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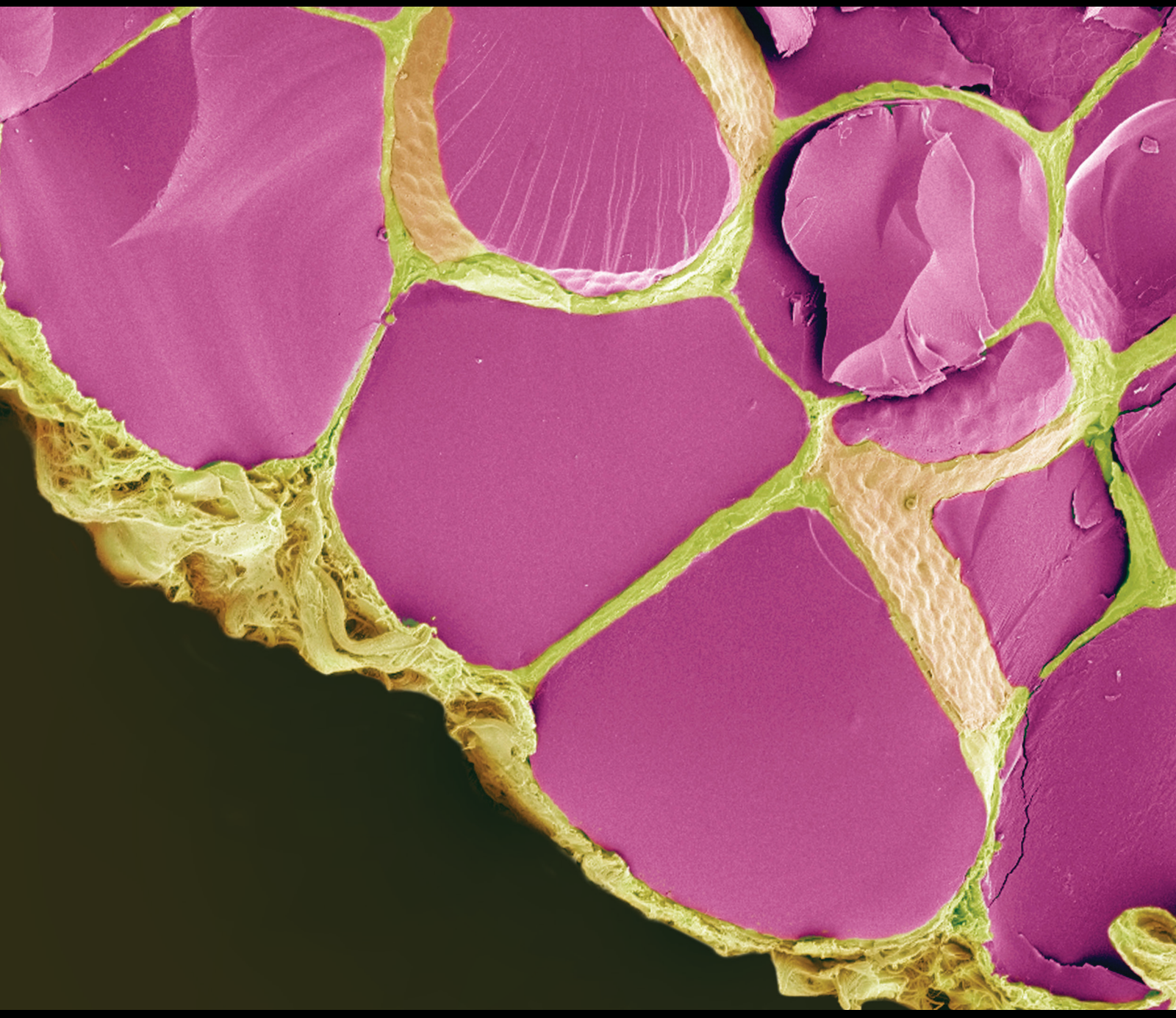

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Review Article

Classic and Novel Sex Hormone Binding Globulin Effects on the Cardiovascular System in Men

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In men, 70% of circulating testosterone binds with high affinity to plasma sex hormone binding globulin (SHBG), which determines its bioavailability in their target cells. In recent years, a growing body of evidence has shown that circulating SHBG not only is a passive carrier for steroid hormones but also actively regulates testosterone signaling through putative plasma membrane receptors and by local expression of androgen-binding proteins apparently to reach local elevated testosterone concentrations in specific androgen target tissues. Circulating SHBG levels are influenced by metabolic and hormonal factors, and they are reduced in obesity and insulin resistance, suggesting that SHBG may have a broader clinical utility in assessing the risk for cardiovascular diseases. Importantly, plasma SHBG levels are strongly correlated with testosterone concentrations, and in men, low testosterone levels are associated with an adverse cardiometabolic profile. Although obesity and insulin resistance are associated with an increased incidence of cardiovascular disease, whether they lead to abnormal expression of circulating SHBG or its interaction with androgen signaling remains to be elucidated. SHBG is produced mainly in the liver, but it can also be expressed in several tissues including the brain, fat tissue, and myocardium. Expression of SHBG is controlled by peroxisome proliferator-activated receptor γ (PPARγ) and AMP-activated protein kinase (AMPK). AMPK/PPAR interaction is critical to regulate hepatocyte nuclear factor-4 (HNF4), a prerequisite for SHBG upregulation. In cardiomyocytes, testosterone activates AMPK and PPARs. Therefore, the description of local expression of cardiac SHBG and its circulating levels may shed new light to explain physiological and adverse cardiometabolic roles of androgens in different tissues. According to emerging clinical evidence, here, we will discuss the potential mechanisms with cardioprotective effects and SHBG levels to be used as an early metabolic and cardiovascular biomarker in men.

1. Introduction

The incidence of cardiovascular mortality is higher in men than in women [1–3], and gender differences are highly related to circulating plasma levels of sex-steroid hormones [4, 5]. Estrogens have cardioprotective effects in women prior to menopause, but in adult men, the main gender-related steroid hormone is testosterone [6]. A study from the Mayo Clinic (2018) exhaustively reviewed and analyzed the main clinical publications in the last 10 years related to plasma testosterone levels, testosterone administration therapies, and their impact on the cardiovascular system.

Evidence indicates that physiological testosterone levels are beneficial for the male cardiovascular system, while testosterone deficiency is associated with an unfavorable metabolic profile and increased cardiovascular risk [7].

Sex hormone binding globulin (SHBG) transports testosterone within the blood stream and regulates its bioavailability and access to extravascular target tissues [8, 9]. In men, plasma testosterone levels fluctuate throughout life and begin to decrease in middle age and continue to decline with age [4]. Low plasma testosterone levels in men have been associated with the concept of "andropause," which rapidly has become a worldwide epidemic condition related to an adverse cardiometabolic risk profile [4, 10, 11]. There is substantial clinical evidence indicating that androgen signaling plays a key role for the cardioprotective benefits elicited by physiological testosterone levels in men.

Several cross-sectional and cohort studies have shown that low SHBG levels are associated with an increased risk of developing metabolic diseases [12, 13]. An association has also been described between SHBG actions unrelated to the transport of sex hormones and metabolic disorders. A meta-analysis examining different concentrations of SHBG showed that low levels of SHBG in men are a predictor of metabolic syndrome and type 2 diabetes [14]. Metabolic abnormalities are closely associated with cardiovascular disease [15–17]. Despite previous evidence, a recent cohort study including about 150,000 middle-aged and aged adult men (40–69 years old) concluded that low circulating SHBG levels are associated with diminished mortality in "all-cause" included in this study, particularly those related with cancer and cardiovascular diseases (CVDs). For total and calculated free testosterone, the expected inverse association with SHBG levels was observed only for "all-cause" and cancer mortality and not for CVD deaths [18]. Another cohort study showed, in a group with age ranging from 35 to 80 years, that elevated levels in SHBG were positively associated with increased incidental cardiovascular disease risk in men over 65 years [19]. However, it is still unknown whether changes in the circulating plasma levels of SHBG, the local expression of SHBG and androgen-binding proteins in tissues such as the heart, or the secondary effects of SHBG level fluctuations in free testosterone are responsible for these effects. In this review, we are going to discuss the mechanisms currently proposed for SHBG cardioprotective effects and how the use of circulating SHBG levels can be useful as an early metabolic and cardiovascular biomarker.

2. SHBG Is not Only a Passive Carrier for Sex-Steroids

In men, approximately 70% of circulating plasma testosterone binds with high affinity to circulating SHBG, 20–30% to albumin, and the remaining 1-2% circulates in free form [20]. In women, the majority of plasma estradiol and testosterone are bound to SHBG and other proteins and is not bioavailable; only about 2% of these sex hormones are free to bind to receptors and have an impact on the body [21]. Circulating SHBG modulates the level of free sex-steroid hormones that can enter to diverse target cells [22]. The endocrinological concept known as "free hormone hypothesis" states that the "bioavailable" steroid hormone, i.e., the one that has an effect when bound to its receptor, is the unbound or "free" fraction of steroid hormones [23]. However, recent evidence indicates that circulating SHBG not only is a passive carrier of male sex hormones but also actively regulates testosterone uptake and androgen signaling [24]. Because circulating SHBG binds to sex hormones, the relative plasma levels of this protein can modulate the concentrations of sex-related hormones accessible for use by the body, which has an impact on the processes regulated by the sex hormones [25]. SHBG can also release hormones in specific tissues and cells directly, which can influence both production and effects of sex hormones as well as the expression and function of circulating SHBG. Also, sex hormones bound to circulating SHBG can change the affinity of SHBG to its peripheral receptors. Moreover, intracellular expression of SHBG in testicular proximal tubule cells increases uptake of dihydrotestosterone and prolongs the expression of androgen-responsive genes [26].

One case description of a patient with a homozygous missense mutation in SHBG, which abrogates protein secretion in a 27-year-old man showed low total testosterone but normal free testosterone levels. Despite this, no alterations were seen in sexual development. However, fatigue, muscle weakness, and impaired exercise tolerance were part of the patient's symptoms. Faced with normal levels of free testosterone, this phenotype suggests that circulating SHBG may affect tissues in a manner dependent or independent of testosterone [27]. Frairia et al. studied tissue distribution of the SHBG membrane receptor either in estrogen/androgen-dependent tissues and proposed that the actions of SHBG in tissues are not strictly sex-steroid-dependent [28]. There is evidence suggesting that circulating SHBG interacts with specific proteins in the plasma membrane and it can be internalized once it is accumulated [29]. In steroidogenic tissues, SHBG can be internalized to activate transduction signaling pathways different and independent of those induced by the classical action mechanism based on intracellular androgen receptors [30]. There is also evidence indicating that circulating SHBG, through LG domains, binds membrane receptors with tyrosine kinase activity and G-protein-coupled receptors [20]. Functional plasma membrane receptors for SHBG have been identified in cardiac tissue [31], and SHBG is expressed in the myocardium [32].

2.1. Circulating SHBG Internalization. Circulating SHBG protein may be internalized through the low-density lipoprotein-related protein 2 receptor also known as megalin receptor in rat yolk cells. Megalin-deficient mice display defects resembling animals treated with androgen receptor antagonists [30]. Megalin is expressed in several tissues including derived cardiac cells [33]. The human megalin promoter gene possesses PPARs-responsive elements, suggesting a metabolic regulation in the protein expression [34]. In fact, megalin expression is reduced in Ren2 rats, a model of metabolic syndrome [35]. Megalin facilitates the uptake of several

ligands, many of which are cataloged as intracrine, including SHBG [36]. These extracellular molecules can act by initiating intracellular signals after internalization. The main intracellular target sites described for intracrine actions include the nucleus and mitochondria. In C2C12, a mouse myoblast cell line, megalin KO, decreases the respiratory and glycolytic capacity [37]. Megalin mediates the retrograde trafficking of TGF-β and angiotensin II to mitochondria through the retrograde early endosome-to-Golgi transport pathway and Rab32 [37], all of this playing a role in mitochondrial physiology. Whether the metabolic effects of SHBG are related to its retrograde transport and if this trafficking is related to mitochondrial modulation in cardiac cells are unknown.

2.2. SHBG as an External Ligand. SHBG activates several signaling pathways depending on a putative membrane receptor coupled to a G-protein [38], increasing the intracellular cAMP levels in COS-1 cells. An increase in cAMP in MCF-7 cells results as a receptor-mediated action of sex-binding protein [39]. Although stimulation of the cAMP pathway has positive effects on cardiac function, long-term activation produces detrimental effects in the myocardium inducing hypertrophy and heart failure [40]. In lymphocytes, incubation with SHBG induces the phosphorylation of ERK and Akt kinases, an effect that is increased by coincubating with estradiol [41]. All these pathways have been implicated as possible targets in metabolic disorders [42–45].

3. Signal Transduction Pathways Involved in SHBG Expression

SHBG is a glycoprotein synthesized and secreted by the liver [46–48] that transports sex-steroids (androgens and estrogens) from steroidogenic organs to their target tissues [6, 20, 24, 49, 50]. The structural organization of SHBG genes is evolutionarily conserved and is expressed in most vertebrates [51, 52]. Additionally, SHBG mRNAs exhibit alternative splicing that encodes the androgen-binding protein (ABP) [22, 53], which differs from SHBG mRNAs by the presence of an exon I (exon A) that does not influence the post-translational modifications required for SHBG secretion [54]. In humans and rats, ABP is produced in the liver [22], Sertoli and Leydig cells [55], and cardiomyocytes [31, 56]. The physiological role of ABPs in peripheral tissues remains poorly understood; however, studies indicate that ABPs regulate the local bioavailability of androgens [56–58]. SHBG/ABPs are polypeptides of 43-44 kDa [20, 50]. The steroid binding domain is in the N-terminus, whereas the regulatory domains can interact with a plasma membrane receptor for SHBG [9, 29, 38, 59, 60]. The human SHBG is a polypeptide of 373 amino acids that constitute a tandem repeat of laminin G-like (LG) domains [9].

Locally produced SHBG modulates the expression of androgen-responsive genes in prostate tissue [61]. Expression of SHBG protein may enhance or inhibit the uptake of androgens in a cell- and tissue-specific manner [62]. SHBG exerts protective roles against excessive androgen exposition during embryonic and fetal cardiogenesis [63]. Hepatic secretion of SHBG is controlled by circulating sex-steroid levels [64, 65]. Others have argued that higher levels of circulating SHBG are compensated *in vivo* by hypothalamic-pituitary feedback, resulting in higher total sex-steroid concentrations [66]. This is a controversial point, and there is a continuing debate over whether—and by which mechanisms—circulating SHBG regulates total, free, and/or bioavailable sex-steroid concentrations and their physiological responses. Expression of ABP has been described in the human heart, and it has been suggested that this protein influences the bioavailability of gonadal steroids in the myocardium [31].

Transcriptional regulation of SHBG has been mainly studied in fetal liver and hepatocyte cell lines [67]. SHBG protein expression is controlled by peroxisome proliferator-activated receptor γ (PPARγ), a coactivator for several nuclear transcription factors, including the androgen receptor [68]. PPARs regulate cell metabolism and improve ATP generation [69, 70]. A key metabolic regulator is AMP-activated protein kinase (AMPK), which acts as an energy sensor [71]. AMPK/PPPAR interaction is critical to upregulate SHBG expression by controlling hepatocyte nuclear factor-4α (HNF4α) [72]. HNF4α affects the transcription of many genes involved in lipid metabolism, and this fact may contribute to explain the reported correlations between circulating SHBG levels and lipid metabolism, glucose metabolism, and in consequence to cardiovascular risks [73, 74]. The SHBG promoter contains PPAR-response elements (PPAR-RE), which are required for SHBG expression. PPARγ acts as a transcriptional inhibitor of SHBG gene expression in the liver [75]. Some controversial results have been reported since women receiving troglitazone (a pharmacologic PPARγ agonist) showed increased circulating SHBG plasma levels [76], while treatment of HepG2 cells with rosiglitazone reduced the secretion of SHBG [75]. It has been reported that GW9662, a different PPARγ antagonist, increased the synthesis of SHBG in HepG2 liver cells [74]. A potential explanation was that footprinted region 3 (FP3) in the SHBG promoter gene contain binding domains for HNF4α, PPARγ, and RXR retinoic acid receptors [77, 78]. Thus, during normal physiological state, HNF4α may bind with high affinity, whereas PPARγ may act as transcriptional inhibitors during alterations of lipid metabolism [75].

Although these studies may homologate certain *in vivo* conditions, they do not consider that gene expression of SHBG can also be regulated by testosterone [64, 65]. In this context, transfection of NB16 cells, which do not express sex-steroid receptors, with a plasmid expressing the androgen receptor showed that incubation with SHBG-testosterone or different hormone or carrier concentrations induces the expression of a reporter androgen-responsive gene in a concentration-dependent manner [30]. Therefore, transcriptional regulation of SHBG seems to be regulated by testosterone levels and transactivation mediated by androgen receptors [64, 65].

Adiponectin is a protein produced in the white adipose tissue, negatively related with body mass index (BMI), and their plasma levels are decreased in obese

patients. In a study performed in HepG2 human cells, adiponectin increases the levels of SHBG through activation of AMPK-HNF4α signaling [79]. In obese patients, there is a chronic low-grade inflammatory state with high levels of TNFα and IL-1β. TNF-α levels are negatively related with SHBG expression. In hepatoblastoma human cells, TNFα induces reduction of the levels of SHBG through the activation of NFκB and the inhibition of HNF4α P1 promoter activity by the p65 subunit [80]. In streptozotocin-induced diabetic rats, the plasma levels of adiponectin were decreased and have an increase in TNFα and IL6 levels [81]. Also, they showed that adiponectin receptor 1 was increased in the heart of diabetic rats, but the levels of adiponectin did not change. In line with this, the systemic levels of adiponectin in plasma were not able to induce the signaling by adiponectin receptor 1, showing a decrease of pAMPK and GLUT4 expression in cardiomyocytes [81]. This information can be hypothetically related with SHBG levels in the heart and plasma of obese and diabetic patients because they share the same signaling factors (Figure 1).

In cardiomyocytes, it has been reported that testosterone activates AMPK to modulate energy production through GLUT4-dependent glucose uptake [82]. AMPK interaction is critical for upregulating SHBG expression in HepG2 cells [79]. Furthermore, upstream AMPK regulates the activity of PPAR-alpha [83]. In the nucleus, androgen signaling stimulates PPAR activity and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) to increase the expression of various nuclear-encoded metabolic genes, including oxidative phosphorylation genes [84, 85]. Since cardiac cells express SHBG and HNF4α gene [86], and testosterone activates the transcriptional machinery to express SHBG protein, including PPAR, AMPK, and HNF4α [31, 32, 87], we hypothesize with the possibility that these pathways may be activated to induce cardiac SHBG expression. Transcriptional regulation of SHBG expression involving the AMPK/PPARs pathway also modulates metabolic networks for fatty acid and glucose metabolism during adaptive cardiomyocyte responses [88]. In addition, the protein deacetylase sirtuin 3 (SIRT3) plays cardinal roles in modulating the metabolic network of fatty acid and glucose metabolism for ATP production [89, 90]. Notably, SIRT3 favorably modifies cellular mechanisms implicated in cardiovascular diseases [91, 92]. SIRT expression is controlled by PGC-1α and AMPK [93, 94] and testosterone activates PGC-1α [95], but it remains unknown whether cardioprotective actions of androgens involve SIRT3 signaling.

4. Effects of SHBG and Testosterone on Cardiac Function: Mechanistic Evidence from Animal and Human Research

Most of the research linking SHBG and androgens has been focused on their circulating levels. If altered levels of circulating SHBG are causally related to high cardiovascular risk, raises the question, what is the potential mechanism?

In humans, plasma SHBG levels are influenced by nutritional state, metabolism, and hormonal factors [9, 58, 96, 97]. Patients with obesity and insulin resistance show reduced circulating SHBG levels [74, 98]. Importantly, circulating SHBG levels are strongly correlated with plasma testosterone concentrations and low testosterone levels are strongly associated with metabolic disorders [99–101]. In addition, decreases in SHBG levels are linked to high cardiovascular risk factors [102]. Although metabolic disorders increase the incidence of cardiovascular disease, it is unclear whether SHBG expression has an impact on SHBG receptor signaling or androgen actions in cardiac cells.

Morbidity and mortality in patients with metabolic and cardiac diseases remain high [11] mainly because of the lack of effective cardioprotective strategies to handle cardiometabolic disorders. A 5-year-long follow-up study indicated that men >65 years of age with elevated SHBG and lower total testosterone were independently associated with an increase of both CVD risk and mortality [19, 103]. Circulating SHBG stands as an available marker for assessment of cardiovascular health, especially in the female population, whereas in men similar effects are less known. Future implications of cardiovascular risk assessment and the importance of plasma SHBG in cardiovascular pathophysiology might be even broader since cardiomyocytes of patients suffering from dilated cardiomyopathy produce cardiac SHBG and appear to be internalized, possibly representing a mechanism for delivering sex hormones to the heart [32]. Thus, an interesting concept is that locally expressed SHBG controls testosterone levels in the myocardium to activate additional androgen signaling pathways. Therefore, abnormal locally produced SHBG function might help explain the adverse cardiac metabolic effects of androgen deficiency.

An interesting study in patients with dilated cardiomyopathy demonstrated that cardiomyocytes express an androgen-binding protein, similar to SHBG, and the subcellular distribution matched with androgen receptor location [31]. Moreover, studies in megalin knock-out mice demonstrate a crucial role for this receptor during cardiac development [104]. Immunohistochemical and 3D reconstitution assays showed that these animals had severe cardiovascular anomalies in structures such as aortic arch, common arterial trunk, coronary arteries, and ventricular septum, as well as a marked thinning of the ventricular myocardium [104]. SHBG has been associated with megalin-induced internalization of the protein into the cells; megalin-deficient mice showed defects resembling androgen deficiency [20, 30]. Although reduced levels of circulating SHBG decrease total testosterone levels, the effect of low testosterone on SHBG expression is not fully understood [105].

Low SHBG levels correlated with measures of heart failure severity and were associated with a higher risk of cardiac death. Interestingly, impaired hepatic SHBG expression impacts testosterone levels, and its deficiency is independently linked to cardiometabolic diseases [106]. In pathological conditions with reduced circulating SHBG levels—such as obesity or insulin resistance—the symptoms of testosterone deficiency in men can be exacerbated [8, 32, 58, 98]. Obesity is considered an independent risk

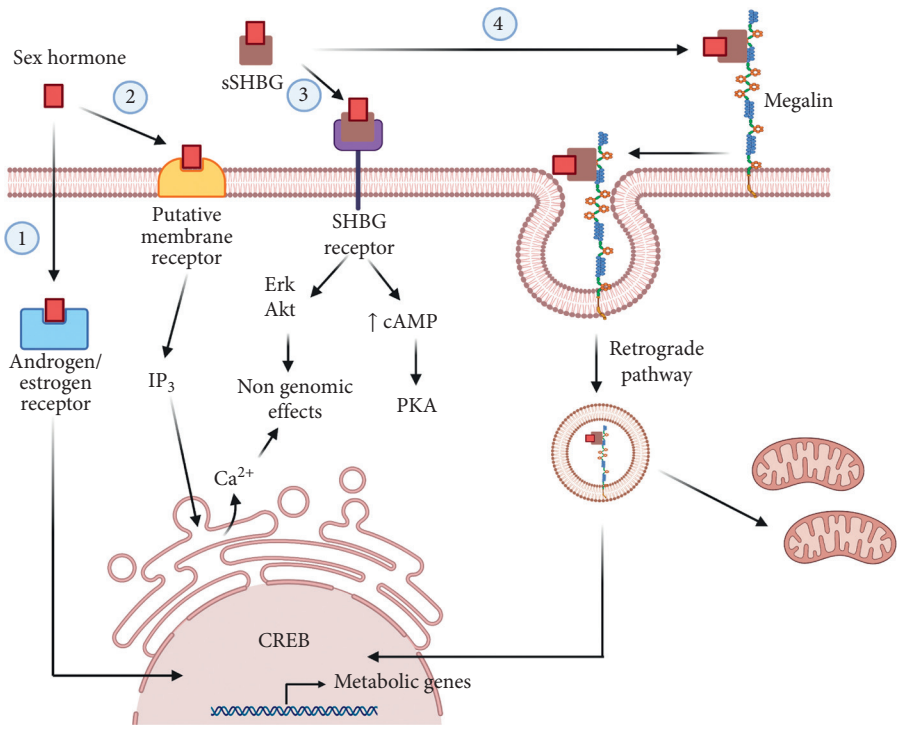

Figure 1: Signaling pathways activated by SHBG. The figure illustrates the action mechanism of SHBG as a hormone carrier and SHBG direct actions. (1) Free circulating androgens and estrogens that correspond to the bioavailable portion of sex hormones can cross the plasma membrane and bind intracellular sex hormone receptors, thus activating the "classic," genomic sex hormone intracellular pathways. (2) As described in the literature, free circulating sex hormones can also bind to putative membrane receptors activating "fast, nongenomic intracellular signaling pathways." (3) Another putative membrane receptor, for SHBG, can also activate intracellular signaling pathways, leading to fast, nongenomic effects. (4) The megalin receptor, which induce the internalization of SHBG and a retrograde pathway that affects nuclear and mitochondrial function, can also account for some SHBG-induced intracellular effects.

factor for heart failure [17], and mice fed with high-fat diet (HFD) for 16 weeks developed obesity that adversely affects the function and structure of the heart and induces cardiac dysfunction [107]. Moreover, low concentrations of total testosterone and SHBG were strongly associated with an increased likelihood of having metabolic syndrome, independent of other cardiovascular risk factors [108]. It has been proposed that myocytes may produce and secrete ABP in a paracrine manner perhaps to influence the bioavailability of sex-steroids in the myocardium [31]. Low plasma levels of SHBG are associated with several sex-steroid hormone-dependent diseases [109] and have been reported to be an early indicator of cardiovascular risk in individuals suffering from obesity and metabolic syndrome [110–112]. Experimentally, circulating SHBG suppression causes cardiac disorders, partly by mimicking low testosterone level conditions, whereas physiological levels of SHBG and testosterone show cardioprotective effects [14, 25, 73, 102]. Jänne et al. showed that decreased SHBG levels in the kidney by castration can be restored with a treatment with dihydrotestosterone [113]. This experiment shows us that SHBG concentration can be modulated by testosterone but is not fully understood the dependence of testosterone to the variations of SHBG levels and how we can differentiate the effects in cardiometabolic function. Laurent et al. reported that SHBG-tg male mice that overexpress SHBG exhibit an increase in total testosterone concentration compared with wild-type mice, although free levels of testosterone do not change. This result changed when the mice were castrated, eliminating the hypothalamic feedback of luteinizing hormone over Leydig cells, showing a decrease in free testosterone levels [114]. Furthermore, *SHBG-tg* mice showed a significant decrease in the weight of the seminal bladder and levator ani/bulbocavernosus muscle, organs that are sensitive to androgens [114]. In accordance with that, Rastrelli et al. demonstrated that high SHBG levels are related to lower PSA and hematocrit, markers of androgen deficiency, and increase ANDROTEST scores, an androgen-dependent clinical parameter, demonstrating that high SHBG levels in humans can be associated with hypogonadism [115]. In line with this observation, Nokoff et al. reported that boys with obesity have lower levels of SHBG and total testosterone in comparison with normal weight controls, but free testosterone levels do not change [116]. This type of data gives us information about the dependency of testosterone levels with SHBG variations and presents the metabolic and physiological effects that SHBG levels can induce in the body, independent of free concentration of testosterone, challenging the free hormone hypothesis. With these antecedents, the dependence of SHBG on cardiometabolic effects is incomplete and future research is needed to probe this question. An interesting hypothesis is that testosterone levels regulate cardiac SHBG expression to positively influence cardiometabolic responses (Figure 2).

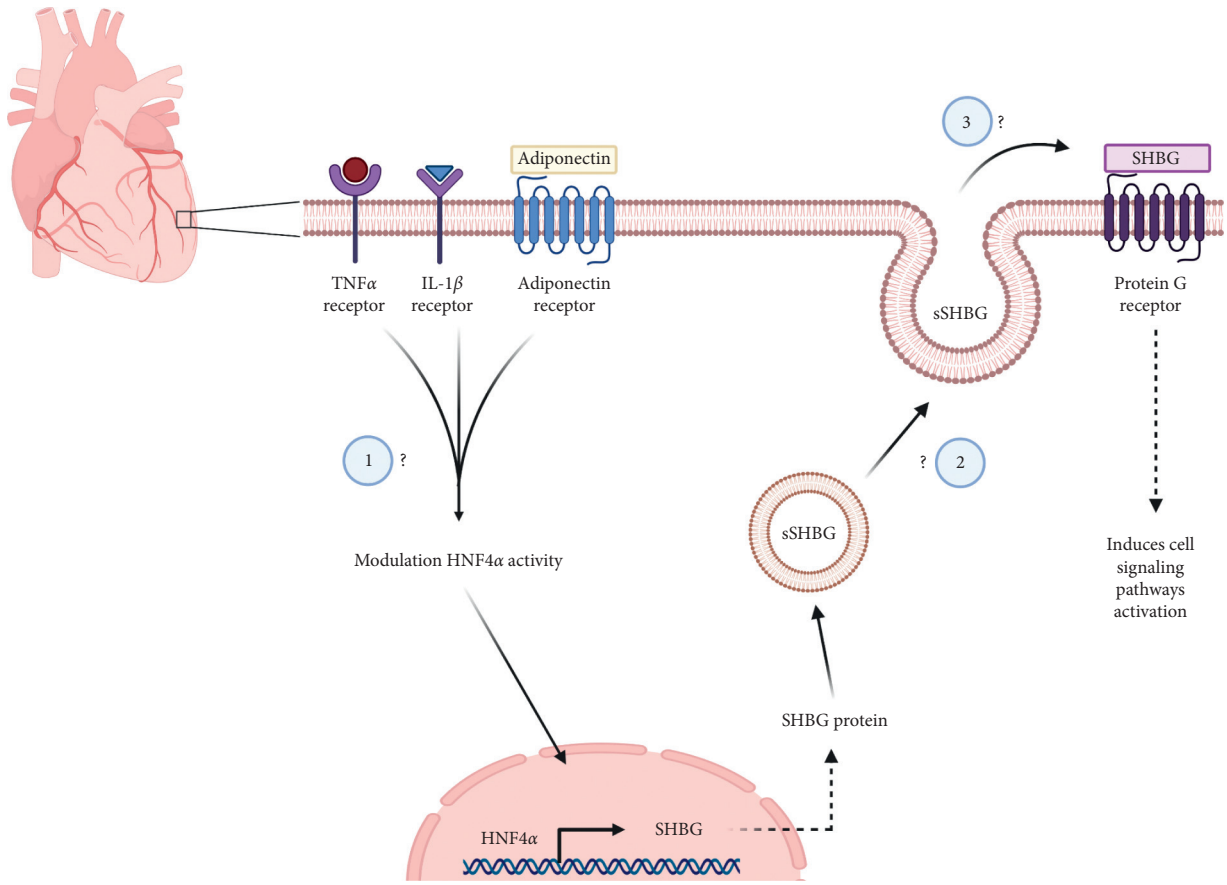

Figure 2: Hypothetical pathways leading to SHBG expression and their modulation in the heart. A hypothetical intracellular pathway for SHBG expression in the cardiomyocytes. The same membrane receptors modulating SHBG expression in the liver could be expressed in the heart. Metabolic cues modulate the activity of the transcription factor hepatocyte nuclear factor 4-α (HNF4α), which leads to an increase in SHBG gene expression. The following are some of the questions that arise relating to the possible production and secretion of SHBG as an endocrine or paracrine mediator: (1) Can TNFα, IL-1β, and adiponectin modulate SHBG expression in cardiac tissue? (2) Is the heart involved in the release of soluble SHBG in a paracrine or endocrine way? (3) Can soluble SHBG trigger intracellular signaling pathways in the heart?

Humanized transgenic mice expressing the human SHBG have been used to study the function of this protein. In this *in vivo* model, SHBG overexpression prevents both the weight increase and fat accumulation induced by high-fat diet. Additionally, SHBG overexpression also abolishes the increase in insulin, leptin, and resistin and protects against high-fat diet-induced obesity [80, 117]. SHBG overexpression does not change food and water intake or intestinal lipid absorption; however, the author did not measure the testosterone levels [117].

On the other hand, elevated levels of circulating SHBG bind more estrogens and may be beneficial by reducing the ability of estrogens to promote breast cancer growth. Also, plasma SHBG levels can directly affect cancer growth [118]. High circulating levels of SHBG have also been associated with better cardiovascular health and metabolic status in postmenopausal women [119, 120]. Therefore, cardiac SHBG expression may be associated with intra-cardiomyocyte testosterone signaling, allowing cardioprotective effects or, otherwise, producing cardiac dysfunction under metabolic disorder conditions. Thus, expression of cardiac SHBG may restrict the anabolic activity of testosterone on the heart, which impairs cardiac SHBG expression and metabolic adaptations. Then, the relationship between low circulating SHBG and low free testosterone may represent early markers of poor cardiovascular health. Physiological effects of testosterone in cardiac cells includes handling of energy substrates and increased gene expression of key enzymes involved in glucose uptake and glycolysis; and regulation of critical transcription factors related to stimuli that affect cardiomyocyte function.

5. Human Diseases and Medications Related to Circulating SHBG

Various human diseases have been associated with altered circulating levels of SHBG, many of which also are linked with high CVD risk (Table 1). In humans, there is diverse information that reflects the bidirectional nature of the relationship between the SHBG levels and metabolic impairment. Different SHBG-gene polymorphisms have been

Table 1: Pathologies and circulating levels of SHBG.

	Author	Condition	Sex	Age (years)	SHBG (nmol/l)	p value	Change
Obesity	Kopelman et al. [121]	Lean control	Women	28	60 ± 8	Non reported	Decreased
		Obese	Women	29	30 ± 4		
	Cupisti et al. [122]	BMI < 25 kg/m^2	Women	26.41 ± 6.09	53.42 ± 23.1	<0.0001	Decreased
		BMI > 25 kg/m^2	Women	29.23 ± 7.08	30.03 ± 14.52		
	Nokoff et al. [116]	Normal weight	Women	10	59.5	<0.0001	Decreased
		Obese	Women	10	18.5		
		Normal weight	Men	12	57	<0.0001	Decreased
		Obese	Men	12	18		
Diabetes	Lindstedt et al. [123]	Control	Women	38–60	88 ± 55	<0.001	Decreased
		Diabetes	Women	38–60	55 ± 31		
	Laaksonen et al. [25]	Control	Men	51.3 ± 6.7	34.5	<0.001	Decreased
		Metabolic syndrome	Men	51.4 ± 6.8	28.2		
		Diabetes	Men	52.2 ± 5.6	26.2	<0.001	Decreased
	Ding et al. [110]	Normal	Women	60.3 ± 6.1	36.9 ± 17.4	<0.001	Decreased
		Type 2 diabetes	Women	60.3 ± 6.1	22.3 ± 13.8		
		Normal	Men	63.7 ± 7.6	27.3 ± 10.7	<0.001	Decreased
		Type 2 diabetes	Men	63.7 ± 7.6	19.6 ± 7.2		
PCOS	Ferk et al. [124]	Control	Women	25.3 ± 3.8	61.0 ± 14.7	<0.001	Decreased
		PCOS	Women	24.4 ± 4.4	44.4 ± 19.1		
	Baldani et al. [125]	Control	Women	31.3 ± 4.8	71.6 ± 21.7	<0.001	Decreased
		PCOS	Women	28.3 ± 5.7	38.4 ± 19.9		
Hypothyroidism	Leger et al. [126]	Euthyroid	Boys and girls	7.1 ± 0.5	77.8 ± 7.9	<0.01	Decreased
		Hypothyroid	Boys and girls	7.1 ± 0.5	48.2 ± 6.5		
Klinefelter syndrome	Plymate et al. [127]	Normal	Men	24–40	6.5 ± 1.2	Not reported	Increased
		XXY Klinefelter's	Men	20–45	16.4 ± 2		
	Estour et al. [128]	Normal weight	Women		25.6 ± 62.9	<0.001	Increased
		Anorexia	Women	20 (14–35)	90.8 ± 32.6		
Malnutrition	Pascal et al. [129]	Control	Boys and girls	16 ± 8 months	0.11 ± 0.03	<0.0005	Increased
		Kwashiorkor patients	Boys and girls	20 ± 8 months	0.18 ± 0.07 μmol/l		

The table shows different diseases and some studies that describe the associated circulating SHBG levels. Gender, mean age (±standard deviation), and p value change between different conditions are also presented.

related with metabolic effects in case-control studies. Low levels of SHBG were correlated with cardiac risk since HDL-L levels were lower than the normal threshold in patients with coronary heart disease, and this correlation might be affected by SHBG polymorphism [130]. Carriers of the SHBG polymorphisms rs6257 and rs6259 present a higher risk of diabetes than carriers of other alleles and present low levels of SHBG [131]. Metabolic diseases have been related to abnormal levels of this protein in plasma. SHBG mRNAs in liver and protein levels in serum were lower when the hepatic triglyceride concentration was high and decrease with the increase of body mass index [132].

Metabolic diseases have been related with abnormal levels of this protein in plasma. In obesity, circulating SHBG levels are decreased to 50% in obese adult women (non-menopause) compared with lean control patients [121, 122]. Also, girls and boys with obesity have near 70% of circulating SHBG compared with nonobese [116]. In patients with diabetes and metabolic syndrome, plasmatic SHBG levels also show decreased levels compared with controls [25, 110, 123]. Laaksonen et al. showed that an entire cohort of patients without diabetes or metabolic syndrome have 34.5 nmol/l of plasma SHBG; nevertheless, some patients that develop diabetes or metabolic syndrome present low levels of SHBG ranging to 26.2 and 28.2 nmol/l, respectively [25]. On the other hand, malnutrition such as anorexia [128] and Kwashiorkor patients with protein and energy malnutrition show increased plasma SHBG levels compared with control normal weight individuals [129]. One example is a longitudinal cohort of patients in whom SHBG levels were evaluated in anorexia and after a treatment to gain weight, showing that the levels of SHBG decrease in the gain weight therapy [129]. This evidence shows that circulating SHBG levels have a negative correlation with the development of obesity/overweight patients and have a positive correlation in malnutrition patients; therefore, plasma SHBG levels are correlating with the nutritional state of patients.

Circulating SHBG is also correlated with endocrinology diseases such as polycystic ovary syndrome (PCOS) and hypothyroidism. In PCOS, the levels of circulating SHBG are decreased compared with control women in a 25%–45% as compared with normal levels [124, 125], whereas in hypothyroidism, the relation is almost the same [126]. Otherwise, in Klinefelter syndrome patients (XXY), there are increased levels of circulating SHBG [127]. In other endocrine diseases such as hypothyroidism, decreased levels of circulating SHBG have been observed, whereas hyperthyroidism leads to increased plasma SHBG levels. This relation has been explained at the level of the transcription factor HNF4α being increased by hyperthyroidism [133].

Nokoff et al. showed that obese children in early puberty state have a decrease in the circulating SHBG levels and total testosterone and have an increase in estrone metabolites, probably by the aromatization of androgens in adipose tissue that can lead to develop hypogonadotropic hypogonadism in these boys and affect the reproductive function in the future [116]. There is incomplete information about the impact of plasmatic SHBG and development of cardiometabolic disease in youth, but the evidence showed a correlation between circulating SHBG, hypogonadism, insulin resistance, cardiac metabolism, and dyslipidemia, which are related with low circulating SHBG levels as a result of altered SHBG hepatic production.

Besides hormones and diseases, some medications and dietary compounds alter SHBG liver production [134, 135]. Antiepileptic drugs such as carbamazepine and phenytoin induce an increase in SHBG circulating levels in men and women [134]. Likewise, thiazolidinediones, and oral contraceptives in women, also increase SHBG plasma levels [136]. To the best of our knowledge, only one prospective study has analyzed the effects of changing circulating SHBG levels on cardiometabolic outcomes. In this prospective study, lifestyle interventions directed to obtain favorable changes in circulating levels of SHBG in men and women could not show to influence the risk of developing type 2 diabetes mellitus in the participants [137].

According to our current understanding, the cardioprotective effects of androgens in men have been poorly studied and the deleterious effects exerted by testosterone appear to be controversial. Recent research indicates that administration of testosterone in physiological doses to individuals with metabolic syndrome improves insulin sensitivity and reduces central obesity [138]. Additionally, development of heart failure in individuals with metabolic syndrome is partially reduced by treatment with testosterone in physiological doses [139, 140]. Likewise, subjects with low plasma testosterone levels develop insulin resistance and diabetes, as well as central obesity and heart failure [99, 100, 141, 142]. Moreover, plasma testosterone levels decline with age, while SHBG levels increase, which in turn leads to progression of testosterone deficiency and age-related cardiovascular pathologies [25, 143].

6. Conclusion and Future Research

Given the important roles of androgens in normal men physiology, abnormal levels must be considered one of the main causes implicated in several disorders and pathological conditions [108, 144–146]. According to a 2017 update demography report from the American Heart Association, almost one in three adult men have some type of cardiovascular disease [147]. In the context of human disease relevance, the international expert consensus panel that convened in 2015 concluded that there is a need for a major research initiative to explore the possible cardioprotective benefits of testosterone therapy, implying that there is sufficient evidence regarding the safety of testosterone therapy in hypogonadal men and that the direction of future research should be set toward defining suitable therapeutic options for cardiovascular disease [148, 149]. Research in the field of androgen signaling will provide a considerable understanding of the physiological and pathological roles of SHBG and sex-steroid hormones. Thus, an appropriate description of testosterone signaling considering circulating and cardiac SHBG expression might help explain both physiological and adverse cardiac metabolic roles of androgens (particularly androgen deficiency). Research directed to elucidate whether plasmatic and cardiac SHBG expression is associated with physiological testosterone levels could represent novel research approaches to study insulin resistance, obesity, diabetes, and heart failure.

Data Availability

The data used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

All the authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Review Article

Screening for Sarcopenia (Physical Frailty) in the COVID-19 Era

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Although the numbers of aged populations have risen considerably in the last few decades, the current coronavirus disease 2019 (COVID-19) has revealed an extensive vulnerability among these populations. Sarcopenia is an age-related disorder that increases hospitalization, dependencies, and mortality in older adults. It starts to develop in midlife or even earlier as a result of unbalanced diet/poor nutrition and low levels of physical activity, in addition to chronic disorders such as obesity and diabetes mellitus. Given that social isolation is adopted as the most protective measure against COVID-19, the level of physical activity and the intake of adequate diet have considerably declined, especially among older adults—denoting an increased possibility for developing sarcopenia. Research also shows a higher vulnerability of sarcopenic people to COVID-19 as well as the development of wasting disorders such as sarcopenia and cachexia in a considerable proportion of symptomatic and recovering COVID-19 patients. Muscular wasting in COVID-19 is associated with poor prognosis. Accordingly, early detection and proper management of sarcopenia and wasting conditions in older adults and COVID-19 patients may minimize morbidity and mortality during the current COVID-19 crisis. This review explored different aspects of screening for sarcopenia, stressing their relevance to the detection of altered muscular structure and performance in patients with COVID-19. Current guidelines recommend prior evaluation of muscle strength by simple measures such as grip strength to identify individuals with proven weakness who then would be screened for muscle mass loss. The latter is best measured by MRI and CT. However, due to the high cost and radiation risk entailed by these techniques, other simpler and cheaper techniques such as DXA and ultrasound are given preference. Muscle loss in COVID-19 patients was measured during the acute phase by CT scanning of the pectoralis muscle simultaneously during a routine check for lung fibrosis, which seems to be an efficient evaluation of sarcopenia among those patients with no additional cost. In recovering patients, muscle strength and physical performance have been evaluated by electromyography and traditional tests such as the six-minute walk test. Effective preventive and therapeutic interventions are necessary in order to prevent muscle loss and associated physical decline in COVID-19 patients.

1. Introduction

Aging involves deterioration of cellular processes, inability to maintain homeostasis, and increased vulnerability to stressors [1–4]. This condition is known as frailty. Because it embroils accumulation of subclinical declines in physical, psychological, social, and nutritional aspects, frailty is not easy to identify in clinical settings or research [1, 5]. Therefore, researchers commonly focus on the physical aspect of frailty, which is usually referred to as "physical frailty/frailty phenotype" or "sarcopenia." In fact, both sarcopenia and physical frailty represent an early stage of physical dependence and disability in old age [6]. Sarcopenia is an age-bound condition that entails a progressive loss of skeletal muscle mass along with declines in muscle strength and physical performance [3, 7].

Sarcopenia is reported in around 60% of older adults aged 80 years or above [8–12]. Its prevalence is increasing because of the rising life expectancy. Statistics predict that the number of sarcopenic older adults will double in the next 30 years [13–15]. Likewise, the number of older adults in need for long-term rehabilitation will quadruple by 2050 [12, 16], primarily because of the increased prevalence of musculoskeletal disorders, frailty, sarcopenia, visual and hearing impairments, fatigue, cognitive decline, sleeping disorders, and depression [15–18].

Loss of muscle mass contributes to a countless number of adverse effects including chronic pain (e.g., back pain), increased risk for chronic debilitating disorders (e.g., obesity and type 2 diabetes), frailty, and progressive decline in functional capacity and independence [15, 16, 19, 20]. Old people with muscle weakness have 4.3-fold greater risk for slow gait speed and 2.6-fold greater risk for severe mobility limitation [16]. Physical disability in sarcopenic people is common regardless of ethnicities, health behaviors, fat mass, and comorbidities [21]. The term "sarcopenia with limited mobility" refers to sarcopenic patients in need for therapeutic interventions because of loss of function resulting from skeletal muscle weakness [14]. Sarcopenic individuals are highly prone to falls, hospitalization, and mortality [8, 22]. A recent meta-analysis reports poor overall survival and relapse-free survival among sarcopenic patients with head and neck cancers compared with nonsarcopenic counterparts [22].

The current coronavirus disease-2019 (COVID-19) pandemic has been associated with a remarkable increase in key risk factors associated with sarcopenia such as decreased physical activity and unhealthy eating patterns (e.g., frequent snacking and skipping meals), which are most noticed in older adults [23–26]. On the other hand, sarcopenia is a disorder that entails chronic inflammation, malnutrition, and metabolic and endocrinal dysregulation, as well as several systemic dysfunctions (e.g., motor nerve degeneration) [3, 7]. Moreover, diaphragmatic muscle thickness is considerably reduced in sarcopenic individuals, which may evoke respiratory failure in critically ill patients [15]. Therefore, sarcopenia stands for a key risk factor that heightens the vulnerability of older adults to COVID-19. In fact, older adults represent the vast majority of patients with symptomatic COVID-19, and their prognosis is rather poor [27, 28]. Preexisting sarcopenia is associated with progressing to disease severity in COVID-19 patients reflected by polypharmacy, multiple organ failure, intensive-care unit (ICU) admission, increased need for mechanical ventilation, and mortality [29–34]. Among community-dwelling older adults, muscle mass is an independent correlate of maximum expiratory pressure, an indicator of respiratory muscle strength [35]. Myokines produced by wasting muscle (e.g., interleukin- (IL-) 6 and IL-7) can considerably alter immunity [36].

Cytokine storms associated with COVID-19 infection activate oxidative and catabolic signaling, which accelerate muscle protein degradation leading to skeletal muscle loss [37, 38]. Body protein loss, indicated by higher levels of blood urea nitrogen and reductions in serum total protein and albumin levels, is evident in severe and fatal COVID-19 patients, and it is associated with the development of multiple organ failure and mortality [37]. Significant weight loss is recorded in 61% of recovering COVID-19 patients; in 26.2% of these patients, weight loss was greater than 10% of body weight [39]. Although weight loss is higher in ICU-admitted patients [39, 40], it was also recorded in patients treated at home [41]. Fatigue and myalgia are common symptoms in severe and fatal COVID-19 patients while markers of muscle damage (e.g., creatine kinase, CK) are rather high [3, 28, 37, 38]. Body composition estimation in ICU-admitted COVID-19 patients denotes excessive loss of muscle mass following their ICU stay [42]—highlighting the high vulnerability of these patients to ICU-acquired weakness [38]. Respiratory symptoms are usually more severe in COVID-19 patients expressing muscle injury, which denotes a possibility of respiratory muscle insult [43, 44]. Postmortem analysis of muscle tissues of COVID-19 patients demonstrates massive necrosis and atrophy of myofibers along with myofibril disarray and Z-disc streaming [45]. In the meantime, COVID-19 survivors express marked reductions in hand grip and physical functioning—they suffer mild fatigue and dyspnea while they perform activities of daily living (ADL) [46]. Altogether, skeletal muscle failure seems to be one of the multiple organ failures that strike COVID-19 patients either due to the dystrophic effects of cytokine storms or due to direct viral binding to its angiotensin-converting enzyme 2 (ACE2) receptor on the surface of skeletal myocytes [47, 48]. Because muscle wasting in hospitalized patients is associated with serious adverse effects including premature death [37, 38], researchers emphasize the importance of screening hospitalized COVID-19 patients, especially older adults, for wasting disorders such as sarcopenia and cachexia [3, 45, 49].

Sarcopenia can be mitigated at early stages by physical activity, high-protein diets (especially those rich in milk soluble proteins), and supplements (e.g., amino acids, vitamin D, and polyphenols in bee products) [3, 7, 20]. Meanwhile, protein and amino acid supplementation to COVID-19 patients who are prone to sarcopenia may improve recovery, decrease ICU admission, and probably prevent muscle loss that frequently occur in patients with prolonged disease course [38]. Therefore, early identification of vulnerable groups through proper screening may be necessary to facilitate efforts for the prevention and/or treatment of sarcopenia during the current COVID-19 crisis. Screening for physical frailty in non-COVID-19 patients and in older populations has been integrated in primary healthcare in some countries as an attempt to target immune vulnerability among these individuals during the current outbreak [50–52]. Likewise, identification of COVID-19 patients liable to skeletal muscle injury may be necessary to provide adequate nutritional and physical rehabilitation therapies in order to facilitate recovery and prevent post-recovery disability [28, 38, 46]. Given that routine clinical care for COVID-19 patients focuses primarily on promoting survival, the estimation of muscle quantity or muscle strength is less considered, which may be associated with extreme later suffering after discharge out of the

development of disability, even in young individuals [15]. Nonetheless, the novelty of the disease as well as its acute and progressive nature entail lack of knowledge regarding the most effective measures for assessing muscle condition in severe cases. Sarcopenia and similar wasting conditions (e.g., cachexia and malnutrition) are widespread in community-dwelling elders and in a variety of non-COVID-19 patients (e.g., diabetics and obese). However, measures that flag disruptions in muscle strength and muscle quantity are lacking in most primary-care settings, albeit anthropometric measures are conducted in some diabetes clinics [15]. To bridge the gap, this review sheds light on conditions in which the assessment of skeletal muscle damage may be necessary. It also provides a detailed illustration of different measures used for detecting sarcopenia, with a focus on measures appropriate for use among patients struck by COVID-19.

2. Relevance of Body Composition to Sarcopenia and COVID-19

Body composition plays a role both in sarcopenia and COVID-19. In particular, high fat mass in obese and overweight older adults is associated with extensive muscle breakdown, which is hidden behind the fat bulk, and therefore, it frequently goes unnoticed [15, 53]. Adipokines such as leptin stimulate an excessive release of cytokines such as interlukin-6, which activate muscle fiber remodeling resulting in alterations in anthropometric measures, even in older people with cancer [54]. The co-occurrence of sarcopenia and obesity is a condition known as sarcopenic obesity [3]. Renal injury is common in COVID-19 patients even after recovery [55]. Longitudinal data show increased risk for renal dysfunction in sarcopenic and obese people [56]. These patients also demonstrate poor respiratory muscle performance, which is not frequently checked in routine care even for people with symptoms such as dyspnea, and it is associated with respiratory dysfunction in these individuals when they contract COVID-19 [15, 35, 57].

Both general and central obesity are associated with a high risk for COVID-19 infection [58]. Body mass index (BMI) is higher in severe COVID-19 patients (mean difference = 1.6, 95% confidence interval (CI): 0.8–2.4; $p = 0.0002$, $I^2 = 75\%$) [59]. Indeed, obesity is the most prevalent chronic comorbidity among symptomatic COVID-19 patients (42%, 95% CI: 34–49%, $p = 0.034$, $I^2 = 69.2\%$) followed by hypertension (40%, 95% CI: 35–45%, $p = 0.001$, $I^2 = 55.6\%$) and type 2 diabetes (17%, 95% CI: 15–20%, $p = 0.001$, $I^2 = 32.6\%$) [60]. Among different comorbidities, COVID-19 patients who are obese express the highest overall pooled event rate for severe complications (56.2%, 95% CI: 35.3–75.1, $p = 0.015$, $I^2 = 71.5\%$) [61]. Several meta-analytic reviews associate BMI $\geq 30\,\mathrm{kg/m^2}$ in COVID-19 patients with composite poor outcome, which consist of hospital admission, ICU admission, ARDS, severe COVID-19, higher use of mechanical ventilation, and mortality. Odds ratio reported in these studies range between 1.4 and 2.0 (p values <0.001) [59, 60, 62], with consistency of the findings across different primary studies ($I^2 = 0\%$) [59].

3. Identification of Individuals with/or Prone to Sarcopenia (Target Groups)

Loss of muscle mass and associated muscle weakness can be reverted, especially during early stages, through sound exercise and nutritional interventions [19, 63–69]. Evidence from animal studies shows that nutritional interventions for muscle wasting yield better results when administered early during the development of muscle atrophy [7, 70]. Therefore, proper screening and management of sarcopenia may support efforts aimed to slow or reverse the progression of physical frailty in old seniors [1]. Indeed, the latest available guidelines for frailty management strongly recommend screening older adults for physical frailty and its underlying causes via rigorous tools, treating sarcopenia as a main cause of weight loss, and addressing nutritional needs in this group [14, 71]. In the same way, current guidelines emphasize the need for assessing sarcopenia and physical frailty in patients with COVID-19 who get hospitalized [30, 31, 33, 72]. Screening for sarcopenia and physical frailty has the merit of identifying potentially remedial conditions while enjoying a noninvasive nature [73]. Thus, timely identification of skeletal muscle wasting in people with potentially modifiable risk factors is of indispensable importance for a successful development of interventional strategies that can restore motor functioning and prevent disability in older individuals and in COVID-19 patients [11, 74].

Sarcopenia has a multifaceted dynamic that entails undernutrition, inflammaging, oxidative stress, motor neuron injury, satellite stem cell dysfunction, metabolic alterations, decreased physical activity, and dysregulation of gut microbiota [3, 7]. Frailty is closely linked to comorbidity, poor cognition, functional dependence, institutionalization, and hospitalization [3, 75]. Therefore, it is recommended to screen for sarcopenia in people with recent functional decline, recent history of reduced appetite that resulted in poor food intake, unintentional weight loss of more than 5% per month, low muscle mass, repeated falls, depression, cognitive decline, and chronic wasting disorders such as chronic heart failure, chronic obstructive pulmonary disease, diabetes mellitus, chronic kidney disease, connective tissue disease, and tuberculosis [14, 76, 77]. Patients with chronic illnesses who undergo surgery may also be screened for sarcopenia both pre- and postoperatively. Sarcopenia is associated with malignancies, and it contributes to greater complications and death postoperatively in these patients [78, 79].

The literature documents sex differences in the onset of sarcopenia. Aging of skeletal muscle develops earlier and faster in women than in men [80–82]. The drop of estrogen, which starts as early as the age of 45 years, is a direct read out for senescence in women, and it promotes the development of several age-related pathologies, including sarcopenia [2, 4, 18]. Estrogen deficiency alters glucose and lipid metabolism, fosters skeletal muscle apoptosis, and hinders processes involved in muscular force generating capacity such as myosin phosphorylation and satellite cell function, which are highly sensitive to estrogen [20, 83]. Evidence

reveals greater age-related dysfunction in myosin and myosin ATP turnover in females than in males [82]. A recent meta-analytic review shows that higher levels of plasma IL-6 are associated with worse changes in muscle quantity and quality in aged women than in aged men probably due to the protective role of testosterone [80]. Among hospitalized older adults with COVID-19, frailty is higher in women than in men (75.2% vs. 59.4%, $p < 0.001$) [84]. DNA methylation of genes that regulate energy metabolism and oxidative stress is more evident in myoblasts and myofibers of women than in those of men [81]. Sarcopenia evaluated by criteria based on the percentage of skeletal muscle mass is associated with three single-nucleotide polymorphisms (SNPs) in women: FTO rs9939609, ESR1 rs4870044, and NOS3 rs1799983 [85]. Therefore, women demonstrating these SNPs as well as menopausal women and young women with ovulation failure should be addressed as candidate targets for the prevention and treatment of sarcopenia.

COVID-19 patients either treated at home or in the hospital, especially in the ICU, are prone to wasting and muscle loss [39, 41]—in particular, patients with polypharmacy, prolonged hospital stay, immobility (in the prone position for a long time), body protein loss, and gastrointestinal symptoms that decrease food intake or increase nutrient loss (anorexia, vomiting, and diarrhea) [28, 37, 38]. Therefore, skeletal muscle assessment may be necessary in severe patients exhibiting weight loss and malnutrition or a high risk for malnutrition [28, 38].

4. How to Screen for Sarcopenia

To screen for sarcopenia in research and clinical settings, researchers have developed a wide range of predictive risk models, which consider numerous variables such as age, sex, race, and comorbidities [86, 87]. The main muscle-related elements that should be evaluated in sarcopenia are the amount of muscle (mass) and its ability to generate force (strength) or function properly (physical performance) [21, 76]. Beyond these measures, current guidelines recommend interventional studies addressing sarcopenia to assess a number of outcome indicators that take into account the progressive nature of sarcopenia [16, 76].

Based on the recommendations declared by the Asian Working Group for Sarcopenia (AWGS), European Working Group on Sarcopenia in Older People (EWGSOP), and many others [21, 76, 88], we attempted to summarize screening measures that can uncover aspects of failure in skeletal muscle quantity and quality (Figure 1). These measures were broadly categorized into two groups: direct and indirect measures of muscle loss. The former group represents primary indices that directly reflect qualities of muscular mass, strength, and function/physical performance [21, 76]. They can also portray overtime changes that result from muscle fiber transformation in response to aging and environmental factors [16, 76]. The second group comprises a large set of secondary parameters, some of which can speak for age-related molecular and cellular changes taking place in skeletal muscle (e.g., various biomarkers), and some others reflect a trail of sarcopenia-related adverse effects, which take physical, cognitive, emotional, and social forms [8, 76, 89, 90].

4.1. Direct Measures of Sarcopenia

4.1.1. Measures of Skeletal Muscle Mass. Skeletal muscle mass can reflect changes in quality of life (QoL) [91]. A number of body-imaging techniques can provide data necessary to estimate muscle mass [76, 87, 92] (Table 1). The five key aspects of sarcopenia evaluation that are addressed by imaging techniques include the thickness, cross-sectional area, echogenicity, fascicle length, and pennation angle of the addressed muscle segments [93, 94]. The literature reports wide variations in the rates of sarcopenia owing to limitations intrinsic in its assessment tools as well as heterogeneity of the assessed populations (e.g., critical patients, geriatric patients, and community-dwelling elderly) [95–97]. Moreover, proper measurement of muscle amount and strength is rather challenging given that the available assessment techniques demonstrate a range of advantages/disadvantages with regard to their reliability of measurement, cost, safety, availability, and ease of use [21, 87, 92]. Magnetic resonance imaging (MRI) and computed tomography (CT) are considered gold-standard techniques for estimating body composition, including muscle density and fat infiltration. They are preferably applied to certain muscles, but they can also scan the whole body [94, 98].

MRI produces high-resolution images that detect muscle, fat mass, and water contents of the body based on the different molecular properties of these anatomical compartments. It simultaneously detects qualitative abnormalities such as muscle disruption, edema, muscle-fat infiltration, and fibrosis [98]. Technically, MRI identifies variations in body structures by detecting radiofrequency signals (T1 and T2) emitting from nuclear spinning towards the direction of an external magnetic field. Mid-thigh or the abdomen at lumbar level mid-L3 are the key areas used to reflect on muscular condition of the whole body. Intermuscular and intramyocellular lipid depots are noted by a short T1 and a long T2 proton relaxation time [99]. A systematic review reports good-to-excellent manual slice-by-slice segmentation reliability in eight studies and moderate-to-good validity against dissection in one study, while the validity of automatic techniques combined with different statistical shape or Atlas-/image-based methods was good in four studies. Manual segmentation is used as a gold-standard method for muscle quantification; however, it is associated with greater errors in volume and shape estimations [96]. MRI types vary based on their molecular basis and scanning techniques such as 23 Na MRI (based on skeletal muscle tissue sodium concentration, TSC), CT muscle attenuation, diffusion tensor MRI, Dixon MRI, and proton magnetic resonance spectroscopy (MRS) (e.g., 13C and 31P) [93, 94, 100]. MRI has high repeatability [100]. It assesses fat infiltration, edema, and specific patterns of muscle involvement. MRI measurements are reported to correlate with disease severity in spinal bulbar muscular atrophy and amyotrophic lateral sclerosis, two key disorders of motor

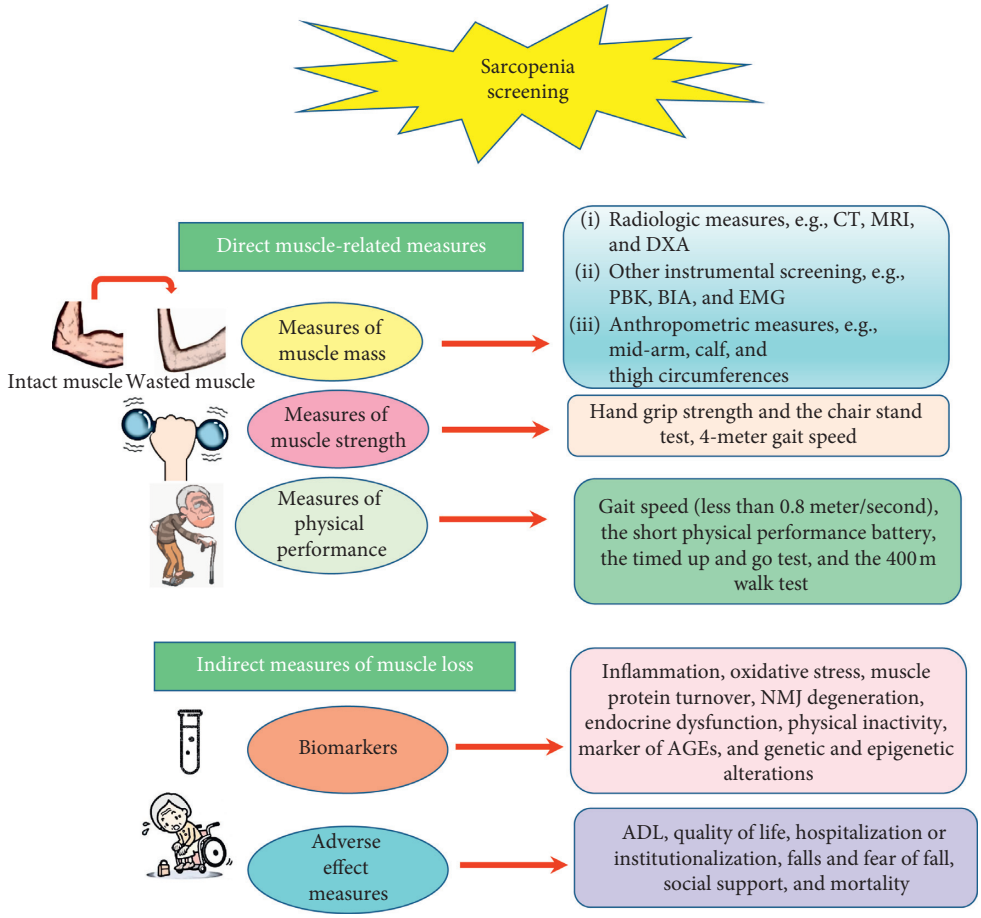

Figure 1: Schematic illustration of the algorithm used for sarcopenia screening. Abbreviations: CT: computed tomography, MRI: magnetic resonance imaging, DXA: dual-energy X-ray absorptiometry, PBK: partial body potassium, BIA: bioelectrical impedance analysis, EMG: electromyography, and NMJ: neuromuscular junction. The most commonly used algorithm for the diagnosis of sarcopenia involves direct assessment of muscle strength and physical performance using gait speed and handgrip strength. When the evaluation reveals muscle weakness and poor muscle function, direct estimation of muscle mass via CT, MRI, or DXA is necessary to confirm the diagnosis. However, muscle mass can be evaluated by cheaper and less sophisticated techniques such as BIA and even by anthropometric measures when other techniques are not available. A variety of indirect measures of muscle quality can be employed in research and clinical settings in order to evaluate the effectiveness of therapeutic protocols that target sarcopenia. Pathologies underlying muscle failure as well as response of sarcopenic muscle tissues to treatment can be detected via a wide range of biomarkers. In addition, numerous health-related outcomes that affect sarcopenic people can be used as indicators of treatment effectiveness, e.g., falls and hospitalization.

neuron loss [101]. MRI involves no radiation risk, but it is expensive, and it requires technical skills and space, which make its use limited to research facilities [99]. The reproducibility and concordance of abdominal skeletal muscle area segmentation analyses by CT and T2-weighted (T2w) MRI abdomen/pelvis are documented as a measure of sarcopenia in renal cell carcinoma patients, denoting that these measures may be used interchangeably [78].

Several studies report reductions in skeletal muscle mass measured by CT in hospital-admitted and critical patients with various health problems, denoting a significant association of muscle mass loss with mortality within one month after hospital admission [102, 103]. Some indices are even based on the quantification of the mass of certain muscles by CT such as the psoas muscle index, which is based on the quantification of the psoas muscle located at the base of the fourth lumbar vertebra. This index is a reported predictor of morbidity, length of hospital stay, and in-hospital complications such as mortality in the elderly and in trauma population [103, 111]. CT effectively identified muscle mass loss in COVID-19 patients over the course of ICU stay [42]. CT may simultaneously measure muscle mass in patients using it for other purposes, e.g., in cancer patients who frequently have this procedure as a regular part of their standard management [79]. CT is used to assess the extent of lung fibrosis in COVID-19 patients. The cross-sectional areas of the pectoralis muscles (PMA, cm^2) can be automatically measured on axial CT images. Pectoralis muscle index (PMI) is calculated as: PMI = PMA/patient's height square (m^2). PMI significantly predicted the length of hospital stay, intubation, and mortality in COVID-19 patients [57]. Sarcopenia assessed by PMI is an independent risk factor for mortality in patients with lung cancer [112]. Cumulative literature shows significant variations in CT techniques regarding contrast use, selected skeletal muscle areas, ranges of radiodensity delimitation, and their cutoff

Table 1: Advantages and possible limitations of different techniques used for assessing sarcopenia and the likelihood for their application in COVID-19 patients.

Muscle assessment techniques	Advantages	Limitations	Relevance to COVID-19	Reference
Computed tomography (CT)	It reliably measures muscle quantity and fat infiltration. It can be applied to the whole body or specific muscles and serves as a base for several reliable muscle mass indices. CT routinely used for other purposes (patients with tumors) can simultaneously measure muscle mass. It predicts mortality in critical patients.	Relatively expensive and time consuming, with high radiation risk. No cutoff values for diagnosing sarcopenia.	It has successfully identified muscle loss in ICU-admitted COVID-19 patients screened for lung fibrosis, albeit patients still encounter radiation risk.	[42, 94, 102, 103]
Magnetic resonance imaging (MRI)	It reliably measures muscle quantity and quality (e.g., fat infiltration). It has no radiation risk, and it can be applied to the whole body or specific muscles.	Expensive and not portable or widely available, with technical difficulties and space requirement. There are no cutoff values for diagnosing sarcopenia. Its use is limited to research facilities.	Despite its high accuracy, its use may be impractical because of its limitations.	[94, 99]
Dual-energy X-ray absorptiometry (DXA)	It correlates with low muscle mass measured by MRI, and it is cheap, quick, and widely available, with low radiation risk.	Not portable and inaccurate in patients with obesity and edema, while variations between protocols limit comparison of results.	May be used as a cheaper and quick alternative of CT. However, it should not be used in obese or edematous patients.	[98, 99, 104]
Ultrasound	Simple, cheap, safe, portable, and noninvasive, with a high reproducibility.	Its measurement lacks standardization, and it is not clear which anatomical sites can best predict total skeletal muscle mass.	Can be a safe and cheap method for frequent muscular assessment over the course of hospital/ICU stay.	[93, 94, 99, 105]
Bioelectrical impedance analysis (BIA)	It yields results concordant with CT, and it is cheap, noninvasive, and widely available.	Results are confounded by body water distribution. It reports higher SMM in patients who are males or have edema than CT. Its equations and cutoff values are population and device specific.	Not preferred in ICU patients because of changes in the hydration status.	[95, 98, 106]
Electromyography	It is a noninvasive way to assesses neuromuscular transmission denervation and deposition of endomysial connective tissue and fat.	Not widely available and requires special technical skills.	It has been used to measure maximal voluntary contraction for quadriceps and biceps in recovering patients.	[46, 99, 107]
Total/partial body potassium (TBK/PBK)	It is a cheap and simple alternative of CT and MRI with less radiation exposure. It yields results consistent with DXA.	It is based on assumptions that may not hold in old and diseased conditions, e.g., fixed muscle content of nitrogen and hydration coefficient of lean body mass.	May not accurately reflect on muscle mass because critical COVID-19 patients exhibit nitrogen loss as well as multiple micronutrient deficiencies and electrolyte imbalance.	[28, 99, 108, 109]
Anthropometric measures (body mass index and circumferences of the calf, thigh and mid-arm)	They are cheap and safe techniques that fit in low-resource facilities because they do not require special skills.	They do not accurately quantify muscle mass and may be confounded by edema and adiposity.	They detected malnutrition and wasting in ICU-admitted COVID-19 patients, especially among diabetics with cytokinemia and hypoproteinemia.	[21, 28, 87, 110]

points. A key reason for such variations is lack of precise information about the correlation between skeletal muscle radiodensity by CT and its molecular composition. Nomenclature uniformization may be established by CT studies that involve direct measures of muscle composition such as MRI [97].

Muscle density can be measured by simpler, safer, and cheaper techniques. For example, dual-energy X-ray absorptiometry (DXA) is a relatively safe technique that entails low radiation exposure [21, 76, 105, 110]. DXA scans the body depending on variations in the absorption of low- and high-energy X-rays by different body components [93, 99]. It is the most frequently used imaging technique for muscle mass estimation. It estimates appendicular lean mass (ALM), the sum of lean mass at the upper and lower limbs. ALM is used to calculate appendicular lean mass index ($\text{ALMI} = \text{ALM}/\text{height}^2$) [98]. It also assesses body mineral content and fat mass [98, 113], which make it the most favorable technique for assessing osteosarcopenic obesity—a combination of osteoporosis, skeletal muscle loss, and obesity [3, 113]. DXA is reported to quantify changes in body composition (fat mass and muscle mass) faster and at a greater precision than nuclear MRI [104]. Indeed, the latest consensus report of the EWGSOP recommends DXA as the technique of choice for the clinical assessment of muscle wasting [114]. Despite these merits, DXA has some limitations: the machine used is not portable, DXA scanners are not available in most primary-care settings, various protocols associated with various hardware and software packages between manufacturers make the comparison of results from different settings impractical, DXA does not detect qualitative changes in muscles (e.g., fat infiltration), it is confounded by body size and hydration state because it does not differentiate between water and lean tissue, and it should be avoided during pregnancy [99].

Ultrasound is a cheap, portable, and noninvasive technique that has a high reproducibility. It is also safe because it does not use ionizing radiation [94]. It quantifies tissue thickness based on the detection of an echo reflected back from tissues exposed to ultrasound beams from a transducer [99]. Total quadriceps volume measured by the ultrasound-derived rectus femoris cross-sectional area is a simple index of muscle size. Its high correlation with MRI pinpoints its high reliability [105]. However, lack of standardization, in terms of the most representative muscle sites, and its high dependence on the expertise and skills of the operator are major drawbacks [93]. Moreover, the interpretation of muscle-fat interfaces is limited due to similar acoustic impedance of muscle and fat tissues. In addition, applying the transducer to the skin with excessive pressure may compress the muscle, which may cause measurement errors [99].

The amount of contractile tissues can be quantified via bioelectrical impedance analysis (BIA), a nonimaging tool that quantifies total muscle mass based on the conduction of an electric current applied across the body [98]. BIA is considered a cheap and easy-to-use alternative of DXA [21]. A current systematic review shows that sarcopenia entails low BIA and both are independent predictors of survival in aging samples. However, the extent to which BIA may be valuable in detecting low muscle quality and/or identifying sarcopenia is not clear [115]. Comparisons between CT and BIA in critically ill patients show higher values of skeletal muscle mass than that calculated by CT, especially among males and patients with mild edema [106]. Similarly, comparisons between CT and BIA among geriatric inpatients based on the EWGSOP case-finding algorithm show systematic overestimation of muscle mass by BIA [116]. BIA is considered a proxy of water distribution [94, 115]; however, it may not accurately reflect on body cell mass, especially in critical patients [106, 116]. In addition, BIA is not a standardized technique because BIA equations and cutoff values are population and device specific. Therefore, the prevalence rates of sarcopenia assessed by BIA vary according to equations and devices used for their estimation [95].

Electromyography (EMG) is a nonimaging, noninvasive technique that may evaluate neuromuscular transmission, the degree of denervation indicated by the number or size of motor units and myocytes, and integrity motor units (captured by near-fiber jiggle and jitter), along with edema and deposition of endomysial connective tissue and fat [99, 117]. EMG involves applying a low-intensity (50-kHz) alternating current to the skin to evoke a surface voltage pattern while certain electrodes placed on the sites of muscle of interest monitor the electrical activity of muscle contraction [99, 105, 118]. It revealed significant differences in maximal voluntary strength and motor stability in older adults with presarcopenia, sarcopenia, and severe sarcopenia. However, it detected no differences in the number of motor units in these groups [117]. It also reflected on muscle fiber loss in seven myositis patients similar to diffusion-weighted MRI [119]. An available systematic review indicates that surface electromyography reliably estimates muscle fiber conduction velocity across multiple sessions in sport science, rehabilitation, physiological, and clinical studies [107].

Because 60% of the potassium content of the body exists in skeletal muscle, the estimation of total-body potassium (TBK) has been used as a cheap and safe nonimaging technique that indirectly measures muscle mass [120]. Given that the isotope ^{40}K exists at a known and constant natural abundance of 0.0012%, a scintillation counter can effectively measure ^{40}K [99]. Therefore, the technique operates by detecting isotropic emissions of gamma rays resulting from the decay of ^{40}K, which occurs at a rate of ~200 gamma rays per second per gram of natural K [99, 109]. Estimations of muscle mass by TBK yielded results consistent with those estimated by DXA among healthy men and women aged 60 years or above [108]. The partial body potassium (PBK) system is a simpler alternative to TBK. It requires subjects to put their arms or legs inside a cavity that comprises a gamma ray detector (4π liquid scintillation counter) for 15 minutes [109]. Despite their simplicity and low cost, TBK and PBK are not favorable for estimating muscle mass because muscle mass calculations are based on many assumptions that may not hold in aged or diseased individuals such as constant intracellular potassium content, nitrogen content, and

hydration coefficient of lean body mass [99, 120]. In this regard, this technique may not suit COVID-19 patients because they exhibit hypoproteinemia, multiple micronutrient deficiencies, and electrolyte imbalance [28, 37].

Techniques commonly used for muscle quantification (both imaging and nonimaging) are not universally available, especially in low-resource communities, due to their high cost and related technical complexity [21, 92]. Therefore, a number of studies validated the ability of simple anthropometric measures to quantify muscle size and to reliably reflect physical performance and predict survival in old people when sophisticated measures of sarcopenia assessment are not available [21, 87, 110]. These measures are based on the notion that muscle mass of the lower limb is strongly linked to the level of functional impairment in old age [14]. Vastus lateralis, one of the muscle groups of the quadriceps in the thigh that is primarily type II fibers, is most vulnerable to shift to oxidative metabolism, fibrosis, and atrophy with advanced age. Meanwhile, the soleus muscle in the calf largely comprises type I fibers, which naturally undergo higher protein turnover [15, 121, 122]. Low appendicular skeletal muscle mass (ASM) in older individuals could be examined as accurate as relative skeletal muscle index (RSMI) obtained by DXA imaging through simple anthropometric measures such as thigh circumference, calf circumference, mid-arm muscle circumference (MAMC), and total skeletal muscle mass estimated by Lee's formula (eTSMM) [21, 87, 110]. For proper evaluation, these measures should be conducted with a contextual regard for sex, race, and age [87]. Cutoff points for low ASM adjusted for body mass index are <0.789 and <0.512 for men and women, respectively [88]. These cutoffs slightly vary according to ethnicities (e.g., between Asian and Caucasian), body size, life style, and culture [76].

Several models can portray muscle wasting. ASM comprises the mass of the extremities (e.g., measured by DXA) after excluding the mass of bone and fat. The latter is automatically excluded by DXA [91]. According to FNIH guidelines, ASM less than 19.75 kg and 15.02 kg or alternatively ASM adjusted for body mass index less than 0.789 and 0.512 can diagnose sarcopenia in men and women, respectively [16]. Skeletal muscle mass index (SMI) is a common measure of muscle mass. It can be calculated by dividing the skeletal muscle mass of the upper and lower limbs by height squared [8]. ASM/height^2 less than 7.0 kg/m^2 and 5.5 kg/m^2 represent recommended cutoff points for diagnosing sarcopenia in men and women, respectively [12, 76]. Moon et al. diagnosed sarcopenia via a novel index based on lower extremity skeletal muscle mass (LESM, excluding mass of fat and bone). This index is calculated by dividing LESM by lower extremity body weight [kg] × 100 or squared height (LESM [kg]/Ht^2 [m]) [91].

4.1.2. Measures of Skeletal Muscle Strength. Measures of muscle mass do not consider contraction potential and muscle power generation. Loss of muscle force output is a result of multiple alterations in muscle composition such as fat infiltration and decreased motor units and neural activity, as well as decreased quality of contractile fibers. In fact, dysfunctional or denervated fibers (which are unable to generate force) count; they contribute to muscle mass when assessed by size quantification measures [123]. Functional techniques can express the ability of a muscle to recruit fibers incorporated in motor unit arrays by capturing the kinematic and/or kinetic output exerted during a dynamic muscle action [123, 124]. However, a recent meta-analysis reports no gold-standard technique [124].

Physiological cross-sectional area (PSCA) is a reliable composite measure of the strength and change in strength of leg extension [125]. It is one of the measures involved in the calculation of "relative muscle strength"—a measure of muscle quality that combines muscle size and strength. Relative strength refers to muscle force generation relative to muscle or body size. It is a sound evaluation tool of architectural and functional characteristics of skeletal muscle. However, PSCA is not easy to measure since it requires an apparatus that might not be easily incorporated in clinical settings [123]. On the other hand, muscle strength is frequently measured by reduced hand grip strength for age and gender, the chair stand test, and 4-meter gait (walking) speed [12, 14, 110, 126]. Hand grip to leg extension strength strongly correlates with gait speed, and both are reported to be equally suitable for screening elders for muscle weakness [127]. Cutoff points commonly used to diagnose sarcopenia are usual gait speed less than 0.8 meter/second and hand grip strength less than 26 kg for men and less than 18 kg for women [16, 76]. It is worth noting that measurement of muscle strength may be confounded by non-muscle-related factors such as levels of cognitive function and motivation [21]. Strength measures such as grip strength correlate with functional mobility and incident mobility impairment [123]. Moreover, strength measures positively respond to various interventions for sarcopenia in old people with evident muscle weakness, even with the persistence of low muscle mass [123, 128].

4.1.3. Measures of Muscle Functioning/Performance. Measures of muscle strength and function are interrelated. Physical performance can be evaluated by usual gait speed (less than 0.8 meter/second), the 6-min walk test, 400 m walk, the Timed Up and Go test, the stair climb power test, and the short physical performance battery [1, 14, 21, 110]. The latter evaluates gait, balance, strength, and endurance. It comprises several tests, e.g., standing with feet together in side-by-side, semitandem, and tandem positions; time to walk 8 feet, and time to rise from a chair and return to the seated position 5 times [1, 21].

According to the algorithm set by the European Geriatric Medical Society (EUGMS), Consensus Committee of defining sarcopenia, EWGSOP, and AWGS, the flow of the screening process starts by assessing muscle strength and physical performance using tests of gait speed and handgrip strength. If initial screening uncovers muscle weakness and poor muscle function, muscle mass should be estimated in order to confirm the diagnosis [1, 14, 21, 76].

4.2. Indirect Measures of Sarcopenia

4.2.1. Biomarkers. Researchers identified numerous biomarkers for early detection of both sarcopenia and physical frailty as well as for a detailed identification of their main pathophysiological mechanisms, which take place at molecular and cellular bases [110, 122, 129]. For instance, The International Conference on Frailty and Sarcopenia Research (ICFSR) Task Force has recently declared that measuring creatinine excretion via the D3-creatine dilution (D3Cr) method is a more reliable biochemical measure of functional muscle mass than assessment by DXA [129]. Judgment about therapeutic strategies for sarcopenia as either promising or not can be made after a relatively shorter time when treatment effects are assessed at a molecular level rather than at a behavioral level. For example, skin autofluorescence, a marker of advanced glycation end products (AGEs), represents an independent determinant for SMI, hand grip strength, and knee extension strength in older individuals [8]. If an intervention manages to decrease glycation stress at a relatively early stage of treatment, it could be easy to predict improvements in muscle status after an expected duration. Therefore, trials recruiting sarcopenic subjects are recommended to include various biomarkers as indicators of effectiveness [16, 76, 130].

Possible markers of sarcopenia include biomarkers of inflammation and oxidative stress (e.g., interleukin- (IL-) 6, IL-1, tumor necrosis factor-α, butyryl-cholinesterase, isoprostanes, oxidized low-density lipoprotein, and vitamins C and E), muscle protein turnover (e.g., creatinine and sarcomeric proteins such as actin, myosin, troponin, and tropomyosin), neuromuscular junction (NMJ) degeneration (e.g., C-terminal again fragment, CAF), endocrine dysfunction (testosterone, DHEA, and GH–IGF-1), growth factors (e.g., transforming growth factor-β, myostatin, and activin A and B), physical inactivity (e.g., complement protein C1q, hemoglobin, albumin, leptin, and uric acid), and glycation stress (e.g., skin autofluorescence) [1, 8, 131, 132]. In addition, epigenetic biomarkers of aging (also known as epigenetic clocks) may play an indispensable role in the evaluation of muscle strength and physical performance during old age and their change across various treatment strategies [90]. Sound treatments may induce changes in sarcopenia signature such as the expression level of genes that regulate pre-mRNA splicing, localization, and modification of RNA such as galectin-1, glutamine transporter SLC38A1, and membrane-bound transcription factor protease S2P [89].

4.2.2. Other Measures. The AWGS suggests a range of secondary outcomes to be assessed by interventional studies that address sarcopenia [76]. Sarcopenia is progressive in nature, which implies deterioration of overall health status, e.g., developing back pain (especially when atrophy affects back muscle) and physical dependence, which entails inability to perform ADL. Such drawbacks can seriously alter QoL of sarcopenic patients [1, 11, 14, 19, 53, 90]. Therefore, parameters recommended by the AWGS involve evaluating the dynamic changes in frailty status, basic and instrumental ADL over a given period of time, QoL, and social support. In addition, a number of adverse effects associated with sarcopenia can be assessed such as hospitalization or institutionalization, falls, fear of fall, and mortality [76].

4.3. Sarcopenia Assessment in COVID-19 Patients. Despite the current lack of agreed-upon treatment pathways of COVID-19, proposed guidelines highlight the importance of an integrative management, which includes defining and monitoring conditions that heighten immune dysregulation and lead to poor clinical outcomes in COVID-19 patients, such as the nutritional status and body composition, particularly muscle condition [42, 133, 134].

Several studies used clinical measures for the assessment of frailty in COVID-19 patients such as the FRAIL scale [31], Frailty Index (FI) [32], and Clinical Frailty Scale (CFS) [29, 30, 33, 135] to stratify COVID-19 patients according to their need for ICU admission [32], mechanical ventilation, and prolonged hospital stay [33], as well as developing multiple organ failure [31, 33] and mortality [29, 30, 135]. Indeed, a scoping review shows that CFS—the most widely used measure of frailty in COVID-19 patients—is predictive for comorbidity, mortality, complications, length of stay, falls, poor cognition, and functional dependence in 87%, 73%, 100%, 75%, 71%, 94%, and 91%, respectively, of hospitalized older patients [75]. Nonetheless, comorbidities are strong independent predictors for poor prognosis in COVID-19 patients [5, 27, 28]. Around half the items on most frailty assessment tools incorporate the assessment of comorbidities [5, 31–33]. Comorbidity, the accumulation of clinically manifest diseases, is another distinct construct, which represents one etiology of frailty, and it is likely to confound the association of poor skeletal muscle condition with COVID-19 prognosis [5].

Measures of physical frailty are interwoven with those of sarcopenia, which is considered a part and a definite biological basis of the frailty spectrum [1]. Nevertheless, frailty measures mainly address functional independence, which mirrors the physical performance aspect of skeletal muscle quality [33, 75]. However, physical performance may not be seriously altered despite muscle wasting/weakness [7]. Therefore, measures of frailty may be unfavorable for assessing skeletal muscle injury, which is the result of excessive protein degradation that leads primarily to muscle loss/weakness in hospitalized COVID-19 patients, usually over short periods of time [3, 28, 37, 38]. This notion gets support from the findings of a large-scale study predicting COVID-19 prognosis based on two measures of frailty: (1) FI, a 49-item scale that describes aspects of health, disease, disability, and mental wellbeing and (2) the frailty phenotype defined by Fried et al. based on five criteria: weight loss, exhaustion, physical activity, walking speed, and grip strength [49]. Analyses adjusted for sociodemographic and lifestyle factors revealed a higher risk for COVID-19 in prefrail (risk ratio (RR) = 1.47, 95% CI: 1.26–1.71) and frail (RR = 2.66, 95% CI: 2.04–3.47) individuals defined by FI compared to those classified as having robust frailty evaluated based on the criteria of the frailty phenotype [49].

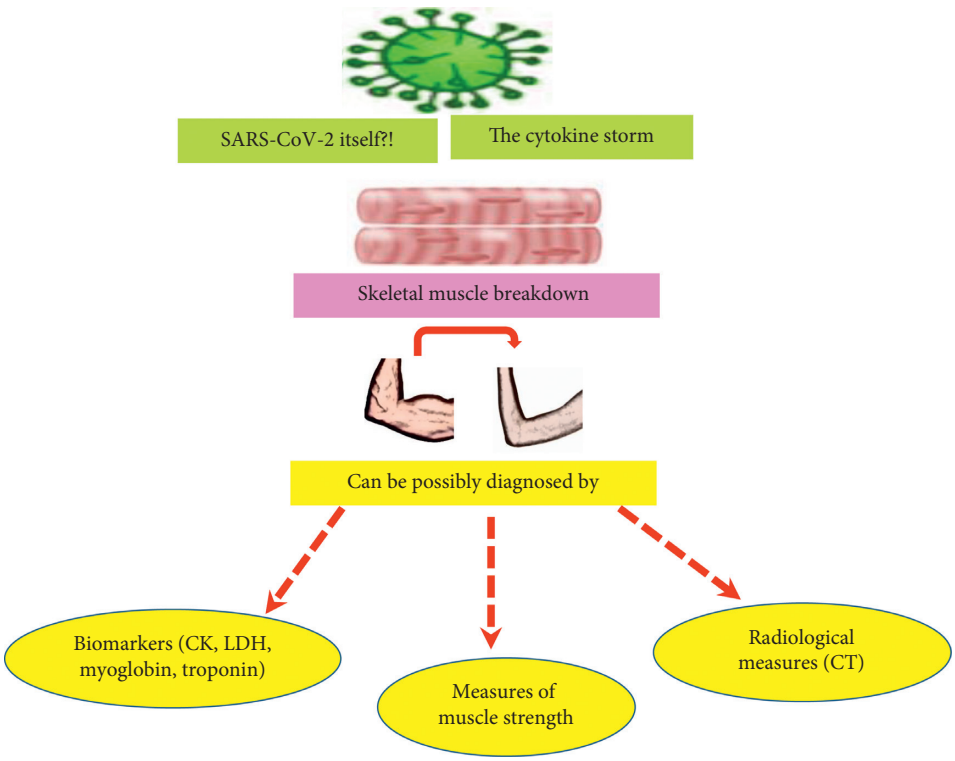

Figure 2: Possible approaches to detect muscle injury in symptomatic COVID-19 patients. Abbreviations: SARS-CoV-2: severe acute respiratory syndrome coronavirus-2, CK: creatine kinase, LDH: lactate dehydrogenase, CT: computed tomography.

CT can be effectively used to evaluate muscle composition change over the course of hospitalization in severe COVID-19 patients [42]. It may facilitate the detection of frailty among those patients by evaluating PMA [57]. However, the adverse effects associated with the exposure of patients to the ionizing radiation of CT are not known. As shown in Table 1, ultrasound and DXA may be faster and safer alternatives of CT taking into consideration that these measures may be confounded by the patient's body size, weight, and hydration status [99]. Maximal voluntary contraction for quadriceps and biceps was measured by electromyography in COVID-19 patients recovering from pneumonia who had no locomotor disability before contracting COVID-19 [46]. Meanwhile, the use of BIA as a cheaper alternative of CT to diagnose physical frailty in hospitalized COVID-19 elders may not be favorable, at least after the first week of hospitalization. Evidence shows that BIA measures in hospitalized geriatric patients are most reliable during the first week of hospital admission. However, older adults with prolonged ICU stay develop hydration abnormalities, which alter the credibility of this diagnostic technique [133].

Although various biomarkers can be used to signify muscle pathology, several lines of evidence show that the evaluation of CK as a biomarker of muscle injury may be a quick and cheap method to signify the development of muscle atrophy in hospitalized COVID-19 patients [134, 136–140]. The levels of lactate dehydrogenase (LDH) also get altered in severe COVID-19 patients, and they predict poor prognosis [136, 138, 141]. A meta-analysis reports significant differences in LDH between severe and nonsevere COVID-19 patients while changes in CK and other muscle biomarkers (e.g., troponin I and myoglobin) were not significant [141]. However, severe COVID-19 patients, especially those who develop rhabdomyolysis, express exponential alterations in CK [134, 140, 142, 143] and myoglobin [143], which denotes the importance of assessing these parameters in COVID-19 patients who are emaciated or experiencing myalgia.

In addition, grip strength and other indicators of muscle strength and physical performance (e.g., gait speed) are suggested to be used as cheap and quick methods to identify frail people with high risk for adverse effects during the COVID-19 crisis [72]. In particular, global measures of maximal strength of respiratory muscles (e.g., maximal inspiratory pressure) are inversely associated with grip strength. In addition, grip strength can effectively predict disability, morbidity, and mortality in older adults as well as in middle-aged and young people [72]. Indeed, the 6-minute walk test, Chester step test for predicting maximal oxygen uptake (VO_2 max), spirometry, cardiopulmonary exercise test (for predicting integrative responses of the respiratory, cardiovascular and skeletal muscle systems), and musculoskeletal testing (endurance testing (e.g., push up test) and proximal and distal muscle strength tests (e.g., rising from a squatting position or stepping onto a chair and walking on the heels and on tiptoes)) have been used to detect functional impairments in recovering SARS and COVID-19 patients. Such impairments were associated with reduced QoL, particularly perceived role-physical [55, 144]. Likewise, the

number of chair rises in the one-minute sit-to-stand test was associated with reduced strength of the quadriceps and biceps in remitting COVID-19 patients [46].

Muscle weakness and poor physical performance usually result from muscle dystrophy; measures of muscle strength and physical performance in COVID-19 were used in recovering patients following discharge [46]. Because rapid deteriorations affect severe COVID-19 patients during the acute phase of the disease (e.g., general wasting and malnutrition), imaging techniques (e.g., CT) and laboratory biomarkers may be more desirable for early detection of frail cases than muscle strength measures. Figure 2 summarizes possible measures that may detect skeletal muscle failure in COVID-19 patients.

5. Conclusions

Sarcopenia and related conditions (e.g., osteosarcopenic obesity) are widespread in aged and diseased populations; they are major risk factors for SARS-COV-2 infection. They contribute to the severity of COVID-19 by potentiating cytokine storms and respiratory failure. The detection of sarcopenia in vulnerable groups, mainly older adults and persons with chronic noncommunicable diseases, should proceed from the evaluation of muscle strength by simple measures such as grip strength to the evaluation of muscle mass in those with proven weakness. Muscle mass loss may be best detected by MRI and CT. However, due to the high cost and radiation risk entailed by these techniques, other simpler and cheaper techniques such as DXA and ultrasound are given preference. CT and electromyography were used to evaluate muscle loss in COVID-19 patients. During the acute phase, measuring the Pectoralis muscle mass can be simultaneously performed when lung fibrosis is routinely checked by CT, which entails an efficient evaluation of sarcopenia among those patients with no additional cost. In recovering patients, muscle strength and physical performance have been evaluated by electromyography and traditional tests such as the 6-minute walk test. Effective preventive and therapeutic interventions are necessary in order to prevent muscle loss and associated physical decline in COVID-19 patients.

Abbreviations

ADL: Activities of daily living
AGEs: Advanced glycation end products
ASM: Appendicular skeletal muscle mass
AWGS: Asian Working Group for Sarcopenia
BIA: Bioelectrical impedance analysis
CFS: Clinical Frailty Scale
CK: Creatine kinase
COVID-19: Coronavirus disease 2019
CT: Computed tomography
D3Cr: D3-creatine dilution
DXA: Dual-energy X-ray absorptiometry
EMG: Electromyography
eTSMM: Total skeletal muscle mass estimated by Lee's formula
EUGMS: European Geriatric Medical Society
EWGSOP: European Working Group on Sarcopenia in Older People
FI: Frailty index
IGFs: Insulin-like growth factors
ICFSR: International Conference on Frailty and Sarcopenia research
IL: Interleukin
ICU: Intensive-care unit
LDH: Lactate dehydrogenase
LESM: Lower extremity skeletal muscle mass
MAMC: Mid-arm muscle circumference
MRI: Magnetic resonance imaging
NMJ: Neuromuscular junction
PBK: Partial body potassium
PSCA: Physiological cross-sectional area
QoL: Quality of life
RR: Risk ratio
RSMI: Relative skeletal muscle index
SMI: Skeletal muscle mass index
SNPs: Single-nucleotide polymorphisms.

Data Availability

No data were used in this study.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Both authors conceptualized the topic and wrote and revised the manuscript.

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Research Article

Serum Levels of Irisin and Omentin-1 in Breast Neoplasms and Their Association with Tumor Histology

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Breast cancer is associated with obesity, possibly due to direct effects of adipokines and myokines, such as omentin-1 and irisin. In this study, we aimed to evaluate omentin-1 and irisin levels in women with benign and/or malignant breast neoplasms vs. healthy controls. Disease-free individuals ($N = 56$) and patients with histologically proven benign ($N = 61$) or malignant tumor ($N = 96$; subdivided into recently diagnosed/treatment-naïve ($N = 72$) and chemotherapy-treated ($N = 24$) subgroups) were enrolled in this study. Demographic, biochemical, and tumor histological characteristics were recorded. Body composition parameters were assessed using bioelectrical impedance. Serum irisin and omentin-1 levels were quantified with ELISA kits. In adjusted models, irisin levels were higher in both benign and malignant cases compared to controls but were comparable between neoplasms. Further adjustment for omentin-1 levels showed that age (odds ratio (OR) = 1.05, 95% confidence interval (95% CI) = (1.02, 1.08), $p < 0.01$) and irisin levels (OR = 5.30, 95% CI = (1.24, 22.38), $p = 0.03$) were independent predictors of the presence of malignancy. These molecules were associated with each other and with other anthropometric and demographic parameters. Irisin was associated with tumor histological characteristics including Ki67% levels, Elston-Ellis grading system, and estrogen receptors status. Omentin-1 was also associated with the Elston-Ellis grading system. In conclusion, serum irisin is increased in patients with both benign and malignant diseases of the breast. When combined with omentin-1, irisin concentration was associated with the presence of breast malignancy. This molecule's role as a potential diagnostic and/or prognostic agent in breast malignancies warrants further investigation in larger prospective studies.

1. Introduction

The modern epidemic of obesity has been associated with various comorbidities, including diabetes mellitus, cardiovascular diseases, and cancer [1]. During recent years, an increasing number of malignancies of different origins, including colon, endometrial, and postmenopausal breast cancer were shown to be associated with body fat accumulation and sedentary lifestyle [2]. It is reported that moderate exercise may decrease breast cancer risk up to 20–30% [3].

One of the proposed mechanisms to link obesity, lack of exercise, and tumor development is the direct effects of adipose tissue secreted molecules, named adipokines, which are altered in the obesity state. While leptin, the prototype adipokine, was increased in patients with breast malignancies and was positively associated with disease progression in vitro [4], serum adiponectin, a beneficial adipokine, was lower in breast cancer patients [5] and induced apoptosis and cell death in breast cancer cell lines [6].

With the increasing number of adipokines being discovered, novel molecules have been proposed to

contribute to obesity-related carcinogenic mechanisms. One of them, omentin-1, is a novel adipokine with similar properties to adiponectin [7]. Omentin-1 treatment induced p53-dependent apoptosis of hepatocellular carcinoma cell lines in vitro [8]. In clinical studies, serum omentin-1 levels were increased in patients with prostate [9] and colorectal [10] cancer and decreased in patients with renal cell cancer [11] compared to controls. Most recently, *omentin* gene expression and circulating levels were lower in patients with breast cancer compared to healthy controls [12]. However, no previous studies have explored its levels in benign diseases of the breast or in response to neoadjuvant chemotherapy.

In addition, in the last decade, muscle tissue was also found to secrete hormone-like peptides, termed myokines [13]. Irisin, the best-known member of the myokine family, was initially proposed as a skeletal muscle-derived hormone, which is proteolytically cleaved from its precursor molecule termed fibronectin type III domain containing 5 (FNDC5) and is subsequently released into the circulation in response to exercise, improving glucose homeostasis by inducing browning of white fat thereby increasing its thermogenic activity [14]. Further studies have elucidated its multifaceted involvement in many pathophysiological diseases, though results are inconclusive. In particular, in vitro data have shown a possible anticarcinogenic profile of irisin in highly malignant breast cancer cell lines [15]. However, irisin protein expressions in breast cancer tissue specimens were higher compared to nonmalignant tissues [16]. In humans, irisin levels were lower in breast cancer patients compared to healthy controls, but in the same study, a positive association with tumor histology was shown [17]. Thus, the exact role of irisin in breast neoplasms is still poorly understood. Furthermore, it is currently unknown whether these molecules could be used as noninvasive diagnostic markers for the timely diagnosis of breast benign or to discriminate between benign growths and malignancies.

Therefore, the main aim of this study was to evaluate irisin and omentin-1 levels in patients with benign vs. malignant treatment-naïve and chemotherapy-treated breast neoplasms vs. apparently healthy controls and study associations with tumor aggressiveness features.

2. Subjects and Methods

2.1. Study Participants. Adult females attending the Breast Cancer Clinic, Department of Surgery, Theaghenio Cancer Hospital in Thessaloniki, Greece, due to recent diagnosis of highly suspicious breast growth and scheduled for a biopsy or surgical removal of the tumor were consecutively enrolled in the study, as previously described [18]. Subjects were subclassified as treatment-naïve cancer patients or individuals with benign breast diseases following histological examination of the tissue specimen. A subgroup of patients who had received neoadjuvant chemotherapy within the last year for reduction of tumor burden and scheduled for surgical removal of the residual tumor was additionally recruited to study the potential effects of neoadjuvant chemotherapy on the biomarkers of interest. Disease-free individuals, with no clinical and/or imaging evidence of breast neoplasms, followed up in the clinic as part of a breast cancer prevention program, were recruited as controls. Exclusion criteria were age less than 18 years, history of other malignancy of any origin, and presence of life-threatening, and muscle wasting diseases.

For all participants, demographic, anthropometric, and medical history data were individually recorded. Body composition parameters were assessed using bioelectrical impedance (BIA), as previously described [19].

After surgery, tumor pathology features including the presence of malignancy; tumor size (cm); the presence of local or disseminated disease; estrogen (ER), progesterone (PR), and human epidermal growth factor-2 (Her-2) receptors' status; Ki67 levels; and the Elston-Ellis modification of Scarff-Bloom-Richardson grading system [20] were recorded for cancer patients.

Early morning, fasting blood samples were collected the day before surgery for the benign and cancer group participants, and on a routine visit for the controls. The study protocol was approved by the Theagenio Cancer Hospital and the Aristotle University of Thessaloniki Ethics Committees in accordance with the Declaration of Helsinki. All participants provided a written informed consent.

2.2. Biochemical and Hormonal Measurements. Metabolic and biochemical parameters including serum glucose and lipid levels were measured using routine laboratory methods. Commercially available ELISA kits were used for the quantification of serum irisin (Phoenix Pharmaceuticals, Burlingame, CA, #EK-067-29, assay sensitivity 1.29 ng/ml, linear range 1.29–27.5 ng/ml) and omentin-1 (EMD Millipore Co., Burlington, MA, #EZH0MNTN1-29K, assay sensitivity 2 ng/ml). Intra-assay and interassay coefficients of variations were less than 10% for both assays.

2.3. Statistical Analysis. Statistical analysis of the data was performed with SPSS v20.0 for Windows (IBM Corp., Armonk, NY). Data for continuous variables are presented as mean ± standard deviation (SD), unless otherwise stated. The normality of distribution of the continuous variables was assessed with the Kolmogorov-Smirnov test. Not normally distributed variables were logarithmically transformed for comparisons, when appropriate. For between-group comparisons, the independent-T test or Mann–Whitney U test was performed in cases of two groups, and one-way analysis of variance (ANOVA) or Kruskal-Wallis test was used for between-group comparisons in cases of more than two groups, with post hoc Bonferroni correction, if needed. Pearson's or Spearman's correlation and partial coefficient was calculated for unadjusted and adjusted bivariate associations, respectively. Linear regression models were performed to identify independent predictors of continuous variables, e.g., tumor size. Univariate and multivariate logistic regression analyses were used to identify independent predictors of categorical outcomes, such as the presence of malignancy. To assess the diagnostic potential of the biomarkers of interest, we selected to run a number of comparisons between (1) cancer-free participants (control and

benign groups) vs. treatment-naïve cancer patients to identify predictors of the presence of cancer in untreated populations, (2) cancer-free participants (control and benign groups) vs. all cancer patients (treatment-naïve and chemotherapy groups) to identify predictors of the presence of breast malignancy in the general population including participants under treatment, and (3) benign and treatment-naïve malignancy group to assess the potential of a hormone of interest to discriminate between a benign breast mass and breast malignancy. The level of statistical significance was set at 0.05 for all analyses.

3. Results

3.1. Descriptive Characteristics and Comparisons between Study Groups. Descriptive characteristics and case-control comparisons of the study variables are depicted in Table 1. Reflecting breast cancer epidemiology, healthy controls were younger compared to subjects of both cancer groups ($p < 0.001$ for both), but not the benign group. The percentage of total body fat (TBF%) obtained by BIA was significantly higher in the treatment-naïve cancer group compared to healthy individuals ($p = 0.03$). Regarding biochemical markers, we found significantly lower glucose levels in the disease-free control group compared to both treatment-naïve ($p = 0.02$) and chemotherapy-treated ($p = 0.001$) cancer groups and lower glucose levels in the benign group compared to the chemotherapy-treated group only ($p = 0.03$). Total cholesterol in both cancer groups was higher than that in the control group ($p = 0.04$ for both), but not the benign group (Table 1).

In raw comparisons (overall p for trend: $p = 0.01$), treatment-naïve cancer group patients had significantly higher logarithmically (Ln) transformed omentin-1 levels compared to healthy controls ($p = 0.02$) (Figure 1(a)). Post hoc comparison between healthy control and chemotherapy-treated group subjects was borderline significant ($p = 0.06$), but significance was lost after adjustment for age ($p = 0.13$), and this result remained unaffected after further adjustment for Body Mass Index (BMI), serum glucose, and total cholesterol (p for trend >0.05 for all) (Figure 1(a)).

Furthermore, regarding irisin (overall p for trend $p < 0.01$) treatment-naïve cancer group patients exhibited higher Ln-transformed irisin levels compared to healthy controls ($p < 0.01$) (Figure 1(b)). Unadjusted post hoc comparison between healthy controls and the benign group was borderline significant ($p = 0.07$). However, adjustment for age (Model 1, overall p for trend: $p < 0.01$) resulted in a significant pairwise comparison between healthy controls and the benign group participants ($p = 0.04$). The significant comparison between the control group and treatment-naïve cancer group remained unaffected ($p = 0.001$). Further adjustment for BMI (Model 2, overall p for trend: $p < 0.01$) did not impact significance. When glucose levels were added to the model (Model 3-overall p for trend: $p = 0.001$), irisin concentrations in the control group were significantly lower compared to both benign ($p = 0.02$) and treatment-naïve groups ($p = 0.001$). The same results were evident when BMI was replaced with TBF% in Model 3, or when total cholesterol was added to the model (Model 4, overall p for trend: $p = 0.01$), i.e., $p = 0.04$ for benign and $p < 0.01$ for treatment-naïve cancer group vs. controls, respectively. Interestingly, when Ln-transformed omentin-1 levels were added to Model 4 (Model 5, overall p for trend: $p = 0.02$), with either BMI or TBF%, in post hoc pairwise comparisons, irisin levels in the control group remained significantly lower compared to the treatment-naïve cancer group only ($p = 0.02$) (Figure 1(b)).

3.2. Associations of the Molecules of Interest with Other Study Variables. Both hormone levels were similar in patients with vs. without hypertension, with vs. without hyperlipidemia, with vs. without diabetes mellitus, with vs. without chronic kidney disease, with vs. without the chronic obstructive pulmonary disease, and with vs. without a history of pregnancies, breastfeeding, and/or history of familial breast cancer or malignancy of any origin ($p > 0.05$ for all).

In the whole cohort, Pearson's bivariate coefficient analysis revealed a positive association between irisin and omentin-1 ($r = 0.28$, $p < 0.001$). Positive associations between omentin-1 and age ($r = 0.26$, $p < 0.001$), WHR ($r = 0.19$, $p = 0.01$), and glucose levels ($r = 0.15$, $p = 0.04$) were also found (Table 2).

Partial correlation coefficients were performed to identify associates of the study variables after adjusting for potential confounders (Table 2). Adjustment for age did not affect the positive association between irisin and omentin-1 ($r = 0.29$, $p < 0.001$) but resulted in a significant negative association between omentin-1 and BMI ($r = -0.19$, $p = 0.01$). Significance in the correlation between omentin-1 and WHR was initially lost but reestablished after further controlling for BMI and blood glucose (Table 2). The strong positive association between irisin and omentin-1 remained unaffected even after further adjustments for BMI and/or serum glucose levels ($p < 0.001$ for all) (Table 2).

3.3. Logistic Regression Analyses to Identify Predictors of the Presence of Cancer. Univariate and multivariate models were used to identify independent predictors of the presence of malignancy in a number of comparisons. In the pooled group analysis comparing cancer-free participants to patients with either recently identified or known breast malignancy under treatment, the only independent predictors were age (odds ratio (OR) = 1.05, 95% confidence interval (95% CI) = (1.02, 1.08), $p < 0.01$) and irisin levels (OR = 5.30, 95% CI = (1.24, 22.38), $p = 0.03$) (Table 3). Similar results were observed when cancer-free individuals were compared to the recently identified treatment-naïve cancer group, i.e., for age (OR = 1.04, 95% CI = (1.01, 1.08), $p = 0.02$) and for irisin (OR = 5.05, 95% CI= (1.14, 22.40), $p = 0.03$) (Table 3). However, there were no independent predictors that could discriminate between benign mass and malignancies (Table 3).

3.4. Associations with Tumor Histological Features in the Treatment-Naïve Cancer Group. Regarding associations with tumor aggressiveness characteristics, in the treatment-

Table 1: Descriptive characteristics of the study variables and unadjusted case-control comparisons.

Variable	Control group	Benign group	Treatment-naïve cancer group	Chemotherapy cancer group	p value for trend
N	56	61	72	24	
Age (years)	48.05 ± 10.79^	52.25 ± 12.28	57.61 ± 12.29	61.21 ± 10.64	**<0.001**
Weight (kg)	71.08 ± 12.59	69.85 ± 13.12	74.39 ± 14.57	74.26 ± 13.94	0.21
Height (cm)	162.20 ± 5.84	159.84 ± 6.97	161.22 ± 7.13	156.88 ± 6.23	**0.01**
Body Mass Index (kg/m^2)	27.38 ± 5.18	27.45 ± 5.52	28.59 ± 5.15	30.14 ± 5.16	0.07
Total body fat (%)	34.90 ± 7.01*	36.77 ± 8.28	39.30 ± 9.98	39.00 ± 8.40	**0.03**
Waist-hip-ratio	0.86 ± 0.71	0.86 ± 0.77	0.87 ± 0.67	0.89 ± 0.57	0.27
Glucose (mg/dL)	94.48 ± 10.35^	100.37 ± 17.32#	105.70 ± 23.45	115.24 ± 33.80	**0.01**
Triglycerides (mg/dL)	104.27 ± 49.49	98.89 ± 47.93	106.21 ± 55.80	118.76 ± 39.60	0.26
Total cholesterol (mg/dL)	207.89 ± 39.79^	220.77 ± 37.18	208.04 ± 35.92	236.14 ± 50.90	**0.02**

Data are presented as mean ± standard deviation (SD). ANOVA or Kruskal-Wallis tests were used in unadjusted comparisons. Post hoc comparisons were determined with Bonferroni correction. For comparisons, logarithmically transformed variables were used for Body Mass Index and triglycerides. Significance is highlighted in bold. *Control significantly lower ($p < 0.05$) compared to the treatment-naïve group only (in the unadjusted model): control significantly lower ($p < 0.05$) compared to the benign and treatment-naïve group. ^Control significantly lower ($p < 0.05$) compared to both treatment-naïve and chemotherapy-treated cancer group. #Benign significantly lower ($p < 0.05$) compared to chemotherapy-treated cancer group.

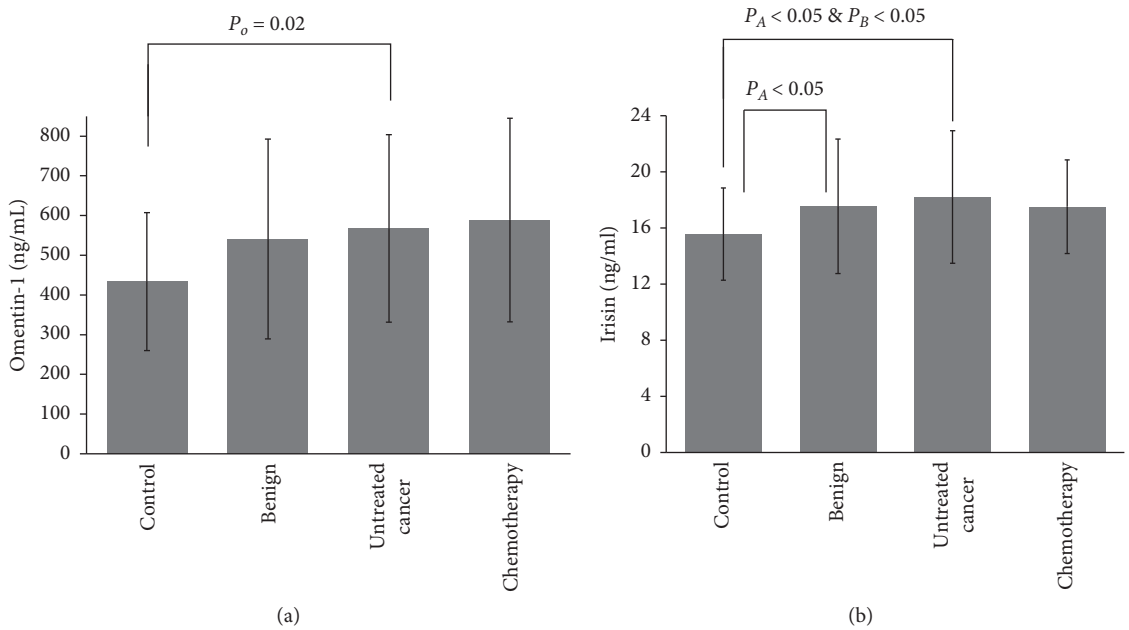

Figure 1: Omentin-1 (a) and irisin (b) serum levels across study groups. Post hoc unadjusted and adjusted comparisons were employed. Analysis of variance (ANOVA) or Kruskal-Wallis tests were used in unadjusted comparisons. Analysis of covariance (ANCOVA) was used in adjusted comparisons. Logarithmically transformed variables were used for both molecules. Adjusted models included: Model 1: adjusted for age. Model 2: adjusted for age and Ln-Body Mass Index (BMI). Model 3: adjusted for age, Ln-BMI and glucose levels. Model 4: adjusted for age, Ln-BMI, glucose levels, and total cholesterol levels. Model 5: adjusted for age, Ln-BMI, glucose levels, total cholesterol levels, and Ln-omentin-1. P_O: control significantly lower ($p < 0.05$) compared to treatment-naïve cancer group only (in the unadjusted model). P_A: control significantly lower ($p < 0.05$) compared to both benign and treatment-naïve cancer group (Model 1- Model 4). P_B: control significantly lower ($p < 0.05$) compared to treatment-naïve cancer group only (in unadjusted model and Model 5).

naïve cancer group, omentin-1 was associated with neither tumor size nor Ki67% levels, whereas irisin was positively correlated with the latter ($r = 0.28$, $p = 0.03$). Both hormones showed an increasing pattern with the Elston-Ellis grading system, i.e., for omentin-1, Grade 1: 304.61 ± 132.26 ng/ml vs. Grade 2: 530.65 ± 243.29 ng/ml vs. Grade 3: 661.61 ± 204.25 ng/ml, $p = 0.01$ (with borderline significantly lower levels in Grade 1 compared to Grade 3, $p = 0.05$) (Figure 2(a)) and for irisin, Grade 1: 12.37 ± 0.14 ng/ml vs. Grade 2: 17.40 ± 4.24 ng/ml vs. Grade 3: 20.04 ± 5.08 ng/ml, $p = 0.01$ (with post hoc Bonferroni correction showing significantly lower levels in Grade 1 compared to Grade 3, $p = 0.03$) (Figure 2(b)). No differences were observed regarding progesterone and Her-2 receptors status for both molecules. However, patients with ER+ breast cancer exhibited lower irisin

Table 2: Bivariate correlation matrices. Unadjusted Pearson's or Spearman's coefficients and partial correlation coefficient after sequential adjustment for age, Body Mass Index (BMI), and glucose levels in the overall study group.

	OMENTIN-1 (ng/mL)				IRISIN (ng/mL)			
	Unadjusted	Adjusted for age	Adjusted for age & BMI	Adjusted for age, BMI & glucose levels	Unadjusted	Adjusted for age	Adjusted for age & BMI	Adjusted for age, BMI & glucose levels
	R		*R*	*R*	*R*	*R*		*R*
Age (years)	0.26***	—	—	—	0.01	—	—	—
Weight (kg)	−0.07	−0.14	0.06	0.06	0.01	−0.04	−0.04	−0.04
Height (cm)	−0.01	0.07	−0.05	0.05	−0.03	−0.06	−0.06	−0.06
Body Mass Index (kg/m^2)#	−0.07	**−0.19***	—	—	0.01	−0.02	—	—
Total body fat (%)	−0.01	−0.14	−0.01	0.01	0.02	−0.03	−0.02	−0.02
Waist-hip-ratio	0.19**	0.13	**0.18***	**0.18***	0.11	0.06	0.06	0.07
Glucose (mg/dL)	0.15*	0.02	0.08	—	−0.05	−0.12	−0.11	—
Triglycerides (mg/dL)#	−0.01	−0.13	−0.08	−0.08	−0.09	−0.08	−0.08	−0.08
Total cholesterol (mg/dL)	−0.04	−0.04	−0.04	−0.03	0.09	0.09	0.09	0.06
Omentin 1 (ng/mL)#	—	—	—	—	**0.28*****	**0.29*****	**0.29*****	**0.30*****
Irisin (ng/mL)#	0.28***	0.29***	0.29***	0.30***	—	—	—	—

Significant correlations are highlighted in bold. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. #Logarithmically transformed variables were used for analyses.

Table 3: Independent predictors for the presence of malignancy.

	Univariate		Multivariate	
	OR (95% CI)	*p*	OR (95% CI)	*p*
(a) Comparison between cancer-free (controls and benign group individuals) vs. cancer (treatment-naïve and chemotherapy-treated) combined groups.				
Age (years)	1.06 (1.03, 1.09)	**<0.001**	1.05 (1.02, 1.08)	**<0.01**
BMI (kg/m^2)*	6.01 (1.29, 28.07)	**0.02**		
TBF (%)*	4.21 (1.28, 13.80)	**0.02**		
Glucose (mg/dL)	1.03 (1.01, 1.04)	**0.001**		
Total cholesterol (mg/dL)	1.00 (0.99, 1.01)	0.90		
Omentin-1 (ng/mL)*	2.23 (1.18, 4.21)	**0.01**		
Irisin (ng/mL)*	4.81 (1.42, 16.23)	**0.01**	5.30 (1.24, 22.69)	**0.03**
(b) Comparison between cancer-free (controls and benign group individuals) vs. treatment-naïve cancer group.				
Age (years)	1.06 (1.03, 1.08)	**<0.001**	1.04 (1.01, 1.08)	**0.02**
BMI (kg/m^2)*	3.81 (0.74, 19.52)	0.11		
TBF (%)*	4.46 (1.20, 16.61)	**0.03**		
Glucose (mg/dL)	1.03 (1.01, 1.04)	**0.01**		
Total cholesterol (mg/dL)	0.99(0.99, 1.004)	0.30		
Omentin-1 (ng/mL)*	2.07 (1.04, 4.1 1)	**0.04**		
Irisin (ng/mL)*	4.98 (1.38, 18.03)	**0.01**	5.05 (1.14, 22.40)	**0.03**
(c) Comparison between benign group individuals vs. treatment-naïve cancer group.				
Age (years)	1.04 (1.01, 1.07)	**0.02**	1.03 (0.99, 1.07)	0.10
BMI (kg/m^2)*	3.83 (0.56, 26.44)	0.17	1.84 (0.05, 66.32)	0.74
TBF (%)*	2.69 (0.66, 10.93)	0.17	1.07 (0.09, 13.24)	0.96
Glucose (mg/dL)	1.01 (0.995, 1.03)	0.16	1.00 (0.98, 1.03)	0.76
Total cholesterol (mg/dL)	0.99(0.98, 1.001)	0.07	0.99 (0.98, 1.004)	0.19
Omentin-1 (ng/mL)*	1.30 (0.62, 2.74)	0.49	0.79 (0.33, 1.87)	0.59
Irisin (ng/mL)*	1.95 (0.46, 8.23)	0.36	1.65 (0.25, 10.98)	0.60

Multivariate model includes age, glucose, BMI, %TBF, total cholesterol levels, serum omentin-1, and serum irisin levels. Significance is highlighted in bold. OR: Odds Ratio, BMI: Body Mass Index, TBF: total body fat. *Logarithmically transformed values were used for comparisons.

levels compared to ER-breast cancer patients (ER+: 17.84 ± 4.84 ng/ml vs. 19.99 ± 3.69 ng/ml, $p = 0.04$).

4. Discussion

Obesity is now considered a major modifiable risk factor for cancer development [21]. Therefore, understanding the mechanisms that link obesity with carcinogenesis is important for the identification of novel diagnostic markers and/or therapeutic targets. In this study, we explored serum levels of muscle- and adipose-tissue-derived molecules, namely, irisin and omentin-1, in patients with benign and malignant breast neoplasms of different stages compared to healthy individuals. We showed that serum irisin

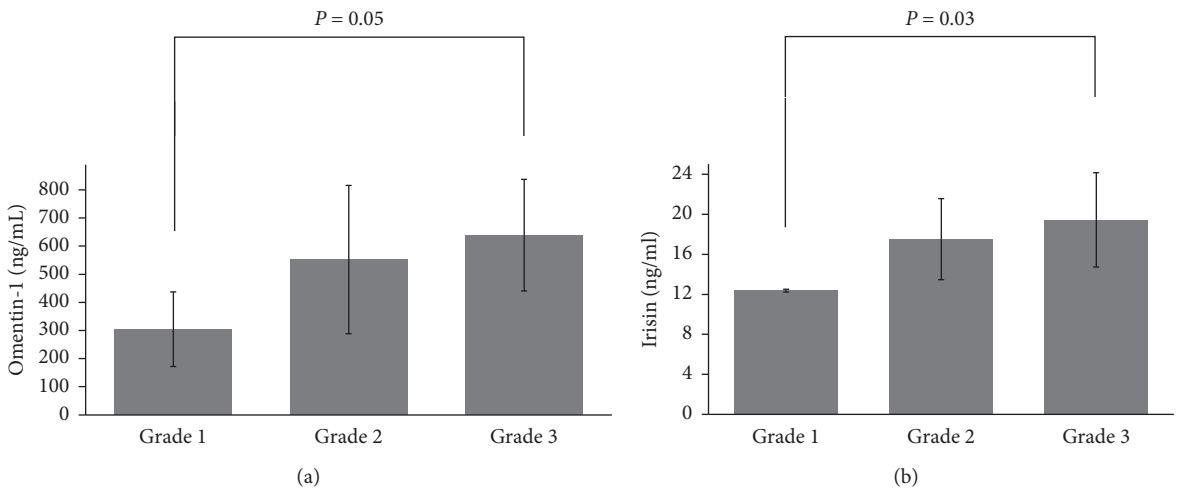

Figure 2: Omentin-1 (a) and irisin (b) serum levels according to the Elston-Ellis modification of the Scarff-Bloom-Richardson grading system. Data are presented as mean ± SD. Analysis of variance (ANOVA) and post hoc comparisons were employed. Logarithmically transformed variables were used for both molecules.

concentrations are increased in patients with both benign and malignant proliferative diseases of the breast independently of potential confounders and are also associated with tumor aggressiveness. In the fully adjusted model, adjusting for omentin-1 showed that irisin was an independent predictor of the presence of malignancy when compared to cancer-free individuals.

Irisin is a recently discovered adipomyokine that was initially suggested as a browning factor of white adipose tissue, orchestrating the beneficial effects of exercise in metabolism, and, thus, raising high expectations as a potential therapeutic agent against metabolic diseases [14]. In follow-up studies, our group was one of the first to elucidate irisin regulation in humans [22, 23]. However, increasing controversy regarding the accuracy and specificity of irisin assays has later emerged, questioning previous findings [24, 25].

Regarding cancer studies, irisin's role in carcinogenesis was recently debated [26]. Preliminary in vitro data showed no effect of irisin treatment in the colon, thyroid, esophageal, and endometrial cancer cell lines [27], while later data supported potential anticarcinogenic properties of irisin in malignant breast [15], lung [28], osteosarcoma [29], and pancreatic [30] cancer cell lines. In contrast, in a recent study, it was found that irisin might increase cell proliferation and migration potential of human hepatocellular carcinoma (HCC) cells [31]. Interestingly, in an animal study, FNDC5 gene expression in white adipose tissue and serum irisin levels were increased in mice with experimentally induced gastric cancer, compared to noncancer or control groups [32]. In human studies, FNDC5 hepatic gene expression was higher in HCC patients undergoing liver transplantation compared to deceased donors [33]. Protein expression of FNDC5/irisin was higher in breast and ovarian cancer tissue specimens [16], as well as in oncocytic papillary thyroid carcinoma [34] and in a series of gastrointestinal cancers compared to nonneoplastic tissues [35]. In addition, elevated irisin expression was observed in cancer cells and tumor stromal fibroblasts of non-small-cell lung carcinoma [36]. Furthermore, in clinical studies recruiting colorectal [37], prostate [38], and bladder [39] cancer patients, serum irisin levels were lower compared to healthy controls. Conversely, increased irisin concentrations were found in renal cancer patients compared to disease-free individuals [40].

Regarding breast cancer, it was suggested that cancer patients exhibit low irisin concentrations [17] and irisin is also protective against metastatic disease [41]. However, in the former study, the ELISA kit used reported irisin levels 100-fold higher than our observations, and based on previous validations, it is less specific. In contrast, the ELISA kit we have used herein has been validated with Tandem Mass Spectrometry [42]. In that study, irisin concentrations were reported in the range of 3–5 ng/ml [42] which is lower than the values reported herein. However, our findings are of a similar magnitude with other published studies using the same ELISA kit [43–46] and indeed slightly higher than the Tandem Mass Spectrometry but significantly lower compared to assays used in different studies. In fact, our assay's linear range is 1.29–27.5 ng/ml, and the assay can detect irisin levels between 10 and 50 ng/ml. Values reported in the present study fall well within this range. In addition, Jedrychowski et al. assessed irisin levels in young, healthy, and normal-weight individuals [42] which is different from our study sample recruiting older individuals with higher BMI and other metabolic comorbidities, which are generally positively associated with higher irisin concentrations [47, 48]. In any case, as irisin was measured with the same kit in all groups and as the conclusions of our study were based on the observed differences of irisin concentrations between the groups, the actual irisin values do not influence the interpretation of the results.

Moreover, in accordance with our observations, a positive association with tumor histology was observed [17]; the authors did not recruit patients with benign diseases of the breast and suggested that these controversial results might be partly explained due to complex and less understood pathophysiological changes occurring in malignancy. In our

study recruiting patients with benign diseases of the breast as well as cancer patients that had received neoadjuvant chemotherapy, we found significantly higher serum irisin levels in both benign and newly diagnosed breast cancer subjects compared to healthy individuals after adjusting for potential covariates and a positive association with tumor behavior. We also showed that irisin concentrations could independently predict the presence of malignancy in the general population. Our results are consistent with immunohistochemistry studies, and discrepancies with other serum studies may be explained by the use of less-validated methods to assess irisin concentrations as well as differences in study design and study sample selection. Our novel findings indicate a possible involvement of FNDC5/irisin in breast tumorigenesis, even from very early stages of development of benign growths, as well as in processes of cancer progression, thereby pointing toward its use as a potential diagnostic and prognostic marker for breast neoplasms; this, however, needs to be confirmed in future prospective studies. When we adjusted our analyses for omentin-1 levels, a surrogate marker of visceral obesity, the comparison between healthy controls and patients with benign tumors failed to reach significance while comparison with cancer patients was persistent, suggesting that the association between irisin concentration and presence of breast malignancy is not affected by visceral adiposity. However, this needs to be confirmed in larger cohorts and/or future longitudinal studies.

Omentin-1, initially termed as intelectin-1, was suggested as visceral fat-derived adipokine with anti-inflammatory and insulin-sensitizing properties [49] which is reduced in obesity, in a similar manner with adiponectin, and drives the higher burden of excessive body fat in patients with increased cardiovascular risk or type 2 diabetes [7]. In a previous study recruiting a rather limited group of individuals at higher cardiovascular risk, we found that omentin-1 was negatively associated with irisin and also closely associated with lipoprotein subparticle profile, possibly indicating a role as a marker of increased cardiovascular risk [50]. These results are not directly comparable to those of the present study, in which we found a positive association between these two molecules, due to differences in the study sample characteristics and study aims. Therefore, this association requires further research in future studies with larger populations. Regarding cancer, although in vitro data have shown possible anticarcinogenic effects of this molecule, findings from clinical studies were contradictory, with most studies showing increased omentin-1 serum concentrations in cancer patients compared to disease-free controls (as reviewed in [51]). However, in a very recent study, omentin gene expression and circulating levels were lower in patients with breast cancer compared to healthy controls [12]. However, to the best of our knowledge, no previous study has assessed omentin-1 levels across the spectrum of proliferative diseases of the breast. Therefore, we have conducted the present study in which we found that omentin-1 levels are increased in treatment-naïve breast cancer patients compared to healthy individuals in unadjusted comparisons. These results are in accordance with a meta-analysis suggesting that malignancies are generally associated with increased omentin-1 levels [52]. However, in our study, the significance of this comparison did not persist after adjustments for covariates, indicating that anthropometric and metabolic parameters, rather omentin-1, are the main predictors of the presence of malignancy. In addition, we reported that serum omentin-1 levels are increased in patients with more aggressive disease burden based on the Elston-Ellis grading system, which may be explained by activation of Akt signaling pathways and/or inflammatory processes [10].

The strengths of this study include its novelty and the simultaneous assessment of two hormones with promising metabolic properties in a group of subjects with breast neoplasms with a wide age range. To the best of our knowledge, this is the first study to explore serum irisin and omentin-1 levels across the spectrum of breast neoplasms, recruiting patients with benign breast lesions as well as newly diagnosed, treatment-naïve cancer patients and individuals who received neoadjuvant chemotherapy and were scheduled for surgical removal of the residual tumor, comparing their levels to healthy controls. We showed that irisin is elevated in either benign or malignant breast diseases, independently of possible covariates. In addition, we have assessed irisin levels with a Tandem Mass Spectrometry-validated kit and examined associations with several aspects of tumor histology. However, due to the case-control design of our study, the causality of the reported associations cannot be supported. However, our results provide an impetus for the design and interpretation of larger studies exploring the involvement of these adipomyokines in neoplasms of the breast. Our rather small study sample may also be considered as a limitation and, as patients were recruited from a single center, a selection bias may be committed. Therefore, larger multicenter studies are needed to further elucidate the role of irisin and/or omentin-1 in breast tumorigenesis.

5. Conclusions

In conclusion, we reported for the first time that serum irisin is increased in both benign and malignant neoplasms compared to healthy controls and, after controlling for potential confounding factors including omentin-1, irisin was associated with breast cancer presence. Irisin was also associated with tumor aggressiveness and may, therefore, hold potential as a future diagnostic and/or prognostic marker. Our results warrant further investigation in future mechanistic and longitudinal studies and/or clinical trials.

Data Availability

Anonymized datasets used to support the findings of the present manuscript are available from the corresponding author, upon reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest. ST is now employed in the Breast Surgery Department, Genesis Clinic, Thessaloniki, Greece.

Authors' Contributions

GP, BCT, and EP contributed to the study conception and design. GP collected data, performed experiments and statistical analysis, and drafted the manuscript. ST collected data. BCT revised the manuscript for important intellectual content. EP interpreted data and drafted the manuscript. All authors read and approved the final manuscript.

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Research Article

Serum Irisin Level Is Positively Associated with Bone Mineral Density in Patients on Maintenance Hemodialysis

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Background. Irisin is a circulating hormone-like myokine that plays an important role in bone metabolism. We performed a cross-sectional study to investigate whether serum irisin levels correlated with bone mineral density (BMD) in patients on maintenance hemodialysis (MHD). *Methods*. Blood samples were obtained from 80 patients on MHD, and serum irisin concentrations were determined using a commercially available enzyme-linked immunosorbent assay. BMD was measured by dual-energy X-ray absorptiometry of the L2–L4 vertebrae. *Results*. In the study cohort, 10 (12.5%) and 19 (23.8%) patients had osteoporosis and osteopenia, respectively, and 51 (63.75%) patients had normal BMD. Lumbar T-score was negatively associated with body height ($P = 0.010$), body weight ($P = 0.002$), body mass index (BMI, $P = 0.010$), and serum irisin ($P < 0.001$) and was positively associated with advanced age ($P = 0.031$), female sex ($P = 0.001$), alkaline phosphatase (ALP, $P = 0.010$), urea reduction rate ($P = 0.018$), and fractional clearance index for urea ($P = 0.020$). Multivariable forward stepwise linear regression analysis revealed that high serum logarithmically transformed irisin (log-irisin, $\beta = 0.450$, adjusted R^2 change = 0.258; $P < 0.001$), female sex ($\beta = -0.353$, adjusted R^2 change = 0.134; $P < 0.001$), and serum ALP level ($\beta = -0.176$, adjusted R^2 change = 0.022; $P = 0.049$) were significantly and independently associated with lumbar BMD in patients on MHD. *Conclusions*. In addition to female sex and serum ALP level, serum irisin level was positively associated with lumbar BMD in patients on MHD.

1. Introduction

It was well known that mineral and bone disorders are associated with increased fracture risk and substantial morbidity and mortality in patients with chronic kidney disease (CKD) compared with the general population [1–3]. Osteoporosis, characterized by low bone mass and density, is associated with an increased fracture risk and significant public health burden globally in the general population as well as in patients with end-stage renal disease (ESRD) [3, 4]. In Taiwan, an epidemiological study of the general population aged above 50 years reported that the prevalence of osteoporosis increased from 17.4% in 2001 to 25% in 2011 [5]. Abnormal bone turnover is common and gradually declines with worsening renal function in patients with CKD. Indeed, the 2017 Kidney Disease: Improving Global Outcomes (KDIGO) guidelines recommend bone mineral density (BMD) examination by dual-energy X-ray to assess fracture risk in patients with CKD based on studies showing that decreased BMD is common in patients on maintenance hemodialysis (MHD), with osteoporosis and osteopenia rates of 9.5%–23% and 16.7%–45%, respectively [6–8].

To elucidate the increased predisposition of patients with CKD and low BMD to osteoporosis and fractures,

several studies investigated factors associated with BMD including age, sex, nutrition, physical activity, body composition, and myokine levels [9,10]. Evidence showed that inflammation could influence the mechanism of osteoclastogenesis as well as bone resorption in late-stage CKD and MHD patients [11]. Irisin, a newly discovered myokine, which is cleaved from the precursor fibronectin type III domain-containing 5 (FNDC5), is primarily known for its modulatory role in phenotypic changes of white adipose tissue to brown adipose tissue to increase energy expenditure and improve glucose homeostasis; irisin also activates promyogenic genes for subsequent activation of satellite cells and increased protein synthesis [12, 13]. Based on its increased production and release by myocytes after exercise, irisin is considered as an anabolic mediator between the muscle and bone both *in vitro* and *in vivo* [14, 15]. In athletes and healthy individuals, irisin positively correlates with total body and hip BMD and strength [16, 17]. However, irisin has also been reported to exhibit a negative relationship with osteoporosis and fracture independently of BMD, body composition, and physical activity in postmenopausal women [18, 19]. A meta-analysis showed that serum irisin was decreased in elderly women with osteoporosis and exhibited a positive correlation with BMD [9].

Overall, these studies highlight the potential role of irisin as a marker and modulator of abnormalities in muscle and bone, but its role on bone density in MHD patients remains unclear. Therefore, we evaluated risk factors for osteoporosis and the relationship between serum irisin levels and BMD in patients on MHD.

2. Materials and Methods

2.1. Patients. This cross-sectional study conducted at Hualien Tzu Chi Hospital, a medical center in Hualien, Taiwan, between June 2015 and August 2015 included patients above 50 years of age on MHD using the standard 4-hour hemodialysis three times a week with standard bicarbonate dialysate and high-flux polysulfone disposable artificial kidney (FX class dialyzer, Fresenius Medical Care, Bad Homburg, Germany). Patients fulfilling the following criteria were excluded: treatment with antiosteoporotic medication (bisphosphonates, teriparatide, or estrogen medications), history of lumbar fracture or surgery, acute infection, malignancy, acute myocardial infarction, pulmonary edema, heart failure at the time of blood sampling, and refusal to provide informed consent. A total of 80 patients fulfilling these criteria were included in the final analyses. The study was approved by the Research Ethics Committee of Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation (IRB106-62-B), and is conducted in accordance with the World Medical Association Declaration of Helsinki.

2.2. Anthropometric Analysis. Body weight and height were measured to the nearest half kilogram and half centimeter, respectively, with patients in light clothing and without shoes. Body mass index (BMI) was calculated as body weight (kg) divided by body height squared (m^2) [20–22].

2.3. Biochemical Analyses. Fasting blood samples (approximately 5 mL) were immediately centrifuged at 3000 g for 10 minutes after collection before the hemodialysis. Serum samples were stored at 4°C. Biochemical analyses were performed within one hour of collection. Serum levels of blood urea nitrogen, creatinine, alkaline phosphatase (ALP), total calcium, and phosphorus were measured using an autoanalyzer (Siemens Advia 1800, Siemens Healthcare, Henkestr, Germany). Fractional clearance index for urea (Kt/V) and urea reduction ratio (URR) were measured before and immediately after hemodialysis using a formal, single-compartment dialysis urea kinetic model. Commercial enzyme-linked immunosorbent assays were used to measure serum levels of irisin (Phoenix Pharmaceuticals, CA, USA; catalogue number: EK-067-29) and intact parathyroid hormone (PTH) (IBL International GmbH, Hamburg, German; catalogue number: NM59041) [22]. The interassay and intraassay coefficient of variation of irisin is less than 15% and 10% and interassay and the intraassay coefficient of variation of intact PTH is 5.7% and 4.8%, respectively.

2.4. Measurement of Bone Mineral Density. BMD was measured immediately after blood sampling before MHD. The BMD of L2–L4 vertebrae was measured using dual-energy X-ray absorptiometry (QDR 4500, Hologic, MA, USA) and expressed using absolute values (g/cm^2) and T-scores (deviation from peak BMD) [20–22]. T-score was defined as the number of standard deviations from the mean BMD of sex-matched young control subjects. Compared to the mean BMD of controls, a lumbar bone T-score less than −2.5 was used as the diagnostic cutoff for osteoporosis and a lumbar bone T-score between −1.0 and −2.5 was used for the diagnosis of osteopenia, according to the World Health Organization criteria [23].

2.5. Statistical Analysis. Data were tested for normal distribution using the Kolmogorov–Smirnov test. Data were expressed as means ± standard deviation for normally distributed data and as medians with interquartile ranges for nonnormally distributed data. Categorical variables were analyzed using the χ^2 test. The significance of differences among the normal, osteopenia, and osteoporosis groups was determined using the Kruskal–Wallis or one-way analysis of variance, based on the normality of data and Dunn's multiple comparison test or post hoc Bonferroni test for multiple comparisons. The duration of hemodialysis and the levels of albumin and irisin exhibited skewed distributions; therefore, the data were log-transformed to achieve normality. Clinical variables that correlated with lumbar BMD in patients on MHD were evaluated by simple linear regression analysis and multivariable forward stepwise linear regression analysis. All statistical analyses were performed using SPSS for Windows (version 19.0; SPSS, Chicago, IL,

USA). A P value of <0.05 was considered to indicate statistical significance.

3. Results

Based on BMD measurements, the study cohort comprised 51 (63.75%), 19 (23.75%), and 10 (12.5%) patients in the normal, osteopenia, and osteoporosis groups, respectively (Table 1). Compared with the normal group, the rates of female and older patients were significantly higher in the osteopenia and osteoporosis groups ($P = 0.001$ and $P = 0.031$, respectively). Additionally, the mean BMI and mean serum irisin levels were lower ($P = 0.010$ and $P < 0.001$, resp.) and the mean ALP, URR, and Kt/V ($P = 0.01$, $P = 0.018$, and $P = 0.02$, respectively) were higher in the osteopenia and osteoporosis groups compared with the normal group. The rates of diabetes mellitus and hypertension as comorbidities did not differ among the three groups.

By simple linear regression analysis, body height ($r = 0.440$, $P < 0.001$), body weight ($r = 0.462$, $P < 0.001$), BMI ($r = 0.332$, $P < 0.003$), serum creatinine ($r = 0.297$, $P < 0.007$), and log-transformed irisin level (log-irisin, $r = 0.517$, $P < 0.001$) were positively correlated with lumbar BMD whereas female sex ($r = -0.430$, $P < 0.001$), age ($r = -0.268$, $P = 0.016$), serum ALP level ($r = -0.294$, $P = 0.008$), URR ($r = -0.271$, $P = 0.015$), and Kt/V ($r = -0.268$, $P = 0.016$) were negatively correlated with lumbar BMD (Table 2). The multivariable forward stepwise linear regression analysis including variables that were associated with lumbar BMD in the simple linear regression analysis (sex, age, BMI, creatinine, ALP, URR, Kt/V, and log-irisin) revealed that serum log-irisin level ($\beta = 0.450$, adjusted R^2 change = 0.258; $P < 0.001$), female sex ($\beta = -0.353$, adjusted R^2 change = 0.134; $P < 0.001$), and serum ALP level ($\beta = -0.176$, adjusted R^2 change = 0.022; $P < 0.049$) were significantly and independently associated with lumbar BMD in patients on MHD.

4. Discussion

The present cross-sectional analysis of 80 patients on MHD revealed that the serum irisin levels were lower in patients with osteopenia or osteoporosis compared to those with normal BMD. Furthermore, in addition to female sex, serum ALP level was negatively correlated, while serum irisin level was positively correlated lumbar BMD in patients on MHD.

Several studies aimed to identify risk factors for low BMD in patients with CKD or ESRD who are at a higher risk of osteoporosis and fracture-related mortality compared to the general population [1, 10, 24, 25]. In a cohort study of patients on CKD not undergoing dialysis, Hyun et al. showed that low BMD was prevalent, with osteopenia and osteoporosis rates of 33% and 8%, respectively; the authors also reported that low BMD was significantly associated with female sex, old age, low BMI, and decreased renal function as well as increased risk of ESRD [26]. In another longitudinal cohort study, Nickolas et al. found that patients on CKD experienced progressive loss of cortical density and thickness along with increased porosity in the bone, which were driven by increased bone turnover based on PTH and bone-specific ALP levels; the authors also found that PTH and bone-specific ALP levels predicted the decreases in the cortical area (2.2% and 2.8%, respectively.) and cortical thickness (2.0% and 2.5%, respectively.) [27]. Moreover, another study utilizing dual-energy X-ray absorptiometry reported that 13.6%, 22.2%, and 33.3% of patients on dialysis exhibited osteoporosis of the spine, hip, and any site (hip and spine), respectively [28]. In addition, the authors found that BMD was negatively associated with age, female sex, and bone-specific ALP level and positively associated with BMI; however, BMD did not show significant associations with dialysis duration, diabetes mellitus, and smoking [28]. In agreement with these studies, we also found that 23.75% and 12.5% of the patients on MHD had osteopenia and osteoporosis, respectively, and that MBD was negatively associated with age, female sex, and ALP and positively associated with BMI.

Physical exercise is crucial for the healthy development of the skeleton, whereas muscle disuse in patients with sarcopenia can lead to osteoporotic hip fractures, which are associated with reduced life expectancy and increased mortality [29]. Serum levels of irisin, a myokine cleaved from FNDC5, increases after exercise, indicating the link between muscle and bone activity [30]. Exercise was shown to induce irisin expression compared to the resting state in mice, and conditioned medium from exercised myoblasts was demonstrated to target osteoblasts and enhance the differentiation of bone marrow stromal cells, indicating the osteogenic effect of muscle [14]. Moreover, injection of recombinant irisin at a lower dose before the induction of browning in white adipose tissue of mice was associated with improved cortical mineral density, bone bending strength, and geometrical architecture as well as with a reduction in osteoclasts [15]. In mice subjected to mechanical unloading, recombinant irisin induced the preservation of cortical and trabecular BMD and bone volume by reducing sclerostin and increasing osteoprotegerin to the levels measured in normally ambulated mice and by restoring osteoblastogenesis [31]. Recently, Zhu et al. reported that the novel *Fndc5*/irisin knockout mice exhibited lower BMD, bone surface, and bone volume, with delayed bone development and hypomineralization [32]. The authors also showed that the treatment of bone marrow-derived mesenchymal cells with recombinant irisin induced osteoblastogenesis through the activation of Wnt/β-catenin pathway and inhibited osteoclastogenesis through the inhibition of receptor activator of nuclear factor-κB ligand-induced AKT cascade and suppression of NFATc1 activation. Altogether, these findings indicate that exercise-induced release of irisin has profound effects on bone remodeling as an anabolic mediator between muscle and bone. In clinical studies, irisin was shown to be a stronger determinant of bone mineral status compared with PTH and bone ALP in children [33]. In athletes and older individuals, serum irisin levels were positively correlated with total body and subregional anatomical BMD [16, 17]. Furthermore, irisin was reported to be negatively correlated with osteoporosis and fracture

Table 1: Clinical characteristics according to different lumbar T-score cutoff points (normal, osteopenia, and osteoporosis) of the 80 hemodialysis patients.

Characteristics	All patients ($n = 80$)	Normal ($n = 51$)	Osteopenia ($n = 19$)	Osteoporosis ($n = 10$)	P
Age (years)	66.93 ± 10.27	64.71 ± 10.10	70.11 ± 9.95	72.20 ± 9.00	0.031*
Hemodialysis duration (months)	57.66 (23.82–122.34)	56.88 (19.44–114.96)	81.00 (34.44–145.20)	47.58 (15.72–164.61)	0.484
Height (cm)	159.74 ± 8.28	161.80 ± 7.80	156.47 ± 7.18†	155.40 ± 9.63	0.010*
Body weight (kg)	64.13 ± 14.55	67.95 ± 14.32	60.41 ± 10.13	51.75 ± 15.15‡	0.002*
Body mass index (kg/m^2)	24.99 ± 4.66	25.88 ± 4.78	24.64 ± 3.76	21.12 ± 3.77‡	0.010*
Lumbar bone mineral density (g/cm^2)	0.95 ± 0.20	1.07 ± 0.14	0.80 ± 0.06†	0.65 ± 0.05‡,a	<0.001*
Lumbar T-score	−0.51 ± 1.60	0.45 ± 1.10	−1.73 ± 0.44†	−3.03 ± 0.38‡,a	<0.001*
Lumbar Z-score	0.57 ± 1.06	1.08 ± 0.76	0.08 ± 0.61†	−1.09 ± 0.87‡,a	<0.001*
Systolic blood pressure (mmHg)	136.60 ± 24.37	140.72 ± 24.56	132.16 ± 21.27	124.00 ± 25.36	0.091
Diastolic blood pressure (mmHg)	73.23 ± 13.78	73.35 ± 15.10	71.16 ± 9.67	66.30 ± 11.14	0.124
Albumin (mg/dL)	4.10 (3.90–4.40)	4.10 (3.90–4.40)	4.10 (4.00–4.10)	4.20 (3.68–5.25)	0.630
Blood urea nitrogen (mg/dL)	58.74 ± 14.47	57.71 ± 13.65	61.00 ± 10.11	59.70 ± 16.30	0.687
Creatinine (mg/dL)	9.22 ± 1.79	9.49 ± 1.81	8.78 ± 1.67	8.65 ± 1.98	0.186
Alkaline phosphatase (U/L)	83.86 ± 29.95	78.65 ± 27.50	84.42 ± 29.87	109.40 ± 31.74‡	0.010*
Total calcium (mg/dL)	8.96 ± 0.78	8.91 ± 0.77	9.15 ± 0.82	8.96 ± 0.78	0.464
Phosphorus (mg/dL)	4.62 ± 1.21	4.71 ± 1.13	4.67 ± 1.25	4.07 ± 1.54	0.310
Intact parathyroid hormone (pg/mL)	225.72 ± 182.18	208.59 ± 178.27	266.92 ± 185.17	234.84 ± 202.18	0.491
Irisin (ng/mL)	39.99 (12.51–105.84)	54.92 (17.46–161.30)	34.19 (12.99–56.16)†	3.01 (1.52–9.03)‡,a	<0.001*
Urea reduction rate	0.73 ± 0.04	0.72 ± 0.04	0.75 ± 0.04†	0.75 ± 0.04	0.018*
Kt/V (Gotch)	1.33 ± 0.17	1.29 ± 0.16	1.40 ± 0.16	1.41 ± 0.18	0.020*
Female, n (%)	39 (48.8)	17 (33.3)	15 (78.9)	7 (70.0)	0.001*
Diabetes mellitus, n (%)	33 (41.3)	25 (49.0)	6 (31.6)	2 (20.0)	0.145
Hypertension, n (%)	35 (43.8)	24 (47.1)	7 (36.8)	4 (40.0)	0.722

Values for continuous variables given as means ± standard deviation and test by one-way analysis of variance; variables not normally distributed given as medians and interquartile range and test by Kruskal–Wallis analysis. *$P < 0.05$ was considered statistically significant after the Kruskal–Wallis analysis or one-way analysis of variance.†Compared with the normal group and osteopenia group, ‡compared with normal group and osteoporosis group, and [a]compared with osteopenia group and osteoporosis group was and <0.05 considered statistically significant after Dunn's multiple comparison test or post hoc Bonferroni test. Kt/V, fractional clearance index for urea.

Table 2: Correlation of lumbar BMD levels and clinical variables by simple regression or multivariable linear regression analyses among the 80 hemodialysis patients.

Variables	Lumbar BMD (g/cm^2)				
	Simple regression		Multivariable regression		
	r	P	Beta	Adjusted R^2 change	P
Female	−0.430	<0.001*	−0.353	0.134	<0.001*
Diabetes mellitus	0.164	0.146	—	—	—
Hypertension	0.162	0.152	—	—	—
Age (years)	−0.268	0.016*	—	—	—
Log-HD duration (months)	−0.054	0.631	—	—	—
Body mass index (kg/m^2)	0.332	0.003*	—	—	—
Systolic blood pressure (mmHg)	0.208	0.064	—	—	—
Diastolic blood pressure (mmHg)	0.200	0.075	—	—	—
Log-albumin (mg/dL)	0.022	0,848	—	—	—
Blood urea nitrogen (mg/dL)	0.035	0.757	—	—	—
Creatinine (mg/dL)	0.297	0.007*	—	—	—
Alkaline phosphatase (U/L)	−0.294	0.008*	−0.176	0.022	0.049*
Total calcium (mg/dL)	−0.022	0.849	—	—	—
Phosphorus (mg/dL)	0.149	0.188	—	—	—
Intact parathyroid hormone (pg/mL)	−0.137	0.244	—	—	—
Log-irisin (ng/mL)	0.517	<0.001*	0.450	0.258	<0.001*
Urea reduction rate	−0.271	0.015*	—	—	—
Kt/V (Gotch)	−0.268	0.016*	—	—	—

Data of HD duration, albumin, and irisin showed skewed distribution and therefore were log-transformed before analysis. Analysis of data was done using the univariate linear regression analyses or multivariate stepwise linear regression analysis (adapted factors were female, age, body mass index, creatinine, alkaline phosphatase, urea reduction rate, Kt/V, and log-irisin). HD, hemodialysis; Kt/V, fractional clearance index for urea. *$P < 0.05$ was considered statistically significant.

independently of BMD, body composition, and physical activity in postmenopausal women [18, 19]. In a meta-analysis, there was a weak association between irisin and lumbar BMD as well as femoral neck fracture; decreased irisin levels were associated with osteoporosis by pooled analysis and irisin levels were even lower in postmenopausal women and those with a history of fracture [9], suggesting that irisin might have utility as a marker for the assessment of metabolic bone disease. However, Kim et al. found irisin could induce the expression of sclerostin in vitro in a dose-dependent manner, which indicated suppression of the activity of osteoblast [34]. Moreover, FNDC5 knockout mice displayed suppressed RANKL activity, which was correlated with the reduction of bone resorption [34]. According to the consideration by Kim et al., irisin could be a molecule similar to parathyroid home which could exert both beneficial and harmful effects through mechanisms such as constant high level or intermittent pulse levels on bone health. Taken together, we found that there was a positive association between lumbar BMD and serum irisin level independently of baseline comorbidities, dialysis duration, female sex, and age. These findings highlight the potential role of irisin as a modulator and marker of bone health in patients on MHD. But the mechanism of irisin on bone health in MHD should be further studied to delineate the actual role of irisin as a mediator of negative feedback control or a marker of decreased muscle mass as well as impaired physical activities in this frail population.

One major limitation of the present study was the cross-sectional design and the inclusion of a limited number of patients on MHD. In addition, evidence showed daily activities would influence the serum levels of irisin but there were no available data on the daily activity of patients. Therefore, the causal relationship between irisin and BMD and the underlying mechanisms should be confirmed by future longitudinal studies.

In the present study, lumbar BMD was positively correlated with serum irisin level in addition to female sex and serum ALP level in patients on MHD. These findings indicate that irisin might serve as an endocrine signal in the pathogenesis of osteoporosis and might be considered as a novel therapeutic target to restore bone mass in osteoporosis caused by muscle disuse in certain chronic diseases. Further, our findings suggest that irisin should be considered as a useful biomarker for osteoporosis in patients on MHD, which requires further confirmation in specifically designed studies.

Data Availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Disclosure

This manuscript was presented in the 2019 World Congress of Nephrology (Melbourne, Australia) held from April 12 to 15, 2019. The funding source had no role in the conception and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation of the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Chia-Wen Lu and Bang-Gee Hsu conceived and designed the experiments; Chia-Wen Lu, Chih-Hsien Wang, Yu-Li Lin, Chiu-Huang Kuo, and Yu-Hsien Lai performed the experiments; Bang-Gee Hs and Jen-Pi Tsai analyzed the data; Chia-Wen Lu and Bang-Gee Hsu contributed to reagents; Chia-Wen Lu, Bang-Gee Hsu, and Jen-Pi Tsai wrote the paper. All authors have read and approved the manuscript. Bang-Gee Hsu and Jen-Pi Tsai contributed equally to this study.

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Research Article

DPP4 Activities Are Associated with Osteopenia/Osteoporosis and Fracture Risk in Newly Diagnosed Type 2 Diabetes

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Background. Recent studies have shown the beneficial effect of dipeptidyl peptidase-4 (DPP4) inhibitor on bone turnover in diabetes mellitus. However, little clinical evidence for DPP4 activity in newly diagnosed type 2 diabetes is available. This study was designed to investigate the relationship between plasma DPP4 activity and osteoporosis/osteopenia and fracture risk in newly diagnosed type 2 diabetes. *Methods*. A total of 147 subjects with newly diagnosed type 2 diabetes were enrolled for this cross-sectional study. The bone mineral density (BMD) at the lumbar spine (L1-4) and femoral neck (FN) was measured by dual-energy X-ray absorptiometry (DXA). The 10-year probability of major osteoporotic fracture (MOF) and hip fracture (HF) was assessed by a modified fracture risk algorithm (FRAX) tool. The plasma DPP4 activity and clinical variables were measured. Correlation analyses between DPP4 activity and osteoporosis/osteopenia and fracture risk were performed. *Results*. Elevated plasma DPP activities were significantly associated with a higher proportion of osteoporosis/osteopenia (50% for quartile-1, 56.4% for quartile-2, 65.8% for quartile-3 and 72.2% for quartile-4). With increasing plasma DPP activities, the incidence rate of osteoporosis/osteopenia is gradually increasing (P for the trend between quartiles = 0.04). Of note, a statistically significant linear correlation was found between plasma DPP4 activities and modified FRAX MOF ($r = 0.20$, $P = 0.02$). Moreover, plasma DPP4 activities were also positively related to modified FRAX HF in newly diagnosed type 2 diabetic patients ($r = 0.21$, $P = 0.01$). *Conclusions*. Elevated plasma DPP4 activity tended to be associated with a higher proportion of osteoporosis/osteopenia and increased the fracture risk in newly diagnosed type 2 diabetes.

1. Background

The prevalence of diabetes mellitus is increasing worldwide with diabetes-related complications, imposing a tremendous burden on all health-care systems [1]. Of note, fragility fractures are increasingly recognized as an important complication of diabetes mellitus and are associated with substantial morbidity and mortality [2]. Diabetic osteopathy may experience various musculoskeletal disorders, such as osteoporosis, osteopenia, and diabetic foot syndrome, which is an underlying condition characterized by micro-architectural changes that can reduce bone quality and increase the risk of bone fractures [3]. Osteoporosis/osteopenia is the most common metabolic disorder in the bone characterized by decreasing the density of normally mineralized bone. Although evidence from both the bench and the bedside has shown a strong interaction between glucose homeostasis and bone metabolism, the mechanisms underlying the detrimental effects of diabetes on skeletal health remain not clearly defined [4].

Poor diabetes management can have adverse consequences such as heart disease, renal failure, osteoporosis, and even death, which are potentially preventable by optimal metabolic control. Antidiabetic drugs are very important for glycemic control in all diabetic patients; however, they may increase osteoporosis and fracture risk in diabetes mellitus [5]. In contrast, it has been reported that recently marketed antidiabetic drugs including incretins and dipeptidyl peptidase-4 (DPP4) inhibitors can potentially improve bone quality [6]. DPP4, also known as CD26, is a crucial factor in

the regulation of insulin secretion and glucose homeostasis. Notably, there is growing evidence that DPP4 may have an important role in bone formation, bone resorption, and bone microstructure [6, 7]. The recent meta-analysis of randomized clinical trials also suggests that treatment with DPP4 inhibitors could be associated with a reduced risk of bone fractures in type 2 diabetes [7]. Besides, previous studies have shown that the beneficial effect of glucagon-like peptide-1 has an in vivo half-life of only a couple of minutes because of rapid inactivation by DPP4, on the bone formation or resorption [8]. Preliminary data on animals and preclinical studies suggest the hypothesis that DPP4 inhibitors could have a positive effect on bone metabolism by a direct effect on bone cells; however, clinical studies are needed to elucidate the association of DPP4 activity with bone metabolism [9].

The fractures risk algorithm (FRAX) is an online tool widely used by many professional institutions for fracture risk assessment [10]. There is evidence showing that diabetes is recommended to replace rheumatoid arthritis in FRAX to effectively improve FRAX performance in diabetic patients [11–13]. The modified FRAX employs clinical risk factors such as age, body mass index (BMI), previous fractures, and other factors combined with femoral neck (FN) bone mineral density (BMD) determined by dual X-ray absorptiometry (DXA) to estimate the 10-year probability of major osteoporotic fracture (MOF) and hip fracture (HF) [13].

Despite knowledge of the importance of DPP4, little is known about the relation between circulating DPP4 activities and diabetic osteopathy in newly diagnosed type 2 diabetic patients. Accordingly, we set out to explore the associations of plasma DPP4 activities with osteoporosis/osteopenia and the ten-year probability of major osteoporotic fracture (MOF) and hip fracture (HF) estimated with modified FRAX in new onset type 2 diabetic patients.

2. Materials and Methods

2.1. Study Population. To explore the association between plasma DPP4 activities and osteoporosis/osteopenia and the ten-year probability of major osteoporotic fracture (MOF) and hip fracture (HF) estimated with modified FRAX, 158 newly diagnosed type 2 diabetic patients were enrolled from subjects undergoing routine health checkup at Shengjing Hospital of China Medical University (Shenyang, China) from December 2017 to May 2019. All of the enrolled subjects underwent the oral glucose tolerance test (OGTT). Type 2 diabetes was diagnosed based on the American Diabetes Association guideline [14]. They had no history of taking medications such as blood pressure medications and lipid-lowering medications. Participants with gestational diabetes, cerebrovascular diseases, chronic renal diseases, and hepatic diseases were excluded from this study. None of the participants received insulin therapy or antidiabetic medication, and therefore, their blood glucose was not affected. Exclusion criteria also included the use of agents that may affect bone metabolisms, such as thiazolidinediones, vitamin K, warfarin, vitamin D, calcium supplement, bisphosphonates, and estrogen, and agents that may lower lipid levels. In this study, type 1 diabetic patients were carefully excluded from clinical grounds, based on fasting C-peptide levels and islet-associated negative autoantibodies, and from a review of medical records, All participants had signed informed written consent prior to participating in the study. The institutional review board of Shengjing Hospital has approved the present study, and all procedures were carried out following the principles expressed in the Declaration of Helsinki.

2.2. Collection and Definition of Clinical Variables. The medical information was collected based on the medical records. BMI was calculated as weight in kilograms divided by height in meters squared. To avoid potential confounding effects, samples of venous blood were drawn after an overnight fast. Clinical biochemical variables were determined at the Department of Medical and Chemical Laboratory Diagnostics of Shengjing Hospital according to routine procedures. DPP4 activity in plasma was assayed as previously reported [15]. Briefly, plasma DPP4 activity was determined as the rate of cleavage of 7-amino-4-methylcoumarin (AMC) from the synthetic substrate H-glycyl-prolyl-AMC (H-Gly-Pro-AMC; Biovision, San Francisco, California, USA). It is expressed as the amount of cleaved AMC per minute per ml (nmol/min/ml). The insulin resistance was evaluated by homeostasis model assessment of insulin resistance (HOMA-IR), and the beta cell function was evaluated by homeostasis model assessment of insulin secretion (HOMA-IS) as previously reported [16].

2.3. BMD Measurement and Fracture Risk Assessment. The areal bone mineral density (BMD) (g/cm^2) of all participants was measured at the lumbar spine (L1–L4) and femoral neck (FN) by dual-energy X-ray absorptiometry (Lunar Prodigy). Accordingly, osteoporosis is diagnosed by a T-score $\leqq -2.5$ SD and osteopenia is diagnosed by a $-1 \geqq T$-score > -2.5 SD at any of the sites on the lumbar spine or FN [17]. The 10-year probability of fractures was determined with the modified FRAX tool (https://www.sheffeld.ac.uk/FRAX/tool.aspx?country=2), with the following parameters: age, sex, weight, height, fracture history, parental history of hip fractures, glucocorticoid usage, RA (diabetes in the present study), smoking status, and alcohol intake [11–13]. A China-specific FRAX algorithm with FN-BMD was selected to evaluate the 10-year probability of major osteoporotic fracture (MOF) and hip fracture (HF) [13].

2.4. Statistical Analysis. Continuous variables are presented as means ± standard deviation (SD), median (25th and 75th percentiles). Categorical variables are presented as percentage. Normal distribution of continuous variables was determined using the one-sample Kolmogorov–Smirnov test. Continuous variables with a normal distribution were assessed by one-way ANOVA with post hoc Tukey's test. Nonnormally distributed data were tested for nonparametric distribution. Correlation analysis between continuous variables was performed by Spearman's analysis. Categorical

variables were examined by the chi-squared test. The Statistical Package for Social Science (SPSS) version 15.0 was applied to perform all statistical and association analyses. Two-tailed tests were adopted throughout, and P values less than 0.05 were considered.

3. Results

3.1. Clinical Features. A total of 147 newly diagnosed type 2 diabetic patients were evaluated. They were divided into three groups, normal bone mineral density group ($n = 57$), osteopenia group ($n = 64$), and osteoporosis group ($n = 26$), according to T-score by dual-energy X-ray absorptiometry. The baseline clinical characteristics of the three groups are depicted in Table 1. We observed significant differences in gender among these groups. The osteoporosis group had a significantly higher ratio of females than the osteopenia group and normal bone mineral density group (65.4% vs. 45.3% and 35.1%, respectively; $P = 0.02$). As expected, compared with the normal bone mineral density group and osteopenia group, the average age in the osteoporosis group was significantly increased (53.4 ± 3.2, 55.7 ± 4.1, and 57.6 ± 5.0, respectively; $P < 0.01$). Of note, plasma DPP4 activity tended to be marginally higher in the osteoporosis group compared with the osteopenia group and normal bone mineral density group (7.7 ± 0.8 vs. 7.5 ± 0.9 and 7.3 ± 0.8 nmol/min/ml, respectively; $P = 0.07$). There were no statistically significant differences in other clinical characteristics among these groups (all $P > 0.05$).

3.2. Correlations between DPP4 Activity and Osteoporosis/Osteopenia. To achieve an even distribution in each group, the subjects were divided into subgroups using DPP4 activity quartiles: Q1: <6.78 (nmol/min/ml); Q2: 6.78–7.39 (nmol/min/ml); Q3: 7.40–8.06 (nmol/min/ml); Q4: >8.06 (nmol/min/ml). Although there was a positive correlation between DPP4 activity and the prevalence of osteoporosis, the trends were not statistically significant ($P > 0.05$). However, higher DPP activities were significantly associated with a higher proportion of osteoporosis/osteopenia in newly diagnosed type 2 diabetic patients (50% for Q1, 56.4% for Q2, 65.8% for Q3, and 72.2% for Q4) (Table 2). The prevalence of osteoporosis/osteopenia showed an increasing trend with the increase in plasma DPP4 activity (P for the trend between quartiles = 0.04) (Figure 1).

3.3. Correlations among DPP4 Activity and 10-Year Probability of MOF and HF. Spearman correlation analysis was used to determine the relationship between plasma DPP4 activity and the 10-year probability of MOF and HF. We also studied correlations between DPP4 activity and clinical variables. As shown in Table 3, plasma DPP4 activities were marginally positively correlated with HbA1c in all subjects ($r = 0.17$, $P = 0.04$). In contrast, no significant correlation was found between plasma DPP4 activities and clinical parameters in newly diagnosed type 2 diabetic patients (all $P > 0.05$). Of note, a marginal linear correlation was found between plasma DPP4 activities and modified FRAX MOF ($r = 0.20$, $P = 0.02$) (Figure 2). Furthermore, plasma DPP4 activities were also positively related to modified FRAX HF in all subjects ($r = 0.21$, $P = 0.01$) (Figure 2).

4. Discussion

The present study cross-sectionally examined the relationship of plasma DPP4 activities to osteoporosis/osteopenia and fracture risk in newly diagnosed type 2 diabetes. Evidence exists in the literature that DPP4 has been identified as a novel protease playing crucial roles in the development of dyslipidemia, inflammation, and insulin resistance, all of which have been suggested to be involved in the pathogenesis of osteoporosis [18]. Our findings extend these observations by demonstrating that elevated plasma DPP4 activities were closely associated with a higher proportion of osteoporosis/osteopenia in newly diagnosed type 2 diabetic patients. Furthermore, our data for the first time indicate that plasma DPP4 activities were also positively related to the 10-year probability of major osteoporotic fracture and hip fracture estimated by modified FRAX in newly diagnosed type 2 diabetic patients.

The findings are consistent with previous data [19] and also indicate that plasma DPP4 activities associate positively with HbA1c. Chronic hyperglycemia may lead to the activation of DPP4, and long-term exposure to high glucose levels may lead to endothelial damage with a consequent increase in DPP4 secretion. In a prospective study from Italy, variations in DPP4 activity over 3 months in type 2 diabetic patients showed a significant positive correlation with variations in HbA1c [20]. Although its overall significance for the normal physiological regulation of glucose homeostasis in humans and its role in the pathogenesis of the metabolic disease remain to be established, it is evident that DPP4 has the potential to influence glycemic control [21].

Although obesity is being an important risk factor for type 2 diabetes and DPP4 being a protease, the association between circulating DPP4 activity and obesity remains debatable. However, no significant correlation was found between circulating DPP4 activity and BMI in our study. This is in line with results from a previous study, which found that adipose tissue-derived DPP4 does not significantly contribute to the active pool of plasma DPP4 activity [22]. It is noteworthy that plasma DPP4 enzyme activity was shown to be positively correlated with BMI in young healthy Japanese subjects [23]. The explanation for the existence of contradictory results lies largely in the dynamic plasma DPP4 activity from adolescence to adulthood [24].

The discovery of the incretins opens up a novel therapy in the treatment of diabetes. Incretins are gut-derived hormones that exert their actions through activation of incretin receptor signaling. In addition to its well-known glycemic control and cardioprotective effects [25, 26], it has also been identified as a novel protease playing crucial roles in bone metabolism [8]. DPP4 is a widely expressed multifunctional serine peptidase that exists as a membrane-anchored cell surface protein or in a soluble form in the plasma and degrades incretin hormones to inactive metabolites [21]. Bone cells, including osteoblasts and

Table 1: Comparison of baseline characteristics by bone status across cohorts with newly diagnosed type 2 diabetes.

Variables	Normal ($n = 57$)	Osteopenia ($n = 64$)	Osteoporosis ($n = 26$)	P value
Female, n (%)	20 (35.1)	29 (45.3)	17 (65.4)	0.02
Smoking, n (%)	20 (35.1)	27 (42.2)	7 (26.9)	0.38
Age (years)	53.4 ± 3.2	55.7 ± 4.1	57.6 ± 5.0	<0.01
BMI (kg/m^2)	25.3 ± 2.1	25.0 ± 1.7	24.9 ± 1.7	0.66
HbA1c (%)	6.5 ± 0.4	6.6 ± 0.4	6.6 ± 0.4	0.85
FBG (mmol/L)	8.2 ± 1.4	8.2 ± 1.4	8.1 ± 1.1	0.96
HOMA-IR	5.4 ± 1.6	5.4 ± 1.2	5.4 ± 1.1	0.98
HOMA-IS	69.2 ± 25.0	71.8 ± 30.7	71.7 ± 23.3	0.86
TG (mmol/L)	1.6 ± 0.7	1.7 ± 1.0	1.6 ± 0.7	0.75
TC (mmol/L)	4.7 ± 1.2	5.0 ± 1.1	4.7 ± 1.1	0.42
LDL (mmol/L)	2.8 ± 0.7	2.8 ± 0.6	2.6 ± 0.7	0.51
HDL (mmol/L)	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.3	0.83
BUN (mmol/L)	5.1 ± 1.5	5.1 ± 1.4	5.4 ± 1.9	0.71
Cr (μmol/L)	73 ± 23	76 ± 20	70 ± 18	0.44
UA (μmol/L)	409 ± 80	409 ± 78	416 ± 85	0.90
DPP4 activity (nmol/min/ml)	7.3 ± 0.8	7.5 ± 0.9	7.7 ± 0.8	0.07

Data are presented as mean ± SD or percentages. BMI, body mass index; HbA1c: hemoglobin A1c; FBG: fasting blood glucose; HOMA-IR: homeostatic model assessment of insulin resistance; HOMA-IS: homeostasis model assessment of insulin secretion.

Table 2: Prevalence rate of osteoporosis/osteopenia according to plasma DPP4 activity quartiles.

	Q1 ($n = 34$)	Q2 ($n = 39$)	Q3 ($n = 38$)	Q4 ($n = 36$)	P_{trend}
DPP4 activity (nmol/min/ml)	<6.78	6.78–7.39	7.40–8.06	>8.06	—
Osteoporosis, n (%)	4 (11.8)	7 (17.9)	7 (18.4)	8 (22)	0.28
Osteoporosis/osteopenia, n (%)	17 (50.0)	22 (56.4)	25 (65.8)	26 (72.2)	0.04

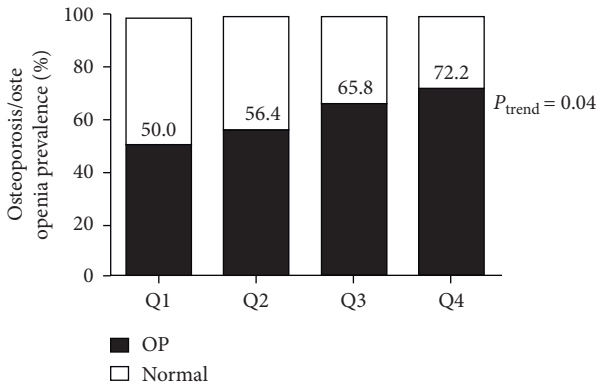

Figure 1: Prevalence rate of osteoporosis/osteopenia according to plasma DPP4 activity quartiles. Q1: <6.78 (nmol/min/ml), Q2: 6.78–7.39 (nmol/min/ml), Q3: 7.40–8.06 (nmol/min/ml), and Q4: >8.06 (nmol/min/ml). Normal, normal bone mineral density (T-score ≥ −1); OP, osteoporosis/osteopenia (T-score < −1) (linear-by-linear association for the trend test).

Table 3: Correlations of plasma DPP4 activity and modified FRAX and other clinical parameters in newly diagnosed type 2 diabetes.

Variables	r	P
Age (years)	0.14	0.08
BMI (kg/m^2)	−0.06	0.45
HbA1c (%)	0.17	0.04
FBG (mmol/L)	0.08	0.35
HOMA-IR	0.15	0.08
HOMA-IS	0.03	0.70
BUN (mmol/L)	0.05	0.56
Cr (μmol/L)	−0.03	0.74
UA (μmol/L)	0.03	0.70
MOF (%)	0.20	0.02
HF (%)	0.21	0.01

BMI, body mass index; HbA1c: hemoglobin A1c; FBG: fasting blood glucose; HOMA-IR: homeostatic model assessment of insulin resistance; HOMA-IS: homeostasis model assessment of insulin secretion; MOF: the 10-year probability of major osteoporotic fracture; HF: the 10-year probability of hip fracture; FRAX: fracture risk algorithm.

osteoclasts, have been shown to express receptors for incretins. Many studies indicate that glucagon-like peptide (GLP) can act as an antiresorptive and anabolic hormone [27]. Furthermore, the GLP-1 receptor is essential for the control of bone resorption as mice deficient in GLP-1 receptor present with cortical porosity as a result of increased osteoclastic bone resorption activity [5]. These experimental studies indicate that incretins have a beneficial effect on bone mass and protective effects on bone quality. As the pharmacological effect of DPP4 inhibitors is to prolong the action of GLP-1, their effect on bone is assumed to be similar to that of GLP-1. Thus, the DPP4 inhibitor seems to have an anabolic effect on bone, attenuating bone loss and potentially reducing fracture risk in type 2 diabetic patients. However, clinical data on the association between DPP4 and human bone are limited.

The present study showed for the first time that high plasma DPP4 activity was associated with the prevalence of osteoporosis/osteopenia in newly diagnosed type 2 diabetic patients (P_{trend} = 0.04). Similarly, the previous study showed a positive correlation between plasma DPP4 activity and

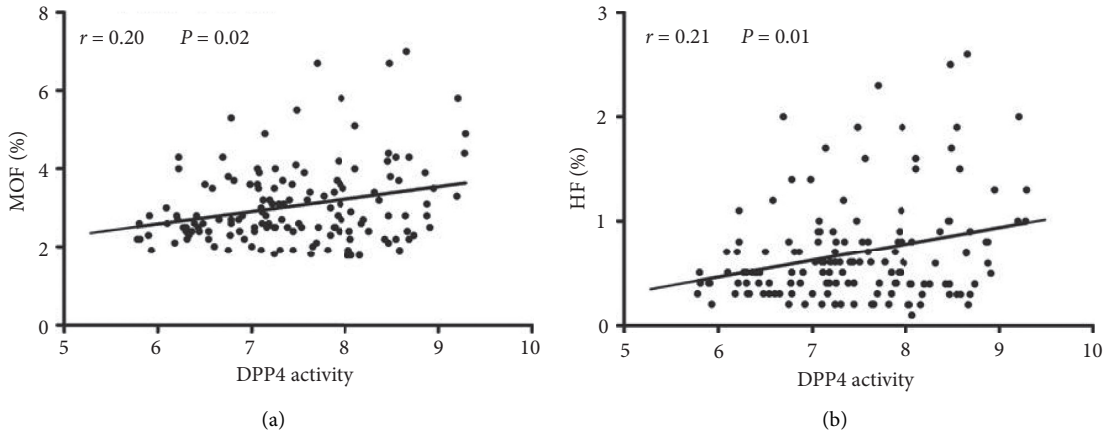

Figure 2: Correlations of plasma DPP4 activity with the 10-year probability of MOF (a) and HF (b) in all participants (calculated by Spearman's correlation analysis).

osteoporosis in postmenopausal women with normal glucose tolerance [18]. Furthermore, our results revealed that plasma DPP4 activity was also positively related to fracture risk determined with modified FRAX in newly diagnosed type 2 diabetic patients. In line with this, the previous meta-analysis suggests that treatment with DPP4 inhibitors could be associated with a reduced risk of bone fractures [7].

Accumulating clinical evidence has demonstrated that plasma DPP4 activity is significantly increased in human subjects with polycystic ovary syndrome and metabolic syndrome [28]. Moreover, plasma DPP4 activity is reported to increase in individuals with type 2 diabetes and associate with signs of endothelial dysfunction such as impaired flow-mediated dilatation [29]. The previous study also showed that excessive activity of plasma DPP4 is independently associated with subclinical left ventricular systolic and/or diastolic dysfunction in type 2 diabetes [30]. Interestingly, there is evidence showing that increased DPP4 activity is associated with a high risk of mild cognitive impairment in elderly type 2 diabetes [31]. These evidences indicate that DPP4 has become an important molecule associated with a variety of diseases. At present, the underlying molecular mechanism how elevated plasma DPP4 activity is involved in diabetic bone and fractures in new onset type 2 diabetic patients has not been clear yet. Therefore, future studies should be performed to elucidate the function of DPP4 in the pathogenesis of bone metabolism in diabetes mellitus.

Several limitations of this study should also be considered. The incidence of osteoporosis/osteopenia and bone fractures is closely related to the hormones in postmenopausal women. It was not to discriminate between genders and between premenopausal and postmenopausal women in the present study. Furthermore, several confounders, such as daily dietary calcium intake and consumption of vitamin D, were difficult to be obtained in this epidemiological study. Osteoporosis/osteopenia was also found to be significantly associated with body mass index. Of note, most of the subjects were lean mass in this study. Finally, the present study fails to address the precise role of DPP4 in the pathogenesis of osteoporosis/osteopenia which is needed to be elucidated by the future investigation.

5. Conclusion

The present study revealed that elevated plasma DPP4 activity tended to be significantly associated with osteoporosis/osteopenia and the fracture risk in newly diagnosed type 2 diabetic patients. Even though the biological mechanism has not been clear yet, the current findings provide a clue that elevated plasma DPP4 activity could suggest osteoporosis/osteopenia risk and future fracture risk in new onset type 2 diabetes.

Data Availability

The datasets generated for this study are available upon request to the corresponding author.

Ethical Approval

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Shengjing Hospital.

Consent

Written informed consent was obtained from individual or guardian participants.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

All authors helped to write the report and commented on the manuscript. Q. M. researched data, contributed to the discussion, and wrote the manuscript. Z. S. researched data and contributed to the discussion. L. D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Review Article

Androgen Effects on the Adrenergic System of the Vascular, Airway, and Cardiac Myocytes and Their Relevance in Pathological Processes

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Introduction. Androgen signaling comprises nongenomic and genomic pathways. Nongenomic actions are not related to the binding of the androgen receptor (AR) and occur rapidly. The genomic effects implicate the binding to a cytosolic AR, leading to protein synthesis. Both events are independent of each other. Genomic effects have been associated with different pathologies such as vascular ischemia, hypertension, asthma, and cardiovascular diseases. Catecholamines play a crucial role in regulating vascular smooth muscle (VSM), airway smooth muscle (ASM), and cardiac muscle (CM) function and tone. *Objective*. The aim of this review is an updated analysis of the role of androgens in the adrenergic system of vascular, airway, and cardiac myocytes. *Body*. Testosterone (T) favors vasoconstriction, and its concentration fluctuation during life stages can affect the vascular tone and might contribute to the development of hypertension. In the VSM, T increases α1-adrenergic receptors (α_1-ARs) and decreases adenylyl cyclase expression, favoring high blood pressure and hypertension. Androgens have also been associated with asthma. During puberty, girls are more susceptible to present asthma symptoms than boys because of the increment in the plasmatic concentrations of T in young men. In the ASM, β_2-ARs are responsible for the bronchodilator effect, and T augments the expression of β_2-ARs evoking an increase in the relaxing response to salbutamol. The levels of T are also associated with an increment in atherosclerosis and cardiovascular risk. In the CM, activation of α_{1A}-ARs and β_2-ARs increases the ionotropic activity, leading to the development of contraction, and T upregulates the expression of both receptors and improves the myocardial performance. *Conclusions*. Androgens play an essential role in the adrenergic system of vascular, airway, and cardiac myocytes, favoring either a state of health or disease. While the use of androgens as a therapeutic tool for treating asthma symptoms or heart disease is proposed, the vascular system is warmly affected.

1. Introduction

1.1. Metabolic Pathways of Steroids. Testosterone (T), the main testicular hormone, is produced by Leydig cells in high concentrations (95%). However, smaller amounts of T are also synthesized by the adrenal cortex [1–4]. The production and secretion of this androgen are regulated through luteinizing hormone (LH) stimulation. Cholesterol is the precursor of T, and the steroidogenesis is carried out through cytochrome P450 enzymes [5]. The conversion of cholesterol to pregnenolone is the first step in producing T and is accomplished by the P450 side-chain cleavage enzyme (P450cc/CYP11A1) [4, 5]. Subsequently, this progestogen is biotransformed either to 17α-hydroxypregnenolone or to progesterone via P450 17α-hydroxylase (P450c17/CYP17A1) and 3β-hydroxysteroid dehydrogenase type 2 (3β-HSD2), respectively. Afterward, 17α-hydroxypregnenolone is converted to dehydroepiandrosterone (DHEA) by cytochrome P450c17/CYP17A1 [5–7]. The conversion of DHEA to androstenedione via 3β-HSD2 or to androstenediol via 17β-hydroxysteroid dehydrogenase (17β-HSD3) is followed by the biotransformation to T by 17β-HSD3 or 3β-HSD2, respectively [5]. Furthermore, T is either reduced to 5α-dihydrotestosterone (5α-DHT) by 5α-reductase or to

5β-dihydrotestosterone (5β-DHT) by 5β-reductase [8–10]. Additionally, T can be converted to 17β-estradiol (E2) via the aromatase (P450aro/CYP19A1) action, and 17β-HSD3 catalyzes the formation of E2 from estrone (Figure 1) [5].

In women, T is produced and secreted by the ovarian stroma, particularly by theca and granulosa cells (25%), the adrenal zona fasciculata (25%), and from circulating androstenedione (50%) [11, 12]. Peripheral tissues such as placenta, liver, skin, prostate, and adipose tissue possess the specific enzymes (or the isoforms) required for the novo synthesis of androgens or their activation from circulating precursors [13]. Furthermore, in the vascular smooth muscle (VSM), airway smooth muscle (ASM), and heart (the tissues that this review is focused on), the expression of some steroidogenic enzymes has been demonstrated. For instance, CYP11A1 and 3β-HSD are expressed in cardiac [14, 15], vascular [15, 16], and lung tissue [17]. Nevertheless, CYP17A1, which is required for the conversion of pregnenolone into 17-hydroxypregnenolone, was not found in the heart [14, 15], and it has not been reported in vascular or ASM. Therefore, de novo androgen biosynthesis is unlikely to occur in those tissues. However, the expression of 17β-HSD5 in the fetal lung [18, 19] and 17β-HSD1,2 in the heart [20] can lead to the biotransformation of pre-existing precursors to T. Interestingly, no significant expression of 17β-HSD3 was found in the heart and the lung since this enzyme is considered to be testis-specific [21]. Furthermore, the presence of 5α-reductase in the cardiac tissue allows the formation of 5α-DHT [20]. Additionally, P450aro has been found in vascular tissues [22, 23], heart [20], and lung epithelial cells [24].

Men usually have much higher levels of T serum concentrations than women. In men from 13 to 80 years old, values of serum T are between 6 and 50 nM [25–27]. 5α-DHT (a more potent androgen) represents about 9-10% of the plasma T levels in males of most species [26, 28]. In women, stable serum values of T (0.7–2.5 nM) are maintained except during pregnancy when T concentrations increment (3.5–5 nM) [27]. Also, 5α-DHT is essentially produced in peripheral tissues and circulates in very low concentrations in women plasma (0.069 nM) [29].

1.2. Nongenomic and Genomic Actions of Androgens. The androgen signaling comprises nongenomic and genomic pathways. The nongenomic effects of androgens are independent of the binding to the cytosolic AR and occur in seconds to minutes [30]. Importantly, these effects are not altered by inhibitors of transcription and seem to be carried out by the androgen binding to plasma membrane lipids or ionic channels [2, 31–35]. Recently, two distinct membrane proteins have been suggested as membrane androgen receptors (mARs): G protein-coupled receptor family C group 6-member A (GPRC6A) and zinc-regulated transporter [Zrt]-protein 9 (ZIP9); both of them may stimulate intracellular pathways via G proteins or mitogen-activated protein kinases (MAPKs) [31, 36–38].

GPRC6A is a member of the C family of G protein-coupled receptors (GPCRs) activated by several ligands such as extracellular Ca^{2+}, cations, basic amino acids, osteocalcin, and T [31, 39–41]. Pi et al. in 2010 showed that the stimulation of GPRC6A triggers the inhibitory G protein α-subunit (Gαi), phosphatidylinositol 3-kinase (PI3K), protein kinase C (PKC), proto-oncogene c-Src kinase (Src), and Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathways [42]. Most recently, the same authors reported that the activation of GPRC6A by testosterone induces cell proliferation and inhibits autophagy through the mammalian target of the rapamycin complex 1 (mTORC1) signaling cascade in prostate cancer cells [43]. ZIP9 is a protein that possesses seven membrane-spanning domains and was first identified as a member of the SLC39A zinc transporter family in Atlantic croaker ovaries [44]. The stimulation of ZIP9 leads to the activation of the Gq protein α-subunit (Gq11) in spermatogenic cells, the stimulatory G protein α-subunit (Gαs) in ovarian follicle cells, and the inhibitory G protein α-subunit (Gαi) in prostate cancer cells [36, 37, 44]. Moreover, the activity of ZIP9 (dependent on **T** stimulation) has also been explored in a Sertoli cell line, where this receptor modulates the phosphorylation of ERK1/2 [45]. While the MAPKs signaling pathway can lead to transcription modulation [46], the role of the mARs in the physiology of cardiac and smooth muscle cells is still unrevealed.

The genomic effects of T occur from hours to days and involve the binding of the androgen to a cytosolic androgen receptor (AR). This hormone receptor, also known as NR3C4, is a member of the nuclear receptor family [47, 48]. As in other nuclear receptors, the protein structure of the AR comprises the *N*-terminal domain (NTD), the DNA-binding domain (DBD), the hinge domain (HD), and the ligand-binding domain (LBD) [49]. The stimulation of the AR by T or 5α-DHT elicits the dissociation of chaperone proteins and the formation of a complex that is transferred to the nucleus where it modulates gene transcription and protein synthesis [2]. 5β-DHT, the other reduced metabolite of T, possesses minor androgenic activity due to a lower binding affinity than 5α-DHT [50]. The AR is expressed in several mammalian tissues, including vascular and airway smooth muscles and cardiac myocytes [2, 51–56]. Furthermore, the activity of the AR has been implicated in cardiovascular and respiratory ailments such as vascular ischemia [53], hypertension [57, 58], asthma [52], and cardiac hypertrophy [54].

In the last years, numerous AR splice variants have been molecularly identified and characterized in humans. Although the function of these alternative AR transcripts in the human physiology is not completely understood, these variants have been related to pathological conditions such as prostate cancer (PCa) and androgen insensitivity syndrome (AIS) [59–62]. In 2005, Ahrens-Fath et al. reported the existence of an NTD-truncated AR isoform with a molecular weight of 45 kDa (AR45) in the heart, skeletal muscle, uterus, prostate, breast, and lung [63]. However, the expression level of AR45 compared with the wild-type AR in these tissues is arguable since a semiquantitative RT-PCR was performed by Ahrens-Fath et al. Also, this receptor variant is expressed in

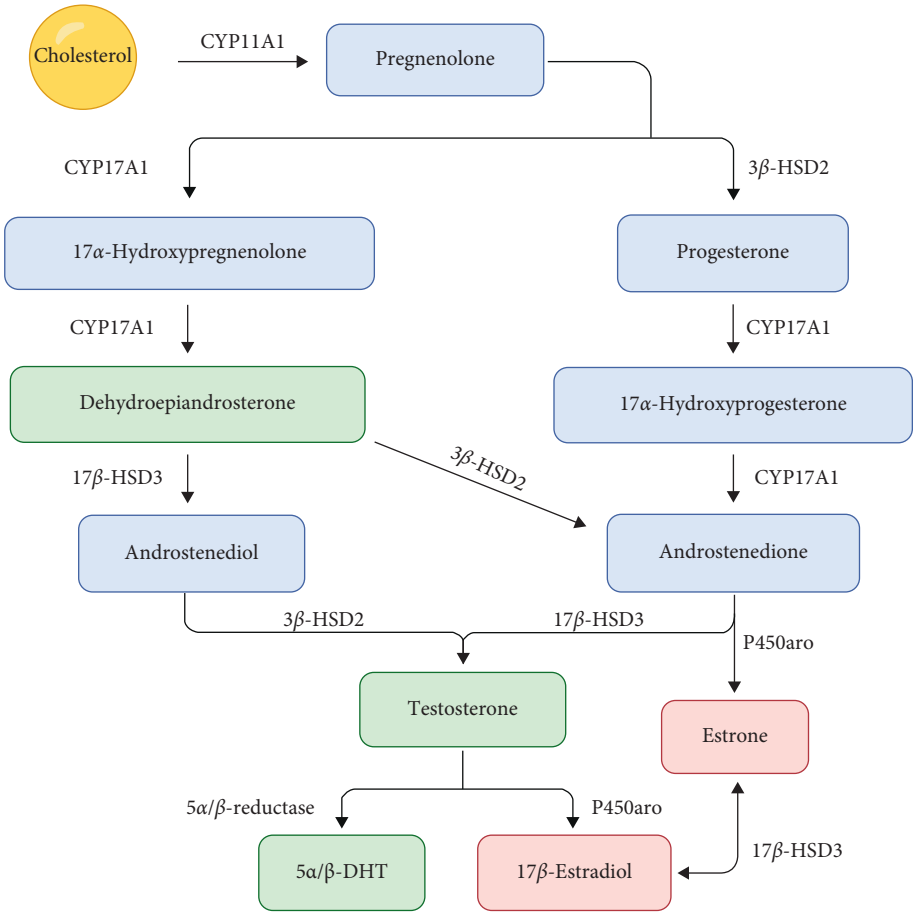

Figure 1: Androgen synthesis from cholesterol. Steroidogenesis in males is carried out mainly by Leydig cells and in females by theca and granulosa cells. Cholesterol is the precursor of all sex steroids, and its conversion to pregnenolone is mediated by the cholesterol side-chain cleavage cytochrome P450 enzyme (CYP11A1/P450scc). Once formed, this progestogen is converted into progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD2). Then, 17α-hydroxylase/17,20 lyase (CYP17A1/P450c17) hydroxylates pregnenolone to produce 17α-hydroxypregnenolone and subsequently removes the acetyl group to form dehydroepiandrosterone (DHEA). This last product can be either converted into androstenedione via 3β-HSD2 or into androstenediol by 17β-hydroxysteroid dehydrogenase (17β-HSD3). Androstenedione and androstenediol are further biotransformed to testosterone by 17β-HSD3 and 3β-HSD2, respectively. Furthermore, testosterone can be reduced to 5α- or 5β-dihydrotestosterone (5-α/β-DHT) by 5-α/β-reductases. Furthermore, P450 aromatase (P450aro) may convert testosterone into 17β-estradiol and androstenedione into estrone. Finally, 17β-HSD3 catalyzes the formation of 17β-estradiol from estrone.

the normal prostate tissue and in human prostate adenocarcinoma derived from the left supraclavicular lymph node metastasis (LNCaP) cells [64, 65]. Additionally, it has been shown that AR45 may repress or stimulate wild-type AR activity [63]. Interestingly, 12 AR variants lacking the LBD (ARV1-12) have been identified in PCa cell lines [64–67]. Among all the ARV isoforms, ARV7 (also known as AR3) has gained relevance due to its demonstrated capability of mediating constitutively AR functions, i.e., constitutive gene transcription in the absence of androgen stimuli. Moreover, ARV7 has been suggested as a predictive biomarker in castrate-resistant PCa since it promotes cancer progression and androgen depletion-resistant growth by regulating serine/threonine kinase 1 encoding gene (AKT1) [64–67]. In spite of the emerging evidence about AR splice variants, further studies are imperative in order to elucidate the possible expression and the physiological role of these alternative transcripts in vascular and airway smooth muscles and cardiac muscle.

Noteworthy, it has been proposed that androgen nongenomic and genomic actions may converge. For instance, in the vascular smooth muscle, the regulation of K^+ channels is dependent on nongenomic and genomic effects of androgens [30]; however, cellular mechanisms and signaling pathways displayed in both types of actions are entirely different and carried out by distinct effector proteins.

1.3. Androgens and Vascular, Airway, and Cardiac Muscles. Vascular smooth muscle (VSM), airway smooth muscle (ASM), and cardiac muscle (CM) cells are excitable entities, with the primary function of contracting and relaxing [68]. Several research groups have shown that androgens interact with the contraction and relaxation mechanisms of different

muscular cell types from distinct species through nongenomic and genomic effects.

With respect to nongenomic actions, in the VSM, numerous authors have reported that androgens induced vasorelaxation in different arteries [69–74]. In this regard, in the ASM, our group and others have observed that DHEA, T, 5α-DHT, and 5β-DHT induced-relaxation through nongenomic actions [33, 75–78].

In relation to the genomic actions, it has been reported that T and DHT induced in the VSM, the genic expression of proteins such as adenylyl cyclase (AC), Ca^{2+}-activated K^+ channels of high conductance (BK_{Ca}), and L-type voltage-dependent Ca^{2+} channels (L-VDCCs) [73, 79]. Most recently, we found in the ASM that T augmented the expression of β_2-ARs, favoring an increase in the relaxing response to salbutamol [51]. In the CM, it has been described that androgens (via a genomic effect) increased the expression of the voltage-dependent delayed rectifier K^+ channel 1.5 ($K_V1.5$), leading to shortening of the action potential duration in mice ventricular cardiomyocytes [80], and also enhanced the expression of $K_V1.7$ diminishing the QT intervals in rats [81]. Testosterone nongenomic and genomic actions and their association with the adrenergic system of vascular, airway, and cardiac myocytes are discussed in the next sections of this manuscript.

1.4. Adrenergic Receptors in Vascular, Airway, and Cardiac Muscles. Under physiological conditions, the adrenergic system plays a critical role in regulating vascular, airway, and cardiac function. In the VSM and CM, sympathetic innervation modulates contraction [82] and the intrinsic conduction system [83, 84], respectively. The ASM tone is partly regulated through circulating catecholamines such as epinephrine released from the adrenal medulla [85, 86]; this hormone acts as an adrenergic receptor agonist. The adrenergic receptors or adrenoceptors are members of the superfamily of G protein-coupled receptors (GPCRs) and modulate several pathways through effectors such as AC or phospholipase C (PLC) [87]. Adrenergic receptors have been classified into three major categories: alpha-1-adrenergic receptors (α_1-ARs), alpha-2-adrenergic receptors (α_2-ARs), and beta-adrenergic receptors (β-ARs). Moreover, each of these groups has been further subclassified into multiple subtypes defined by the differences in their genetic sequences and their pharmacological action: α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, and β_3 [88, 89].

α_1-ARs are coupled to a heterotrimeric Gq protein and PLC signaling pathway. PLC triggers the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), resulting in the increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and the activation of protein kinase C (PKC) [87, 90–92]. Also, the stimulation of α_1-ARs promotes an extracellular Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCCs) [93] and triggers extracellular signal-regulated kinases 1 and 2 (ERK1/2) [94, 95]. In humans, α_{1A}, α_{1B}, and α_{1D} adrenergic receptors are encoded by distinct genes located on chromosomes 8, 5, and 10, respectively [87]. The three subtypes of α_1-ARs are present in most blood vessels modulating smooth muscle contraction and vascular tone. α_{1A} is the most prevalent subtype in human arteries; nevertheless, the expression levels of α_1-ARs depend on the vascular bed studied. α_{1D}-AR subtype predominates in large conduction vessels as the aorta and carotid arteries, whereas α_{1A}-AR subtype is involved in regulating vascular tone of mesenteric, splenic, pulmonary, and caudal (in mice and rats) arteries controlling organ blood flow [96–101]. While α_{1A} and α_{1D}-ARs are the main subtypes involved in vascular contractions, α_{1B}-AR subtype is also expressed in several blood vessels, and it was thought that it did not require extracellular Ca^{2+} to activate smooth muscle contraction [99, 100]. Unfortunately, studies related to α_{1B}-AR function in the VSM have been restrained by the lack of selective antagonists. However, this receptor subtype has been proposed to be involved in the regulation of systemic BP [102–104] and coronary blood flow [105].

The evidence of α-ARs in the ASM is also present; nonetheless, these receptors seem not to be relevant in the functionality of this tissue. In this context, norepinephrine-induced contraction has been observed in guinea pig [106, 107], rabbit, cat, and rat [107] tracheal preparations but only after β-AR blockade. Interestingly, Kneussl and Richardson in 1978 found that human and dog ASM did not contract in response to norepinephrine, unless they were previously stimulated with histamine or KCl [108]. These insights confirm the predominance of relaxant β-AR function in the ASM of most mammals. However, in 1985, Montaño et al. revealed that the *Erythrocebus patas* monkey possesses α-AR predominance in this tissue [109].

In cardiomyocytes of species such as mice, rats, and humans, all three α_1-AR mRNAs have been detected with the predominance of α_{1A}- and α_{1B}-AR subtypes [105, 110–112]. Although the stimulation of these receptors in cardiomyocytes can evoke muscle contraction, most works have been focused only on ventricular heart sections. In this context, it has been observed that norepinephrine and epinephrine can induce positive inotropic and chronotropic effects in the right atrium from mice, probably through α_1-AR signaling [113]. Nevertheless, different studies have shown that, in the heart, α_1-ARs are mainly involved in processes such as hypertrophic responses, upregulation of myosin light chain-2, modulation of the atrial natriuretic factor (ANF), and heart failure [114–120].

α_{2A}, α_{2B}, and α_{2C} adrenoceptor genes are located on human chromosomes 10, 4, and 2, respectively. The encoded products share about 50% of amino acid identity and show the same affinity for norepinephrine and epinephrine [121]. All α_2-ARs are coupled to the pertussis toxin-sensitive G proteins such as Gi/Go and to the inhibition of the AC. The consequence of the inhibition of this enzyme is a decrease in the production $3', 5'$-cyclic adenosine monophosphate (cAMP), reducing the activity of protein kinase A (PKA) [121–125]. Additionally, Gi/Go signaling cascade modulates Ca^{2+} [126, 127] and K^+ [128, 129] channels without the involvement of other second messengers. VSM cells express all subtypes of α_2-ARs, and their stimulation is related to contraction and vasopressor effects [124, 130–132]. α_{2A}-Adrenoceptor is the most predominant subtype in this tissue

and participates in the regulation of the muscular tone in the aorta [133] and carotid (possibly controlling cerebral blood flow) [134, 135] and mesenteric arteries [136] and in the peripheral vasoconstriction related to the skin blood flow [137]. α_{2B}-AR is more involved in the vascular tone of smaller arteries [138] but contributes to BP regulation to a greater extent than α_{2A}- and α_{2C}-ARs [139]. Furthermore, α_2-adrenoceptors seem to play a minor role in cardiac contractility compared to β- and α_1-ARs. Recently, it was demonstrated in ventricular cardiomyocytes that the stimulation of α_2-ARs could modify $[Ca^{2+}]_i$ and induce myocardial contraction [140, 141].

β-ARs, like all other adrenergic receptor subtypes, are composed of seven transmembrane spanning helices. The three subtypes (β_1, β_2, and β_3) are found in VSM [87], ASM [142], and CM [143] cells. Their coding genes are located in human chromosomes 10, 5, and 8, respectively [87, 144, 145]. The stimulation of the β-AR mediates the activation of AC and the subsequent increment in the production of cAMP [146, 147]. In its active state, β-AR is associated with the α-subunit of Gs protein. In the VSM, the activation of β-ARs induces the relaxation of the tissue, regulating the peripheral vascular resistance and controlling the organ blood flow and vascular tone [87]. Among all β-ARs, β_2-AR is the predominant expressed subtype in most vascular beds, while a minor proportion of β_1-ARs is also present. Apparently, β_1-adrenoceptors play an essential role in the function of coronary and cerebral arteries [87, 148–151]. β-Adrenoceptors also occur in endothelial cells where they mediate vasodilation through nitric oxide (NO) production [152]. In airways, β_2-agonists are well known as the most effective bronchodilators. The β_2-agonist binding to the β_2-AR in the cell membrane of the ASM triggers the formation of cAMP by the action of the AC [153–155]. Subsequently, the increment of the cAMP levels activates PKA, a phosphorylating protein, which favors K^+ channel opening and bronchorelaxation [156]. In the heart, β_1 and β_2 are the most valuable adrenoceptor subtypes with a predominance of β_1-ARs over β_2-ARs (ratio of ~80/20). The stimulation of these receptors in cardiomyocytes mediates positive chronotropic, inotropic, and lusitropic effects [157–159]. Gs-PKA signaling in cardiomyocytes promotes the phosphorylation of phospholamban (PLB), L-VDCCs, ryanodine receptors (RyRs), and cardiac myosin-binding protein C leading to an increase in $[Ca^{2+}]_i$ and favoring muscle contraction [160]. Interestingly, it has been demonstrated that a sustained activation of β_1-ARs may induce cardiotoxic effects, and β_2-ARs switch their natural Gs coupling to Gi protein coupling, opposing the positive β_1-AR effects [157, 161, 162].

It is well known that adrenoceptors play a key role in maintaining vascular, airway, and cardiac muscular function. In this regard, the modulation by T of the adrenergic receptor signaling pathway has been investigated, and the observed effects appear to be dependent on the studied tissue and the predominance of the adrenergic receptor subtypes either favoring muscle relaxation or contraction [51, 79, 163, 164]. This review focuses on the effects of T on the adrenergic system in the vascular, airway, and cardiac muscles and its relevance in pathological processes related to this system.

2. Vascular Smooth Muscle

The maintenance of the vascular tone is due to the balance between vasoconstriction and vasorelaxation modulated by several neurotransmitters and hormones [165]. The VSM found in the medial layer of the blood vessels is responsible for controlling vascular tone and blood pressure (BP) [166]. The regulation of the VSM membrane potential and the vascular tone is mainly determined by Ca^{2+} and K^+ channels [167, 168]. The main K^+ channels expressed in the VSM are the voltage-dependent delayed rectifier K^+ channels (K_V), Ca^{2+}-activated K^+ channels of high conductance (BK_{Ca}), ATP-sensitive K^+ channels (K_{ATP}), and inward-rectifier K^+ channels (K_{IR}) [169, 170]. VSM constriction is caused by increments in $[Ca^{2+}]_i$ [171]. Vasoconstrictor agonists act on GPCRs coupled to the $_q\alpha$ subunit (GPCR-$_q\alpha$) such as α_{1A}-, α_{1B}-, and α_{1D}-ARs, bradykinin, histamine H_1, and thromboxane-A_2 receptors, among others [172–174]. These receptors activate the PLC enzyme and IP_3 signaling pathway, inducing the release of Ca^{2+} from the sarcoplasmic reticulum (SR) and the influx of this ion through VDCCs [175]. In the VSM, two major subtypes of VDCCs with distinct electrophysiological properties are present. L-VDCCs are activated by large depolarizations and inactivated relatively slowly. T-type voltage-dependent Ca^{2+} channels (T-VDCCs) are activated by small depolarizations and inactivated rapidly [176, 177].

Moreover, VDCCs are not the only source of extracellular Ca^{2+}. The influx of this ion is also carried out by nonselective cation channels such as receptor-operated Ca^{2+} channels (ROCCs), store-operated Ca^{2+} channels (SOCCs), and transient receptor potential (TRP) channels. The Ca^{2+} influx exerted by these channels is thought to be triggered by agonists such as norepinephrine, vasopressin, and acetylcholine via GPCRs linked to the phospholipase C_β (PLC_β) signaling pathway and the formation of IP_3 and DAG. This last second messenger regulates the activity of ROCCs, and IP_3 induces depletion of internal Ca^{2+} stores leading to capacitative Ca^{2+} entry through SOCCs [178–181]. Additionally, TRP channels have been classified as ROCC subtypes, and transient receptor potential canonical channels 3, 6, and 7 (TRPC3, TRPC6, and TRPC7) have been shown to be susceptible to DAG stimulation promoting its opening and contributing to Ca^{2+} influx. Afterward, Ca^{2+} complexes with calmodulin to activate myosin light-chain kinase (MLCK) causing vasoconstriction [168, 182]. Conversely, the decrease in cytosolic Ca^{2+} leads to vasorelaxation [183]. Vasodilator agonists that stimulate GPCRs coupled to the $_s\alpha$ subunit (GPCR-$_s\alpha$) such as β_1- and β_2-ARs, histamine H_2, prostaglandin E_2, and adenosine A_2 receptors, among others [184], induce the synthesis of cAMP and $3', 5'$-cyclic guanosine monophosphate (cGMP); therefore, they activate PKA and protein kinase G (PKG), respectively [185], leading to a decrease in the vascular tone [183]. In the last two decades, the evidence about the relationship between androgens and vascular reactivity has increased. The

nongenomic effects of T in the VSM can be due to its action on ion channels resulting in vasorelaxation. In 1996, Perusquía et al. postulated that T, 5β-DHT, and 5α-DHT induced vasorelaxation in the rat aorta [186]. Later on, the same group observed that T was capable of blocking the extracellular Ca^{2+} influx inducing vasorelaxation of the precontracted human umbilical artery [72]. More recently, it was demonstrated that 5β-DHT and T induced vasorelaxation by blocking L-VDCCs in the rat thoracic aorta [70]. In addition to blocking Ca^{2+} entry through L-type Ca^{2+} channels, T is capable of activating K^+ channels. The efflux of K^+ evokes membrane hyperpolarization and closes Ca^{2+} channels leading to vasorelaxation in pig [187] and rabbit [188] coronary arteries. In this regard, different types of K^+ channels have been proposed as targets for T modulation. In the dog coronary artery [189] and rat aorta [190], K_{ATP} channels have been shown to be involved in the T-associated relaxant effect. BK_{Ca} channel activation in the human internal mammary artery [191] and pig coronary artery [187] is also implicated in T-induced vasorelaxation. Moreover, Saldanha et al. demonstrated that T produced relaxant responses in human umbilical artery rings precontracted with serotonin (5-HT), histamine, and KCl, and these effects were dependent on both BK_{Ca} and K_V channel activity (Figure 2(a)). They also studied the long-term effects of androgens in the same model, founding that DHT, through genomic actions, decreased the mRNA expression of the α-subunit of L-VDCC and upregulated the β_1-subunit of BK_{Ca}, favoring relaxation [73].

2.1. The Effects of Testosterone on Adrenergic Receptors in the Vascular Smooth Muscle. Sex differences in cardiovascular diseases, i.e., hypertension, have been broadly studied. Men are more likely to develop hypertension or coronary heart disease (CHD) than women [192–194]. Hypertension is defined as persistent systolic BP $\geq$ 140 mmHg and or diastolic BP $\geq$ 90 mmHg, according to 2018 ESC/ESH guidelines [195]. The World Health Organization has rated hypertension as one of the deadliest causes of premature death worldwide due to its asymptomatic behavior that can result in concomitant diseases after years. In this regard, sex differences in the development of hypertension have been reported. Female sex hormones, such as estrogens, have been widely implicated in the hypertension-related gender differences [57]; however, several authors have pointed out a prohypertensive role for androgens [58]. Studies in humans and castrated rats revealed that androgens exert a prohypertensive effect, while estrogens appear to oppose the increase in BP [196]. In this context, Torres et al. found in castrated male Wistar rats an increment in aortic vasodilation, indicating a sex hormone influence [197]. Another research group observed that gonadectomized hypertensive rats, both males and females, showed a reduced BP, and the administration of T restored it in the castrated male experimental group [198]. Moreover, it has been proposed that the effect of T on VSM does not benefit a state of relaxation but rather favors vasoconstriction. Fluctuations in androgen concentrations throughout life stages can affect the vascular tone, and T may contribute to developing hypertension [58]. In this sense, hyperandrogenism (HA) in pre- and postmenopausal women has been associated with an unfavorable metabolic profile, obesity, and hypertension [199–201]. HA is defined as an excess of androgen production and secretion by adrenal glands or the ovaries [202]. Moreover, the development of HA in females has shown to be associated with ovarian disorders, e.g., ovarian hyperthecosis (OH) [203], virilizing ovarian tumors (VOTs) [204], and polycystic ovary syndrome (PCOS) [199–201]. PCOS is one of the most common endocrine disorders affecting women of reproductive age [205]. The metabolic phenotype in PCOS is characterized by increased LH compared with the follicle-stimulating hormone (FSH) and HA [205, 206]. Furthermore, evidence points out that hyperandrogenemia in women suffering from PCOS is associated with an increased systolic and diastolic BP, and this relation is independent of other risk factors such as obesity and insulin resistance [207].

During aging, the vascular tone is led to vasoconstriction, and β-ARs have been proposed as targets of several drugs related to hypertension disease [208]. Aged animals have a weak vascular response to β-AR agonists, and possibly, mechanisms of the β-AR signaling pathway are altered [209]. Vascular tone is modulated through the action of the sympathetic nervous system (SNS) on β-ARs promoting the increase in cAMP levels [142, 210]. It has been reported that androgens promote vasoconstriction by increasing catecholamine (mainly norepinephrine) levels [57]. In 2005, Martin et al. demonstrated that the adrenergic system (through norepinephrine action) reduced the mean arterial pressure in castrated male spontaneously hypertensive rats (SHR) [211]. In other studies, vascular tone at different stages of rat growth was compared to explore the role of T in β-adrenergic-induced vasodilation [79]. In aortic rings of mature rats, vasorelaxation response induced by isoproterenol (a well-known unspecific β-adrenergic agonist) showed an impairment of this response compared to aortic rings obtained from younger rats. According to the authors, this impaired relaxing response could be related to higher plasma T-levels in older rats. The authors elegantly demonstrated that T reduced the β-AR-elicited vasorelaxation without any alteration in the expression of the β_2-AR but interfering downstream in the signaling cascade. Furthermore, the authors exhibited that T (via a genomic effect) diminished the expression of AC and yielding of cAMP in castrated rats [79]. These findings point out that changes in the levels of T could lead to high BP and hypertension.

Furthermore, the vessel tone is also regulated by α-ARs. These receptors promote vasoconstriction and might contribute to hypertension development [92]. In this context, the modulation of the α_1-AR by T has been reported. Testosterone replacement therapy increased BP in gonadectomized SHR and the number of α_1-ARs in the tail artery [164]. Furthermore, in 1999, it was found that the incubation for 24 hr with T (0.1 nM–1 μM) increased the abundance of α_{1B}-AR mRNA in VSM cells through a genomic action. The same study reported that glucocorticoids, such as dexamethasone, increased catecholamine-mediated

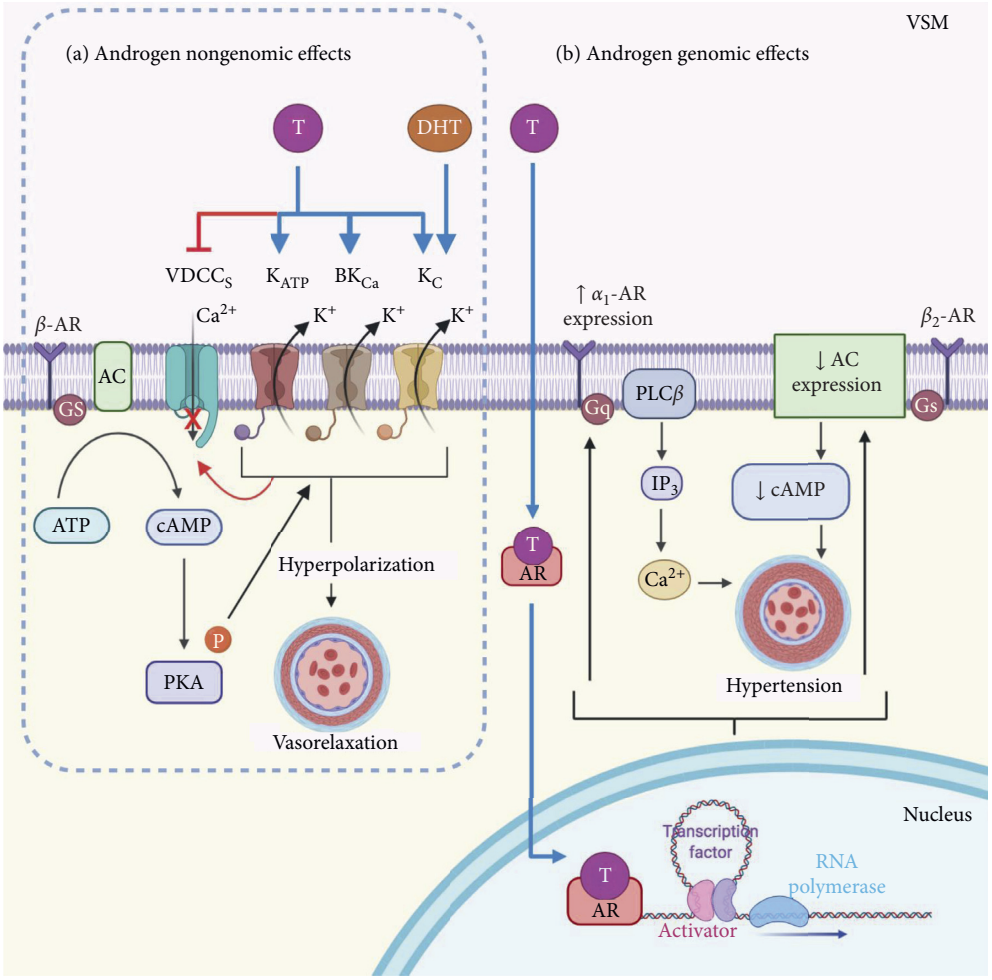

Figure 2: Androgen effects on the adrenergic system in the vascular smooth muscle (VSM). (a) Stimulation of the β-adrenergic receptor (β-AR) leads to an increase in the activity of the K^+ channels and to plasma membrane hyperpolarization. β-AR receptor is coupled to a Gs protein (Gs) that activates adenylyl cyclase (AC), which enhances the synthesis of 3′,5′-cyclic adenosine monophosphate (cAMP) and consequently promotes the protein kinase A- (PKA-) induced phosphorylation of the K^+ channels. K^+ channel phosphorylation increases their open probability and evokes membrane hyperpolarization that closes Ca^{2+} channels, leading to vasorelaxation. Testosterone, via a rapid response (nongenomic), activates ATP-sensitive K^+ channels (K_{ATP}), Ca^{2+}-activated K^+ channels of high conductance (BK_{Ca}), and voltage-dependent delayed rectifier K^+ channels (K_V). Dihydrotestosterone (DHT, a reduced metabolite of T) enhances the activity of the K_V channel. T also blocks VDCCs. Androgen-induced vasorelaxation mediated by the activation of K^+ channels and the blockade of VDCCs might improve the response of β-AR signaling. (b) The genomic androgen receptor (AR) signaling involves androgen crossing the plasma membrane, entering the cytoplasm, dissociation of chaperone proteins, and binding to its cytosolic receptor. AR stimulation by T results in a decrement of AC expression and a reduction of cAMP synthesis. Moreover, T increases the α_1-adrenergic receptor (α_1-AR) expression. This receptor is coupled to a Gq protein (Gq), which, through phospholipase C_β (PLC_β), catalyzes the formation of inositol-1, 4, 5-triphosphate (IP_3) and triggers intracellular calcium release from the sarcoplasmic reticulum (SR). The genomic effects of T favor vasoconstriction in the VSM and might lead to hypertension development.

vasoconstriction due to an increased α_{1B}-AR expression [212]. In this context, T is not the only steroid hormone related to vascular physiology. High concentrations of glucocorticoids (such as cortisol) promote the retention of sodium and decrease the activity of prostaglandins leading to a contracted state of the VSM [213].

Although the regulation through norepinephrine of the vessel tone is essential for both females and males, the existence of sex differences in vessel vasoconstriction and vasodilatation has been reported. In 2017, Al-Gburi et al. demonstrated that the α-adrenergic vasoconstriction was weaker in female than male rats. They also found that the stimulation of β_1-, β_2-, and β_3-ARs evoked a greater response of relaxation in females than in males [214]. The diminished vasoconstriction and the enhanced vasorelaxation were due to the upregulated expression of the β_1- and β_3-ARs mainly in an endothelial location in female rats. Later on, Riedel et al. confirmed the overexpression of the β_1- and β_3-ARs in endothelial cells of the blood vessel by the action of the estrogens. The endothelial adrenergic stimulation caused an enhanced NO-dependent vasorelaxation in female rats [215], counteracting the vasoconstrictive outcomes

modulated by the α-ARs [215]. These findings could explain very well the sex and age differences on the role of the adrenergic response in the VSM.

In conclusion, **T** reduces the β-AR-elicited vasorelaxation by interfering downstream in the signaling pathway and upregulates the α-AR expression (Figure 2(b)). These hormonal effects are carried out principally through genomic actions leading to vasoconstriction and might be involved in the development of hypertension. Nevertheless, androgen nongenomic actions have opposite outcomes in the VSM, yielding their effects to vasorelaxation. However, the genomic actions of androgens (long-term effects) seem to be the predominate deleterious effects favoring hypertension. Therefore, the possible use of androgens, due to their nongenomic actions, as a therapeutic tool for the treatment of hypertension could not be appropriated based on their long-term genomic actions.

3. Airway Smooth Muscle

The maintenance of proper air flux through the airways results from the balance between contraction and relaxation of the ASM. The response of the ASM to physiological and pathophysiological stimuli determines the airway caliber in order to regulate the airflow [216]. The basal tone of the ASM is maintained by the influx and efflux of Ca^{2+} across the cell membrane, keeping an intracellular basal Ca^{2+} concentration (${}_b[Ca^{2+}]_i$) around 100–150 nM [33, 77, 217–219]. The SR, ion channels, GPCRs, ATPases, and other mechanisms preserve ${}_b[Ca^{2+}]_i$ in the ASM cells. The mechanisms responsible for the Ca^{2+} influx are carried out by transient receptor potential canonical 3 (TRPC3), L-VDCCs and T-VDCCs, ROCCs, SOCCs, and reverse-mode Na^+/Ca^{2+} exchanger (NCX_{REV}) [77, 219]. Endogenous agonists such as acetylcholine, histamine, and leukotrienes act through the GPCRs-${}_q\alpha$ pathway. These receptors activate the PLC_β enzyme, which catalyzes the formation of DAG and IP_3, favoring SR Ca^{2+} release through the IP_3 receptor [220]. Increased Ca^{2+} in the cytosol promotes the release of more Ca^{2+} (Ca^{2+} sparks) through RyRs; this event is known as Ca^{2+}-induced Ca^{2+} release (CIRC) [221, 222]. Increase in $[Ca^{2+}]_i$ is restored by two ATPases: sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and plasma membrane Ca^{2+}-ATPase (PMCA) [223, 224]. Airway smooth muscle relaxation is predominantly mediated by the sympathetic system. Circulating epinephrine is more important in mediating relaxation in human airways than norepinephrine. In the ASM, β_2-AR is the main adrenoceptor subtype responsible for the bronchodilator effect [156]. Activation of this receptor triggers the formation of cAMP and, consequently, the activation of PKA [156]. PKA-mediated phosphorylation modulates proteins involved in the control of the airway muscle tone by regulating the Ca^{2+} availability and inactivating myosin light-chain kinase [225]. Furthermore, it is well known that the activation of the β_2-AR favors hyperpolarization and relaxation of the ASM through the opening of different K^+ channels [156, 226]. In the ASM, the main K^+ channels are the Ca^{2+}-activated K^+ channels (K_{Ca}) and K_V [227, 228]. K_{Ca} are activated by increases in $[Ca^{2+}]_i$ and through the cAMP-PKA signaling pathway [229, 230]. There are three subfamilies of K_{Ca}, all of them occurring in airways: high conductance (BK_{Ca}), intermediate conductance (IK_{Ca}), and low conductance (SK_{Ca}) [229]. Moreover, K_V have been characterized as $K_V1.2$, $K_V1.5$, and $K_V7.5$ in the ASM [227, 231]. Several agonists can lead to the bronchodilation of the ASM involving the opening of distinct K^+ channels. In this regard, it has been shown that the most critical channels in bronchodilation induced by 5-HT and ATP are BK_{Ca} [226, 228]. Most recently, our research group demonstrated that both K_{Ca} and K_V are implicated in salbutamol-induced relaxation in guinea pig airways [51].

Sex hormones play a role in the development of lung diseases. Androgens have been associated with asthma. During puberty, girls are more vulnerable to present asthma symptoms than boys, until the fifth life decade, when men become more susceptible than women [232, 233]. It has been reported that the variations in sex hormones during the menstrual cycle, hormone replacement therapy, and pregnancy have an influence in asthma patients [234–236]. Asthma is a chronic and inflammatory disease, characterized by hyperresponsiveness of the airways (AHR). This phenomenon is presented as an increased reactivity of the ASM to different agonists that leads to exaggerated bronchoconstriction. In addition, this disease is conducted by a type 2 immune response through eosinophils, basophils, mast cells, etc. [237]. However, not all asthma patients course with type 2 inflammation; instead, they can display interleukin-17- (IL-17-) mediated neutrophil inflammation [238].

Several studies have exposed that T induces a potential ASM relaxation effect through a nongenomic effect. An early work was conducted in the rabbit tracheal smooth muscle previously contracted with cholinergic agonists. The addition of T relaxed the ASM in an epithelium-dependent way involving NO production [78]. Later on, it was found that T relaxed precontracted guinea pig and bovine tracheal smooth muscles in an epithelium-independent way by blocking L-VDCCs [76]. In this context, our group demonstrated that T blocked L-VDCCs and SOCCs in the guinea pig ASM [34]. Additionally, the same study revealed that T induced the synthesis of prostaglandin E_2 (PGE_2), the main relaxing prostanoid in the airways [34]. Our studies pointed out that the blockade of the L-VDCCs and SOCCs and the production of PGE_2 are the main components of the T-induced relaxation in guinea pig precontracted airways. Then, we observed that T did not only relax the guinea pig ASM but lowered ${}_b[Ca^{2+}]_i$ and the muscular tone through the inhibition of L-VDCCs and TRPC3 [77, 219]. Most recently, our research group found that T interfered with the IP_3 receptor, decreasing the cholinergic-induced guinea pig ASM contraction [33]. Noteworthy, all the previously mentioned effects of T on ASM were carried out through nongenomic effects. Likewise, T, via a genomic action, negatively regulates type 2 inflammation and the expression of IL-17A [239, 240]. Furthermore, it was found that androgens, via AR activation, mediate the regulation of intracellular Ca^{2+} increment induced by proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) or interleukin-13 (IL-13) in the human ASM [52]. All these

androgen effects contribute to diminishing the ASM reactivity and favor the absence of asthma symptoms.

3.1. The Effects of Testosterone on Adrenergic Receptors in the Airway Smooth Muscle. Treatment with β-agonists to reverse airway obstruction, as seen in asthma and chronic obstructive pulmonary disease (COPD), has an essential role in controlling exacerbations. Therapeutically, there are two types of β-agonists: long-acting β-agonists to manage asthma together with glucocorticoids and short-acting β-agonists to relieve exacerbations [241]. Physiologically, the circulating catecholamines mediate the relaxation of the airways in humans. The androgen effects on the expression or function of the β-AR in the ASM have been scantly studied. In 1972, Salt and Iverson reported that T, via a nongenomic action, acted as an inhibitor of the extraneuronal uptake for catecholamines in the CM [242]. In this context, it was found that T potentiated the relaxation induced by isoprenaline (a nonselective β-adrenergic agonist) in pig bronchus, also via a nongenomic effect. The authors claimed that the potentiation effect observed was due to the inhibition of catechol-O-methyl transferase (COMT) or abolition of extraneuronal uptake [243]. In 2008, Bordallo et al. showed that 5α-DHT (a reduced metabolite of T) potentiated the relaxation induced by salbutamol, a β_2-adrenergic agonist, in the bovine tracheal ASM [76]. However, the effect of 5α-DHT seemed not to be related to a direct interaction with β_2-AR. Although the authors did not define the cause of the potentiation, it might be related to the inhibition of both the uptake of catecholamines and COMT (Figure 3(a)). Most recently, our group studied the genomic effects of T on β_2-AR. We found that chronic guinea pig ASM exposure to T augmented the expression of β_2-AR and evoked an increase in the relaxing responses to salbutamol (Figure 3(b)). Interestingly, this effect was abolished by flutamide (antagonist of the AR) [51]. We also observed that T potentiated salbutamol-induced potassium currents (I_K) involving the K_V and K_{Ca} upregulation (Figure 3(b)). Contrasting with other studies in the VSM [79], we did not find any modification of the adenylyl cyclase 6 (AC-6, the main isoform in the ASM) expression in tissues chronically exposed to T [51]. In summary, in the ASM, T and its metabolites, through nongenomic and genomic actions, have complementary effects. Consequently, androgens might play an important role as potential physiological modulators of the ASM tone, facilitating relaxation via β_2-AR, and therefore could be a therapeutic alternative for asthma treatment, although further research is needed (Figure 3).

4. Cardiac Muscle

Traditionally in the CM, autonomic control is derived by extrinsic signals or electrical stimulation of peripheral nerves. Moreover, neurocardiac control is maintained by an extensive network of intrinsic cardiac neurons, i.e., the intrinsic cardiac nervous system (ICNS) [244–246]. The ICNS comprises collections of neuronal somas residing on supraventricular tissues and the epicardial surface. This system is also composed by connecting nerve fibers known as ganglionic plexuses (GPs) [222, 246]. GPs are distributed in 5–7 regions comprising the right dorsal atrial, ventral right atrial, left dorsal, ventral left atrial, middle dorsal, right coronary, and left coronary plexuses [247]. Neuronal activity is modified by the activation of sensory nerves [248] and neuroactive chemicals, including acetylcholine, histamine, α- and β-adrenergic agonists, NO, neuropeptide Y (NPY, coreleased alongside norepinephrine), and calcitonin gene-related peptide (CGRP) [246, 249].

Similar to other muscular cells, $[Ca^{2+}]_i$ determines the contractile function of the heart through distinct Ca^{2+}-handling proteins. In the sinoatrial node, the pacemaker cells start depolarization of the cardiac myocytes. This process is regulated by the parasympathetic nervous system (PNS) and the SNS [250]. The self-depolarization produces action potentials along the CM, allowing the influx of Ca^{2+} through L-VDCCs and T-VDCCs. Furthermore, the Ca^{2+} influx elicits calcium release from the SR via RyR isoform 2 (RyR_2) [251]. Cardiac contraction results from a sudden increase in $[Ca^{2+}]_i$ and the formation of the Ca^{2+}-calmodulin complex with the further activation of MLCK. Afterward, Ca^{2+} is sequestered to the SR by SERCA, and the cell takes it out by the Na^+-Ca^{2+} exchanger in its forward mode (NCX). In addition, K_{Ca} channels are activated, leading to membrane hyperpolarization. These are the main mechanisms responsible for CM relaxation [252, 253]. Physiologically, catecholamines, through β-ARs, induce the synthesis of cAMP and the activation of PKA. This kinase promotes cardiac contraction by phosphorylating the L-VDCCs and RyR_2 since they increase their open probability and therefore the increment in $[Ca^{2+}]_i$ [254, 255]. PKA is also capable of evoking the relaxation of the CM by phosphorylating PLB, allowing SERCA to pump Ca^{2+} into the SR more rapidly [256]. In human ventricular cardiomyocytes, β_1- and β_2-ARs enhance cardiac frequency and contractility; meanwhile, β_3-ARs mediate negative inotropic effects [257]. The β_2-ARs essentially trigger the G_s/AC/cAMP/PKA pathway but are also involved in nonclassical G_i signaling displaying adverse effects on PKA activation and the inotropic response mediated by G_s [258].

There is increasing evidence that gender is highly related to cardiovascular states of health and disease. Whether androgens play a significant role in these dissimilarities is still investigated. Moreover, the genomic effects of T on ventricular cardiomyocytes' performance have been demonstrated. In this regard, Golden et al. showed that this androgen increased the mRNA expression of several critical Ca^{2+}-handling proteins. Treatment of rat ventricular cardiomyocytes with T increased the levels of gene expression of L-VDCC, β_1-AR, and NCX with 8 and 24 hours of exposure [259]. The T-induced changes in the mRNA expression levels of the mentioned proteins could be related to the improvement of the function of the cardiomyocytes and also be implicated in the development of hypertrophy and heart failure. These results point out an essential role of T in sex-related differences in the cardiac function.

Besides their electrophysiological properties, VDCCs are also classified using a standard nomenclature based on their

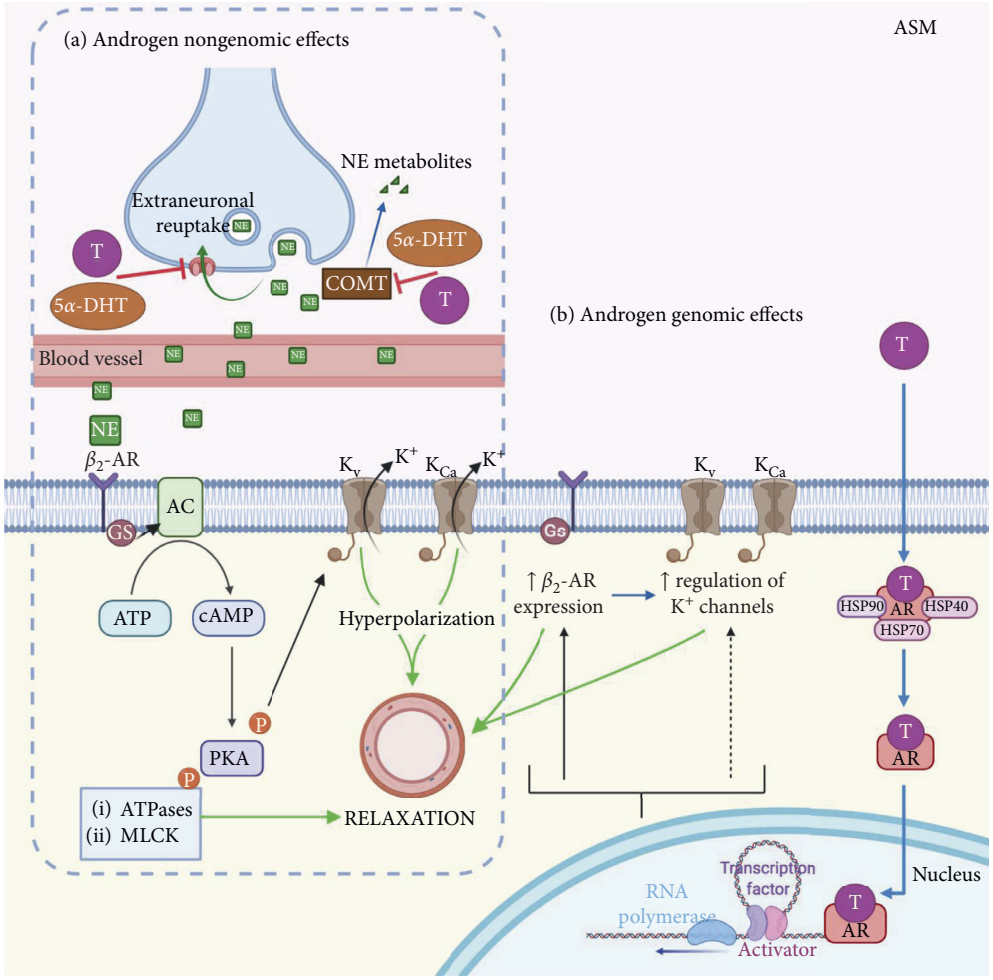

Figure 3: Androgen effects on the adrenergic system in the airway smooth muscle (ASM). (a) Testosterone (T), through a nongenomic effect, potentiates the relaxation induced by the β_2-adrenergic agonist through the inhibition of catechol-O-methyl transferase (COMT) or by the abolition of extraneuronal uptake of catecholamines. The inhibition of these mechanisms by T or 5α-dihydrotestosterone (5α-DHT, a reduced metabolite of T) leads to the accumulation of catecholamines such as norepinephrine (NE). The β_2-adrenergic agonist activates adenylyl cyclase (AC), leading to the activation of protein kinase A (PKA), which phosphorylates K^+ channels, ATPases, and myosin light-chain kinase (MLCK). These targets promote the relaxation of the ASM. (b) The androgen receptor (AR) signaling involves androgen crossing the plasma membrane, entering the cytoplasm, dissociation of chaperone proteins, and binding to the AR. Testosterone stimulation increases the β_2-adrenergic receptor (β_2-AR) expression in the guinea pig ASM and upregulates the Ca^{2+}-activated K^+ channels (K_{Ca}) and the voltage-dependent delayed rectifier K^+ channels (K_V). K_{Ca} are activated by increases in intracellular Ca^{2+} and through the cAMP-PKA signaling pathway. In the ASM, the main K^+ channels are the Ca^{2+}-activated K^+ channels of high conductance (BK_{Ca}). The K_V channel subtypes characterized in the ASM are $K_V1.2$ and $K_V1.5$. T (nongenomic and genomic effects) favors the relaxation of the ASM and might contribute to decreasing asthma symptoms.

molecular features of the pore-forming α1-subunit. Therefore, VDCCs are named using the chemical symbol of the permeating ion (Ca) with the physiological modulator (voltage) indicated as a subscript (Ca_V). A numerical identifier resembles the channel α1-subunit gene subfamily (1 to 3) and the order of discovery of the α1-subunit within that subfamily (1 through n). According to this nomenclature, the L-VDCCs are represented by $Ca_V1.1$–$Ca_V1.4$ subunits, and T-VDCCs correspond to $Ca_V3.1$–$Ca_V3.3$ [260]. In this regard, it has been reported that chronic administration of T enhanced Ca^{2+} influx through L- and T-VDCCs due to an increased expression of the $Ca_V1.2$, $Ca_V3.1$, and $Ca_V3.2$ subunits in ventricular cardiomyocytes, initially upgrading their performance but subsequently bringing the cell into new ${}_b[Ca^{2+}]_i$ [261, 262]. These studies suggest that augmented ${}_b[Ca^{2+}]_i$, via the upregulation of the Ca^{2+} channels aforementioned, might contribute to chronic cardiac pathogenesis when T levels are elevated.

While the nongenomic effects of T on the CM have been reported, the studies are scarce and seem to be contradictory regarding the Ca^{2+} handling and the cardiac contraction/relaxing outcomes. On the one hand, a group of researchers demonstrated in cultured rat cardiomyocytes that the acute exposure to T rapidly increased $[Ca^{2+}]_i$, and this augment was not abolished by an antagonist of the androgen receptor. Elegantly, the authors confirmed that the mechanism involved in the T-induced increase in $[Ca^{2+}]_i$ was mediated by the activation of a plasma membrane AR associated with a

pertussis toxin- (PTX-) sensitive G protein (Gi/o) and with the activation of the PLC-IP_3 signaling pathway leading to cardiac hypertrophy and failure [263]. The activation of PLC may be mediated through the action of $\beta\gamma$-subunits of the Gi/o proteins [264, 265]. On the other hand, it has been shown that acute exposure of T decreased the L- and T-VDCC activity by reducing their open probability [262, 266].

4.1. The Effects of Testosterone on Adrenergic Receptors in the Cardiac Muscle. Gender-related differences in cardiovascular disease (CVD) seem to be affected by age. It is well documented that the risk of dying for men between ages 45 and 64 from a CVD is higher than for women in the premenopausal period; however, there is a slight increase in the risk of CVD death in women after menopause [267].

The role of sex hormones in CVD is still unclear; particularly, a controversy about the effects of T in cardiovascular (CV) health and disease currently exists in the medical community. It is generally accepted that normal levels of T are beneficial for CV health in men, and a decline in these levels is related to an increase in CV events [268]. Nevertheless, a potential risk of developing CV events in patients receiving testosterone therapy has been reported [269, 270]. In postmenopausal women, endogenous elevated levels of total T (>0.9 nM) have been associated with CVD risk factors, such as high blood pressure and insulin resistance [271–275]. In this context, it has been postulated that estrogen augments the levels of sex hormone-binding globulin (SHBG). After menopause, the loss of ovary function leads to a general decline in sex steroid levels. Moreover, the fall of estrogen may lead to decreased levels of SHBG and higher free androgen levels [276]. Therefore, higher androgen levels and decreased estrogen levels in postmenopausal women have been suggested to be partially responsible for the CDV risk [268, 275]. Contrastingly, a study performed by Kaczmarek et al. demonstrated that low T levels are associated with coronary artery disease (CAD) in postmenopausal females [277]. During the menopausal transition, obesity is closely associated to CVD since it favors the secretion of proinflammatory cytokines, reactive oxygen species (ROS), and prothrombotic mediators [278–282]. Obesity promotes an unfavorable lipid profile which is associated with the development of CVD in elderly women [283, 284]. This profile is characterized by low high-density lipoprotein (HDL), higher total cholesterol (TC), and triacylglycerol (TAG) plasma levels [285]. In this regard, it was proved that oral DHEA therapy increased HDL and reduced TAG and LDL in adrenal-androgen-deficient postmenopausal women [286]. Also, obesity is a common feature of PCOS, exacerbating its symptoms and conferring a greater risk for CVD. The androgenic status, i.e., hyperandrogenism, needs to be considered when evaluating the metabolic and CV risk in PCOS women [200]. The ongoing controversy regarding the role of T in CVD might be moderately explained by the interaction between T and adrenoceptors in cardiac myocytes as discussed in the following.

It has been proposed that low plasmatic levels of T in older men are associated with an increase in atherosclerosis and cardiovascular risk, suggesting that this androgen plays a cardioprotective role against CVDs, such as coronary heart disease (CHD) and chronic heart failure (CHF) [287]. Moreover, it has been reported that an association between younger age at menopause and a greater risk of CDH in women coursing a natural menopause process [288]. Furthermore, T could confer cardiac protection against ischemic injuries by increasing the effects of the α_1-AR signaling pathway. The activation of α_1-AR improves the myocardial performance after an infarction, reducing injury and arrhythmias [289]. CHD is characterized by myocardial ischemia and cardiac injury [290]. In this regard, it has been shown that patients with CHD have lower androgen levels than healthy men and that low doses of T improved ischemic threshold in men suffering from angina [291, 292]. Furthermore, the administration of T enhanced the function recovery of the myocardium after a no-flow ischemia challenge in rats [293]. These observations point out to a reduction induced by T in the susceptibility to present myocardial ischemia and favor dilation of the coronary artery [294].

The SNS (through norepinephrine) activates α- and β-ARs controlling the CM tone. However, during myocardial ischemia, the release of norepinephrine increases the risk and contributes to cardiac injury [295, 296]. In this regard, it has been shown that the T-induced overexpression of the β_1-AR triggered proapoptotic pathways, weakening the cardiac structure and accelerating heart injury and failure progression [259, 297]. This overexpression also led to muscle hypertrophy in mice while producing an initial increase in contractility followed by a progressive dysfunction (Figure 4) [298].

On the contrary, the α_1-ARs may play an important role in cardioprotection, specifically, the α_{1A}-subtype. The overexpression of α_{1A}-AR can improve the outcome after myocardial infarction [299], cardiac contractility, and reduced arrhythmias [119, 300]. In 2008, Tsang et al. demonstrated in rat ventricular myocytes that T replacement therapy (TRT) upregulated the α_1-AR expression and augmented the cardiac responses, leading to a reduction in ischemia and cardiac injury [289]. Later on, in 2009, the same research group demonstrated that T enhanced the contractile function induced by the stimulation of both α_1- and β_1-AR in perfused rat hearts (Figure 4). Also, T treatment accelerated the relaxing response of the cardiac tissue. Interestingly, both phenomena were mediated by the AR [301]. The enhanced contractile response was explained since T augmented the function of RyR, leading to increased Ca^{2+} release from the SR. Otherwise, the augmented relaxing response was due to a more efficient activity of NCX regarding α_1-AR stimulation and a heightened SERCA activity, accompanied with increased phosphorylation of PLB in the case of β_1-AR stimulation [301]. Interestingly, they additionally found that the absence of T downregulated the expression of β_2-AR in rat hearts, indicating that this androgen may also interact with this receptor subtype [289]. Moreover, it has been documented that the activation of β_2-AR reduced apoptosis and increased the contractile mechanisms but did not accelerate relaxation as α_1-AR and β_1-AR

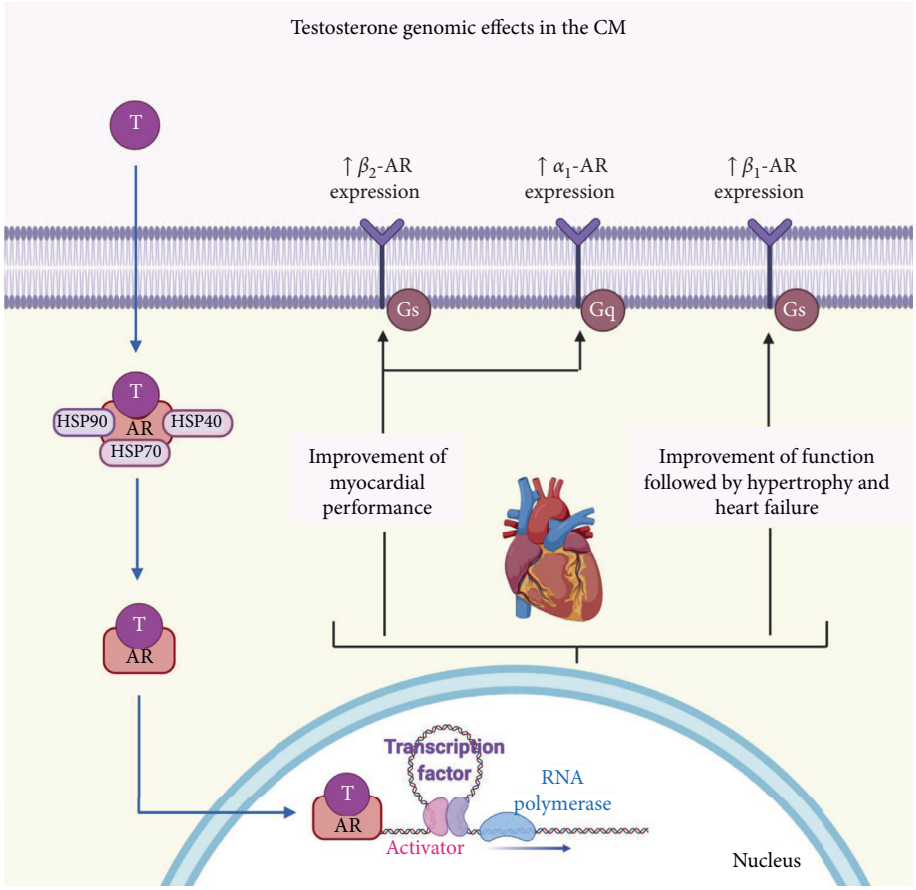

Figure 4: Genomic androgen effects on the adrenergic system in the cardiac muscle (CM). The genomic AR signaling involves androgen crossing the plasma membrane, entering the cytoplasm, dissociation of chaperone proteins, and binding to the AR. Testosterone (T) stimulates the expression of the α_1-adrenergic receptor (α_1-AR) and β_2-adrenergic receptor (β_2-AR), improving ionotropic activity and leading to the development of contraction without cardiotoxic effects. In addition, T increments the expression of the β1 adrenergic receptor (β1-AR), acutely improving the cardiomyocytes' function, but chronically leading to hypertrophy and heart failure.

stimulation did [302]. Although several studies have conducted about the relationship between cardiac function and androgens, more information is required to determine if T might play a key role in CHD.

Testosterone has also been associated with CHF [303]. This disease is a metabolic syndrome characterized by endocrine and inflammatory alterations, including elevated circulating catecholamine levels [304]. Testosterone deficiency (in hypogonadal subjects) has been demonstrated in 26% to 37% of male patients with CHF [305, 306]. Moreover, drugs used in CHF treatment, e.g., spironolactone and β-blockers, may diminish the function of Leydig cells, leading to a decline in the production of T [307, 308]. The low levels of T have been associated with reduced ejection fraction and increased systemic vascular resistance [309]. In this regard, the effects of T on β-AR have been investigated. In 2011, Sun et al. demonstrated that a TRT in a heart failure rat model reversed the damage (decrease in contractility, apoptosis, and fibrosis in cardiomyocytes) through the protection of the cardiac β-adrenergic system. Notably, the stimulation of the AR by T upregulated the expression of β_2-AR, improving the myocardial performance (Figure 4) [163]. Furthermore, it has been proposed that TRT in men with CHF induced an increase in the cardiac output and afterload [310].

The genomic regulation of the β-AR has been associated with cardiac remodeling and heart failure [311, 312]. In this regard, it has been shown that exercise training in rats reverses β-AR dysfunction by reducing the levels of G protein-coupled receptor kinase-2 (GRK2), an enzyme implicated in β_1-AR and β_2-AR dysregulation in CHF [313–315]. Moreover, exercise seems to restore the adrenal GRK2/α_2-AR/catecholamine production axis [313]. Also, exercise augments vascular β-AR responsiveness and diminishes the activity of GRK2 [316]. Interestingly, β_1-AR expression in the heart would be directly influenced by anabolic-androgenic steroids (AAS, synthetic derivatives of T) [317]. The use of AAS in combination with resistance training frequently improves the physical performance and helps athletes gain muscle mass and strength [318, 319]. However, numerous AAS abuse side effects include endocrine (hypogonadism) and detrimental cardiovascular issues [320–322]. For instance, vigorous training, anabolic steroid abuse, and the sympathetic nervous system's stimulation in mice increased cardiac levels of IL-1β and TNF-α and plasmatic levels of total cholesterol [320]. Furthermore, it

has been demonstrated that the use of AAS induced cardiac hypertrophy and increased myocardial susceptibility to ischemia injury [322, 323]. In this context, the administration of nandrolone (AAS) to male rats under an exercise training protocol increased the expression of β_1- and β_2-AR in the cardiac right atrium, provoked the prolongation of the QTc interval, and increased the BP [324]. In addition, the exposure of nandrolone augmented hypertension in SHR rats and β_1-AR protein expression in the left ventricle [317]. These data suggest that myocardial injury may be predisposed by high-performance training, steroid abuse, and the sympathetic nervous system's stimulation. Moreover, these insights may explain cardiac ailments and deaths in athletes under an AAS regimen.

Given the differences between studies showing the protective role of T in CV events and reports pointing out adverse CVD outcomes, it has been remarkably proposed that the use of **T**, as a treatment in CVD, should only be considered for male patients with a diagnosis of hypogonadism. Moreover, due to the increase of T therapy for postmenopausal women, the potential risk of developing CDV events needs further research [268, 275].

5. Conclusions

The adrenergic system plays a pivotal role in the control of vascular, airway, and cardiac physiology. A relationship between androgens with the adrenergic system of these tissues is proposed. This review summarizes that, in the vascular smooth muscle, T, via the androgen receptor, reduces the AC expression and increases the α_1-AR expression, leading to high BP and hypertension. Moreover, in the airway smooth muscle, T, via nongenomic action, potentiates the β-adrenergic-induced relaxation through the inhibition of COMT or by the abolition of extraneuronal uptake. This androgen, via a genomic effect, also augments the expression of β_2-AR and induces an increase in the relaxing responses to salbutamol. In the cardiac muscle, T upregulates the expression of α_{1A}-AR and β_2-AR mediated by the AR signaling, improving the myocardial performance. Moreover, T also increments β1-AR expression, improving the cardiomyocytes' function; however, the enhancement in muscle work during a long period ends up developing hypertrophy and heart failure.

Consequently, we might argue that androgen genomic actions have deleterious effects in the VSM favoring hypertension. Nevertheless, in the ASM, nongenomic and genomic actions of androgens contribute to diminish the hyperresponsiveness of this tissue, favoring the absence of asthma symptoms. Therefore, androgens could be a therapeutic alternative for asthma treatment. However, in heart diseases, further research is required to determine the possible therapeutic use of androgens in these ailments.

Finally, the use of T and DHEA as a therapeutic tool for the treatment of asthma symptoms or some cardiovascular diseases, is questionable. T has virilizing adverse effects, androgenic actions that favor prostate cancer, and its aromatization leads to the production of estrogens. Additionally, DHEA is further biotransformed into various sex steroids, such as T and estrogens, with their subsequent effects. However, 5β-DHT, a well-known T metabolite without genomic effects, could be a prospective therapeutic agent for the treatment of these illnesses.

Abbreviations

AAS:	Anabolic-androgenic steroids
AC:	Adenylyl cyclase
AC-6:	Adenylyl cyclase 6
AHR:	Airway hyperresponsiveness
α_1-ARs:	Alpha-1-adrenergic receptors
α_2-ARs:	Alpha-2-adrenergic receptors
ANF:	Atrial natriuretic factor
AR:	Androgen receptor
ASM:	Airway smooth muscle
Basic bFGF:	Fibroblast growth factor
β-ARs:	Beta-adrenergic receptors
BK_{Ca}:	Ca^{2+}-activated K^+ channels of high conductance
BP:	Blood pressure
cAMP:	3′, 5′-Cyclic adenosine monophosphate
cGMP:	3′, 5′-Cyclic guanosine monophosphate
CGRP:	Calcitonin gene-related peptide
CHD:	Coronary heart disease
CHF:	Chronic heart failure
CIRC:	Ca^{2+}-induced Ca^{2+} release
CM:	Cardiac muscle
COMT:	Catechol-O-methyl transferase
COPD:	Chronic obstructive pulmonary disease
CV:	Cardiovascular
CVD:	Cardiovascular disease
DAG:	Diacylglycerol
DHEA:	Dehydroepiandrosterone
5α-DHT:	5α-Dihydrotestosterone
5β-DHT:	5β-Dihydrotestosterone
EGFR:	Epidermal growth factor receptor
ERK1/2:	Extracellular signal-regulated kinases 1 and 2
FSH:	Follicle-stimulating hormone
GDP:	Guanosine diphosphate
GPRC6A:	G protein-coupled receptor family C group 6-member A
GPCR-$_q\alpha$:	GPCRs coupled to the $_q\alpha$ subunit
GPCRs:	G protein-coupled receptors
GPCR-$_s\alpha$:	GPCRs coupled to the $_s\alpha$ subunit
GRK2:	G protein-coupled receptor kinase-2
GPs:	Ganglionic plexuses
GTP:	Guanosine-5′-triphosphate
HA:	Hyperandrogenism
HDL:	High-density lipoprotein
HGFR:	Hepatocyte growth factor receptor
3β-HSD:	3β-Hydroxysteroid dehydrogenase
17β-HSD:	17β-Hydroxysteroid dehydrogenase
ICNS:	Intrinsic cardiac nervous system
I_K:	K^+ currents
IK_{Ca}:	Ca^{2+}-activated K^+ channels of intermediate conductance
IL-13:	Interleukin-13
IL-17:	Interleukin 17

IP_3: Inositol-1, 4, 5-triphosphate
$_b[Ca^{2+}]_i$: Intracellular basal Ca^{2+} concentration
$[Ca^{2+}]_i$: Intracellular Ca^{2+} concentration
K_{ATP}: ATP-sensitive K^+ channels;
K_{Ca}: Ca^{2+}-activated K^+ channels
K_{IR}: Inward-rectifier K^+ channels
K_V: Voltage-dependent delayed rectifier K^+ channels
$K_V1.5$: Voltage-dependent delayed rectifier K^+ channel 1.5
LH: Luteinizing hormone
L-VDCCs: L-type voltage-dependent Ca^{2+} channels
MAPKs: Mitogen-activated protein kinases
MEK: Mitogen-activated protein kinase kinase
MLCK: Myosin light-chain kinase
NCX: Na^+-Ca^{2+} exchanger
NCX_{REV}: Reverse-mode Na^+/Ca^{2+} exchanger
NO: Nitric oxide
NPY: Neuropeptide Y
PCa: Prostate cancer
PCOS: Polycystic ovary syndrome
PDGF: Platelet-derived growth factor
PGE_2: Prostaglandin E_2
PI3K: Phosphatidylinositol 3-kinase
PKA: Protein kinase A
PKC: Protein kinase C
PKG: Protein kinase G
PLB: Phospholamban
PLC: Phospholipase C
PLC_β: Phospholipase C_β
PMCA: Plasma membrane Ca^{2+}-ATPase
PNS: Parasympathetic nervous system
PTX: Pertussis toxin
ROCCs: Receptor-operated Ca^{2+} channels
RyR_2: RyR isoform 2
RyRs: Ryanodine receptors
SERCA: Sarcoplasmic reticulum Ca^{2+}-ATPase
5-HT: Serotonin
SHR: Spontaneously hypertensive rats
SK_{Ca}: Ca^{2+}-activated K^+ channels of low conductance
SNS: Sympathetic nervous system
SOCCs: Store-operated Ca^{2+} channels
SR: Sarcoplasmic reticulum
T: Testosterone
TAG: Triacylglycerol
TC: Total cholesterol
TNF-α: Tumor necrosis factor alpha
TRPC: Transient receptor potential channels
TRPC3: Transient receptor potential canonical 3
TRT: Testosterone replacement therapy
T-VDCCs: T-type voltage-dependent Ca^{2+} channels
VDCCs: Voltage-dependent Ca^{2+} channels
VEGFR-1/2: Vascular endothelial receptors 1/2
VSM: Vascular smooth muscle
ZIP9: Zinc-regulated transporter [Zrt]-protein 9.

Data Availability

No data were used to support this study.

Disclosure

Figures of this review were created with BioRender.com.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Abril Carbajal-García and Jorge Reyes-García contributed equally to this work.

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