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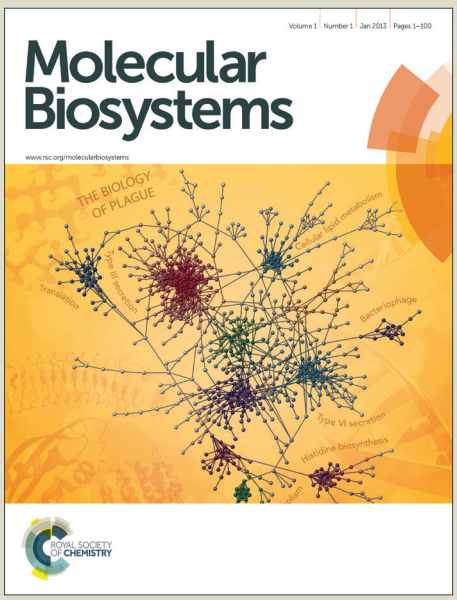

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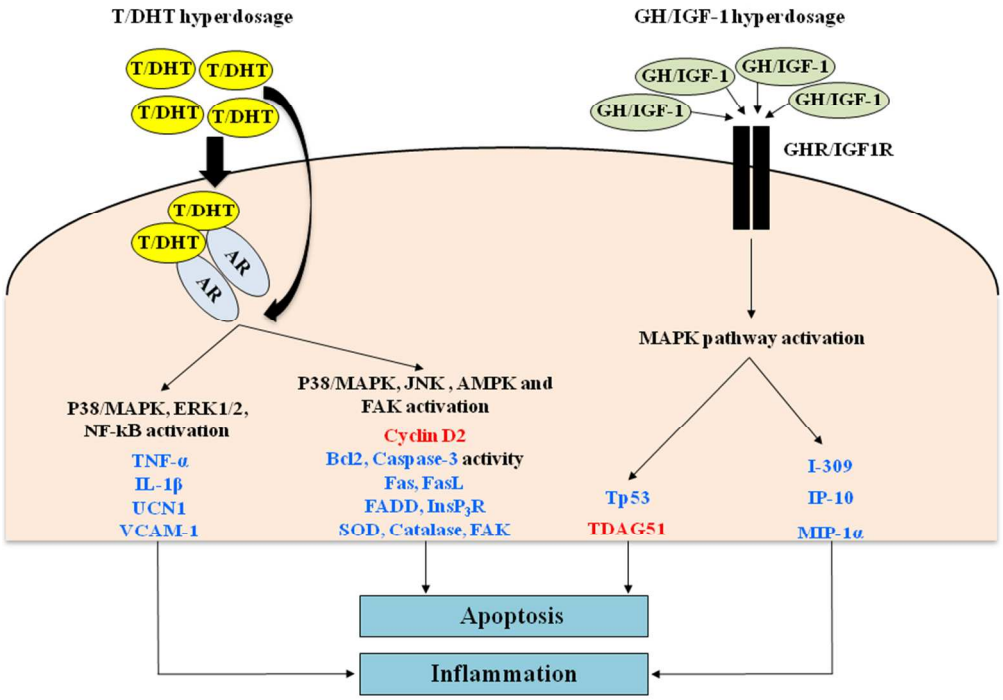

Supraphysiological doses of doping agents, such as T/DHT and GH/IGF-1, affect cellular pathways associated with apoptosis and inflammation.

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Molecular effects of supraphysiological doses of doping agents on health

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Abbreviations: anabolic androgenic steroid (AAS), cardiovascular disease (CVD), cerebellar granulosa cell (CGC), dihydrotestosterone (DHT), growth hormone (GH), GH binding protein (GHBP), GH deficiency (GHD), GH receptor (GHR), human aortic endothelial cell line (HAEC), human hepatocyte cell line (HEPG2), human monocyte-derived macrophages (HMDM), human neuroblastoma cell line (SH-SY5Y), human peripheral blood lymphocyte (PBL), human proximal tubular epithelial cell line (HK-2), human umbilical vein endothelial cell line (HUVEC), high-density lipoprotein (HDL), insulin-like growth factor-1 (IGF-1), IGF-1 binding protein (IGFBP), IGF-1 receptor (IGF1R), low-density lipoprotein (LDL), Performance- enhancing drug (PED), primary human proximal tubular epithelial cells (PTEC), rat pheochromocytoma cell line (PC12), sex hormone binding protein (SHBP), testosterone (T), total cholesterol (TC), world anti-doping agency (WADA).

Abstract

Performance-enhancing drugs (PEDs) gained a wide popularity not only among sportsmen but also among specific subsets of population, such as adolescents. Apart from their claimed effects on athletic performance, they are very appealing due to the body shaping effect exerted on fat mass and fat-free mass. Beside the "underestimated" massive misuse of PEDs, the short- as well as long-term consequences of such habits remain largely unrecognized. They have been strictly associated with serious adverse effects, but molecular mechanisms are far to be elucidated. Here, we analyze the current understanding about the molecular effects of supraphysiological doses of doping agents in healthy biological systems, at genomic and proteomic level, in order to define the molecular sensors of organ/tissue impairment, determined by their misuse. The focus is put on the anabolic androgenic steroids (AASs), specifically testosterone (T) and its most potent derivative dihydrotestosterone (DHT), and on the peptide hormones, specifically the growth hormone (GH) and the insulin-like growth factor-1 (IGF-1). A map of molecular targets is defined and the risk incidence for human health is taken into account.

Introduction

The use of performance-enhancing drugs (PEDs), commonly referred as doping agents, is no longer restricted to sport, but affects also the general population. There is an increasing public-health concern about the widespread misuse of illicit drugs among non-competing amateurs and high-school students in several European countries and in the USA[1,2]. Based on anonymous questionnaires, it has been estimated that the consumers of each country represent about 1% of their respective populations[1]. However, one major problem related to the under- or over-interpretation of these estimates is the sparse information coming from reliable studies of PED abuse in healthy subjects. There is a substantial under-reporting of the numerous side effects of doping agents. The long-term consequences of their misuse remain largely unknown, and, on the other hand, the chronic toxicity from past long-term abusers must be considered nowadays a growing public health problem.

Apart from their claimed role in athletic performance, PED misuse is also strictly associated with serious adverse effects on health, such as cardiovascular diseases and cancer[3-5]. Clinical studies demonstrated that acute myocardial infarction is the most common event among PED abusers[6,7] and that left ventricular hypertrophy may even persist after abuse cessation[8]. The entity of these side effects depends on sex, dose, duration of treatment, fitness condition and on genetic factors. The aim of this review is to analyze the current understanding about the molecular effects of supraphysiological doses of doping agents in healthy biological systems in order to define the molecular sensors of organ/tissue impairment, at both mRNA and protein level, determined by PED misuse. Molecular targets are then discussed in relation to the risk of incidence of specific pathological outcomes. Data concerning the effects of supraphysiological doses of doping agents carried out on unhealthy subjects were not considered in this study. The focus was put on two main categories of doping agents, prohibited by the code of World Anti-Doping Agency (WADA): the anabolic androgenic steroids (AASs), specifically testosterone (T) and its most potent derivative dihydrotestosterone (DHT), and the peptide hormones, specifically the growth hormone (GH) and the insulin-like growth factor-1 (IGF-1).

Testosterone and dyhydrotestosterone

AASs are synthetic derivates of T, the main male sexual hormone. Endogenous T is produced by the Leydig cells in the testes; a small amount can be also secreted by adrenal cortex or obtained by the peripheral conversion of androstenedione[9]. In serum most of circulating T is bound to carrier proteins: sex hormone binding globulin (SHBG) and albumin. Its lipophilic structure allows T to freely cross the plasma membrane of target cells and to bind in the cytoplasm to Androgen Receptor (AR), a 110-kDa member of the nuclear receptor superfamily of ligand-activated transcription factors[10]. By means of 5α-reductase enzymatic activity, in androgen-responsive tissues, such as muscles, internalized T can be rapidly converted into DHT, the most potent derivative, able to bind AR with higher affinity[11]. Ligand-activated AR translocates to the nucleus where it is able to regulate gene expression directly[12] or indirectly through the interaction with some transcription factors, such as c-Jun, Foxa2, Oct1, GATA1, AP-1, p53, RelA, SHP and others[13-14]. Genes, regulated by nuclear ligand-activated AR, encode muscle-specific transcription factors, enzymes and structural proteins[15]; recently, it has been shown that the genomic regulation of ligand-activated AR is exerted also on miRNAs[16-19].

AASs can also regulate cell-specific molecular pathways. In fact, besides to the genomic action, which occurs within an hour, T/DHT show also a rapid (seconds to minutes) non-genomic activity that modulates several signal transduction pathways, including IGF-1 signaling[20-21]. In this context, T/DHT activate second messenger pathways, apart from the classical transcriptional activity, by establishing a crosstalk with signaling molecules, in two main ways: they activate the tyrosine kinase c-Src[22-24] and two members of the MAPK signaling cascade (Raf1 and ERK-2)[23] in an AR-mediated fashion; moreover, T and DHT can also activate cAMP and PKA through the SHBG receptor[21].

Testosterone can also be irreversibly converted by aromatase enzyme to estradiol (E2), the female sexual hormone, equally able to act through genomic and non-genomic mechanisms[25]. The relative amounts between T and E2 is cell specific and their functions, determined by a complex interaction between genomic and non-genomic activities, are distinct, if not conflicting, in different cellular types[9].

AASs gained a wide popularity among sport players due to specific anabolic effects, such as the increase of lean body mass, decrease of fat mass, increase of strength and enhancement of athletic performance; moreover, it cannot be forget that the younger abusers are mainly influenced by aesthetic purposes[26,27]. Several AASs are included in the Prohibited List, published by

WADA yearly.

Such an abuse among professional athletes and amateurs is in contrast with well-documented adverse effects associated to AAS consumption: AAS abuse is reported to be strictly associated to risk increase in cardiovascular disease, liver diseases and reproductive system alterations and changes in behaviour[28-33]. These numerous side-effects as well as the chronic toxicity from past long-term abuse in now middle-aged men must be considered, hence, a growing public health problem.

Timing and protocols of assumption are extremely variable and in general consumers assume multiple drugs for a total androgen dose ranging between 10- and 100-fold above physiological concentration[34]. The average physiological level of T and/or DHT in male serum is 10 nM[35]. Several reports define 100 nM as a supraphysiological concentration (see Table S1).

The improvements in muscle strength observed in response to T administration have been widely described previously[9] and are out of scope of this review.

The aim of the present review is to highlight the molecular effects (genes, proteins) mediated by T/DHT administration on different cells/systems and strictly associated to increased risk for human health.

Growth hormone and Insulin-like Growth factor-1

Growth Hormone (GH) is a single-chain polypeptide hormone produced and secreted by anterior pituitary gland[36,37]. GH secretion is regulated by two hypothalamic factors, the Growth Hormone Relasing Hormone (GHRH) and somatostatin, the first stimulating and the last inhibiting the process[38]. GH secretion takes place in a pulsatile manner with major peaks occurring at the onset of slow-wave sleep and few hours after the meal. Many factors affect GH secretion: gender, age, adiposity, sleep, diet, exercise and other[39]. The levels of circulating hormone are maximal at puberty[39] and decline during adulthood[40]; in aged men, GH levels are 5- to 20-fold lower than young adults, and they are associated to a reduction in GHRH and an increment in somatostatin[38].

Insuline-like Growth Factor 1 (IGF-1), a peptide hormone and a tissue growth factor, is produced by the liver in response to GH action and circulates at nanomolar concentrations. However, GH modulates IGF-1 production in a paracrine/autocrine fashion in healthy individuals in many other GH-responsive tissues[41,42]. IGF-1 mediates many of GH actions, included anabolic functions and growth promoting effects, and exhibits mitogenic and insulin-like metabolic activities[42]. There are two isoforms in humans: IGF-1-liver type and IGF1Ec, mainly produced by the skeletal muscle and known as Mechano Growth-Factor[43].

Circulating GH and IGF-1 are associated to GH- and IGF-binding proteins (GHBPs and IGFBPs, respectively), which regulate hormone half-life and receptor interaction[44-46]. In fact, both hormones activate transduction signaling in target tissues through their membrane receptors: the GH receptor (GHR), a plasma membrane-resident receptor of the cytokine receptor class I superfamily[37], and the IGF-1 receptor (IGF1R), a tyrosine kinase membrane receptor homologous to oncogenes of tyrosine kinase class, along the insulin receptor (IR) [47].

At cellular level, GH/IGF-1 activate the JAK2-Stat5b, the Akt and the MAPK intracellular signalling pathway, particularly important for GH growth-promoting activity[42,48]. Both hormones promote proliferation and survival of a wide range of cell types[49-52] and increase differentiation of cells including myoblasts[53,54].

IGF-1 level in the serum are stable in healthy individuals and its administration inhibit GH release. Subjects with IGF-1 deficiency shows severe growth and mental retardation[55]; on the other hand, higher circulating levels of IGF-1, within the physiological range, are associated to better overall survival compared to subjects with lower physiological levels[56].

The GH/IGF-1 complex signaling network regulates growth, development and differentiation in several tissues[38], and also carbohydrate and lipid metabolism[57-59].

Muscles, along with bones, represent the main target tissue of the GH/IGF-1 axis[60]. Acute administration of GH regulates muscle mass and metabolism by switching fuel utilization toward fat oxidation[61]. In fact, GH stimulates lipolysis, both at resting and during physical activity, determining a rise in plasma levels of FA[62,63] and reducing carbohydrate utilization in healthy and in subjects affected by GH deficiency (GHD)[64,65]. In GHD, lean body mass and muscle mass are reduced[66] but GH treatment ameliorates muscle protein balance by shifting amino acids from oxidative towards synthetic pathways[67-69]; similarly, in healthy subjects, GH supplementation determines anabolic effects, mediated by IGF-1[70,71], not only by reducing amino acids oxidation but also increasing protein synthesis[72,73]. The GH-mediated changes in body protein metabolism is time-dependent with a return to basal protein turn-over within few weeks[74].

The GH/IGF-1 axes contributes also to age-dependent sarcopenia: in muscles, aging is associated to a decrement in IGF1R expression and phosphorylation[75], and to a reduced GHR mRNA expression and to a rise in myostatin expression[76]; in fibroblasts, aging determines a reduction of DNA synthesis and of cell proliferation[77].

Controversial results have been reported regarding GH/IGF-1 role in increasing muscle strength, muscle protein synthesis, fatty acid availability and in sparing of glycogen stores[50,63,78], due to the lack of convincing evidences supporting a direct effect on skeletal muscle. Nevertheless, GH and/or IGF-1 are assumed, at supraphysiological concentrations, alone or in combination with AASs as doping agents[79]; both GH and IGF-1 are included in the WADA list of banned drugs.

Little is known about adverse effects of long-term misuse of GH/IGF-1 at supraphysiological concentrations. Chronic administration for longer periods in healthy athletes may lead to the clinical features associated with acromegaly[79]. Many studies reported that high serum levels of GH/IGF-1 play a key role in CVD risk[80]. A recent study conducted on a large population of middle-aged healthy subjects showed a significant association between higher fasting serum GH-levels and CVD mortality and morbidity risk in man[81]. Moreover, a positive correlation was also observed between slight increments in circulating IGF-1 and the incidence of prostate and colorectal cancers[42,82].

In order to define a clear map of the fuzzy network of actions determined by supraphysiological exogenous administration of GH/IGF-1 on human health, we review the current understanding of their molecular effects, considering recent works focused only on supraphysiological treatments and their consequences at mRNA and protein level (Table S2).

Molecular effects of supraphysiological doses of T and DHT

Vascular effects

Testosterone is a vasoactive hormone that predominantly has vasodilatory actions on several vascular beds[83,84] in a variety of species[85-89]. The proposed molecular mechanism, underlying this action, involve either Ca^{2+} channels and K^{+} channels[90].

Hoppe and co-authors observed a distinct chronic vs acute supraphysiological T effects on single cardiac T-type Ca^{2+} channels from neonatal rat ventricular cardiomyocytes[91]. In particular, the chronic supraphysiological treatment (100 nM and 10 μM for 24-30 h) determined an increase in the whole cell T-type calcium current ($I_{Ca,T}$) density and in the beating frequencies, supported by an increased expression of pore-forming subunits $Ca_V3.1$ and $Ca_V3.2$, both at protein and mRNA levels. Conversely, the administration of acute supraphysiological doses (10 μM) determined a decrease in the $I_{Ca,T}$ current density. Interestingly, the non-genomic acute administration of T on T-type Ca^{2+} channel antagonized the genomic-dependent chronic effect. Such findings were also confirmed in adult rat ventricular cardiomyocytes[92], where similar effects were observed on the L-type Ca^{2+} channels in the chronic (100 nM) and in the acute (100 nM) treatment. Hoppe and co-authors speculate that the non genomic action of T could be explained by its lipophilic nature: in fact, they propose that T might act through the lipid phase of the membrane close to the T- and/or L-type Ca^{2+} channels, similarly to other antagonists of calcium channels.

A different effect of T on voltage-dependent Ca^{2+} channels was reported by Peers and co-authors [93] They evaluated the effects of a physiological (1nM) vs supraphysiological (IC_{50}=275±0.7 nM) T administration on a rat aortic smooth muscle cell line, A7r5. These authors observed that, at 1 nM, T inhibited L-type Ca^{2+} channels, whereas, at high doses, also T-type Ca^{2+} channels were inhibited[93]. Such results differed from Hoppe's group, probably due to the different muscle tissue analyzed in their respective studies (striated muscle vs smooth muscle); anyway, both agreed in suggesting a direct interaction of T with pore-forming Ca^{2+} channel subunits in muscle tissues[94].

In general, the deregulation of calcium homeostasis is often a signal of adverse events and, recently, it has been linked to all cancer hallmarks[95,96]. Hence, we can speculate that supraphysiological T levels might trigger a detrimental cascade of molecular events in muscle tissues, by interfering with calcium current density, eventually leading to major risks of cancer development.

Regarding the effects mediated by T on K^+ channels regulation, Hoppe and co-authors[97] demonstrated a cytoprotective effect of T from ischemic cell death in rat ventricular myocytes. The authors reported that supraphysiological doses of T (10 μM) protected cardiomyocytes against ischemic injury by opening mitoK_{ATP} channels and allowing the oxidation of mitochondrial flavoproteins. Similarly, at supraphysiological concentrations (up to 300 μM), T causes potent and rapid vasorelaxation by activating K_{ATP} channels in the human radial artery[98] and by inhibiting Ca^{2+} influx in rat aorta[99], leading to the hypothesis that T stimulates the K_V and K_{Ca} channels in large conductance vessels and, conversely, the K_{ATP} channel, in small resistance vessels[98].

Vasodilation affecting K^+- and/or Ca^{2+} channels is an endothelium-independent process; on the other hand, this phenomenon can occur also through an endothelium-dependent mechanism that includes the production of endothelium-derived vasoactive peptides. At supraphysiological level, T/DHT plays a role also in this context. Infact, T (up to 3,5 μM) induces an increase both in adrenomedullin (ADM)-secreting endothelial cells and in ADM mRNA expression in a concentration-dependent manner in human aortic endothelial cells[100] (HAECs). On the other hand, high levels of T determines an increase in endothelin-1 (EDN1)-secreting endothelial cells and in the correspondent EDN1 mRNA expression[101]. ADM and EDN1 have potentially contrasting actions on vascular smooth cells, being the first a potent vasodilator[102,103] and the latter a vasoconstrictor peptide[104]; nevertheless, they are similarly regulated by high levels of T in HAECs. Taking in account such findings, it can be considered that, unlike the physiological levels, supraphysiological doses of T affect vascular activity in a complex manner, and it is not easy to predict any specific vascular effect.

An altered vascular responsiveness to hormonal stimuli is considered an hallmark of atherosclerosis. The genesis of an atherosclerotic plaque is a complex process that involves several factors (genetic, enviromental and/or pathophysiological)[105]. A specific set of key risk biomarkers, such as central abdominal obesity, high levels of triglycerides, elevated low-density lipoproteins (LDL) and reduced high-density lipoproteins (HDL), defines the main features predisposing to atherosclerotic plaque formation, a process closely interconnected with the action of pro-inflammatory cytokines.

Androgens have long been considered major contributors to the risk of atherosclerosis. For example, when rhesus monkeys were submitted to repeated injection of T (bimonthly administration; 50 mg/inj; 32 months), to increase serum levels of into supraphysiological range, LDL increased from the 12th month, whereas HDL decreased constantly during the treatment in

treated animals[106]. At the same time plasma glutamate oxaloacetate transaminase (SGOT) and plasma glutamate pyruvate transaminase (SGPT) levels increased and remained elevated up to the end of treatment.

Bhasin and co-authors monitored T effects on eugonadal healthy men (18-35 yrs) by weekly injections varying from low physiological (25 mg/inj/wk; 20 wks) to supraphysiological (600 mg/inj/wk; 20 wks) concentrations. Partecipants received monthly injections of a long-acting GnRH agonist to suppress endogenous T production. Bhasin demonstrated that only at high doses T induced a decrease of HDL and apolipoprotein A1[107]. Similarly, Herbst and coll administred T (600 mg) weekly for 3 weeks to elderly, obese, eugonadal men and observed a reduction of HDL and SHBG together with an increase of LDL density and Hepatic Lipase (HL) activity. The rise in HL activity was responsible for the conversion of HDL_2 to the denser HDL_3, leading to the reduction of HDL[108].

Despite such findings, new emerging evidences are pointing out that T may play a protective role in vascular health, as antagonist of the atherosclerotic process. Indeed, clinical and epidemiological studies confirm that low plasma T levels are positively associated to atherosclerosis[105,109,110]. Furthermore, Androgen Deprivation Therapy (ADT) in prostate cancer patients determines an increase of total cholesterol (TC), LDL and triglycerides in serum[111,112]; conversely, T replacement therapy in hypogonadal men induces a decrement of serum TC, LDL and triglycerides and promotes an increase in HDL[113]. Nevertheless, only few reports addressed the role of supraphysiological doses of T on atherosclerosis in healthy subjects. Langer and co-authors[114] demonstrated that T, both at physiological (10 ng/ml) and at supraphysiological concentrations (100 ng/ml), increases the expression of the scavenger receptor B1 (SR-B1) mRNA and protein in human hepatocyte cell line (HEPG2) and in human monocyte-derived macrophages (HMDM) cells in a dose-dependent manner. SR-B1 is known to mediate selective uptake of HDL-derived cholesterol and cholesteryl ester into the liver and in steroidogenic tissues[115.] Authors hypothesized that T plays a protective role in that context as it intensifies reverse cholesterol transport, by facilitating the transport of excess cholesterol from atherosclerotic plaques of arterial wall to the liver[114]. Likewise, an anti-inflammatory role for T was hypothesized by Corcoran and co-authors[116]: they observed that supraphysiological doses of T significantly reduced the expression of the pro-inflammatory cytokines TNF-α and IL-1β in primary HMDM cell cultures treated with moderately oxidized LDL (50 mg/ml, 48 hours).

Recently, Wan and co-authors[117] reported that supraphysiological levels of DHT (1 μM) determined a converse effect on human umbilical vein endothelial cells (HUVEC) in presence or absence of lipopolysaccharide (LPS), a powerful bacterial virulence proinflammatory factor. It was observed that, in absence of LPS, DHT induced a significantly downregulation of urocortin (UCN1) mRNA and protein expression through an AR-dependent mechanism; conversely, in presence of LPS, UCN1 mRNA and protein expression increased in HUVEC through an AR-independent mechanism, involving p38/MAPK, ERK1/2 and NF-kB activation. UCN1 is a neuropeptide belonging to the corticotropin-releasing factor (CRF), up-regulated by inflammatory cytokines[118], and involved in the vascular inflammatory process. Hence, such opposing experimental evidences suggest that supraphysiological DHT levels exert on vascular cells a differential action, based on the inflammatory status: T/DHT administration would not induce inflammation *per se*, but, it could be able to amplify the pro-inflammatory effect of LPS. Also Annibalini and co-authors confirmed recently that the role on inflammation of sex steroids (T, DHT and E2) is dependent on the inflammatory status of the system under investigation[119]. In fact, they demonstrated that the inflammatory action of TNF- α is magnified by co-administration of supraphysiological doses of T (up to 1 μM) or DHT (100 nM), by increasing the TNF- α-induced vascular cell adhesion molecule 1 (VCAM-1) gene expression; conversely, in absence of TNF- α stimulation, T was unable to modify significantly the expression pattern of VCAM-1 gene. These results are in contrast with Hatakeyama's findings[120]. This latter observed that a similar supraphysiological T treatment (100 nM and 1 μM) on HAECs determined a reduction of TNF- α-induced VCAM-1 expression. The controversial results can be ascribed to the different endothelial system (HUVEC[119] vs HAEC[120]) and to the different TNF-α concentration (1 ng/ml[119] vs 20 ng/ml[120]), used by the two research groups. Anyway, such opposing data underline the complexity of the cross-talk among biologically active species, above all when supraphysiological concentrations of hormones are taken into account.

Apoptotis

Apoptotic damage of vascular endothelial cells is a key event in atherogenesis. Testosterone and its metabolites are able to shift the balance toward cell survival or apoptosis through highly orchestrated mechanisms, not completely elucidated yet.

Ling and co-authors reported that hyperandrogenic states (up to 100 nM) induced apoptosis in serum free HUVECs, through the detection of multiple apoptosis-associated determinants, such as

the reduction of DNA synthesis and Bcl-2 expression, and, on the other hand, the increase in the number of apoptotic cells and in genomic-DNA fragments[121]. Supraphysiological T doses (100 nM) on HUVEC determined an alteration of endothelial cell growth with a strong anti-proliferative effect, leading to apoptosis and affecting intracellular Ca^{2+} levels[122]. Similarly, Powazniak and co-authors on HUVECs confirmed that supraphysiological T concentrations (0.1-9.6 μM) promoted the activation of JNK and p38/MAPK pathways, causing apoptotic cell death[123]. Also Kayampilly and co-authors observed that hyperandrogenic DHT states reduced cyclin D2 mRNA expression and inhibited granulose cell proliferation, all events being mediated by AMPK activation[124]. Similarly, the same authors showed that DHT activates AMPK in a time and dose-dependent manner and reduces FSH-mediated mitogenic signal, leading to the inhibition of granulosa cell proliferation[125]. Furthermore, Verzola and co-authors[126] confirmed a pro-apoptotic behavior of supraphysiological doses of T (up to 1 μM) on immortalized human proximal tubular epithelial cell line (HK-2) and in primary human proximal tubular epithelial cells (PTECs), through upregulation of Fas, FasL and FADD and activation of caspase-dependent apoptotic pathway.

In neurons, T acts as a neurosteroid determining both neuroprotection and neurodegeneration[127-129]. Also in these cell systems T can affect intracellular Ca^{2+} concentration[130], and it is known that prolonged elevated cytosolic calcium concentrations can initiate the apoptotic program in many cell types[131,132]. Estrada and co-authors evaluated the effect of acute supraphysiological T treatments (100 nM, 1 μM, 10 μM) in a human neuroblastoma cell line (SH-SY5Y). Hyperandrogenic states in neuroblastoma cells induced a decrease in cell viability, an increase in DNA fragmentation and the activation of caspase, triggering apoptotic cell death[133]; moreover an activation of inositol 1,4,5-triphosphate receptor ($InsP_3R$) was also observed [130].

Conversely, to such an amount of experimental evidence stating the proapoptotic action of T, a parallel literature convincingly demonstrates its ability to suppress cell death and promote cell survival. Erkkila and co-authors reported that high T levels (100 nM, 1 μM) suppressed apoptosis in seminiferi tubules from human testis tissues in vitro, indicating that T play a critical role in germ cell survival[134]. Ahlbom and co-authors showed that cerebellar granule cells (CGCs) obtained from 7-day old rat pups, pretreated *in vivo* with T (500 μg/0.05 ml injection), are selectively protected *in vitro* from apoptosis induced by oxidative stress (H_2O_2 or S-nitrosocysteine)[135]; these observations were associated to an increased activity of two of the major antioxidant enzymes, SOD and catalase. Similarly, CGCs, treated in vitro with supraphysiological doses of T (1 μM), were less susceptible to oxidative challenges showing up-regulation of cellular antioxidant defences through

an AR-dependent mechanism[136]. Likewise, Pike[137] demonstrated that T or DHT (up to 100 nM) conferred neuroprotection from cell death induced by β-amiloid peptide into PC12 cells and into primary hippocampal neurons cultures from Sprague-Dawley rat pups. These results can partly explain the increased vulnerability of an aged brain to neurodegenerative disorders, such as Alzheimer's disease (AD), and the age-related decline of circulating T levels in elderly men[137]. Nowadays, it is clear that T increases neuronal resilience against AD-related injuries and it is used as anti-aging drug[138], but more studies are needed to define the molecular mechanisms involved and to optimize the hormone therapy[139].

More recently, Imperlini and co-authors found out that chronic supraphysiological DHT treatment (0.7 μg/ml total concentration in three doses) on primary human peripheral blood lymphocytes (PBLs) from male healthy donors, induced an anti-apoptotic effect 7d after the first treatment[140]. In fact, it was observed an over-expression of the pro-survival factor Bcl-2 and a reduced activation of pro-apoptotic caspase-3 in the treated cells compared to the untreated PBLs. Indeed that proteomic study pointed out that the steroid treatment affected the expression profile of more than 30 protein species, half of which were related to apoptosis. Similarly, the same anti-apoptotic effect was registered in human PBLs when DHT treatment was associated to a single IGF-1 supraphysiological administration[141]. Such a double treatment mimicks the ability of supraphysiological T treatments in increasing serum IGF-1 levels in healthy young men[142] and indicates that DHT+IGF-1 hyperstimulation affects cell adhesion, migration and survival through both downregulation of cytokines and paxillin signaling-related proteins, and activation of several pathways downstream FAK.

Biomarkers of T/DHT doping

Supraphysiological doses of T and/or DHT are assumed by athletes and amateurs to enhance sport performance and/or to obtain a better body shape. Up to now it is difficult to find convincing reports describing genomic effects of T/DHT chronic treatment on the health of young sportsmen; sometimes, few gene information can be gained by studies planned with different aims, even if those data need to be confirmed in specific validation assays. But recently, Mancini and co-authors performed a chronic supraphysiological DHT treatment (0.7 μg/ml total concentration in three doses) on human PBLs and analysed the differentially expressed genes by a transcriptomic approach in order to define a putative set of biomarkers of steroid doping[143]. In this study, authors reported that 275 genes (210 up-regulated, 65 down-regulated) were differentially expressed 7

days after the first treatment, most of them matching significantly the “Skeletal and Muscular Disorder” category according to the Ingenuity Pathway Analysis database. The most upregulated genes in this dataset were IDO1, CXCL13, CCL1, GZMB, VDR and IL2RA.

Several factors concur in shaping the effects of supraphysiological doses of T/DHT: i)the age, ii)the gender, iii)the organ system (tissue and source), status and enzymatic profile, iv)the type, dose and duration of a T/DHT treatment, v)the endogeneous T/DHT levels and vi) the putative interactions with other biologically active molecules (es.: hormones, cytokines) or endocrine tissues (es.: adipose tissue). Each parameter modify heavily the outcome of any single treatment; such a multiparameter context explains the extreme variability in published reports. Such a variability gives rise to the present confounding information about the effect of supraphysiological doses of T/DHT, able both to trigger and to inhibit pivotal cellular pathways/functions (vasoactivity, calcium homeostasis, atherosclerosis, inflammation, apoptosis; Fig. 1). Indeed more molecular studies are needed to better define the complex array of effects determined by T/DHT on several organs and apparatus.

Molecular effects of supraphysiological doses of GH and IGF-1

Apoptosis

As a growth factor, IGF-1 controls proliferation and differentiation, and protects cells against apoptosis, as demonstrated in several in vitro and in vivo systems[144,145]. Velazquez and co-authors investigated the effects of physiological and supraphysiological IGF-1 levels on preimplantation bovine embryos, with a particular focus on polycystic ovary syndrome (PCS), characterized by high levels of serum IGF-1. These authors found out that at physiological levels (from 50 to 150 ng/ml), IGF-1 did not affect apoptosis. Conversely, at supraphysiological concentrations (from 950 to 1500 ng/ml), IGF-1 induced several biological/biochemical effects: a) increased apoptosis; b) decreased TP53 protein expression; c) increased number of cells and IGF1R protein expression in the inner cell mass (ICM)[145]. Such findings do not correlate with previous studies, where a downregulation of IGF1R was observed in blastocysts or mouse embryos treated with high IGF-1 levels[146-148].

As for anti-apoptotic effects, it has been reported that treatments with 50nM of IGF-1, up to 48 h, increased T cell death-associated gene 51 (TDAG51) expression, at gene and protein level, through activation of IGF-1R and p38 MAPK pathway, in mouse embryo fibroblasts[149]. In particular, authors demonstrated that TDAG51 plays a regulative role in the anti-apoptotic effects of IGF-1.

Nevertheless, the anti-apoptotic effects of IGF-1 are dose- and system-dependent. In fact, high IGF-1 levels did not determine any apoptotic effects in human PBLs (Orrù personal communication). In this cell system, 6d after a single IGF-1 hyperstimulation, the MAPK signaling pathway was still active; in particular, p70S6K Tyr229, Tyr389 and Tyr421/Ser424 were found all phosphorylated, thus indicating that the acute *in vitro* treatment generated several sustained signaling, including those related to protein synthesis processes[150]. Such proteomic study showed also a consistent cytoskeletal reorganization mediated by Stat-1 and an overproduction of cytokines positively related to immune response and inflammation. All together these data indicated that, following IGF-1 hyperdosage, circulating PBLs could be more prone to transendothelial migration[150].

In the same experimental model, an acute supraphysiological IGF-1 treatment determined the overexpression of 102 genes, involved in skeletal muscle disorders, as well as in cell-mediated immunological response[143]. Among these genes, the most upregulated species are fibronectin 1 (FN1), involved in cell adhesion and migration processes, including host defense and metastasis[151], and RAB31, an oncogene key regulator of intracellular membrane trafficking and associated to

breast cancer[152, 153]. The transcriptomic approach was also adopted by Mitchell and co-authors[154] to evaluate the molecular effects induced by GH abuse in PBLs isolated from male and female recreational athletes. GH treatment (2 mg/inj/die for 8 wks) induced an approximately 2-fold increase in serum IGF-1; RTqPCR validation assays confirmed an upregulation of HSPC159, ITGB3, OLFM4 and TUBB1 genes only in females.

Cancer

The system GH/IGF-1 has been recognized for decades for its role in tumorigenesis and growth[42,155]. IGF-1 plays a key role in tumour formation and proliferation. Several evidences from both humans and animal models demonstrate a link between GH/IGF-1 levels and cancer risk[155]. Transgenic GH overexpressing mice, characterized by elevated circulating IGF-1 levels, exhibit hepatomegaly due to hypertrophy and hyperplasia[156]. The cellular morphological modifications, so-called pre-neoplastic lesions, observed in the liver of GH overexpressing mice are similar to that observed in humans at high risk of liver cancer development. Hence, GH overexpression induces tumorigenesis in the liver of transgenic GH overexpressing mice by stimulating tumor cell proliferation[157]. Recently, Miquet JG et al. investigated the molecular pathogenesis and the signal transduction pathways related to the pro-oncogenic liver pathology induced by prolonged exposure to elevated hepatic GH levels in transgenic mice model[158,159]. In particular, the authors evaluated the mRNA and protein expression and the activation of several signaling mediators and effectors involved in cell growth, proliferation and survival, such as Akt2, NFκB, GSK3β, β-catenin, cyclin D1, cyclin E, c-myc, c-jun and c-fos[158,159]. These studies indicate that prolonged exposure to GH leads to a liver dysregulation of several oncogenic pathways similar to that observed in many human tumors.

Biomarkers of GH/IGF-1 doping

In the last decade, in order to clarify the doping action of the GH/IGF-1 system, few studies were based on gene doping animal models.

In this context, a gene doping model of GH-overexpressing rats has been recently used to evaluate both the molecular effects of GH abuse in healthy animals by using a transcriptome approach and to identify putative biomarkers for the detection of unauthorized GH gene therapy in humans. In particular, a gene expression profile was identified on PBLs from rats subjected to long-term GH gene therapy and sacrificed 24 weeks after the injections[160]. Sixty one genes were found

differentially expressed in GH gene-treated rats 24 weeks after GH gene therapy. These genes were mainly involved in processes as angiogenesis, oncogenesis, apoptosis, cardiac hypertrophy, immune networks, signaling pathways, adipocytokines, arachidonic acid metabolism, CAMs and cytokine-cytokine receptor interaction. Eight differentially expressed genes were selected as candidate biomarkers for the detection of GH abuse, after RT-qPCR validation experiments. Some of the differentially expressed genes are involved in inflammation and immunity, such as: Pla2g2a, a PLA2 group IIa secreted phospholipase A2 involved in many human diseases, including coronary artery disease, colon cancer and inflammation; Rap1B, a small GTPase involved in the platelet activation; and Nfkbia, the NF-kappa B inhibitor alpha, involved in the inflammatory response[160].

Following the same rationale, Macedo A et al. performed a proteomic study to characterize the molecular effects in transgenic IGF-1 overexpressing mice[161]. By delivering the IGF-1-cDNA into multiple muscles of adult animals using adeno-associated virus (AAV) vectors, the muscle whole-proteome changes were analyzed after 15 and 30 days, and they were correlated with morphological and functional modifications. The AAV-IGF-1-injected mice can be properly considered a mouse model of doping, since the measured levels of transduced IGF-1 exceed by more 100- and 10-fold those of the endogenous mouse mRNA and protein, respectively. This supraphysiological condition determined, at morphological and structure levels, a marked muscle hypertrophy, neovascularization and a fiber switch from fast to slow type. These cellular alterations are finely supported by proteomic analysis outputs: in IGF-1-transducted muscles, structural proteins involved in muscle hypetrophy and slow fiber-specific proteins were overexpressed, fast type-ones were underexpressed, and the key proteins controlling energy metabolism were upregulated. In particular, the authors suggest that following IGF-1 delivery, a transition from an anaerobic to an aerobic metabolism might occur in muscles, since some of specific enzymes, belonging to both type of energy metabolisms, are concomitantly induced, but not at the same levels. Such a novelty could have been more convincing if time points longer than 30 days would have been considered.

Although the clinical effects of supraphysiological IGF-1 treatment are well documented, a comparable production of molecular studies on short- and long-term effects on healthy subjects is still missing. In this field, the published papers are mainly aimed to discover new biomarkers for detecting GH/IGF-1 doping. In this context, the unique data, at molecular level, are restricted to the study of effects on the serum levels of IGFBP; for example, it has been reported that IGFBP-4

and IGFBP-5 are increased in healthy adults during one month's treatment with supraphysiological GH doses[162].

Many questions still remain to be clarified before accurate and reliable methods for doping detection are found. Certainly, in this field, further studies with a large number of subjects are needed. At moment, a very limited number of studies assess the molecular modifications induced by high doses of GH/IGF-1 over time in healthy in vitro and in vivo systems. However, those studies often describe GH/IGF-1 effects in pathological rather than healthy condition, at physiological and/or pharmacological concentrations, values that are lower compared to the doses assumed by abusers. Despite this, the current understanding of the molecular effects of GH/IGF-1 abuse shows that supraphysiological doses affect cell function such as apoptosis and cytoskeletal reorganization, and they have implications on the inflammation response and on the skeletal muscle system (Fig. 2).

Future perspective

The extreme heterogeneity of data published so far, regarding the effects of doping agents on health, mirrors the scanty knowledge of PED administration protocols as well as the sparse information on their biological activity at supraphysiological concentrations. There is still a long way to solve such a puzzling tangle, and in this scenario metabonomics studies can provide the missing piece that will allow to gain complementary information to genomics and proteomics. In fact, metabonomics, as defined by Nicholson in 1999, represents "the quantitative measurement of the time-related multiparametric metabolic response of living systems to patho-physiological stimuli or genetic modification"[163]. This approach uses biofluids (urine, plasma, seminal fluid, cerebrospinal fluid, saliva and others)[164] and is mainly based on NMR spectroscopy and/or mass spectrometry procedures[165].

Over the past few years, metabonomic protocols were also applied to the doping field. Dumas and co-authors were the first to demonstrate the potentiality of metabonomics as a tool for the analysis of metabolic perturbations induced by doping agents[166]; they revealed the biological signature of AASs in cattle by using NMR spectroscopy, and, in particular, they found out that the urinary biomarkers, at supraphysiological level, are associated to nitrogen and energy metabolism. Afterwards, advances in mass spectrometry (MS) and bioinformatics contributed to the development of untargeted metabonomics to investigate the urine profiling following doping agents administration in animals. In this context, Kieken and co-authors developed a metabonomic approach based on liquid chromatography-electrospray-high resolution mass spectrometry (LC-ESI-HRMS) to compare horse urine fingerprints collected before and after treatment with recombinant equine growth hormone (reGH)[167]. About 20 metabolites detected by different mass/charge values and retention times were selected as potential biomarkers of GH abuse; interestingly, the results of this preliminary study showed a long-term effect of reGH, demonstrating global modifications of horse urine metabolome mostly 25 days after the first administration. However, this pioneering study remains mainly technical, since the identity of the metabolites responsible for the discrimination between treated and non-treated animals are nowadays unknown. A similar approach based on ultraperformance liquid chromatography in combination with time-of-flight accurate mass spectrometry (UPLC-TOFMS) was used to analyze urine profiles of bovine treated with AASs[168]. In this paper, metabolites, differentially regulated following doping agents administration, were partially identified by accurate mass data and

retention time comparison with commercially available standards or by LC-LTQ-Orbitrap tandem MS.

However, the purpose of these early studies was to set up and assess MS-based metabonomic strategies as new screening tools for doping agents abuse, thus demonstrating the feasibility of such approaches[169,170].

Recently it has been described a semi-automated strategy for the annotation (based on experimental masses and retention times) of metabolites in global fingerprints acquired from untargeted metabonomics approach from tissue samples of bovine treated with AASs[171]; this implementation, in fact, requires specific softwares and is essential for metabolite identification, thus representing the major challenge for the feasibility of metabonomic approach.

The application of metabonomics to the doping in humans is mainly focused on the technological advancements and on the search for the most sensitive protocols serum[172,173]; few reports are focused on the potential risk of PED consumption for human health. One example comes from West and co-authors, who assessed the risk of human exposure to endocrine active compounds, such as T, in human germ-like cells (GLCs)[174]. At the highest dose tested (100 μM), all the steroid hormones determined a decrease in intracellular level of amino acids and an increase in metabolites related to cellular energetics and metabolism, such as glucose and lactate. Moreover, a decrement in the degree of fatty acid saturation and in C14-C20 fatty acids was observed; finally, at 100 μM T caused a reduction of cholesterol and cholesterol-derivatives.

The low number of metabonomic studies evaluating the molecular effects of doping agents on health is related both to ethical concerns and to the great effort in setting up the experimental design. In fact, to perform a metabonomic investigation, whatever the aim pursued, tools, procedures and methods need to be finely scheduled, ranging from the sample collection and preparation to the generation of metabolic profiles/fingerprints, from the raw data processing to the bioinformatic/statistical analysis for metabolite identification.

In the field of doping, a successful experimental design depends on the type of sample (plasma or urine) and on the sample collection from PED abusers and from a control-non doped population. Urine samples are, of course, most feasible and easy to collect rather than plasma, especially in a context of amateur or professional sporting event. However, both types of samples are informative about metabolic changes resulting from doping agents abuse: urine, in fact, is a rich source of hydrophilic metabolites; the plasma composition, on the other hand, is more stable and representative of other kind of molecules.

The sample collection represents a challenging issue in a metabonomic study applied to the analysis of doping agents molecular effects in healthy subjects. In fact, metabolic profiles are influenced by fitness condition besides to sex, food consumption, environmental context as well as by individual genetic profile; as a consequence, the critical step of such experimental designs is related to the definition of exclusion/inclusion criteria for volunteers enrollment in both groups, doped and control. A prerequisite of a good study design is having strong matched-groups in order to observe exclusively doping-dependent phenomena and, hence, to identify putative biomarkers in anti-doping analysis.

Conclusions

AASs and growth factors constitute the most popular prohibited substances among abusers, and it is now increasingly frequent to run into administration protocols that mix molecules belonging to both classes. The consequences on human health determined by a short and/or long-term PED misuse are partly known but the molecular mechanisms underlying such adverse events are still unclear, and sometime confounding. Here, we reviewed the supraphysiological effects of T/DHT or GH/IGF-1 on different cellular pathways/functions, and discussed the consequences of both treatments strictly associated to inflammation processes and apoptosis. In general, the growth factors, at supraphysiological concentrations, exert mainly anti-apoptotic and/or pro-inflammatory effects in different biological systems; conversely, the actions produced by hyperandrogenic states are less defined and sometimes confounding. Surely, several endogeneous and exogeneous factors have to be considered in order to clarify the molecular mechanisms responsible for health risk factors related to PED abuse; further investigations, including metabonomic studies, are needed to define new biomarkers related to the emergent issue of doping-related-dysfunctions.

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Legend to Figures

Figure 1: Schematic representation of the effects induced by supraphysiological doses of T/DHT in several *in vitro* and *in vivo* systems. The main molecular targets (genes in red, proteins in blue) are shown; their main downstream effects are framed.

Figure 2: Schematic representation of the effects induced by supraphysiological doses of GH/IGF-1 in several *in vitro* and *in vivo* systems. The main molecular targets (genes in red, proteins in blue) are shown; their main downstream effects are framed.

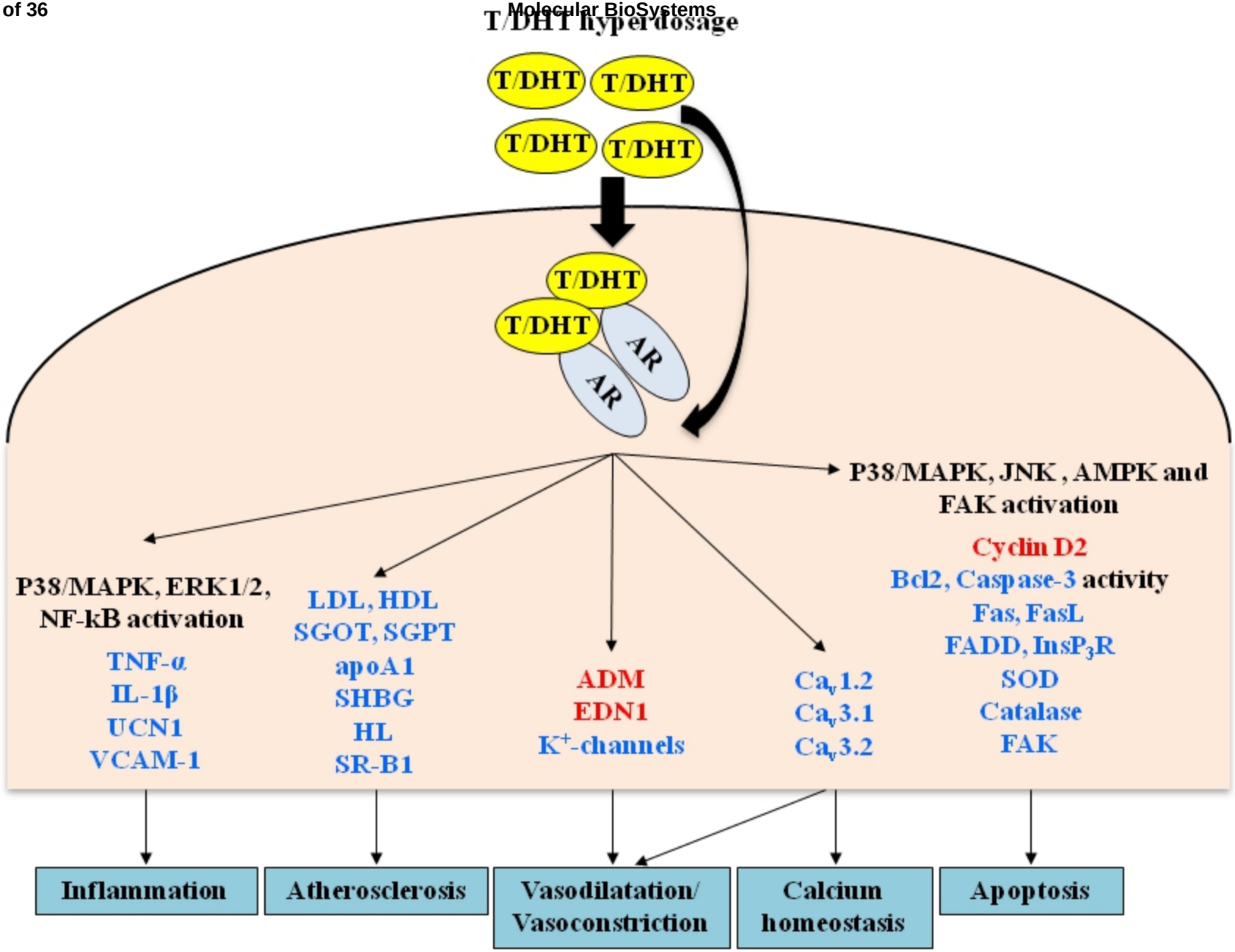

Figure 1

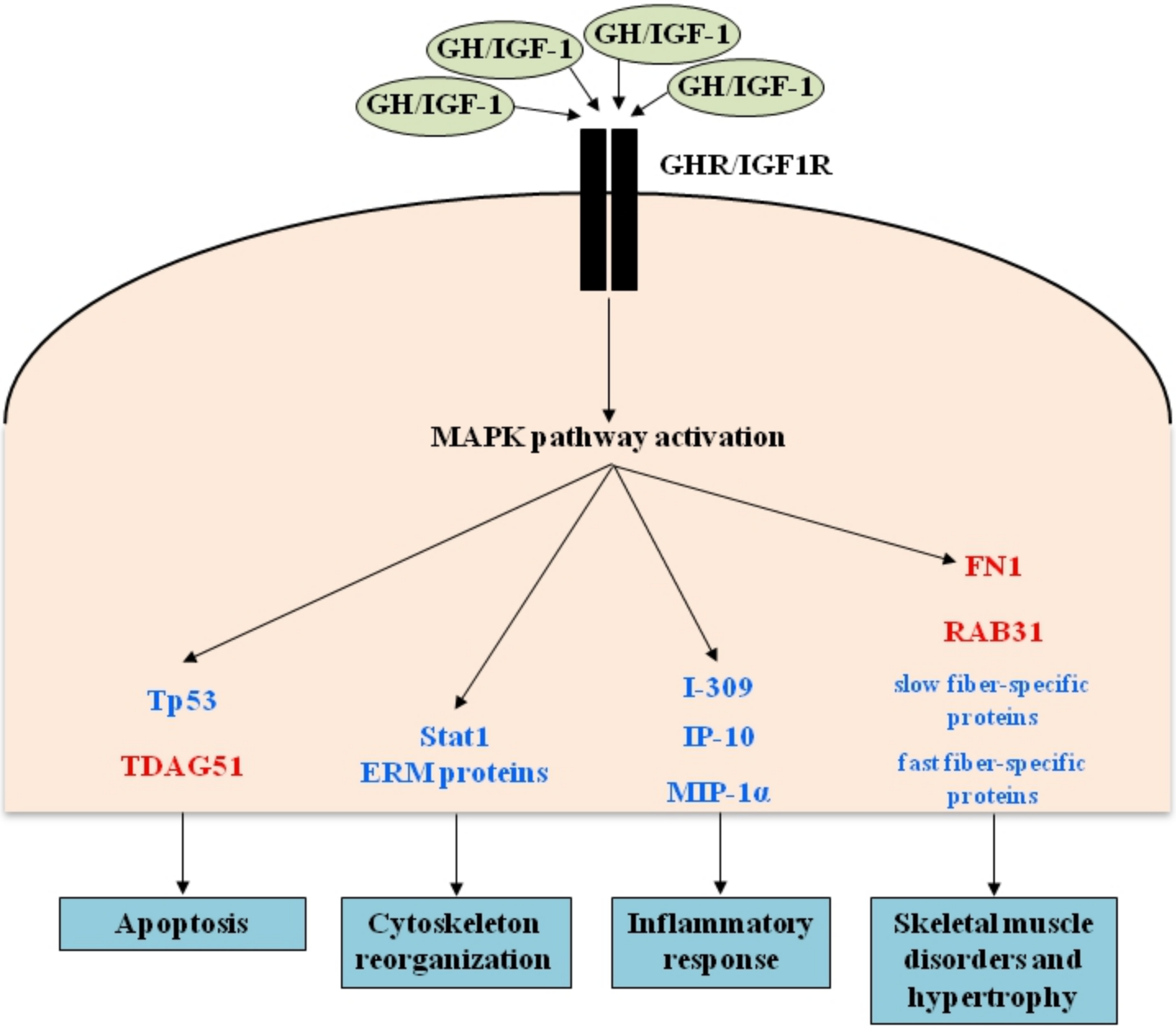

Figure 2