Testosterone recovery therapy targeting dysfunctional Leydig cells

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Abstract

Reduced serum testosterone affects millions of men across the world and has been linked to several comorbidities, metabolic dysfunctions, and quality of life changes. The standard treatment for testosterone deficiency remains testosterone replacement therapy. However, limitations on its use and the risk of significant adverse effects make alternative therapeutics desirable. Studies on the mechanisms regulating and synthesizing testosterone formation in testicular Leydig cells demonstrate numerous endogenous targets that could increase testosterone biosynthesis, which could alleviate

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reduced testosterone effects. Testosterone biosynthesis is facilitated by a conglomerate of cytosolic and mitochondrial proteins that facilitate cholesterol translocation into the mitochondria, the rate-limiting step in steroidogenesis. An effective therapeutic approach would be to increase endogenous testosterone formation by enhancing steroidogenesis in Leydig cells. Numerous ligands for steroidogenic proteins have been developed which increase steroid hormone formation. However, off target effects on neurosteroid and adrenal steroid formation may limit their clinical use. First-in-class biologics, such as voltage dependent anion channel peptides and transplantation of induced human Leydig-like cells offer advances in the development of specific strategies that could be used to enhance endogenous steroid formation in hormone deficient patients.

Introduction

Although some testosterone decline is normal in men of middle and advanced age, some men have significantly decreased testosterone levels known as hypogonadism. Hypogonadism is a condition characterized by severe testosterone deficiency and affects nearly 5 million men in the United States[1]. While hypogonadism is most commonly associated with infertility, it has also been correlated with other numerous conditions, such as cardiovascular disease, depression, fatigue, reduced bone mineral density, increased body fat, metabolic syndrome, and declining muscle mass[2,3]. Hypogonadism can be separated into two categories: primary hypogonadism and secondary hypogonadism. Primary hypogonadal patients present depleted testosterone levels due to a suboptimal response to luteinizing hormone (LH) stimulation; whereas secondary hypogonadism is characterized by low LH levels or low gonadotropin releasing hormone (GnRH) levels, leading to insufficient steroid hormone biosynthesis[4]. Moreover, primary hypogonadal patients display increased LH, suggesting that Leydig cell mechanisms are disrupted[5]. The primary causes of secondary hypogonadism are associated with the pituitary or hypothalamus[4]. These can be congenital, acquired, or caused by damage to gonadotrophs[4].

Given testosterone's essential role in spermatogenesis, hypogonadal patients suffer from infertility[6]. Furthermore, androgen metabolites levels, such as dihydrotestosterone (DHT) and 3α-androstenediol glucuronide (3α-ADG), become imbalanced and cause alterations in secondary sex characteristics, including muscle mass, body mass index, and facial hair[5]. Patients may present with fatigue and declining mood, given the ability of neurosteroids to act as positive or negative regulators of the GABA receptor[7]. There are also numerous congenital and acquired origins of hypogonadism

that may manifest throughout the male lifespan[8]. Therapeutic strategies for endogenous targets to treat hypogonadism from all origins are highly sought.

Testosterone replacement therapy (TRT)[9] and aromatase inhibitors[10] have been used to elevate serum testosterone and alleviate symptoms of hypogonadism. TRT involves administering exogenous testosterone at appropriate intervals, both daily-acting, intermediate acting (1-3 weeks), and long-acting (2-6 months)[9]. However, this exogenous testosterone leads to hypothalamic-pituitary-gonadal axis (HPG) imbalance and suppresses the release of gonadotropins[11]. This represses Leydig cell testosterone biosynthesis, a critical driver of spermatogenesis, and leads to reduced fertility[9,11]. Moreover, intermediate and long-acting injections may produce serious adverse events (SAEs) including pulmonary microembolism, anaphylaxis, and polycythaemia[9,12,13], and an increased risk of cardiovascular disease and stroke may exist in older men receiving TRT as indicated in recent studies[14,15], resulting in the FDA and medical societies cautioning its use[3]. Numerous alternatives to TRT have been considered[16]. The testosterone metabolite DHT is also used strategically to treat hypogonadism in some countries[17]. DHT binds to androgen receptors with a greater affinity than testosterone and provides some relief from symptoms of hypogonadism[18]. The disadvantages of DHT are its price, increased hemoglobin, increased red blood cell count, and inferior clinical results when compared to TRT[17,18]. Aromatase inhibitors are also used to prevent aromatase from converting testosterone to estrogen, thereby, maintaining testosterone levels[19]. In clinical studies with aromatase inhibitor used for hypogonadal patients, LH levels, free testosterone, and sexual desire increased[20]. Moreover, aromatase inhibitors may be suitable for hypogonadal patients with increased estrogen levels[18]. However, concerns regarding the effect of aromatase inhibitors on bone minerals still remain after treatment with the inhibitor letrozole led to vertebrae deformities in 45% of adolescent males with delayed puberty[21]. The selective estrogen receptor modulators clomiphene citrate and tamoxifen are also used off-label for the treatment of primary hypogonadism due to their ability to induce the release of GnRH by the hypothalamus and subsequently increase the production of the gonadotropins LH and FSH by the anterior pitutary[16].

Testosterone regulation and formation:

Testosterone biosynthesis predominantly occurs in testicular Leydig cells and is tightly regulated by the hypothalamus-pituitary-gonadal (HPG) axis, comprised of the hypothalamus, pituitary, and testes[22]. In this system, the hypothalamus secretes GnRH which reaches and stimulates the anterior

pituitary gland to release LH. LH acts on the testicular Leydig cell LH receptor (LHR), a G protein-coupled receptor, and initiates a signaling cascade that mobilizes cholesterol and increases testosterone biosynthesis[22]. LHR stimulation activates adenylate cyclase and increases cAMP production and subsequent cAMP-dependent kinase activation[1]. Mechanistic targets inducing the production of endogenous testosterone in Leydig cells would be most desirable. Viable drug targets should have specificity, a sustainable response, and acceptable safety profiles.

The rate-limiting step in steroid hormone biosynthesis is cholesterol's translocation across the outer and inner mitochondrial membranes (OMM, IMM) into the mitochondria[1]. Cholesterol's translocation into the IMM results in cholesterol side chain cleavage by the cytochrome P450 CYP11A1, producing pregnenolone[23]. This translocation is mediated through a multi-protein scaffold termed the Steroidogenic InteracTomE (SITE)[24]. The SITE is comprised of cytosolic and mitochondrial proteins, of which numerous have become focal points in the search for endogenous targets that induce steroidogenesis. Cytosolic SITE proteins include the acyl-CoA-binding protein (ACBD1/DBI)[25-27], ACBD3[24], Sec23ip[24], steroidogenic acute regulatory protein (STAR)[28-32], 14-3-3 proteins[33-35], and the cAMP-dependent protein kinase (PKA), which is composed of regulatory and catalytic subunits inducing STAR phosphorylation upon cAMP activation[36]. OMM SITE proteins include the translocator protein (TSPO)[1], the voltage dependent anion channel (VDAC1)[1], and ATPase family AAA domain-containing protein 3A (ATAD3A)[24], while IMM SITE proteins include the cholesterol side-chain cleavage enzyme (CYP11A1), ferredoxin (FDX) and ferredoxin reductase (FDR)[24]. The fine details of cholesterol's translocation across the mitochondrial membranes are not yet clear, but there are notable protein-protein interactions that have been elucidated (Fig. 1).

VDAC1 and TSPO are the main anchors of the cytosolic proteins to mitochondrial contact sites[24,37]. ATAD3A bridges the mitochondrial membranes and is involved in contact site formation, mediating access of cholesterol to CYP11A1[38,39]. The adenine nucleotide translocase (ANT) protein interacts strongly with VDAC1 to form a contact site complex between the OMM and IMM, which is involved for the trafficking of molecules across the mitochondrial membranes[40], but does not interact directly with the SITE complex as currently identified[38]. In addition, the IMM optic atrophy 1 (OPA1) protein participates in the formation of contact sites and mitochondrial fusion between mitochondrial membranes, a process essential for steroidogenesis[41]. External response to hormonal stimulation initiates STAR targeting to the SITE complex at the OMM[42]. STAR anchors to the mitochondrial SITE scaffold at VDAC1, a solute-specific transporter to the IMM[40], and STAR becomes phosphorylated by PKA[23]. PKA is targeted to mitochondria by A-kinase anchoring proteins binding

to the regulatory subunits to PKA, such as ACBD3[43], a protein that interacts with TSPO, and AKAP121[44], leading to effective translation and phosphorylation of STAR and conformational changes which would accelerate cholesterol translocation and optimize steroid formation[23,28]. In response to these changes TSPO is polymerized and cholesterol binding is enhanced[45], due to TSPO's high affinity for cholesterol[38,46,47]. TSPO contains five transmembrane domains with separate cholesterol and drug binding domains and is highly abundant in the OMM[48]. ACBD1/DBI is an endogenous ligand of TSPO, involved in hormone-dependent steroid formation[24]. The polymerization of TSPO strengthens the TSPO-VDAC1 interaction, enhancing cholesterol binding and transport[36,38,49]. SITE optimization enhances cholesterol translocation across the mitochondrial membranes to CYP11A1, where FDX and FDR regulate the electrons needed for side-chain cleavage by the enzyme[24,38,48]. 14-3-3γ and 14-3-3ε are hormonally stimulated and act as negative regulators of steroidogenesis by delaying maximal steroid hormone formation[33]. Upon hormone stimulation, 14-3-3γ interacts with STAR, limiting its activity in cholesterol transport[33]. Similarly, stimulation also triggers 14-3-3ε binding to the VDAC1-TSPO complex and regulates cholesterol translocation into the mitochondria by reducing the rate of transport[33]. Other intracellular regulators of steroidogenesis include signaling molecules (PDGF, DHH) kinases (MAPK, PKG, CAMKI, AMPK), and transcription factors (NUR77, MEF2, GATA4)[50,51]. Moreover, numerous nuclear receptors and protein phosphorylation events are involved in steroidogenesis regulation[52,53]. Steroidogenesis is also regulated systemically by the HPG axis[1]. It is imperative that steroid hormone synthesis is precisely regulated, as insufficient or overproduction of steroids is detrimental[1].

Mechanisms of Leydig cell dysfunction:

The physiopathology of numerous diseases related to impaired steroid hormone biosynthesis are mediated by compromised Leydig cell integrity. In aging, the integrity of Leydig cell-specific mechanisms mediating steroid hormone biosynthesis is compromised. Whereas gene mutations in key steroidogenic genes can lead to disease phenotypes or lethality, compromised Leydig cell integrity can be caused by several intracellular factors:

Reductions in steroidogenic enzymes. The steroidogenic machinery tightly regulates and maintains steroid hormone biosynthesis[1]. Declining or aberrant expression of SITE proteins or other proteins

involved in steroidogenesis can occur at the transport, import, or conversion steps[24]. For example, STAR is constitutively expressed in Leydig cells, mediating cholesterol transport from intracellular stores to the mitochondria[54], and extracellular hormonal stimulation of Leydig cells increases STAR expression to upregulate cholesterol translocation[55]. Mutations in *Star* lead to steroid hormone biosynthesis deficiency and the accumulation of lipids in testosterone producing cells[56,57]. Moreover, decreased *Star*/STAR expression with age reduces cholesterol translocation in aged Leydig cells[58].

Mutations to TSPO also alter the ability of steroidogenic cells to import cholesterol into the mitochondria[59]. This results in increased lipid accumulation and disruption of steroid production and has implications for the hormone biosynthesis in the brain, adrenal glands, and testis[59-61]. TSPO's decline in aging Leydig cells showed that alterations in cholesterol import play a role in age-related testosterone decline[62]. Other downstream steroidogenic enzymes that are decreased in aging include CYP11A1, HSD3B, CYP17A1, and HSD17B[63].

Imbalanced antioxidant and reactive oxygen species production. Reactive oxygen species (ROS) are mostly produced by the mitochondria and can compromise the integrity of cellular machinery and structures[64]. Age-related oxidant/antioxidant imbalances are correlated with protein, lipid, and DNA damage, linking integrity of mitochondrial quality control to the development of age-related pathologies[65]. Oxidant/antioxidant imbalance may arise from increased oxidant production in Leydig cells, as mitochondrial superoxide production has been observed in aged rat Leydig cells[66]. While the generation of ATP via the electron transport chain produces ROS ubiquitously in mammalian cells, Leydig cells also produce ROS through hormone biosynthesis via mitochondrial and smooth endoplasmic reticulum P450 reactions[67,68]. Changes in biosynthesis can, thus, alter ROS production. Over time, ROS exposure damages mitochondria and compromises their function, leading to mitochondrial dysfunction[69]. When left uncleared, dysfunctional mitochondria produce excessive amounts of ROS which further damage cellular enzymes and structures[65,70]. Dysfunctional mitochondrial are normally eliminated via mitochondrial autophagy (mitophagy) and replaced by new mitochondria through mitochondrial biogenesis[71]. However, this process, which is disrupted in compromised Leydig cells, causing disruptions to mitochondrial function, cellular homeostasis, and steroidogenesis[58,65].

Reduced mitochondrial function of Leydig cells. Leydig cell steroidogenic function and cellular bioenergetics are integrally linked to one another, as steroidogenesis requires reliable mitochondrial membrane potential and ATP synthesis[72,73]. Mitochondrial dynamics such as fission, fusion, biogenesis, and mitophagy are, therefore, required for sustainable steroidogenic capacity[41]. The clearance of dysfunction mitochondria is mediated by PINK1/PARKIN interactions[69] and the generation of new mitochondria, mitochondrial biogenesis, is regulated by the genes *Nrf1/2* and *Tfam*[74]. The trafficking of molecules across the mitochondrial membranes is mediated through a variety of mitochondrial contact sites, pores, and transporters all of which are regulated by *Mfn1/2*, *Opa1*, and *Drp1*[71]. Aging leads to a decline in these genes' expression systemically across many tissues[75], and the reduction of steroidogenic capacity in aging Leydig cells in particular is driven by this mitochondrial dysfunction[58]. When compared with healthy cells, aged Leydig cells present depressed ATP levels, mitochondrial biogenesis, and mitophagy. Moreover, the expression of genes regulating these mitochondrial dynamics are decreased[58].

Endogenous targets for testosterone recovery therapy:

The role of numerous SITE proteins and steroidogenic regulators have been investigated to identify endogenous therapeutic targets that induce steroid hormone formation. Several proteins within the cytosol and mitochondria mediate cholesterol translocation from intracellular stores to the OMM where the SITE complex resides[38]. Rone et al. investigated the role of numerous steroidogenic and mitochondrial dynamic proteins to elucidate their role in steroidogenesis[38]. Such investigations revealed that knocking down OPA1, VDAC1, and ATAD3A had no effect on membrane permeable steroid formation. However, VDAC1 and ATAD3A knockdowns did reduce hormone induced steroidogenesis, suggesting that OPA1 is not critical for hormone-induced steroidogenesis[38]. Recently it was shown that upregulating OPA1 via pharmacological and transfection methods increased TSPO expression and steroid hormone formation in both basal and hormone-stimulated dysfunctional Leydig cells, suggesting that OPA1 may play a role in the regulation and formation of the SITE complex[76]. Progress has been made in targeting SITE proteins to ameliorate testosterone decline. Studies on TSPO ligands and 14-3-3ε peptides (VDAC1 peptides) have offered potential therapeutic strategies for inducing endogenous testosterone formation. While TSPO ligands enhance cholesterol translocation, VDAC1 peptides are designed to block the negative regulation of steroidogenesis[34,51,77-80].

TSPO ligands: Engagement of the OMM protein TSPO via a drug ligand-induced activation stimulates steroid hormone production *in vitro* and *in vivo* in rats and mice[48,61,77,78,81,82]. TSPO possesses high affinity for cholesterol binding, which leads to its subsequent translocation to the IMM for side-chain cleavage by CYP11A1 producing pregnenolone[83,84]. TSPO's C terminus plays a key role in the uptake of cholesterol from the cytosol and translocation into the mitochondria[85,86], and disruption of the protein within steroidogenic cells disrupts mitochondrial cholesterol transport and steroid formation[84,87]. Steroidogenesis and TSPO expression correlate with one another as shown by disruption of steroidogenesis with TSPO's age-related decline *in vivo* and its ablation *in vitro*[62,87]. Moreover, transfection of TSPO into TSPO-disrupted cells restores steroid formation, demonstrating its indispensable role in steroidogenesis[87].

Numerous studies have shown that drug ligands targeting TSPO produce enhanced steroid levels in both MA-10 tumorigenic Leydig cells and isolated primary Leydig cells, as well as increased serum testosterone levels[77,78,81]. However, serum LH levels may also become increased following TSPO drug ligand treatment likely due to an effect of the ligand on brain TSPO[88,89], suggesting that using this target may enhance testosterone biosynthesis by either stimulating the Leydig cell steroidogenic machinery and/or by elevating LH release[77]. TSPO specific ligands are also known to increase glucocorticoid and corticosteroid levels[61] and have been shown to affect neurosteroid production[90-92]. Accordingly, the use of TSPO ligands as a therapeutic approach to treat neurological and psychiatric disorders have also been investigated[93]. Similarly, the use of TSPO ligands may also induce anxiolytic-like responses, as ligand treatment has been shown to counteract panic attacks in rodents[94]. While molecular entities targeting TSPO elevate serum testosterone levels, adrenal steroids and neurosteroids are also affected. Therefore, TSPO ligands have been proposed as therapeutic agents for the regulation of steroid hormones in the testis and brain. However, this lack of specificity remains an issue, as TSPO is expressed in numerous tissues.

VDAC1 peptides: New insights into the role of 14-3-3ε in the regulation of steroidogenesis have made it a promising therapeutic target. 14-3-3 proteins regulate target proteins by altering activity, post-translational modifications, and subcellular localization[95]. LHR stimulation initiates the translocation of 14-3-3ε to the OMM[33] and its recruitment to the TSPO-VDAC1 complex at Ser167 on VDAC1. There it competes with TSPO for VDAC1 binding and thus reduces cholesterol import[51]. Blocking the

interaction between 14-3-3ε and VDAC1 using cell-penetrating peptides induces steroid formation *in vivo* and *ex vivo*[80]. Aghazadeh et al. fused a component of the HIV transcription factor 1 (TAT) with the predicted Ser167 binding motif on 14-3-3ε, creating a cell permeable VDAC1 peptide, TAT-VDAC1 containing Ser167 (TVS167), which competed with 14-3-3ε for VDAC1 binding[79]. This reduced negative regulation of steroidogenesis by blocking the 14-3-3ε binding to VDAC1, which led to increased steroidogenesis *in vitro* and *in vivo*. Given the homologous mechanisms of 14-3-3ε between species, the TAT-based peptide offers a promising approach in humans. Although TAT peptides penetrate indiscriminately and 14-3-3ε is found in numerous tissues, function is tissue specific[79]. TVS167 treatment did not significantly increase corticosterone levels in rats treated with the compound, demonstrating specificity to testicular Leydig cells[80]. Additionally, the action of the TVS167 peptide induced steroidogenesis independent of LH and would offer a major improvement in safety when compared to TRT[96]. The minimal bioactive sequence of the peptide was recently identified, and we ultimately generated bioactive stable peptide derivatives that can be administered orally and induce T formation in normal and hypogonadal animal models (manuscript in preparation). Moreover, they demonstrate safety, efficacy, and target specificity[34,51,79,80]. In summary, these first-in-class biologics make an excellent candidate for treatment of diseases caused by Leydig cell dysfunction over other pharmacologic or biologic strategies.

Implantation of human Leydig-like cells: The generation of transplantable testosterone-producing cells offers another alternative for treating pathologies related to Leydig cell dysfunction. Previously, it was shown that mesenchymal stem cells (MSCs) were able to differentiate into testosterone producing Leydig cells, suggesting that healthy Leydig cell populations could be transplanted into hypogonadal patients[97]. However, MSC isolation produces limited cell numbers and reduces the clinical application of this method. Recent developments have revealed human Leydig-like cells (hLLCs) can be generated from human induced pluripotent stem cells (hiPSCs), which are highly expandable in cell culture[98,99]. Li et al. demonstrated that hLLCs producing steroidogenic gene expression, steroidogenic enzymes, and testosterone could be generated by differentiating early mesenchymal progenitors from hiPSCs while overexpressing steroidogenic factor 1 (SF-1) in culture with dibutyryl-cAMP (dbcAMP), recombinant desert hedgehog, and human chorionic gonadotropin (hCG)[98]. Given their clinical viability, the implantation of hLLCs would represent a monumental step forward in treating diseases related to Leydig cell dysfunction. This strategy could restore testosterone levels by

replenishing testosterone-producing-cell populations in the testicular environment, leading to the production of endogenous testosterone formation.

Conclusions and future directions

Testosterone deficiency impacts the quality of life and wellbeing for millions of men worldwide, with only limited treatments having undesirable off-target effects[16]. New understanding of the molecular interactions producing testosterone has laid the foundation for the development of novel therapeutic strategies. Identification of the hormonally regulated multiprotein SITE complex[38] and the deeper understanding of hormonal stimulation and cholesterol translocation from cytosolic stores across the OMM and into the IMM for side chain cleavage demonstrates numerous therapeutic targets for various indications related to hormone insufficiency (Table 1)[16,18]. However, their effects on neurosteroids, adrenal steroids, and the HPG axis have remained a barrier to safe and efficacious treatment of testosterone deficiency. Apart from VDAC1 peptides, existing strategies have lacked specificity for testicular Leydig cells and, therefore, have raise concerns regarding off-target effects (Fig. 2). VDAC1 peptides are first-in-class biologics that offer a novel approach for rescuing intratesticular and serum testosterone formation in hormonally mediated diseases[79,80]. These therapeutics could be used to restore endogenous testosterone formation and restore well-being for millions of aging men worldwide.

There are additional mechanisms to uncover. The movement of cholesterol between the mitochondrial membranes, the relationship between aging and the Leydig cell oxidative environment, and age-dependent protein-protein interactions remain elusive and are active areas of research[24]. With more information we may determine the cause of reduced testosterone and develop interventions that may maintain Leydig cell function. Moreover, targeting the molecular deteriorations that differ between aging Leydig cells and other aging steroidogenic tissues could lead to additional testis-specific strategies.

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Authors' contributions

The authors contributed equally as they conceptualized the content of this review, drafted the manuscript, and edited and reviewed the final manuscript.

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Figure Legends

Figure 1. Steroidogenic InTeractomE (SITE) proteins of the Leydig cell. Cytosolic, OMM, IMM, and endoplasmic reticulum proteins interact to facilitate the transfer of cholesterol into the mitochondria and production of numerous steroid hormones, including testosterone in the endoplasmic reticulum. 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; ACBD1, acetyl coenzyme A-binding domain 1 or diazepam binding inhibitor; ACBD3, acetyl coenzyme A-binding domain 3; ATAD3A, ATPase family AAA domain-containing protein 3A; CYP11A1, cytochrome P450 11A1; CYP17A1, cytochrome 17A1; FDX, ferredoxin; FDR, ferredoxin reductase; PKA, cAMP-dependent protein kinase; PKA-R, regulatory subunit; PKA-C, catalytic subunit; Sec23ip, Sec23-interacting protein; STAR, steroidogenic acute regulatory protein; TSPO, translocator protein; VDAC1, voltage dependent anion channel 1.

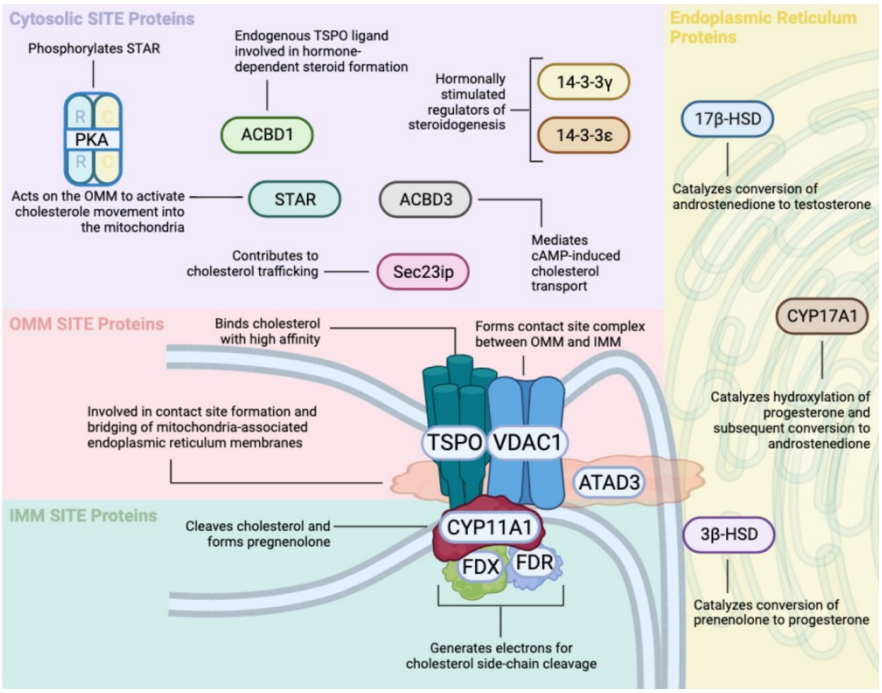

Figure 2. Off-target effects of therapeutic strategies. Numerous therapeutics that are used to treat testosterone deficiency have off-target effects on the hypothalamic pituitary gonadal axis, adrenal gland, and testicular Leydig cells.

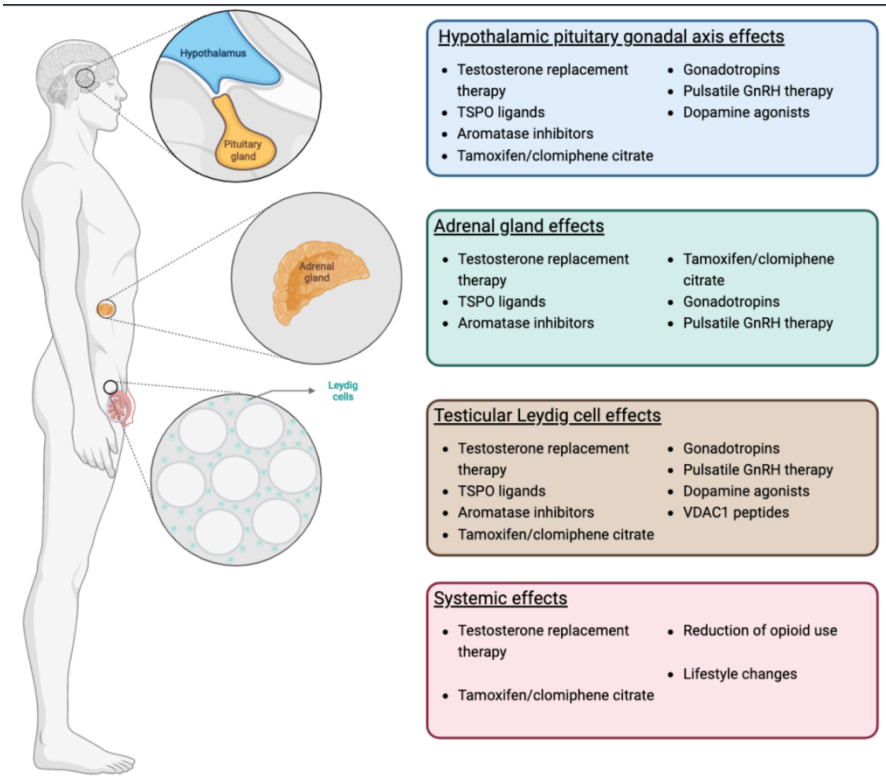

Table 1. Treatment options available for testosterone deficiency and their use in other indications. Adapted from Ide et al. ↑ positive effect, ↓ negative effect; GnRH: gonadotropin releasing hormone. TSPO: Translocator protein. VDAC1: Voltage-dependent anion channel.

Therapy	Indications	Results	Concerns
Testosterone replacement therapy	Hypogonadism Diabetes Osteoporosis Sexual desire	Testosterone ↑ Health ↑ Sexual function ↑ Bone mineral density ↑ Spermatogenesis ↓ Fertility ↓ Mood ↓	Infertility, LH suppression, prostate cancer, fluctuating testosterone levels, lower haematocrit, skin irritation, development of male breast tissue, alterations in mood
Aromatase inhibitors	Hypogonadism (off label) Breast cancer Gynecomastia Ovulation induction	Testosterone ↑ Bone mineral density ↓	hot flashes, weight gain, insomnia, venous thromboembolism, erectile dysfunction, breast pain
Gonadotropins	Hypogonadism Infertility (off label)	Testosterone ↑ Spermatogenesis ↑	Gynecomastia, erythrocytosis
Pulsatile GnRH therapy	Hypogonadism Fertility Prostate cancer Transgender therapy	Testosterone ↑ Spermatogenesis ↑	Erythrocytosis, difficult, expensive
Tamoxifen / Clomiphene citrate	Hypogonadism Dysmenorrhea Breast cancer Infertility	Testosterone ↑ Spermatogenesis ↑	Venous thromboembolism, vision, mood changes, weight gain, not effective in primary hypogonadism, off-label use

	Gynecomastia		
Dopamine agonists	Hypodonadism Psychopathological disorders Parkinson's disease	Testosterone ↑ Sexual function ↑ Semen quality ↑ Bone mineral density ↑	Headaches, hypotension, nausea
Reduction of opioid use	Opioid-induced hypogonadism	Testosterone ↑ Sexual function ↑	Few alternatives for treating chronic pain
Lifestyle changes	Health Wellbeing Hypogonadism Sexual function	Testosterone ↑ Sexual function ↑ Spermatogenesis ↑ Health ↑ Wellbeing ↑	Time, motivational and environmental barriers
TSPO ligands	Psychopathological disorders Hypogonadism Amyotrophic lateral sclerosis (ALS)	Testosterone ↑ Neurosteroids ↑↓ Adrenal steroidogenesis ↑↓	Target specificity, off target effects
VDAC1 peptides	Testosterone deficiency	Testosterone ↑	Development, adoption