S6 kinase, 70 kDa (p70-S6K)[295]. Interestingly, p70-S6K is a substrate of mTOR complex 1, with the latter considered an integrator of nutrients, energy, and growth factor signaling (reviewed in [296]). Indeed, in the same muscle, p70-S6K was activated, as indicated by an increase in its Threonine-389 phosphorylated form. Another substrate of mTOR complex 1, eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP), was hyperphosphorylated. The action of mTOR complex 1 on 4E-BP is the canonical way in which the former stimulates protein synthesis, by abolishing the latter's ability to bind and inhibit the mandatory translation initiation factor eIF4E. Many of these anabolic markers may be paradoxically elevated in acute, non-starving atrophy settings, where BCAA sudden release increases their circulating levels.

In addition to their signaling roles, BCAA are also used by muscle as protein precursors, and as energetic substrates, when preferred energetic substrates are not available[297]. In studies which measure leucine disappearance from the bloodstream or culture medium, a distinction must be made between the leucine used in non-oxidative, anabolic reactions and the alpha-ketoisocaproate-forming, ergogenic usage.

The degree to which energetic needs modulate BCAA usage appears to differ

between humans and rats, reducing the validity of animal models (reviewed in [298]). Amino acid uptake or release rates are perturbed by energy and hormonal factors, to a degree that is still to be measured in humans. An argument has been made for using phenylalanine as a tracer, because muscle catabolism is negligible, and because it has a lower insulin secretagogue effect[299, 300].

Adult muscle remodeling

With aging, muscle gradually shifts from a mitotic to a postmitotic profile, with muscle growth achieved increasingly through hypertrophy, that is, cell size growth. While the typical middle-aged or elderly adult is undergoing net loss of muscle mass, the muscle is still adept at re-growing and remodeling under two common circumstances, exercise and injury.

Humans achieve muscle mass growth following exercise, although some forms of exercise are more suitable at increasing strength or resistance that mass per se. In animals, muscle growth is induced by muscle overload, or muscle unloading and reloading, which may be conceived as forms of aerobic exercise. In healthy volunteers, the acute response to exercise includes increased intramuscular expression of MRFs MyoD and myogenin, and increased circulating IGF-I and IL-6[301, 302, 303]. These signals are associated with increased proliferation of satellite cells and recruitment of neutrophils to the muscle[304, 305]. In the acute phase, the satellite cells colocalize with IGF-I[301]. The negative muscle regulator myostatin is not correlating with the phenotype, that is, it is not decreased by acute exercise[305, 306]. Acute exercise increases the fractional protein synthesis rate in muscle[307, 308]. Interestingly, a single bout of exercise during fast leads to increases circulating cortisol levels and the release of 3-MH, indicative of increased stimulation of protein degradation[309]. Concomitant amino acid feeding extinguishes GC and catabolic response to exercise. Variations in regimens of exercise and timing and

composition of diet led to a plethora of studies. Just as most of the short-term exercise routines do not lead to muscle macroscopic changes, some studies describe an absence of changes at molecular level following short-term exercise (reviewed in [310]). An important future direction in exercise science is establishing what distinguishes an effective brief exercise routine from an ineffective one. In this context, GC may be interesting noninvasive markers.

In the long term, exercise increases fiber CSA, density of satellite cells, and the number of myofiber nuclei, while the level of intramuscular MyoD, IGF-I, myostatin slowly return to normal[311]. On the other hand, long-term exercise induces the expression of catabolic markers, such as the E3 ligases MAFbx and MuRF-1[312]. Taken together, these molecular findings indicate exercise causes muscle remodeling, which manifests as increased muscle turnover, with upregulation of both protein degradation and synthesis. Moreover, post-exercise muscle accretion combines hyperplasia and hypertrophy.

A similar biphasic response is yielded by injury. In the immediate stage after injury, the muscle is infiltrated by pro-inflammatory M1 macrophages, while at later stage, anti-inflammatory (M2) subclass dominates (reviewed in [313]). Although the studies are rather incomplete, it appears that, similar to exercise adaptations, injury triggers a burst of growth factors, probably including IGF-I, basic fibroblast growth factor (bFGF), and transforming growth factor-beta (TGF-β) ([314]). Most of the studies of regeneration provide circumstantial evidence, such as improved healing in the presence of a presumed mediator, rather than impaired healing in its absence. Still unidentified molecules from crushed muscle are able to stimulate myoblast proliferation, above the levels caused by stimulation with known growth factors[315].

This work uses extensively the C2C12 cell line, an immortalized female mouse muscle progenitor line obtained from a muscle recovering after mechanical injury. Less than half of the C2C12 cells in their proliferating, undifferentiated form, express MyoD or Pax7[316]. Preliminary evidence suggest these myoblast-like

cells do not express Pax3 either[317]. Therefore, the C2C12 line are an incomplete model of muscle accretion through proliferative means.

Generation and regeneration of muscle in common scenarios, such as development and adaptation, remain an object of study, due to their complexity. Variable importance of the immune cells, of MRFs and of IGF-I, and concurring redundancy forestall attempts to envisage a common pattern of muscle hypertrophy.

Hormonal control of muscle mass

The variability of muscle mass within population is reflective of the variable needs for muscle strength. Muscle mass and strength are adjusted to the needs of the organism mainly through humoral mechanisms. The most relevant endocrine regulators of muscle mass, AAS, GC, and IGF-I, will be discussed in dedicated sections.

While anabolic role of IGF-I at muscle level is undisputed, insulin's effect is inconsistent, because it is easily altered by external factors. The acute phase of insulin response poses a conundrum, with some studies showing it stimulates protein synthesis in human muscle[318], while others demonstrating that its effect is limited to anti-catabolism[319]. In male rat muscle, 30 minutes in 30 nM insulin or IGF-I are equally able to stimulate protein synthesis and to inhibit protein degradation[320]. Both effects are above 10%, although the effect on protein degradation appears less ample and is marred by higher variability.

Insulin and IGF-I pathways overlap to a some degree. The liver is the main source of circulating IGF-I, under the pituitary stimulation with growth hormone (GH). However, auto- and paracrine secretions may full supplant the absence of hepatic IGF-I in adult conditional knockout mice[321]. In contrast, insulin is secreted solely by the pancreas. Our understanding of the regulation of insulin secretion is improving, dispelling the simplistic view that nutrients alone are its

sole modulators (reviewed in [322]). Therefore, both IGF-I and insulin emerge as anabolic stimuli, responding to increased demand for macromolecules and / or increased supply of nutrients, with IGF-I embracing a more localized and insulin a systemic, integrative role. For both hormones, physiological concentrations are tens of times higher than the half maximal effective concentration (EC50) for their receptor, suggesting that physiological fluctuations cause marginal effects downstream[323, 324]. On the other hand, insulin has the ability to bind and activate IGF-I receptor (IGF-1R), with an EC50 about an order of magnitude lower than physiological insulinemia. The converse is true, with IGF-I being able to bind and activate insulin receptor (IR), isoforms A and B. There is a small, but real, potential for interference between insulin and IGF-I signals.

The levels of bioavailable IGF-I are under complex regulation (reviewed in [325, 326]). IGF-I may be sequestered by IGF-I binding proteins (IGFBP), which are secreted by muscle under IGF-I stimulation ([327]). The interaction with IGFBP may prevents IGF-I from interacting with receptor, or it may extend its circulating half-life by protecting it from degradation. Depending on the isoform and location of IGFBP, the interaction may result in extinction or amplification of the IGF-I signal. IGFBPs levels are modulated by insulin and their affinity is modified through competition by insulin-like growth factor 2 (IGF2). The latter can also stimulate IGF-1R, thus providing its own anabolic and pro-myogenic signals[328]. IGF2 is irreplaceable in fetal development[329], suggesting there might be distinct, unidentified receptors for this hormone family.

There is no consensus with regards to the ability of GH to stimulate muscular secretion of IGF-I. Multiple studies found an upregulation of its mRNA[330, 331], but protein data are lacking. Medium conditioned by GH-stimulated C2C12 cells fails to elicit hypertrophy in other C2C12 myotubes[332], suggesting that, at best, IGF-I has an intracellular autocrine action. In addition to the indirect effect mediated by hepatic and the putative muscular IGF-I, GH has an IGF-I-independent effect on muscle. For example, knockout of GH receptor

impairs body growth further beyond IGF-1R knockout[333]. In the context of pituitary pathology associated with Cushing's disease, the associated GH perturbations may contribute to loss of muscle.

Hypothyroidism is often associated with muscle weakness and pseudohyertrophy[334]. Other hormones such as the parathormone, are likely to have small effects on muscle protein metabolism, essentially irrelevant outside their respective pathologies[335]. In conclusion, muscle mass homeostasis is under a tight, multifactorial hormonal control, whose study is complicated by significant redundancy. The absence of third-party organs, such as glands, from reductionist cell-culture may limit their ability to replicate in vivo phenomena.

Interaction of muscle mass and vascularization

Muscle vascularization is a modulator of muscle mass and contractility. Mice whose muscle VEGF-A secretion was genetically depleted still express a tenth of the muscle VEGF-A protein, but have only half of the capillaries per muscle fiber, compared to their $Cre^{-/-}$ siblings[336]. The muscle-restricted VEGF-A-depleted mice have 12% lighter gastrocnemii, although the muscle loss disappears when muscle mass is normalized to total body weight. The loss does not affect a specific fiber type more than the others. Therefore, the muscle depleted of VEGF-A is less able of endurance effort (80% shorter time to exhaustion on the inclined treadmill) and of brief anaerobic exercise (34% lower maximal running speed).

Conversely, murine muscles injected with VEGF-A-expressing retroviruses display a higher proportion of hypertrophic fibers than those expressing bacterial β-galactosidase[337]. Moreover, in the VEGF-A-overexpressing muscle, many of the fibers have central nuclei, a sign of increased fusion with myoblasts. In the murine C2C12 cell line, VEGF-A causes faster differentiation, into longer myotubes, with more nuclei per fiber, although mitotic rates are in fact diminished. These observations are consistent with direct anti-apoptotic and

profusogenic effects.

It has been suggested that the most direct effect of VEGF-A depletion is partial segregation of muscle from blood-carried endocrine signals. For example, VEGF-A-depleted muscle has lower glucose uptake, a defect which is reversed by explantation [338]. In addition, vascularization defects may induce relative intramuscular hypoxia, which is an independent atrophying, pro-proteolytic factor[339].

The relative importance of VEGF overexpression during myogenesis is still open to debate, as muscle-restricted VEGF receptor (VEGFR) knockout animals were not studied yet. Multiple effects concur to obfuscate VEGF action in hypertrophying muscle. First, VEGF promoter contains three binding sites for MyoD, meaning that growing muscle will express more VEGF[340]. Development of vasculature in growing muscle may be a physiologically meaningful way to ensure vasculature remains competent upon increase circulatory demands. Second, although the canonical upregulator of VEGF is hypoxia-induced factor 1α (HIF 1a), muscle VEGF is also stimulated by PPAR γ coactivator 1 (PGC-1)[341, 342]. Even in the absence of VEGF, PGC-1 facilitates mitochondria biosynthesis, leading to oxidative fiber hypertrophy and improvements in endurance capacity [343]. Therefore, it is difficult to distinguish VEGF-induced muscle changes from the common muscle remodeling program. Finally, VEGFR activation has multiple effects, including phosphorylation of Src family proteins[344], of phospholipase C (PLC) γ[345], and, indirectly, of regulatory subunits of phosphatidylinositol 3-kinases (PI3K)[346] and of the Signal Transducers and Activators of Transcription (STAT) STAT3 and STAT5[347]. Most of these VEGF effects overlap with the effects of many other muscle anabolic agents. Thus, it is unclear whether VEGF plays a mediating role in muscle hypertrophy, or it provides the homeostasis of the vasculature to fiber ratio.

Control of muscle mass through innervation

Limb and trunk muscles are controlled by the lower (alpha) motoneuron, located in the ventral horn of the spinal cord. Their main point of contact is a chemical synapse, the neuro-muscular junction (NMJ). In addition to the direct synaptic activity, muscles and lower motoneurons are involved in a mutually beneficial life-long interaction. The loss of NMJ-mediated stimulation leads to rapid, sizeable loss of muscle mass, commonly seen in spinal cord injury. Experimentally, denervation of a hindlimb or hemidiaphragm is a relatively simple procedure, allowing the use of an animal as its own control. Experimental denervation is the most studied model of muscle atrophy, generating working hypotheses for the study of GAML.

In utero, experimental destruction of motoneurons with bungarotoxin abolishes formation of secondary myotubes, although it has limited effect on the formation of primary fibers[348]. Conversely, motoneuron precursors from the embryo spinal cord degenerate and die if they cannot engage in significant interactions with myofibers[349]. Eliminated motoneurons include neurons whose axons fail to reach myotubes and neurons which eventually fail to maintain contact with myotubes, due to synapse elimination. The latter is a selective, competitive process, whose molecular basis is still unknown, and which ensures injectivity of the correspondence between lower motoneurons and myofibers (reviewed in [350]). For a few days after birth, rat motoneurons go into a particularly sensitive state, when axotomy determines motoneuron death[351]. Thence, axotomy elicits the reprogramming of the neuron into a less differentiated state, followed by axonal regrowth (reviewed in [352, 353]). The re-establishment of contact between the motoneuron and muscle causes the reverse molecular changes, suggesting the existence of muscle-secreted neurotrophic factors. The ability of NMJ to regenerate or even preserve its optimal structure degrades with aging, although it is not clear whether NMJ cause

or are caused by aging-related muscle loss[354]. The improvement in NMJ regeneration through exercise has been seen as an important path towards its understanding. Factors that improve muscle's ability to exercise or even mimic exercise, such as IGF-I or androgens, have been shown to benefit NMJ recovery or to slow down its degradation during aging[355, 356, 357].

The loss of contact with the lower motoneuron elicits similar changes in the muscle, with clinical relevance in the understanding and treatment of spinal cord injury and spinal muscular atrophy. Conceptually, denervation is distinct from disuse, such as that induced by damage to the upper motoneuron. Clinically, the latter manifests differently, through a syndrome termed pyramidal weakness, that mainly affect muscles opposing gravity[358]. In humans, after upper motoneuron damage, disuse response occurs in a few days after injury, and leads to exaggerated spasticity[359]. Understandably, literature does not describe any animal model of disuse by experimental damage to the upper motoneuron. In fact, literature contains multiple examples where "disuse" is taken to mean "absence of action potentials, due to denervation", "lack of work, due to denervation, unloading, immobilization", or even both[360]. Consequently, there are no experimental attempts to disentangle muscle-maintaining effects of work, of the NMJ transmitter, acetylcholine, and of any other musculotrophic neuron-released factor.

Experimental denervation causes rapid and ample loss of muscle mass. For example, three weeks after sciatic nerve removal, the tibialis anterior muscle halves in weight[361]. Fewer than one in 7,000 myofiber nuclei undergo apoptosis in this time[362], indicating that denervation and the fast recovery that follows renervation are more similar to the adult exercise remodeling paradigm, that to the proliferation-dependent regulation of the infantile muscle. Supporting the hypothesis on NMJ-independent trophic factors, denervation did cause massive and rapid apoptosis among stromal cells.

In a seminal study, Goldberg demonstrated that denervation leads to

increased loss of prelabeled muscle protein, proving that denervation upregulates protein degradation[363]. Moreover, denervation increases urinary 3MH, indicating activation of myofibril catabolism[364]. The rate of muscle loss in denervated muscle is halved by the proteasome inhibitor bortezomib[365]. Denervation stimulates persistently proteasome enzymatic activity[366], and upregulates all the ubiquitin-proteasome pathway components, including ubiquitin, the E3 ligases MuRF1 and MAbx, and the proteasomal subunit macropain (also known as A1) [367, 222]. The upregulation of the E3 ligases is induced by multiple independent transcription factors, including myogenin and the Foxo class[368, 369, 370, 371]. In the first few days of denervation, myogenin is induced by histone deacetylase (HDAC) 4[372]. After the first week, Foxo activation is attributed to the downregulation of its negative regulator, Akt[370, 373].

Because the mTOR inhibitor rapamycin prevents fiber hypertrophy that normally follows in vivo injections with a plasmid coding constitutively active Akt[373], the scientific community assumed, since the beginning of century, that denervation-induced Akt inactivation leads to loss of downstream mTOR-mediated effects (for example, [374, 375]). Recent studies contradicted this paradigm. In 2013, Quy and colleagues found that denervation increased Threonine-389 phosphorylation and catalytic activity of p70-S6K, indicating that denervation causes in fact activation of mTOR complex 1[376]. In 2014, Tang and colleagues proved that rapamycin, an inhibitor of mTOR complex 1 which lacks intrinsic anabolic properties, abolishes denervation-induced loss of muscle mass[370]. These experiments prove that mTOR complex 1 should be activated for denervation-associated muscle atrophy to proceed in its later stage. Tang showed that denervation causes phosphorylation of insulin receptor substrate (IRS) 1, proving that, in denervation, activated mTOR complex 1 inhibits Akt through a p70-S6K - IRS 1-mediated negative feedback loop. Quy found that mTOR complex 1 activation is lost upon proteasome inhibition with bortezomib. One can

hypothesize the existence of an mTOR negative regulator which is specifically targeted by the ubiquitin-proteasome system during denervation. However, the late timing of the Akt / mTOR involvement suggests that the most plausible explanation places mTOR activation after the proteasome releases significant amounts of free amino acids. It is therefore more likely that denervation-induced mTOR activation is caused by the proteasome-stimulated amino acid release.

Establishing the role for mTOR complex 1 in denervation-induced loss of muscle mass is crucial. In conceptual frame before Quy and Tang experiments, mTOR complex 1 was thought as being inactivated by denervation, thus downregulating on protein synthesis and removing a restriction on autophagy. This hypothesis was conceptually attractive, because it implied a co-operation between multiple anabolic and catabolic pathways, towards achieving muscle loss. In particular, denervation-induced autophagy appealed to the muscle biologists of the 2000's. For example, in a never-replicated experiment, one group claimed that the lysosomal inhibitor chloroquine prevents denervation-induced muscle loss[**?**]. Others showed that denervation upregulates lysosomal enzyme cathepsin L[377], while a third group claimed that denervation causes buildup of the autophagosomal marker LC3-II [378]. However, most experiments refuted the role of autophagy in GAML. The lysosomal inhibitors leupeptin, methylamine, and E64-c have minimal effects on the release of free tyrosine from denervated muscle[364]. GFP-LC3 transgenic mice exhibited a loss of autophagosomes in denervated muscles[376].

A similar debate surrounds protein synthesis regulation in denervation. In Goldberg's 1969 experiment, the specific activity of the remaining muscle protein was essentially the same as in control limbs. Increased or even unchanged protein synthesis rates would have caused a reduction in specific activity. Therefore, Goldberg concluded that denervation causes protein synthesis decreases, thus opening a debate that is still unsettled. Goldberg's observed reduction in protein synthesis rate is consistent with an indiscriminate loss of translation initiation

and elongation factors, as much as with an active mechanism of protein synthesis cessation. Moreover, more recent functional studies, including some from Goldberg's group, found that denervation stimulates translation[379, 376, 366, 380, 364]. Unlike the 1969 study, contemporary studies measured protein synthesis rate directly and over shorter time intervals (hours, rather than weeks). As in the case of autophagy, follow-up studies found molecular changes in regulators of translation, in some cases supporting a regulated, denervation-triggered mechanism of protein translation shutoff, in others refuting them. Both Tang and Quy found that denervation causes increased phosphorylation of 4E-BP, thus leading to stimulation of protein synthesis. Both also show that the effect on 4E-BP is prevented by rapamycin, demonstrating that protein synthesis is upregulated through an mTOR-dependent mechanism.

Given that rapamycin has essentially no effect on adult muscle mass, it may be that mTOR is not the crucial effector of denervation-induced muscle loss once thought. Recent studies show that its activation, and the downstream net anabolic effects it elicits through autophagy and translation, cannot explain the observed phenotype. Denervation-induced muscle loss is, to great extent, produced by the ubiquitin-proteasome system. Better experiments are needed in order to understand autophagy and of mTOR in denervation-induced muscle loss. The irreconcilable differences between functional and molecular studies of denervation epitomize similar predicaments in the study of muscle atrophy due to other etiologies, including the less-explored GAML.

Ubiquitin-proteasome system as a major effector in GAML

In the 75 years since the discovery of chronic steroid myopathy, scientists attempted, with variable success, to develop multiple animal models. In dogs,

seven days of 0.44 mg/(kg d) cause increased nitrogen excretion, increased glutamine and alanine release from the hindlimb[381]. In rabbits, fourteen days of 3 mg/(kg d) Dexa diminished diaphragm's twitch and tetanic contraction force[382] Interestingly, four days of 1 mg/(kg d) Dexa did not change muscle mass, despite the upregulation of some catabolic markers[383]. Early studies were marred by an absence of meaningful macroscopic outcomes, such as muscle force or mass. Moreover, the modern molecular correlates of muscle loss were not known, and could not have been investigated.

In the era of genome sequencing, where murine species became the most common model, studies of GAML focused on rats, because changes in muscle mas are more readily are more readily observed, compared to mice. The effects of 7-day, 5 mg/(kg d) treatment on rats allow the classification of GCs in two subsets[384]. Members of the short-acting subset, including prednisolone and corticosterone, cause net gains in body weight, through increased adiposity, MR-mediated water retention, and a net neutral effect on muscle mass. In contrast, long-acting, specific GCs, such as Dexa, betamethasone, and triamcinolone, cause loss of body weight, and an even more rapid loss of muscle mass. GAML in rats is an organized process, lacking the microscopic features of necrosis[385]. Although Dexa temporarily reduces food intake, possibly through stimulation of leptin secretion[386], pair-feeding experiments demonstrated that the GAML is not solely the effect of changes in appetite[387, 388]. In response to Dexa, rat muscle fibers undergo reductions in CSA, to an ampler degree in fast twitch fibers[389, 390]. Dexa-induced loss of muscle mass is present, although less manifest, in female rats, possibly because, in males, hypercortisolism is compounded by a reduction in circulating T levels[389]. In line with the section on human steroid myopathy, this section will focus on the effects of Dexa on male adult rats.

The nitrogen imbalance induced by Dexa slows down by the third day, and is compensated around the seventh day of treatment[391, 392]. Given that later

time points are marred by feedback mechanisms and by animal mortality, almost no published experiment on rats extended past 10 days. For an animal weighing 300 times less and aging 20 times faster than humans, the common 5-10 days experiments are comparable to chronic exposure in humans. Rats appear more resistant than humans to GAML, given that reported experiments start at 0.5 mg/(kg d) Dexa, more than twice the muscle-imparing dose in humans.

There are no studies analyzing the role of satellite cells and nuclear density in GAML in situ. Dong and colleagues hypothesize a “glucocorticoid-induced satellite cell dysfunction”, and report that Dexa reduces the number of satellite cells in a post-injury regenerating muscle[393]. One is intrigued to see that Dong et al. had the ability to measure change in satellite cells upon Dexa treatment, yet chose not to investigate or report it. Based on similarity with denervation-induced atrophy, it is likely that GAML is not caused by a satellite cell dysfunction.

The loss of muscle in the Dexa-treated adult rat is the result of an increase in protein degradation. In rats receiving 0.5 mg/(kg d) Dexa for six days, epitrochlearis muscle proteolysis rate increased by 50%, while protein synthesis was essentially unchanged[387]. Dexa-stimulated proteolysis affects the contractile apparatus, as indicated by the doubling of urinary 3-MH output[394]. The upregulation of proteolysis occurs even when explants, rather than animals, are treated with Dexa, suggesting that GAML does not require extramuscular inputs[395]. The increase in proteolytic rate is unchanged when explants are treated with the lysosome inhibitor methylamine and the lysosome / calpain inhibitor E-64. On the other hand, the Dexa-stimulated increase in proteolysis is abrogated when the explant is depleted of ATP by a combination of the mitochondrial decoupling agent dinitrophenol and the unlysable glucose homologue, 2-deoxyglucose[396]. The proteasomal subunits C2 are upregulated by Dexa[387]. Proteasome chymotrypsin-like catalytic activity is doubled by Dexa[397].

Unbiased microarray searches in mice muscles atrophying in various

experimental paradigms have repeatedly yielded two upregulated genes, termed atrogenes, MAFbx and MuRF-1[398, 222]. Both are E3 ligases, pointing to an important role for the proteasome-ubiquitin system in GAML. Recently, the role of MAFbx has been questioned, as unbiased searches in rats failed to identify it as a target of Dexa[399], and its genetic depletion did not reduce mouse GAML[400]. While MuRF-1 knockout reduces GAML, it does not abolish it completely.

Therefore, explant models suggest that GAML is, to a wide extend, the result of upregulation of the proteasome-ubiquitin system. Recent research aims to establish if other proteolysis or protein synthesis regulators contribute to GAML. Alternatively, it may be the case that the proteasome-ubiquitin system initiates GAML by neutralizing a few specific muscle protecting factors.

GC-induced loss of sensitivity on the Akt / mTOR axis

The most comprehensive account on GC-induced changes on protein metabolic regulation came, surprisingly, from the study of associated glucose metabolism changes. Dexa causes systemic insulin resistance, manifested as uncompensated hyperglycemia[388]. On one hand, Dexa induces concerted catabolic changes which eventually converge to hyperglycemia, and consequent hyperinsulinemia. Together, these yield a higher index of homeostatic model assessment - insulin resistance (HOMA-IR)[401]. On the other, Dexa-induced hyperinsulinemia fails to trigger its typical anabolic program in muscle. For example, Dexa overrides insulin to reduce muscle glucose uptake[402], through a reduction in recruitment of glucose transporter (GLUT) 4 to the membrane[402**?**]. Dexa co-administration blunts muscle anabolic and anti-catabolic response to exogenous supplementation with either insulin or IGF-I, at the levels of protein and glucose metabolism[403, 320]. Dexa reduces muscle expression of

IGF-I[404].

The effects of Dexa appear to be mediated by the insulin pathway in an indirect way. As described in the next paragraphs, Dexa causes no direct change on the insulin pathway, yet, at almost every step, abrogates insulin's anabolic and anti-catabolic program. The disconnection between the anabolic insulin signal and the catabolic state in the sarcoplasm starts with the early steps of the signaling pathway. Dexa treatment does not change basal levels of autophosphorylated insulin receptor and phosphorylated IRS1, in absolute or stoichiometric terms[401, 405]. However, insulin's ability to cause IRS 1 and 2 phosphorylation is reduced to less than a third, in mice treated for 5 days with $1\,\mathrm{mg/(kg\,d)}$ Dexa[406]. Dexa hinders the recruitment of PI3K to the membrane complex containing insulin receptor and IRS1[401, 406]. It has been speculated that PI3K catalytic subunits are sequestered in the cytosol by an glut of its regulatory unit, p85[401], whose expression is upregulated by Dexa[399]. These findings suggest that GCs activate a multifaceted anti-insulin program.

In other systems, including muscle cell lines treated with Dexa in vitro, the depletion of membrane-bound PI3K leads to reduced production of 3-phosphoinositides, lower activation of 3-phosphoinositide-dependent protein kinase 1 (PDK1), and lower phosphorylation of the latter's substrate, Thr 308 on the activation loop of Akt. While Thr 308 phosphorylation is not detectable in rat muscle in basal state, its sensitivity to insulin is depressed by Dexa[407]. A similar situation is seen at the other phosphorylation site on Akt, Ser 473, which is stimulated by growth factors, through mTOR complex 2 and possibly through 3-phosphoinositides[408]. One study reported that chronic Dexa induced a reduction in Ser 473 phosphorylation[409], but more commonly, in basal conditions, Ser 473 phosphorylation is essentially undetectable in rat muscle[407, 406]. Nevertheless, the same studies report that Ser 473 sensitivity to insulin is reduced by Dexa, in a pattern similar to Thr 308. It is unclear what brings about the coordination between Ser 473 and Thr 308 phosphorylation in

GAML, because the separation of roles between them is a subject of ongoing research. In vitro manipulations indicate that the two sites synergize for maximal Akt activation[410]. Mutagenic abolition of Ser 473 phosphorylation abrogates Akt activity on substrates belonging to the FoxO class, but does not change Akt ability to phosphorylate tuberin (Tuberous Sclerosis Complex 2; TSC2) and glycogen synthase kinase 3 (GSK3)[411]. This suggests that mTOR complex 2 modulates Akt specificity rather than its activity, although similar results would be seen if specificity does not change, but FoxO substrates are relatively scarcer than tuberin / GSK3. An alternative hypothesis is based on the fact that non-mutagenic in vivo experiments failed to find circumstances when Akt is activated in the absence of Ser 473 phosphorylation (reviewed in [412]). This suggests that in vivo Ser 473 may be a requirement for Thr 308 phosphorylation. In the absence of better experiments, the concerted Dexa-imposed changes in Ser 473 and Thr 308 may be ascribed to either Ser 473 epistasis, or to parallel PI3K-triggered pathways. Both imply that Dexa-induced Akt modulation must involve Ser 473. Since Ser 473 sensitivity to insulin is depressed by Dexa, Dexa may cause intermittent suppression of Akt, especially after feeding.

Chronic Dexa reduces GSK3-β phosphorylation at Ser 9, thus leading to its activation [407]. In turn, phosphorylation of glycogen synthase at Ser 645, 649, 653, 657 is stimulated, leading to increased intramuscular glycogen deposits[413, 407]. Among that tens of substrates of GSK3-β, the subunit ε of the eukaryotic initiation factor 2B (eIF2B) may mediate an anti-anabolic effect[414]. While inactivation of GSK3-β is typically attributed to latent Akt inhibition, the Ser 9 site is also a target for ribosomal protein S6 kinase, 90kDa (p90-RSK), serum and glucocorticoid-inducible kinase-like kinase (SGKL), and p70-S6K[415, 416, 417]. The role of GSK3-β in muscle atrophy was briefly the subject of research at the turn of the century, in the context of a hypothesized atrophic mechanism involving calcineurin and nuclear factor of activated T-cells (NFAT). Since then, evidence that calcineurin is not involved in muscle

anabolism[418] led to an unjustified neglect of GSK3-β.

In other models, Akt inactivation leads to loss of phosphorylation on TSC2 and PRAS40 (reviewed in [419, 420]). Typically, the effects of the former prevail, suggesting that Dexa should lead to mTOR complex 1 inhibition[421]. One group reported that Dexa-induced decrement in protein synthesis is unmodified, in absolute terms, when mTOR complex 1 is inhibited by rapamycin co-administration[403], suggesting that mTOR signaling is dispensable. In a C2C12 microarray study, interference of IGF-I transcriptional program with PI3K inhibitor was virtually identical to the interference induced by rapamycin[422], suggesting that, on the contrary, mTOR is indispensable for PI3K effects. Most likely, both hypotheses are based on reductionist models that do not reflect true in vivo phenomena. Given its multifactorial nature, regulation of mTOR complex 1 cannot be inferred from its direct investigation, but may be better estimated from changes in its substrates. Phosphorylation of mTOR complex 1 substrates 4E-BP and p70-S6K is measurable in rat muscles, but, similar to Akt, do not change in response to Dexa in basal conditions. But Dexa reduces insulin's ability to induce phosphorylation of 4E-BP and p70-S6K[423, 403], demonstrating correlation with Akt changes.

Many published studies documents the effect of Dexa on the rat cell line L6. However, these and other myogenic cell lines have significant limitations, which may cause divergence between in vitro models and the steroid myopathy they aim to describe. As mentioned earlier, in the case of primary cells, experiments with Dexa are impossible, because primary cell survival is GC-dependent. Moreover, Dexa has proliferative and trophic effects on myogenic cell lines. Commonly tested doses of Dexa, in the range of tens of nM, have been shown to synergize with IGF-I [424] and even to act directly towards improved proliferation of L6 myoblasts[425, 426]. C2C12 fusion is more efficient when Dexa is added to IGF-I[90]. Until the end of the 1990s, Dexa was a common ingredient in myogenic culture media. Even contemporary standard proliferation media,

containing 10% fetal bovine serum, provide significant and unpredictable concentrations of GC, insulin, and IGF-I, impairing their study at physiological concentrations. In fusing C2C12 cells, IGF-I and Dexa synergize to upregulate Dexa catabolic effectors, such as REgulated in Development and DNA damage responses-1 (REDD1)[90]. The synergy is also amplifying some of IGF-I effects, such as Akt phosphorylation on Ser 473[427]. Therefore, it is likely that Dexa-induced loss of sensitivity to insulin cannot be replicated in cell culture, especially when non-myotube cells are still present.

Many in vitro studies on myogenic cell lines fail to deplete myoblasts, and are therefore inaccurate models of proliferation-independent in vivo GAML. With this caveat, in vitro studies have been vital in excluding third-party organs, such as the pancreas, from the analysis of GAML. Some features of GAML are more readily observed, and have been initially described in cell culture. Fully differentiated myotubes from L6 and C2C12 cell lines lose more than a quarter of their diameter when treated with 100 nM Dexa[428]. The loss of diameter is reliably underlined by an 20% increase in protein degradation rate[429, 428]. Four fifths of the Dexa-induced proteolysis augmentation is lost, when proteasome inhibitors, such as beta-lactone, or ATP depleting agents, like dinitrophenol, are co-administered. In contrast, lysosomal inhibitors had no effect, while E-64 has minimal effect[429]. Replicating in vivo findings, L6 and C2C12 cells respond to Dexa by upregulating expression of atrogenes, in a Foxo-dependent manner, and in the absence of detectable Akt phosphorylation[430, 431, 432]. Diverging from in vivo studies, L6 and C2C12 myotubes exhibit Dexa-induced downregulation of p70-S6K Thr 389 phosphorylation and activity [433, 399].

In conclusion, the muscle response to GC is associated with molecular changes congruent with Akt / mTOR loss of sensitivity to trophic factors such as insulin and IGF-I. Interestingly, the pathway appears largely unaffected by Dexa in basal conditions, when trophic factors are withheld. It may be the case that mTOR-mediated mechanisms of GAML become relevant during episodes of

hyperinsulinemia, such as after feeding. For continuous, diet-independent GAML to proceed, mTOR-independent are also required.

Based on p70-S6K divergence, it appears that GC causes Akt-mediated mTOR modulation, in contrast with denervation atrophy, where Akt is downstream of mTOR. However, all our knowledge about Akt pathway in GAML is derived from experiments with intrinsic anabolic interventions. Experiments on transgenic mice, unpicking one putative mediator at a time, may invalidate the current model of GAML, as they did in denervation atrophy.

Foxo transcriptional program in GAML

In Dexa-treated atrophying muscle, myogenin, MyD, and myf-5 are upregulated[434]. It is unclear whether myogenin plays any role in upregulating E3 ligases, as in early stages of denervation-induced atrophy. The upregulation of Foxo by Dexa brings many similarities between GAML and the late stage of denervation-induced atrophy.

Evidence for the role of Foxo during GAML is indirect. The combined knockout of the three regulatable Foxo genes led to abrogation of the late, Akt- and Foxo-dependent, stage of denervation-induced atrophy[370]. Transfection with dominant negative Foxo3A partially prevents diameter reduction in disuse of rat soleus and Dexa-treated C2C12 myotubes[435, 436]. Moreover, dominant negative Foxo3A transfection in rat soleus leads to loss of atrogene upregulation[437]. Therefore, Foxo may provide an mTOR-independent pathway for GAML.

The importance of Foxo transcription factors is supported intuitively by their role as integrators of multiple atrophy signaling pathways. Dexa causes their upregulation by multiple mechanisms. First, chronic Dexa doubles Foxo expression[399]. Foxo promoters contain GR-binding sites[438]. In vitro, Foxo induction by GR is facilitated by the histone acetyl transferases p300 and

CREB-binding protein, which are independently upregulated by Dexa[439]. Second, Foxo transcription factors are thought to be potentiated by Dexa through post-translational means. Phosphorylated Foxo transcription factors are exported from nucleus, and eventually marked for ubiquitin-proteasome-mediated degradation (reviewed in [440]). It is believed that the Dexa-induced impairment of their kinase, Akt, contributes to an increase in their activity in GAML, although the evidence for this is weak. In rat levator ani, inhibition of Akt by chronic Dexa cause a reduction in Foxo phosphorylation[441]. Interestingly, this report is also one of the few describing changes in Akt activity in response to Dexa at basal levels, possibly illustrating the peculiarities of male levator ani rather than a common mechanism. In CC12 myotubes, one group reported the replication of this finding[435], while another refuted it[431].

Among the Foxo-induced genes, a special importance was given to the E3 ligases associated with muscle loss, MAFbx and MuRF-1[431]. Both atrogenes contain FOXO-binding regions in their promoter[435, 442]. These Foxo-responsive elements are in close proximity of SMAD3-binding elements, in a shared response element which facilitates synergistic interactions between the two classes of transcription factors[443]. Interestingly, SMAD3 mediates myostatin, a strong negative regulator of muscle mass (discussed later).

REDD1

LKB1

Dexa upregulates myostatin in the atrophying muscle[444]. In explanted myofibers, expression of a dominant negative form of activin A receptor, type IIB (ActRIIB), the receptor for myostatin, causes an increase in CSA, which is halved by rapamycin[445]. In myostatin knockout mice, Dexa induces most of its transcriptional program, including modulations of IGF-I, MuRF-1, MAFbx, FOXO3A, but Dexa-induced muscle loss does not occur [397]. Therefore, myostatin acts, at least in part, through mTOR-and FOXO-independent pathways. Although it is unclear to what degree GAML depends on myostatin-mediated,

mTOR-independent mechanisms, their existence cannot be discounted.

Protein synthesis modulation in GAML

Dexa has a dual effect on 4E-BP, the inhibitor of protein synthesis that binds and inhibits the eukaryotic initiation factor 4B (eIF4B). First, Dexa induces 4E-BP expression[399], presumably through a Foxo-binding sequence in its promoter[446]. (The promoter has been shown in Drosophila, but the paper describing it in mouse cell culture has been withdrawn for unspecified reason.) Second, as mentioned earlier, Dexa reduces mTOR-effected 4E-BP phosphorylation sensitivity to insulin, which is expected to cause reductions in protein synthesis rate expected after feeding. Currently published studies did not account for such fluctuations. Often, animals are euthanized after being fasted. Perhaps for this reason, available data on protein synthesis are inconclusive.

Some of the most exhaustive studies in this field came from Grizard laboratory. In two studies using $0.5\,\mathrm{mg/(kg\,d)}$ Dexa, protein synthesis was unchanged in the epitrochlearis muscle, compared to pair-fed animals[387, 447]. In another study, assaying other glycolytic muscles, the same dose of Dexa was reported as halving of translation rate[448]. In a later study, quadrupling the dose of Dexa lead to a significant, but less ample reduction in FSR[403]. Tomas observed an increase in FSR in response to Dexa, in one of the very few published experiments that failed to observe changes in protein degradation rate[449]. Overall, changes in protein degradation are more reliably seen than those in protein synthesis, suggesting that the latter may be observed only in special conditions, such as immediately after feeding.

In cell cultures, Dexa inhibition of protein synthesis should be more readily detectable, given that, in vitro, Dexa affects 4E-BP phosphorylation directly, even in basal state[430]. However, studies are similarly equivocal. In the most glaring example, the same group, using the same methods, found that protein synthesis is

“not altered” by Dexa in one experiment, and decreased in another[428, 450]. Studies measuring protein synthesis in a mixed population of differentiating and proliferating L6 cells observed a low amplitude decrease in protein synthesis rate[451]. Even when detected, the amplitude of changes in protein synthesis is far lower than that on protein degradation, suggesting a secondary role for the latter[450, 451, 452].

As in human studies, the role of protein synthesis modulation during hypercortisolism is still debated, because some acute effects are detectable. In the first hours after receiving GCs, the rate of protein synthesis in the muscle decreases, as indicated by reductions in incorporation of labeled precursors[453]. Other suggestive data come from indirect evidence, which corroborate with the observed changes in 4E-BP1. For example, acute GC administration causes a reduction in the proportion of polyribosomes[454]. The proportion of polysomes recovers to basal level in less than 24 hours, when the GC is short-acting prednisone[384]. In contrast, polysome downregulation lasts about two days after Dexa. But there are no accounts on polysome profile changes after longer GC exposure.

Putative role of autophagy in GAML

Expression of the lysosome protease cathepsin D and of the calcium-dependent protease m-calpain is trebled by Dexa[387].

Animal models of steroid myopathy

Lol

Ubiquitin expression is stimulated by hours of Dexa treatment[395].

Dexa increases muscle glutamine synthetase activity[455], possibly as an adaptation to increased free amino acids.

Triamcinolone reduces IGF-I expression in diaphragm (PMID: 9872851).

Six or 24 hours of 10-1000 nM Dexa determined increased release of radioactive tyrosine from rat L8 fully differentiated myotubes. In these cells, Dexa upregulated the expression of m-calpain and cathepsins D and B[456].

According to PMID: 9083257, L6 myotubes do not change protein degradation rate when treated with Dexa. Dexa treatment of L6 “myotubes“ upregulates p85 regulatory of PI3 kinase, but decreases PI3 kinase catalytic activity bound to IRS-1, probably because p85 alpha confines p110 to the cytosol (PMID: 9054447). In L6 cells, Dexa downregulates total and phosphorylated IRS-1, and leaves S6 kinase activity unchanged (PMID: 9468291).

C2C12 express MyoD in response to Dexa (PMID: 8744946). C2C12 cells do not express MyoD in response to Dexa (PMID: 9187537).

C2C12 express IGFBP-2 in response to Dexa or to IGF-I (PMID: 8691100).

C2C12 increase protein synthesis in response to IGF-I when they divide, but do not respond any longer when “fusing” (PMID: 9042337).

C2C12 fusion is no affected by Dexa (PMID: 9011578).

C2C12 differentiation is stimulated by Dexa (PMID: 9674941).

Dexa reduces C2C12 calcium influx by non-transcriptional means (PMID: 9756393).

Dexa reduces protein synthesis rate in cultured bovine myotubes (PMID: 9781494).

Models of anabolic alleviation of GAML

In a model where C2C12 cells are treated with Dexa, they proliferate less (rather unusually), downregulate protein synthesis, and upregulate protein degradation (all by tracer). (PMID: 8791198). T has no significant effect on any of these.

Anabolic steroids regulation of muscle mass

Protein synthesis regulation

Protein degradation regulation

Atrophy-associated genes

Autophagy in glucocorticoid myopathy

Other proteolytic mechanisms in glucocorticoid myopathy

IGF-I role in muscle homeostasis

IGF-IEa isoform

Insulin or IGF-I, in concentrations close to the physiological levels, increase glucose uptake about 10-fold[402].

IGF-I expression is GH-independent before the age of 6 months (PMID: 11726733).

Triamcinolone causes muscle loss which co-administration of GH does not compensate[457]; or it does(PMID: 8937196)?

Chapter 3

Hypotheses

Chapter 4

Methods

Literature review

The introduction section was based on review of all literature indexed by PubMed. Search expressions included ‘testosterone OR androgens‘, ‘dexamethasone OR betamethasone OR triamcinolone OR prednisone OR prednisolone OR hydrocortisone OR cortisone OR triamcinolone OR fludrocortisone‘, ‘Cushing‘, ‘ribosome OR polysome OR lysosome OR autophagosome OR proteasome OR ligase OR cathepsin OR FOXO OR IGF1 OR calpain OR mTOR OR AMPK OR Akt‘and combinations thereof. Relevant primary data were summarized.

Literature plots were digitized with WebPlotDigitizer, a web application created by Ankit Rohatgi[458].

Animal studies

Male, 6-8 week old (young adult), C57Bl/6J mice were used, using protocols approved by the Institutional Animal Care and Use Committee of the Boston University School of Medicine. Mice were acclimated for 3-7 days between delivery from The Jackson Laboratories through experimental interventions. Mice were injected subcutaneously every morning for 1-7 days with 200 µL corn-oil based solution, including 14 µL (?) ethanol, which delivered .

Cell culture studies

Immunofluorescence microscopy

In vivo studies

Metabolic measurements at organism level

Measurement of muscle protein synthesis and degradation

Enzymatic assays

Gene expression

Immunoblot

Chapter 5

In vivo experiments

todo

Chapter 6

In vitro findings

todo

Chapter 7

Discussion

todo

Conclusions

Future directions

todo

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Curriculum vitae

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Education

PhD candidate, Boston University School of Medicine, expected graduation 2015

BS (Licentiat in Biofizica), Universitatea Bucuresti, 2005

DM (Doctor in Medicina), Universitatea de Medicina si Farmacie Carol Davila Bucuresti, 2002

Research experience

- 2012-2015: Department of Medicine, Boston University School of Medicine, P.I. Dr. Shalender Bhasin, under the supervision of Dr. Carlo Serra

Theme: Testosterone alleviating glucocorticoid-induced muscle loss.

- 2009-2012: Department of Biophysics, Boston University School of Medicine, P.I. Dr. Assen Marintchev

Theme: Interactions between translation initiation factors eIF1A and eIF5B

- 2005-2008: Center for the Study of Brain, Mind and Behavior, Princeton University, P.I. Dr. Anne Treisman, FRS

Theme: Brain mechanisms for statistical processing of visual scenes

- Summer 2004: Biology Department, Universitatea Bucuresti, P.I. Dr. Gordon Reid, under the supervision of Dr. Iurie Barbu

Theme: The mediation of thermoception by ionic membrane channels

- 2001-2002: Biophysics Department, Universitatea de Medicina si Farmacie Carol Davila Bucuresti, P.I. Dr. Dan Eremia, under the supervision of Dr. Eva Katona,

Theme: The effect of non-ionizing radiation of the mobility of the cell membrane lipids

Peer-reviewed publications

1. Serra C, Tangherlini F, Rudy S, Lee D, Toraldo G, Sandor NL, Zhang A, Jasuja R, Bhasin S. Testosterone improves the regeneration of old and young mouse skeletal muscle. J Gerontol A Biol Sci Med Sci. 2013 Jan;68(1).

2. Serra C, Sandor NL, Jang H, Lee D, Toraldo G, Guarneri T, Wong S, Zhang A, Guo W, Jasuja R, Bhasin S. The effects of testosterone deprivation and supplementation on proteasomal and autophagy activity in the skeletal muscle of the male mouse: differential effects on high-androgen responder and low-androgen responder muscle groups. Endocrinology. 2013 Dec;154(12).

3. Guo W, Bachman E, Vogel J, Li M, Peng L, Pencina K, Serra C, Sandor NL, Jasuja R, Montano M, Basaria S, Gassmann M, Bhasin S. The effects of short-term and long-term testosterone supplementation on blood viscosity and erythrocyte deformability in healthy adult mice. Endocrinology. 2015 Mar 16;en20141784. PMID: 25774550.

Other scientific communications

1. Sandor NL, Hendrickson E, Sandor D, Wagner G, Pestova TV, Marintchev A. Interplay Between Intra- And Intermolecular Interactions Involving Human eIF1A and eIF5B. Abstract presented at the 2010 Meeting of Translational Control, Sept. 2010, Cold Spring Harbor, NY.

2. Sandor NL, Lee D, Toraldo G, Zhang A, Jasuja R, Bhasin S, Serra C. The Role Of Testosterone On The Control Of Muscle Protein Synthesis And Degradation. Abstract presented at the 2011 Evans Center Days, Nov. 2011, Boston, MA.

3. Serra C, Lee D, Sandor NL, Toraldo G, Jang H, Jasuja R, Bhasin S. Characterization of the neuromuscular junction in castrated male mice. Poster presented at ENDO2013, The Endocrine Society's 95th Annual Meeting & Expo, 2013, San Francisco, CA.

4. Sandor NL, Jasuja R, Serra C, Bhasin S. Testosterone alleviates glucocorticoid myopathy by inhibiting the proteolytic machinery. Poster presented at the 2013 Evans Center Days, Nov. 2013, Boston, MA.