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The role of vitamin D in male fertility: A focus on the testis

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Abstract In the last decade, vitamin D has emerged as a pleiotropic molecule with a multitude of autocrine, paracrine and endocrine functions, mediated by classical genomic as well as non-classical non-genomic actions, on multiple target organs and systems. The expression of vitamin D receptor and vitamin D metabolizing enzymes in male reproductive system, particularly in the testis, suggests the occurrence of vitamin D synthesis and regulation as well as function in the testis. The role of vitamin D in the modulation of testis functions, including hormone production and spermatogenesis, has been investigated in animals and humans. Experimental studies support a beneficial effect of vitamin D on male fertility, by modulating hormone production through genomic and non-genomic actions, and, particularly, by improving semen quality essentially through non-genomic actions. However, clinical studies in humans are controversial. Indeed, vitamin D seems to contribute to the modulation of the bioavailable rather than total testosterone. Moreover, although an increased prevalence or risk for testosterone deficiency was reported in men with vitamin D deficiency in observational studies, the majority of interventional studies demonstrated the lack of effect of vitamin D supplementation on circulating levels of testosterone. The most consistent effect of vitamin D was reported on semen quality. Indeed, vitamin D was shown to be positively associated to sperm motility, and to exert direct actions on spermatozoa, including non-genomic driven modulation of intracellular calcium homeostasis and activation of molecular pathways involved in sperm motility, capacitation and acrosome reaction. The current review provides a summary of current knowledge on the role of vitamin D in male fertility, by reporting clinical and experimental studies in humans and animals addressing the relationship between vitamin D and testis function.

Keywords Vitamin D · Male fertility · Testis · Hormone production · Testosterone · Semen quality · Environment · Lifestyle

1 Introduction

The main functions of the testis include the synthesis and secretion of reproductive hormones by Leydig cells and Sertoli cells, and the spermatogenesis, granted by Sertoli cells in the seminiferous tubules, site of the production of the spermatozoa [1]. Proper testis functions require multiple molecular events that might be regulated by several factors, including hormones and vitamins [2]. The term vitamin D refers to a group of liposoluble vitamins, with a biochemical structure similar to steroid hormones, whose main function is the regulation of calcium and phosphorus homeostasis, and pivotal target organs are intestine, skeletal system, kidney and parathyroid glands [3]. Nevertheless, the spectrum of target organs of vitamin D has expanded enormously in the last years, by including adipose tissue [4], thyroid [5], immune system [6], pancreas [7], cardiovascular system [8], central nervous system [9], as well as reproductive system [3, 10, 11]. Vitamin D deficiency is associated to hyperparathyroidism, rickets and osteomalacia, but it has been suggested to be a risk factor for several

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additional clinical conditions, such as obesity, thyroid dysfunction, autoimmune diseases, diabetes mellitus, cardiovascular diseases, dementia, cancer, as well as infertility, although whether a direct role of vitamin D is implicated in such conditions is still a matter of debate [12, 13]. The vitamin D group includes five different components, with the two most biologically relevant being ergocalciferol (vitamin D2) and, mainly, cholecalciferol (vitamin D3) [14]. The inactive form of vitamin D3 is partially derived from the diet, but it is predominantly synthesized in the skin with consequent activation requiring two enzymatic steps, the first operated in the liver, with the formation of 25-hydroxy-vitamin D3, and the second operated in the kidney, with the formation of 1α,25-dihydroxy-vitamin D3, the final active form of vitamin D3 [14], hereafter referred to as VitD. A specific 24-hydroxylase enzyme expressed in the kidney and in different target organs for VitD is deputed to the inactivation of the entire series of circulating forms of vitamin D3 [14]. VitD exerts its multiple actions through the binding and activation of VitD receptor (VDR) [15].

VitD has been suggested to play a role in male fertility, based on the evidence of the expression of VDR and VitD metabolizing enzymes (25-hydroxylase, 1α-hydroxylase and 24-hydroxylase) in the male reproductive system, and, particularly, in the testis and in the spermatozoa, with a consequent impact on semen quality, either in animals or in humans [16].

The current review reports the information concerning the expression and localization of VDR and VitD metabolizing enzymes in the male reproductive system, and critically summarizes data on the role of VitD in male fertility, with particular attention on testis function, by discussing the experimental and clinical evidences supporting a role for VitD in the regulation of testis hormone production, semen quality and, consequently, male fertility. A specific aim of the current review is to clarify whether VitD exerts direct or indirect actions on male fertility, and to describe the mechanisms underlying the entire series of effects exerted by VitD, by differentiating between its genomic and non-genomic actions. Lastly, a brief discussion on the potential role of VitD as an anticancer drug against testis cancer is also included in the current review.

2 Vitamin D synthesis, metabolism regulation and role

The major source of inactive vitamin D3 is the endogenous synthesis, which occurs in the skin, where a cholesterol precursor is converted to cholecalciferol, or vitamin D3, by the ultraviolet B radiation from the sun [14]. In order to be converted in its biologically active form, the inactive vitamin D3 is subjected to two different enzymatic steps [14]. Indeed, cholecalciferol (vitamin D3), released from the skin and bound to vitamin D-binding protein (DBP), is transported to the liver, where it is internalized in hepatocytes, and the hydroxylation at the 25th carbon is catalyzed by the microsomal enzyme 25-hydroxylase, producing an intermediate product, the 25-hydroxy-cholecalciferol (25-hydroxy vitamin D3) [14]; this compound is the major circulating form of vitamin D3 and is currently considered the most accurate surrogate marker of vitamin D status because of its longer half-life and higher concentration compared to VitD [14]. The 25-hydroxy-cholecalciferol released from the liver, is transported, bound to DBP, to the kidney, where it is internalized in the epithelial cells of the proximal tubule, and the hydroxylation at the 1st carbon is catalyzed by the enzyme 1α-hydroxylase, leading to the formation of 1α,25-dihydroxy-cholecalciferol (1α,25-dihydroxy-vitamin D3), which corresponds to the biologically active compound VitD [14, 17, 18]. The enzyme 24-hydroxylase, which is expressed in the kidney and in different target organs of VitD, is involved in the inactivation of the entire series of circulating forms of vitamin D3 [14, 19]. The main regulators of VitD metabolism are parathyroid hormone (PTH), produced by the parathyroid glands, and fibroblast growth factor 23 (FGF23), produced by osteoblasts and osteoclasts, which are modulated by circulating calcium and phosphorus levels, respectively, and VitD level itself [14, 20]. These regulators predominantly target the renal 1α-hydroxylase, with an additional effect on 24-hydroxylase, suggesting that 1α-hydroxylation is the key step in the activation of vitamin D3 or synthesis of VitD [14]. The reduction of circulating levels of calcium and 25-hydroxy-vitamin D3 results in stimulation of PTH secretion that, beyond promoting increase in calcium levels, stimulates 1α-hydroxylase and inhibits 24-hydroxylase expression in the kidney; the subsequent increase in VitD and calcium levels, in turn, suppresses PTH production resulting in inhibition of 1α-hydroxylase and stimulation of 24-hydroxylase, with consequent reduction of VitD levels, therefore completing the feedback loop [14, 21]. FGF23 is induced by the increase in circulating levels of phosphorus and 25-hydroxy-vitamin D3 and, beyond promoting renal phosphorus excretion, inhibits 1α-hydroxylase and stimulates 24-hydroxylase expression, with consequent reduction of VitD levels; the subsequent reduction in VitD and phosphorus levels, in turn, inhibits FGF23, with consequent increase in VitD levels, by closing the feedback loop [14, 22, 23]. VitD has been shown to modulate its metabolism by means of feedback mechanisms targeting renal 1α-hydroxylase and 24-hydroxylase, in particular, by down-regulating 1α-hydroxylase, and up-regulating 24-hydroxylase; these feedback mechanisms have been demonstrated in *in vitro* and *in vivo* studies [14, 24]. The complex regulation of VitD metabolism is necessary for the maintenance of proper calcium-phosphorus homeostasis [25]. Indeed, VitD promotes calcium and phosphorus absorption in the intestine, promotes calcium reabsorption and phosphorus excretion in the kidney, and modulates the balance between bone formation and resorption in strict dependence of circulating calcium levels [25]. The expression of VitD metabolizing enzymes is not restricted to the

liver and the kidney, but also concerns different organs and systems, including the male reproductive system, suggesting that VitD might be locally produced in non classical organs and systems [26]; however, whereas renal VitD synthesis is central to the maintenance of circulating calcium and phosphorus levels, extra-renal VitD synthesis is not essential in this process, but is mainly involved in paracrine and autocrine actions of VitD [26]. The local regulation of VitD synthesis in organs different from the kidney is an extremely challenging field and involves a plethora of factors; the regulation of extra-renal VitD synthesis has been shown to be cell type-specific, since different regulators have been reported in different cell types [14]. A graphic representation of renal VitD synthesis and VitD metabolism regulation is depicted in Fig. 1.

3 Vitamin D mechanisms of actions

VitD has been shown to exert multiple biological functions by means of VDR, which initiates both genomic and non-genomic actions; genomic action results in a direct modification of target genes expression, whereas non-genomic action consists in the activation of intracellular signal transduction pathways, which ultimately modulate several cell functions [15]. Two types of VDR, the classical nuclear VDR and the non-classical membrane VDR, mediate the genomic and non-genomic VitD actions, respectively [15]. The classical VDR belongs to the superfamily of nuclear receptors and is a DNA-binding transcription factor [15]. In target cells, VDR is localized in the cytoplasm and upon ligand binding it translocates to the nucleus and forms a heterodimer with an unoccupied retinoid X receptor (RXR) [15], which might be expressed as three different receptor subtypes: RXRα, RXRβ, and RXRγ [27]. The DNA-binding domain of each heteropartner recognizes a VitD response element (VDRE) in the promoter region of target genes, and regulates gene transcription [15]. The sequence variations in VDRE of different VitD target genes determine a range of different affinities for the VDR-RXR heterodimer and, therefore, different sensitivity to VitD transcriptional activity [15]. Moreover, sequence variations in VDRE of different VitD target genes induce unique conformations in the VDR-RXR complex, by promoting the recruitment of distinct subsets of co-modulators which might determine either gene transcription stimulation or repression [15, 26]. The classical genomic actions of VitD generally takes hours to days for the complete activation mediated by the transcription and the following expression of the target genes; conversely, the non-genomic actions of VitD occur within 1–2 min to 15–45 min, because mediated by the interaction with intracellular signaling pathways. VitD non-genomic actions are driven by the activation of a non-classical membrane VDR, firstly discovered in the cell membrane of intestinal cells [15, 28–30]. Membrane VDR primarily regulates membrane and cytosolic second messengers, converging on kinases and phosphatases; the major intracellular signaling pathways found to be involved in VitD-driven non-genomic response are protein kinase A, protein kinase C, mitogen-activated protein kinase, as well as chloride and calcium channels, although no agreement on the stimuli that initiate the non-genomic actions of VitD exists [15, 28]. Although VitD non-genomic actions do not involve directly the regulation of gene expression, they might indirectly activate intracellular pathways, which ultimately result in downstream modifications of gene expression [28]. A graphic representation of VitD genomic and non-genomic actions is depicted in Fig. 2.

4 Vitamin D and male reproductive system

The relevant role of VitD in male fertility is supported by the expression of VDR and VitD metabolizing enzymes in male reproductive system of animals and humans. Indeed, VitD metabolizing enzymes have been found in virtually the entire series of reproductive organs, especially in the testis, with involvement of somatic or germ cells, suggesting that VitD might be locally synthesized and degraded, and its metabolism locally regulated, independently from systemic VitD metabolism. Moreover, the VDR expression in the testis suggests that the locally produced VitD might exert autocrine and paracrine action, possibly displaying a role in the regulation of testis function, therefore influencing male fertility. The expression pattern of VDR and VitD metabolizing enzymes in male reproductive system of animals and humans has been extensively investigated and is provided in Table 1.

4.1 Vitamin D receptor and vitamin D metabolizing enzymes expression in animal male reproductive system

The expression of VDR has been widely investigated in animal male reproductive system, with the most studied animal model being the rodents [28, 31–48]. VDR messenger expression was poorly investigated compared to its relative protein. Only one study assessed VDR messenger expression in the testis, and, particularly, in selected populations of testis cells, namely Sertoli cells, Leydig cells, spermatocytes and spermatids, demonstrating in the entire series of cells the messenger expression of VDR [32]. VDR protein expression was clearly documented in prostate, seminal vesicles, epididymis, as well as in the testis, particularly in a group of germ cells, including spermatogonia and spermatocytes, and in Sertoli cells, with increasing amount along with Sertoli cell maturation [28, 33, 34, 41, 42, 45–48]. Conversely, VDR protein expression in Leydig cells is controversial [40, 42]. A faint VDR protein expression was detected in spermatozoa, although it seems to be lost during the passage in the tail of the epididymis [46]. RXR expression was demonstrated in rat testis, especially in developing animals [49, 50]. RXRα protein was detected in Sertoli cells, Leydig cells,

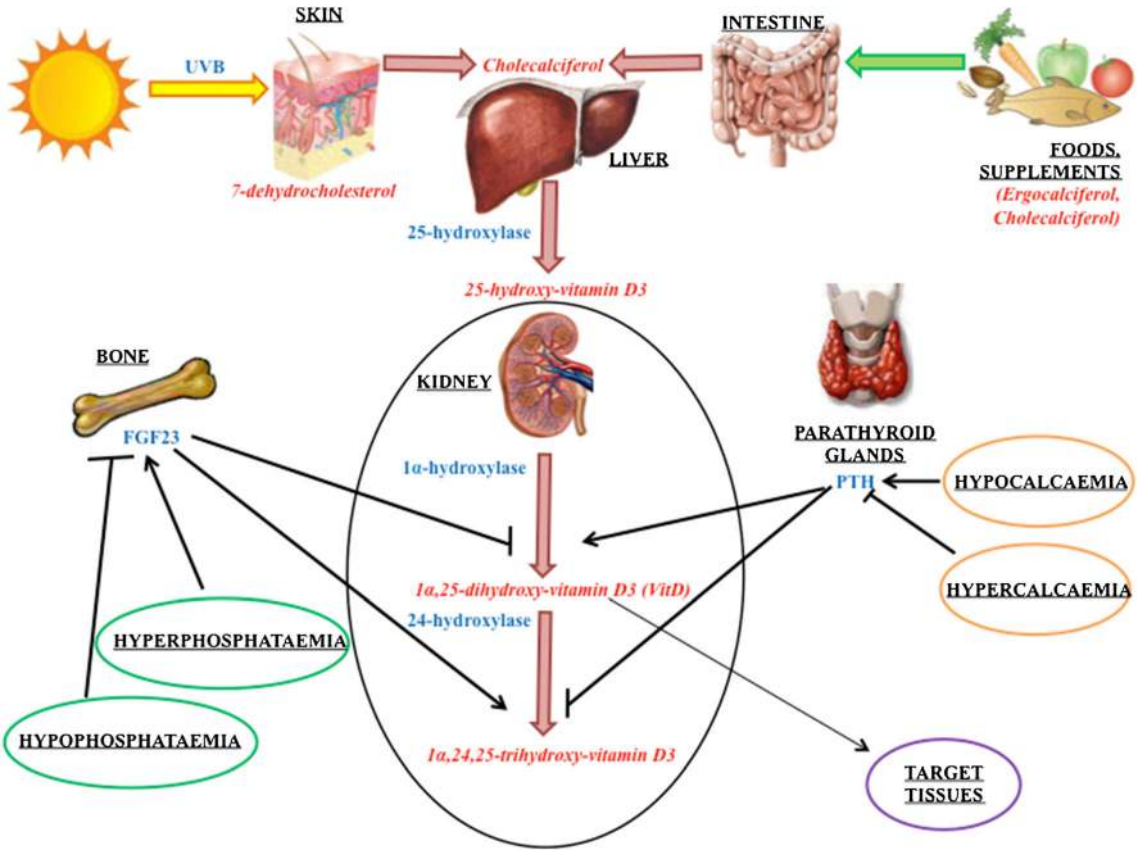

Fig. 1 Vitamin D Metabolism Regulation. The two major forms of vitamin D are ergocalciferol and cholecalciferol. Both forms of vitamin D may be derived from foods or supplements, whereas the major amount of choclecalciferol is synthesized in the skin, where UVB radiations from the sun stimulate the conversion from 7-dehydrocholesterol. Cholecalciferol is biologically inactive and its activation requires two enzymatic hydroxylation reactions: the first step is 25-hydroxylation by the hepatic enzyme 25-hydroxylase, which produces the intermediate product 25-hydroxy-vitamin D3; the second step is 1α-hydroxylation by the renal enzyme 1α-hydroxylase, which produces the biologically active 1α,25-dihydroxy-vitamin D3 (VitD). The adequate turnover of VitD is granted by the renal enzyme 24-hydroxylase, which produces the inactive form 1α,24,25-trihydroxy-vitamin D3. VitD metabolism is regulated by three major factors: parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), which are modulated by circulating calcium and phosphorus levels, respectively, and VitD itself. Hypocalcaemia and reduced circulating levels of 25-hydroxy-vitamin D3 stimulate PTH secretion by the parathyroid gland; PTH induces an increase in calcium levels and 1α-hydroxylase expression and reduces 24-hydroxylase expression in the kidney, by resulting in increased circulating calcium levels and VitD production. VitD and hypercalcaemia, in turn, suppress PTH secretion and modulate 1α-hydroxylase and 24-hydroxylase, by decreasing and increasing their expression, respectively, thus completing the feedback loop. Hyperphosphataemia and increased circulating levels of 25-hydroxy-vitamin D3 stimulate FGF23 secretion by osteoblasts and osteoclasts; FGF23 promotes renal excretion of phosphorus and reduces 1α-hydroxylase expression and induces 24-hydroxylase expression in the kidney, by resulting in decreased circulating phosphorus levels and VitD production. Reduced VitD and phosphorus levels, in turn, suppress FGF23 secretion, thus completing the feedback loop

and germ cells, particularly spermatogonia, spermatocytes, and spermatids [49, 50]. RXRβ protein was detected in Sertoli cells, with increasing amount during Sertoli cell maturation, and Leydig cells, whereas its expression in germ cells is conflicting [49, 50]. RXRγ protein was detected in Sertoli cells, Leydig cells, and germ cells, particularly in spermatogonia, spermatocytes, and spermatids [49, 50]. RXR expression was demonstrated also in mouse testis, either in developing or adult animals [51, 52]. RXRα protein was detected in Leydig cells of the developing animals and in spermatids of adult animals [51, 52]. RXRβ protein was detected in Sertoli cells of developing and adult animals [51, 52]. RXRγ protein was detected in spermatids in adult but not in the developing animals [51, 52]. VitD metabolizing enzymes expression has not been extensively analyzed in animal male reproductive system: 25-hydroxylase and 1α-hydroxylase messenger was detected in testis homogenates; 1α-hydroxylase protein was detected in prostate, seminal vesicles and head, body and tail of epididymis, and in the testis, particularly in Sertoli cells, Leydig cells, spermatogonia and spermatocytes [45, 46, 48].

4.2 Vitamin D receptor and vitamin D metabolizing enzymes expression in human male reproductive system

The expression of VDR messenger and protein has not been extensively investigated in human male reproductive

Table 1 Protein Expression of Vitamin D Receptor and Vitamin D Metabolizing Enzymes in Male Reproductive Organs and Cells

SPECIES	MALE REPRODUCTIVE ORGAN/CELL								
	Prostate	Seminal Vesicles	Epididymis	Leydig cells	Sertoli Cells	Spermatogonia	Spermatocytes	Spermatids	Spermatozoa
VDR									
Rodents	+	+	+	+/−	+	+	+	NA	+/−
Humans	+	+	+	+/−	−	+	+/−	+	+/−
25-hydroxylase									
Rodents	NA	NA	NA	NA	NA	NA	NA	NA	NA
Humans	+	+	+	+	−	−	+	+	+/−
1α-hydroxylase									
Rodents	+	+	+	+	+	+	+	NA	−
Humans	+	+	+	+	−	+	+	+	+/−
24-hydroxylase									
Rodents	NA	NA	NA	NA	NA	NA	NA	NA	NA
Humans	+	+	+	+	−	+	−	+	+/−

+, positive staining; −, negative staining; +/− heterogeneous or inconsistent staining. Abbreviations: NA, Not Assessed

system [53–60]. VDR messenger and protein expression was documented in prostate, seminal vesicles and epididymis [58–60]. In the testis, VDR protein was detected in Leydig cells, in immature Sertoli cells, and in germ cells, where it was found in spermatogonia and spermatids, and, inconsistently, in spermatocytes and spermatozoa [53, 55, 58, 59]. In human spermatozoa, VDR showed a heterogeneous pattern of localization, since it was found in head [53–56] post-acrosomal region [53, 54], neck [53, 56] and/or mid-piece [53–55]. To the best of our knowledge, no studies evaluated the expression of RXR in the human testis. VitD metabolizing enzymes messenger and protein expression has not been extensively analyzed in human male reproductive system. The 25-hydroxylase messenger and protein were found in prostate, seminal vesicles, epididymis and testis, particularly in Leydig cells, spermatocytes and spermatids [53]. In spermatozoa, 25-hydroxylase displayed a heterogeneous expression and was localized predominantly in post-acrosomal region, neck, and tail [53]. The 1α-hydroxylase messenger and protein were found in prostate, seminal vesicles, epididymis and testis, particularly in Leydig cells, spermatogonia, spermatocytes and spermatids [53]. In spermatozoa, 1α-hydroxylase displayed a heterogeneous expression and was predominantly localized in post-acrosomal region, neck and mid-piece [53]. The 24-hydroxylase messenger and protein were found in prostate, seminal vesicles, epididymis and testis, particularly in Leydig cells, spermatogonia and spermatids [53]. In spermatozoa, 24-hydroxylase displayed a heterogeneous expression and was mainly localized in neck and annulus [53].

4.3 Vitamin D synthesis and metabolism regulation in male reproductive system

A series of clinical observational studies in humans suggested the occurrence of VitD synthesis in the testis, as hypothesized from the negative effect of orchiectomy or testis dysfunction on circulating levels of 25-hydroxy-vitamin D3, a surrogate marker of vitamin D status [61–63]. This hypothesis is supported by experimental and clinical studies although the specific cells within the testis producing VitD is still a matter of debate. Indeed, an experimental study in animals demonstrated that mouse Leydig cells basally secrete 25-hydroxy-vitamin D3, which is also stimulated by human chorionic gonadotropin (hCG) [64]. These evidences are in line with a clinical interventional study in humans showing that hCG treatment in men with late-onset hypogonadism, significantly increased circulating levels of 25-hydroxy-vitamin D3 [65], reinforcing the hypothesis that Leydig cells might drive local production of 25-hydroxy-vitamin D3 in the testis. On the other hand, another observational study suggested that germ cells might also contribute to testis VitD synthesis since severe impairment of spermatogenesis or Sertoli cells only syndrome, were associated with significantly lower circulating levels of 25-hydroxy-vitamin D3 [66]. The regulation of testis VitD metabolism has not been extensively investigated; nevertheless, some evidences suggested the existence of testis regulatory mechanisms, which resemble renal regulatory mechanisms of VitD metabolism; these regulatory mechanisms mainly include FGF23 and PTH-related molecules pathways. In an experimental study in FGF23-null mice, testis 1α-hydroxylase expression was significantly increased compared to wild-type animals, therefore suggesting that FGF23

might be implicated in the local regulation of VitD metabolism by modulation of 1α-hydroxylase expression [67]. This hypothesis is supported by the results of an experimental study, showing that the treatment with Klotho protein, a specific cofactor which is essential for the activation of FGF23 signaling, significantly suppressed 1α-hydroxylase messenger expression in mouse Sertoli cells [68]. Nevertheless, endogenous Klotho expression was only detected, within the mouse testis, in spermatids [69], suggesting that this molecular pathway involved in VitD metabolism regulation [70, 71] might be effective only in these specific germ cells. Although, in humans, Klotho expression has not been investigated in the testis, FGF23 receptor expression has been found in germ cells [72], suggesting that the FGF23/FGF23 receptor/Klotho pathway might be implicated in local VitD metabolism regulation in the human testis. PTH-related molecules, such as PTH-related peptide (PTH-rP), as well as their receptors, have been detected in testis lysates, Leydig cells, and germ cells, in animals [73, 74]. Although in humans the expression of PTH-related molecules and their relative receptors has not been fully investigated, PTH-rP expression was detected in Leydig cells [75]. These evidences suggest that a PTH-like pathway might be involved in local VitD metabolism regulation in the testis. Nevertheless, the exact role of FGF23 and PTHrP pathways in testis VitD metabolism regulation has not been completely addressed. Lastly, similarly to VitD metabolism regulation in the kidney, VitD-driven feedback mechanisms have been proposed to apply in the testis. This hypothesis is supported by the evidence that VDR-null mice displayed increase in 1α-hydroxylase and decrease in 24-hydroxylase expression in the testis [76]. However, in rat, circulating VitD or its inactive precursors did not up-regulate VDR expression in the testis [77]. Moreover, in humans, circulating 25-hydroxy vitamin D3 did not correlate with 24-hydroxylase expression in spermatozoa from healthy and infertile men [78]. These evidences reinforce the hypothesis that locally produced VitD, rather than circulating VitD, might be involved in the local regulation of VitD metabolism in the testis. A graphic representation of VitD synthesis and VitD metabolism regulation in male reproductive system is depicted in Fig. 3.

5 Vitamin D and testis function

The role of VitD on male fertility seems to depend predominantly on the effect on testis function. Testis function comprises two interconnected and complementary processes, hormone production and spermatogenesis, which, in coordination with the action of accessory glands, ensure proper potential of male fertility [1]. Hormone production by the testis is a complex function, which requires the participation of both somatic and germ cells, and is crucial to guarantee adequate spermatogenesis [1]. Nevertheless, most of the current knowledge on the role of testis hormones in controlling germ cell differentiation and development has been derived from studies in animal models, and the precise mechanisms by which testis hormones modulate spermatogenesis have not been fully characterized [79]. The main hormones produced by the testis include the steroid hormones testosterone and estradiol, the glycoprotein hormones anti-müllerian hormone (AMH) and inhibin-B (INH-B), and the peptide hormone insulin-like 3 (INSL3) [79]. Spermatogenesis encompasses a complex network of events occurring in the seminiferous tubules that comprises spermatocytogenesis, including spermatogonia proliferation and differentiation in spermatocytes; spermatidogenesis, representing meiotic division of spermatocytes with production of spermatids; spermiogenesis, including the steps of maturation and differentiation of spermatids in mature spermatozoa; and spermiation, consisting in the release of mature spermatozoa into the lumen of the seminiferous tubules [79]. The hormonal milieu within the testis has a pivotal role in the control and coordination of the multiple steps of spermatogenesis [79]. Testosterone is the primary male sexual hormone and an important anabolic hormone, which plays a central role in the development and function of male reproductive system and in the regulation of sexual function; it is considered a well-being hormone since it contributes to the health of the musculoskeletal system, and is involved in several important processes including metabolism, cognition and mood [80]. A relavant role of testosterone is represented by the regulation of spermatogenesis, particularly spermatidogenesis and spermiation performed within the testis, by actions on Sertoli cells [79]. Testosterone is synthesized by Leydig cells under pituitary LH pulsatile secretion, but its production is also modulated by paracrine and autocrine signals provided by growth factors and cytokines secreted within the testis [79, 81]. The concerted endocrine, paracrine and autocrine modulation of testosterone production results in intratesticular testosterone levels, which are 100 fold higher than circulating testosterone levels, and have been suggested to exert the major control on spermatogenesis, compared to circulating testosterone levels [79, 81]. Estradiol is the primary female sexual hormone but it seems to exert a role in male fertility, and, particularly, in the regulation of spermatogenesis [79]. The exact role of estradiol in spermatogenesis is still a matter of debate, although it is well documented that proper spermatogenesis requires a complex balance between testosterone and estradiol concentrations [79]. In the testis, estradiol is mainly produced in Leydig cells by conversion from testosterone, whose last important step is catalyzed by aromatase enzyme; nevertheless, Sertoli cells and germ cells were shown to contribute to local estradiol levels [79, 82, 83]. The balance between circulating testosterone and estradiol levels are assumed to reflect the balance between intratesticular testosterone and

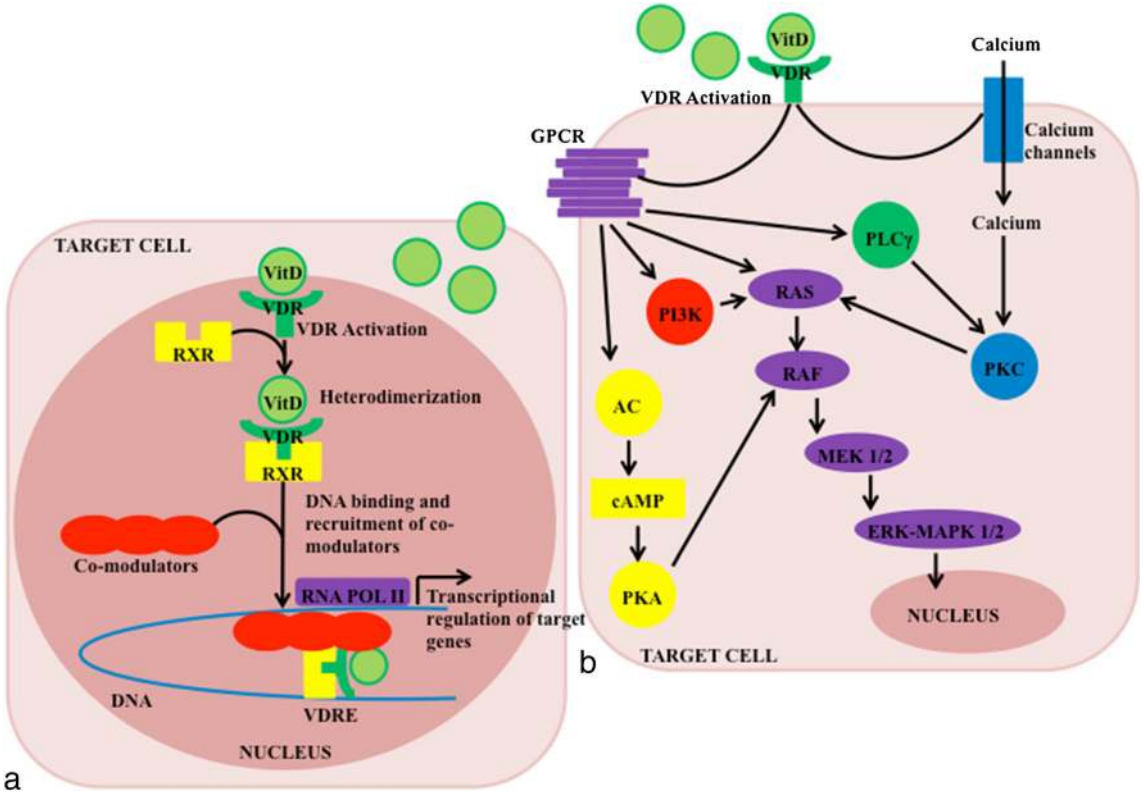

Fig. 2 Vitamin D Genomic and Non-genomic Actions. (**a**) Genomic actions. Genomic action of 1α,25-dihydroxy-vitamin D3 (VitD) determines a VitD receptor (VDR)-mediated modification of target genes expression. The classical nuclear VDR belongs to the superfamily of nuclear receptors and is a DNA-binding transcription factor. In target cells, VDR, which is activated upon ligand binding, translocates to the nucleus and forms a heterodimer with retinoid X receptor (RXR). The VDR-RXR heterodimer recognizes a VitD response element (VDRE) in the promoter region of target genes, and modulates gene transcription. Sequence variations in VDRE of different VitD target genes induce unique conformations in the VDR-RXR complex, by promoting the recruitment of distinct subsets of co-modulators, which results in either gene transcription stimulation or repression. **(b)** Non-genomic actions. Non-genomic action of VitD determines a VDR-mediated modulation of a plethora of intracellular signal transduction pathways; a putative cell membrane-bound VDR mediates these actions. Non-genomic action of VitD is hypothesized to ultimately activate the mitogen-activated protein kinase (MAPK)–extracellular signal-regulated kinase (ERK) 1 and 2 cascade by means of several intermediate effectors, which become activated upon VitD binding to VDR. Activated VDR stimulates calcium influx, which, in turn, activates calcium-driven intracellular pathways, such as protein kinase C (PKC). Moreover, VitD might activate G-protein coupled receptors (GPCRs), which, in turn, stimulate several downstream pathways, including phosphatidylinositol 3-kinase (PI3K), adenylate cyclase (AC), Ras, and phospholipase C gamma (PLCγ). Each of these pathways might converge by different signaling on the activation of ERK-MAPK 1/2, which might engage in cross-talk with the classical genomic VDR-driven pathway, to modulate gene expression

estradiol levels, which are mainly involved in the control of spermatogenesis [79]. The possible role of AMH, INH-B and INSL3 in local regulation of spermatogenesis has not been clearly determined. AMH, which is mainly produced by Sertoli cells in the fetus and pre-pubertal testis under FSH stimulation, plays an important role in the differentiation and development of the male reproductive system, but its role in the adult is not clear [84]. In adults, AMH is produced at very low levels by Sertoli cells under FSH stimulation; nevertheless, AMH levels in seminal plasma are much higher than blood plasma levels, and are influenced by the status of germ cell maturation, which is a measure of the progression of spermatogenesis [84]. INH-B is mainly produced by Sertoli cells under FSH stimulation until puberty, whereas, after the initiation of spermatogenesis, INH-B levels are influenced by the status of germ cell proliferation, which is directly proportional to sperm count [84]. Based on these evidences, AMH and INH-B have been proposed as direct markers of Sertoli cell function and indirect biochemical markers of the quality of spermatogenesis [84, 85]. INSL3 is produced by Leydig cells and reflects their differentiation status, therefore being considered a marker of Leydig cell function [86]. INSL3 has been suggested to modulate testosterone production by an autocrine action, and spermatogenesis, by a paracrine action [86]; although the role of INSL3 in spermatogenesis has not been completely clarified, recent findings suggested that it might exert an anti-apoptotic and pro-survival action on germ cells [86]. It should be noted that, although paracrine and autocrine actions of intratesticular hormones exert a central role in the regulation of spermatogenesis, the endocrine regulation of testis functions is directed by the pulsatile release of GnRH from the hypothalamus, and LH and FSH stimulation by the

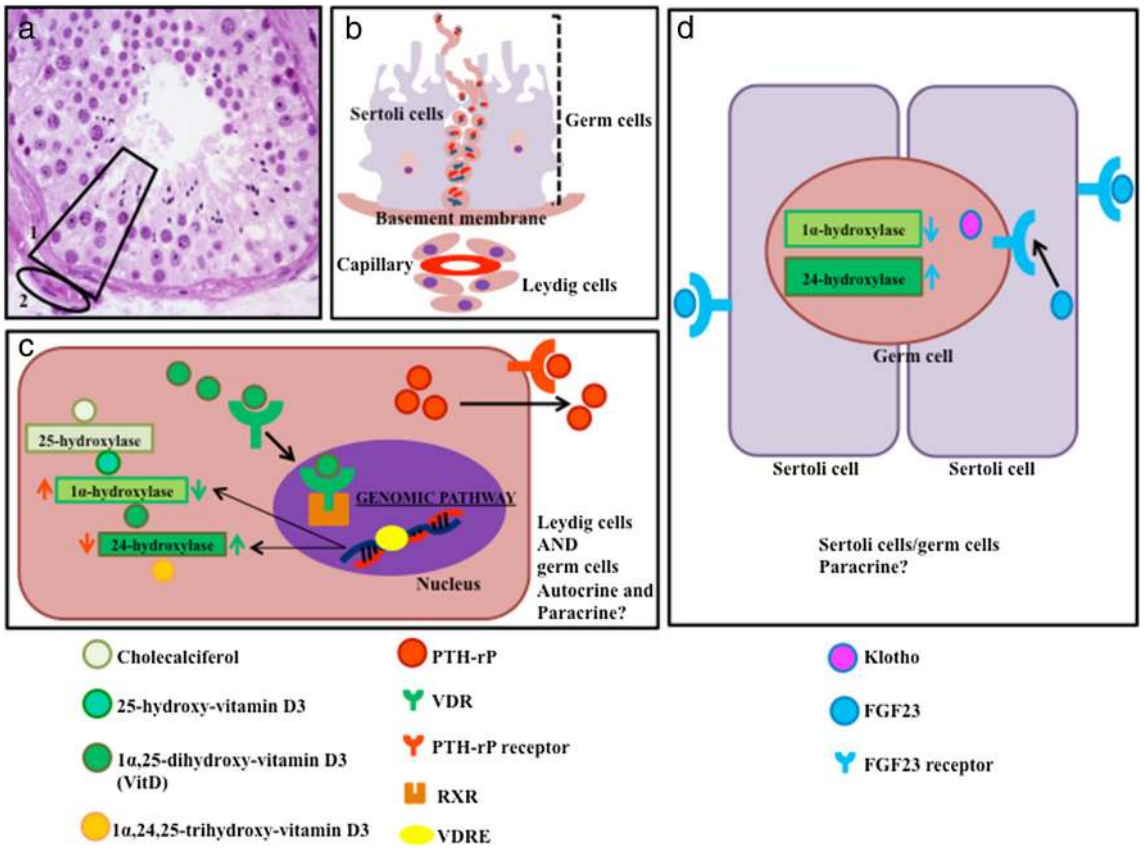

Fig. 3 Vitamin D Metabolism Regulation Within the Testis. (**a**) Cross-section of a seminiferous tubule. **(b)** Detail of Sertoli cells and adjacent Leydig cells, and schematic overview of germ cells undergoing spermatocytogenesis, spermatidogenesis, spermiogenesis and spermiation. **(c, d)** Overview of the proposed mechanisms regulating local 1α,25-dihydroxy-vitamin D3 (VitD) metabolism in the testis. Somatic and germ cells within the testis variably express VitD receptor (VDR), and VitD activating (25-hydroxylase, 1α-hydroxylase) and inactivating (24-hydroxylase) enzymes. The exact identity of the testis cells which produce VitD has not been clearly established, although experimental and clinical evidences suggest that Leydig cells or germ cells, or both, might be possible candidates. Moreover, the mechanisms involved in the local VitD metabolism regulation within the testis have not been extensively investigated. Nevertheless, scattered experimental evidences suggest that regulatory mechanisms which resemble renal VitD metabolism regulation, namely parathyroid hormone (PTH)-related molecules and fibroblast growth factor 23 (FGF23) pathways, might exist within the testis. PTH-related molecules, such as PTH-related peptide (PTH-rP), as well as their receptors, have been found to be expressed in testis lysates, Leydig cells, and germ cells, in rodents, whereas, in humans, PTH-rP expression was detected in Leydig cells. The espression of Klotho, a FGF23 cofactor required for FGF23 signaling, was detected in germ cells in rodents, but has not been investigated in humans; moreover, FGF23 and its receptor have been hypothesized to be expressed in Sertoli cells in rodents, and FGF23 receptor expression has been detected in germ cells in humans. Although incomplete, these findings may suggest that **(c)** PTH-related molecules might exert autocrine and/or paracrine VitD metabolism regulation in Leydig cells and germ cells, whereas **(d)** FGF23/Klotho axis might exert autocrine and/or paracrine VitD metabolism regulation in Sertoli cells and germ cells. The exact effects of PTH-related molecules and FGF23 on local VitD metabolism regulation within the testis remains to be elucidated. Lastly, the evidence that VDR and VitD metabolizing enzymes are co-expressed in Leydig cells and germ cells, suggests that locally produced VitD might exert autocrine and paracrine regulatory actions on VitD metabolism, by VDR-mediated genomic down-regulation of 1α-hydroxylase, and up-regulation of 24-hydroxylase

pituitary gland [81]. Moreover, feedback mechanisms driven by steroid hormones, mainly testosterone, as well as INH-B from the testis, modulate GnRH release from the hypothalamus and GnRH responsiveness in the pituitary, therefore modulating LH and FSH secretion, respectively [81, 87]. Sex hormone-binding globulin (SHBG), a transport glycoprotein with high binding affinity for sexual hormones, is a major factor regulating circulating testosterone and estradiol balance between the non bioavailable SHBG-bound and the bioavailable free fraction, therefore contributing to testis hormone-driven endocrine feedback mechanisms on the hypothalamus and pituitary gland [81].

5.1 Vitamin D and testis function: Hormone production

The effects of VitD on testis hormone production have been investigated in experimental *in vitro*, *ex vivo* and/or *in vivo* studies in animals. In humans, the effect of VitD on testis hormone production has been investigated in three different

types of studies: 1) clinical observational studies evaluated the relationship between vitamin D status and circulating levels of testis hormones; 2) clinical interventional studies evaluated the effect of vitamin D supplementation on testosterone and estradiol production; and 3) experimental studies investigated on the molecular mechanisms underlying VitD effect on testosterone production.

5.1.1 Animal studies

The effects of vitamin D status, determined by the assessment of VitD circulating levels, on testosterone production have been investigated in *in vitro*, *ex vivo*, and *in vivo* studies in animal models. An early *in vivo* study in rats demonstrated that circulating levels of testosterone were significantly lower in vitamin D depleted, compared to vitamin D repleted animals [88]. However, a more recent *in vivo* study in VDR-null mice demonstrated only a trend to a reduction in circulating testosterone/LH ratio, probably sustained by a slight but not significant reduction of testosterone levels, compared to wild-type animals [76]. These evidences support the hypothesis that VitD might have a role in the regulation of testosterone levels, although the mechanisms of action have not been completely clarified. A possible mechanism for the effect of VitD on testosterone production might be indirectly hypothesized from the results of an *in vivo* study in vitamin D-depleted and vitamin D-repleted chickens [89], investigating on the testis expression of calbindin-D_{28K}, a cytosolic calcium-binding protein involved in the regulation of intracellular calcium homeostasis, and proposed to be involved in testis hormone production in rats [90]. Although vitamin D-depleted and vitamin D-repleted chickens had comparable circulating levels of testosterone, in testis homogenates of vitamin D-depleted chickens the expression of calbindin-D_{28K} was significantly reduced, compared to vitamin D-repleted chickens [89], suggesting that VitD might modulate the expression of calbindin-D_{28K} in the testis, by means of a genomic action, without producing a significant change in the circulating levels of testosterone, at least at the specific experimental doses of VitD achieved in the study [89]. However, the study was performed in young chikens, characterized by juvenile circulating testosterone levels and the levels of VitD achieved by manipulating animals diet produced a suboptimal vitamin D status, rather than a definite vitamin D deficiency status; therefore, the hypothesis that a definite vitamin D deficiency would induce a significant change of testosterone production in adult chicken with fully developed testes, producing sustained circulating levels of testosterone, cannot be excluded, but requires further investigation [89]. Taken together, the results of the study suggest the hypothesis that, in animals, testosterone secretion might be modulated by VitD-induced changes in intracellular calcium homeostasis in Leydig cells. Different molecules have been found to mediate the effect of VitD on testosterone production; in particular, osteocalcin, a hormone involved in bone metabolism and produced by osteoblasts, partially as a consequence of the genomic action of VitD, was proposed to be a mediator of such VitD effect [28]. Indeed, the osteocalcin G protein-coupled receptor GPRC6A is expressed in Leydig cells, and osteocalcin was shown to directly induce testosterone production in *in vitro* cultures of primary Leydig cells, in *ex vivo* cultures of whole testis explants and in *in vivo* studies in mice [91]. Moreover, circulating levels of testosterone were markedly decreased in osteocalcin loss-of-function and increased in osteocalcin gain-of-function mice [91]. Furthermore, an *in vitro* study in TM3 mouse Leydig cells demonstrated that osteocalcin induced the messenger expression of steroidogenesis enzyme StAR in cells overexpressing the wild-type GPRC6A, but not in cells overexpressing the loss-of-function F464Y mutant receptor [92]. Consistently, GPRC6A-null mice and mice injected with F464Y mutant showed a decrease of testis messenger expression of steroidogenesis enzymes, with consequent reduction of circulating testosterone levels, and final clinical manifestation of feminization, by demonstrating that the osteocalcin receptor mediated osteocalcin-induced effect on testosterone production through the stimulation of expression of steroidogenesis enzymes by Leydig cells [91–93]. Taken together, these studies suggest that VitD genomic stimulation of osteocalcin expression might have an indirect relevant role in modulating testosterone production by the testis. Beyond the effect on testosterone, *in vitro* and *in vivo* studies in animals investigated the effects of VitD on estradiol production. In an *in vivo* study in VDR-null mice, both aromatase expression and activity in the testis were significantly reduced, although serum estradiol levels showed only a slight reduction, compared to wild-type mice [94], suggesting that estradiol synthesis might be affected by VitD signaling ablation. Furthermore, an *in vitro* study on rat Sertoli cells showed that VitD significantly induced aromatase expression by both genomic and non-genomic mechanisms [28, 32]. *In vivo* studies in VDR-null mice highlighted that the expression of AMH, INH-B and INSL3 in the testis was not directly influenced by VDR status, since comparable levels of the relative messengers were detected in VDR-null and wild-type mice [76]. Further evidences demonstrated that a non specific effect of VitD on intracellular calcium homeostasis might influence testis hormone secretion. Indeed, in *in vivo* studies in rats, VitD induced calcium uptake in the testis via the activation of non-genomic pathways [33]; these findings are consistent with *in vitro* studies on mouse Sertoli cells, which showed increased exocytosis and activated non-genomic pathways, following VitD treatment [44]. These results, however, only indicated that VitD might increase calcium-mediated exocytosis, and, possibly, hormone secretion by Sertoli cells, and did not provide any evidence on the specific hormone which might be influenced by this action. In conclusion, the effect of VitD on testosterone production is

still debated, since results of different studies are not completely consistent, in animal models; however a possible stimulation of testosterone production might be hypothesized, and include an indirect effect of VitD on testosterone synthesis, mediated by a genomic VitD-induced expression of osteocalcin, and a direct effect of VitD on testosterone secretion, probably obtained through the genomic VitD-induced expression of calbindin-D_{28K}. The effect of VitD on estradiol synthesis is more consistent, although not clearly demonstrated but postulated by the direct genomic and non-genomic VitD-induced increase of aromatase expression and activity in the testis. Lastly, VitD does not seem to be implicated in the modulation of AMH, INH-B and INSL3 production. A summary of the effects of VitD on hormone production by the testis, in animal models, is provided in Table 2. A schematic overview of the proposed mechanisms driving the effects of VitD on hormone production by the testis, in animal models, is provided in Table 3.

5.1.2 Human studies

The relationship between vitamin D status, determined by the assessment of the surrogate marker 25-hydroxy-vitamin D3 circulating levels, and the effects of vitamin D supplementation on circulating levels of testis hormones, have been investigated by observational and interventional studies, respectively, but the results are controversial; moreover, experimental studies are scarce. The comparison among clinical studies is challenging, due to discrepancies in subjects cohorts characteristics, such as age, BMI and baseline vitamin D status. The difference in the statistical analysis among the studies also contributes to challenge the interpretation of data, particularly in relation to the presence or absence of adjustment of eventual confounding factors; therefore, when adjustment has been performed, exclusively the data that persisted after adjustments for confounders in the different studies has been considered in the current review. The observational studies in cohorts of men of different ages or in cohorts of young men, showed that circulating levels of 25-hydroxy-vitamin D3 were not associated to circulating levels of total or free testosterone [95–101], except for one study [102]; nevertheless, some of these studies showed a positive association between circulating levels of 25-hydroxy-vitamin D3 and SHBG [95–97, 102], suggesting that VitD might influence the circulating levels of SHBG, rather than the total circulating levels of testosterone, and, consequently, might contribute to the modulation of the bioavailable fraction of testosterone. Similarly, a recent study in young infertile men, showed no significant correlation between circulating 25-hydroxy-vitamin D3 levels and total testosterone, although a positive association with SHBG, and a negative correlation with free testosterone were found [103]. Nevertheless, a study on a mixed cohort of male partners from infertile couples and from couples with desire of parenthood, failed to report any association between circulating 25-hydroxy-vitamin D3 and both

Table 2 Effects of Vitamin D on Testis Function: Hormone Production and Semen Quality

REF.	SPECIES	HORMONE LEVELS				
		Testosterone	*Estradiol*	*AMH*	*INH-B*	*INSL3*
Sonnenberg J. 1986	*Rat*	+	NA	NA	NA	NA
Blomberg Jensen M. 2013	*Mouse*	=	=	=	=	=
Kinuta K. 2000	*Mouse*	NA	=	NA	NA	NA

REF.	SPECIES	REPRODUCTION		SEMINAL PARAMETERS		
		Mating Ratio	*Fertility Ratio*	*Sperm Count*	*Sperm Motility*	*Sperm Morphology*
Kwiecinski G.G. 1989	*Rat*	+	+	NA	NA	NA
Uhland A.M. 1992	*Rat*	+	+	NA	NA	NA
Sood S. 1992	*Rat*	NA	NA	+	NA	NA
Sood S. 1995	*Rat*	NA	NA	+	NA	NA
Kinuta K. 2000	*Mouse*	NA	NA	+	+	NA
Sun W. 2015	*Mouse*	NA	NA	+	+	NA

+, increase; =, no change. Abbreviations: NA, Not Assessed.

Table 3 Proposed Mechanisms Underlying the Effects of Vitamin D on Testis Function

SPECIES	HORMONE PRODUCTION		
	Testosterone		
	Action	***Mechanism of action***	***Effect***
Animals	Direct	G, ↑ calbindin-D_{28K}	NA, ↑*
	Indirect	G, ↑ osteocalcin	NA, ↑*
Humans	Direct	G, ↑ steroidogenesis enzymes	↑
	Indirect	G, ↑ osteocalcin	NA, ↑*
	Estradiol		
	Action	***Mechanism of action***	***Effect***
Animals	Direct	G and NG,↑ aromatase	=
Humans	NA	NA	NA
	AMH		
	Action	***Mechanism of action***	***Effect***
Animals	Na	Na	=
Humans	NA	NA	NA
	INH-B		
	Action	***Mechanism of action***	***Effect***
Animals	Na	Na	=
Humans	NA	NA	NA
	INSL3		
	Action	***Mechanism of action***	***Effect***
Animals	Na	Na	=
Humans	NA	NA	NA

SPECIES	SEMEN QUALITY		
	Action	***Mechanism of action***	***Effect***
Animals	Direct	modulation of calcium homeostasis	↑ sperm motility
	Direct	NG, ↑ cell proliferation, ↓ apoptosis	↑ sperm count
Humans	Direct	NG, ↑ intracellular calcium	↑ sperm motility
	Direct	NG, ↑ activation of intracellular pathways	↑ sperm motility

↑, increase; =, no effect. Abbreviations: G, Genomic; NG, Non-genomic; NA, Not Assessed; Na, Not applicable. *Hypothesized but not demonstrated effect.

total and free testosterone, as well as SHBG levels [104]. The observational studies in cohorts of elderly men are highly controversial; indeed, some studies reported the absence of association between circulating 25-hydroxy-vitamin D3 and total testosterone or free testosterone or SHBG levels [105–107], whereas different studies reported a positive association with total or free testosterone [108–112], one study reported a negative association with free testosterone [113], and two studies reported a positive association with SHBG levels [109, 113]. The differential relationship between vitamin D status and circulating testosterone levels in young or elderly men could be partially explained by the absence or presence of age-related comorbidities, such as metabolic and cardiovascular diseases, as well as lifestyle or physical activity, which might independently affect vitamin D status and circulating testosterone levels, and have been heterogeneously addressed in adjusted statistical models. Anyway, the current available data are still insufficient to suggest a causal relationship between vitamin D status and circulating testosterone levels. Although the relationship between vitamin D status and testosterone production has been investigated by various studies, few observational studies specifically assessed the relationship between vitamin D deficiency and hypogonadism, characterized by testosterone deficiency, by reporting controversial results. Population-based studies [105, 106, 108–110], as well as studies in cohorts of men with primary or secondary hypogonadism [114, 115], reported that men with hypogonadism had significantly lower circulating 25-hydroxy-vitamin D3 levels, compared to eugonadal men [105, 109, 110, 114, 115], and that the prevalence and the odd ratio for hypogonadism were increased in men with vitamin D deficiency, compared to men with vitamin D sufficiency [105, 106, 108–110]. Conversely, different population-based studies [104, 111, 113], and one study in men with congenital hypogonadotropic hypogonadism [116], showed that circulating 25-hydroxy-vitamin D3 levels were not significantly different in hypogonadal compared to eugonadal men [104, 116], and that hypogonadism was not associated to vitamin D deficiency [111, 113]; surprisingly, one study showed an association between hypogonadism and the highest quintile of circulating 25-hydroxy-vitamin D3 levels [104]. Lastly, one study in children with hereditary VitD-resistant rickets, characterized by the expression of non functional VDR, showed that testis stimulation with hCG induced a normal testosterone response, suggesting that testosterone production was independent from VDR status [117]. The interventional studies showed conflicting results. Indeed, the results of one study in young men showed that very short-term (4 days) supplementation with VitD did not influence circulating levels of total testosterone [118]. A lack of effect of supplementation was also highlighted in one study in young men with hypogonadism, which showed similar circulating levels of total testosterone before and after short-term

(3 months) supplementation with vitamin D3 or 25-hydroxy-vitamin D3 [119]. On the other hand, the long-term supplementation (12 months) with vitamin D2 and vitamin D3 in cohorts of men of different ages produced controversial results, since either a significant increase [120] or no change [112, 121, 122] in total testosterone, and a significant increase [122] or no change [121] in free testosterone and SHBG, was reported after supplementation. Lastly, very long-term supplementation (24 months) with 25-hydroxy-vitamin D3 did not modify circulating levels of total testosterone [114]. A complementary set of interventional studies evaluated the relationship between VitD and testosterone through a different approach, namely the assessment of the effect of testosterone replacement on circulating levels of 25-hydroxy-vitamin D3, in men with hypogonadism of different etiology. The majority of studies showed that neither short-term (6 months) [65, 123], nor long-term (1 year up to 5 years) [114, 115, 121, 124, 125] testosterone replacement induced significant changes in circulating levels 25-hydroxy-vitamin D3 in men replaced with testosterone, compared to baseline levels [65, 114, 123–125], or to a placebo-controlled group [115, 121]. Conversely, only two studies showed that short-term (2 weeks to 3 months) testosterone replacement induced a significant increase in circulating levels of VitD [126, 127]. The experimental studies, which might definitely demonstrate whether VitD has a role in regulating testosterone production, are scarce, and the molecular mechanisms that might link vitamin D status to testosterone production have not been fully addressed. One *in vitro* study on primary cultures of Leydig cells, demonstrated that treatment with VitD significantly induced the messenger expression of enzymes involved in testosterone synthesis, and increased testosterone secretion [128], suggesting that VitD might have a direct role in testosterone synthesis by Leydig cells, mediated by VitD genomic actions. Additionally, an indirect effect of VitD on testosterone synthesis, mediated by genomic VitD-driven osteocalcin production, has been proposed. Experimental studies demonstrated that defects in osteocalcin signaling might be a contributor of male infertility, since a study in a group of men referring to a fertility center and subjected to genetic screening, showed that two patients harboring a rare F464Y mutation of osteocalcin GPRC6A receptor displayed testis failure, with low or low-normal circulating testosterone levels, and high circulating LH levels, and oligozoospermia [92]. These observations support the hypothesis that the VitD genomic stimulation of osteocalcin expression might have an important role in modulating testosterone production by the testis. The observational studies assessing the relationship between vitamin D status and circulating levels of estradiol are quite consistent, and showed that circulating levels of 25-hydroxy-vitamin D3 were not associated to circulating levels of estradiol [96–100, 102, 104–107, 111, 120], except for one study in elderly men, which reported a positive association [108], and a study in young infertile men which showed a negative association [103]. One interventional study demonstrated that 24 months of supplementation with 25-hydroxy-vitamin D3 did not influence circulating estradiol levels [114]. Although *in vitro* studies on ovarian, as well as prostate and breast carcinoma cell lines showed that VitD induced a variable cell type-specific effect on aromatase gene expression by means of genomic actions [129, 130], this effect has not been investigated in the testis. The observational studies assessing the relationship between vitamin D status and production of AMH and INH-B are scarce and evidences scattered, whereas no studies focused on the relationship between vitamin D status and production of INSL3. A study in a cohort of middle-aged and elderly men found that circulating levels of 25-hydroxy-vitamin D3 were positively associated to circulating AMH levels [131]. It might be hypothesized that VitD increases AMH gene expression by means of a direct genomic action; this mechanism is supported by the presence of VDRE in AMH promoter, and by the evidence that VitD increased AMH expression in *in vitro* cultured prostate cancer cells [132]. Conversely, no association was found between circulating levels of 25-hydroxy-vitamin D3 and INH-B [97, 131]. These evidences seem to suggest that a vitamin D status might influence AMH, rather than Sertoli cell endocrine function, in men of general population. Conversely, in young infertile men, a positive association between circulating levels of 25-hydroxy-vitamin D3 and INH-B was reported [103]. Inferral of a causal relationship between vitamin D status and production of AMH and INH-B is dampened by the descriptive design of observational studies and by the lack of experimental studies. In conclusion, data from observational and interventional studies on the relationship between vitamin D status and testis hormone production are quite inconsistent or controversial, and experimental studies are scarce. No specific causal relationship between vitamin D status and testosterone production seems to be supported, although VitD might be hypothesized to modulate circulating levels of SHBG, rather than the total circulating levels of testosterone, and, consequently, might contribute to the modulation of the bioavailable fraction of testosterone; nevertheless, *experimental* studies suggest that VitD might have a direct role in testosterone synthesis by Leydig cells, mediated by genomic VitD stimulation of steroidogenesis enzymes expression, and an indirect role, mediated by genomic VitD stimulation of osteocalcin expression. Similarly, no causal relationship seems to occur between vitamin D status and circulating levels of estradiol, and INH-B, whereas a positive association with AMH was reported by one study. In infertile men, a different pattern of relationships seems to occur, namely a negative association with estradiol and a positive association with INH-B, suggesting that in infertile men, VitD might have a different role in regulating endocrine testis function, compared to fertile men. Nevertheless, further experimental research is needed to

Table 4 Summary of Clinical Observational and Interventional Studies - Relationship Between Vitamin D and Hormone Production

REF.	Type of study	HORMONE LEVELS							Notes
		Age	*TT*	*FT*	*SHBG*	*E2*	*AMH*	*INH-B*	
Chin K.Y. 2015	*OBS*	*20+*	=	=	↑	NA	NA	NA	*1*
Valimaki V.V. 2004	*OBS*	*18–20*	=	=	↑	=	NA	NA	*2*
Ramlau-Hansen C.H. 2010	*OBS*	*18–21*	=	NA	↑	=	NA	=	*3*
Hammoud A.O. 2012	*OBS*	*18–67*	=	=	=	=	NA	NA	*4*
Lerchbaum E. 2014	*OBS*	*20–58*	=	=	=	=	NA	NA	*Unadjusted*
Livshits G. 1999	*OBS*	*18–91*	=	NA	NA	=	NA	NA	*5*
Wulaningsih W. 2014	*OBS*	*20+*	=	=	=	=	NA	NA	*6*
Ceglia L. 2011	*OBS*	*30–79*	=	=	NA	NA	NA	NA	*7*
Anic G.M. 2016	*OBS*	*20+*	↑	NA	↑	NA	NA	NA	*8*
Blomberg Jensen M. 2016	*OBS*	*28–38*	=	↓	↑	↓	NA	↑	*9*
Wang N. 2015	*OBS*	*Na*	=	=	=	=	NA	NA	*10*
Lee D.M. 2012	*OBS*	*40–79*	=	=	=	=	NA	NA	*11*
Chen R.Y.T. 2008	*OBS*	*Na*	=	=	=	=	NA	NA	*Unadjusted*
Nimptsch K. 2012	*OBS*	*40–75*	↑	↑	NA	↑	NA	NA	*12*
Wehr E. 2010	*OBS*	*Na*	↑	↑	↑	NA	NA	NA	*13*
Tak Y.J. 2015	*OBS*	*40+*	↑	↑	NA	NA	NA	NA	*14*
Rafiq R. 2015	*OBS*	*65–89*	↑	=	=	=	NA	NA	*15*
Zhao D. 2017	*OBS*	*45–84*	=	↓	↑	NA	NA	NA	*16*
Dennis N.A. 2012	*OBS*	*54–93*	NA	NA	NA	NA	↑	=	*Unadjusted*
HeijboerA.C. 2015	*OBS/INT*	*42–86*	↑/=	NA	NA	NA	NA	NA	*Unadjusted/17*
Jorde R. 2013	*INT*	*21–75*	=	=	=	NA	NA	NA	*17*
Žofková I. 1989	*INT*	*20–38*	NA	=	NA	NA	NA	NA	*18*
Foresta C. 2015	*INT*	*Mean 34*	=	NA	NA	NA	NA	NA	*18*
Pilz S. 2011	*INT*	*Na*	=	↑	↑	NA	NA	NA	*18*
Canguven O. 2017	*INT*	*35+*	↑	NA	NA	=	NA	NA	*18*
Ferlin A. 2015	*INT*	*19–53*	=	NA	NA	=	NA	NA	*18*

↑, increase or positive association; ↓, decrease or negative association; =, no change or no association. Abbreviations: OBS, Observational study; INT, Interventional study; TT, total testosterone; FT, free testosterone; E2, estradiol; Na, Not available; NA, Not Assessed. 1, Adjusted for: age, BMI, race/ethnicity; 2, Unadjusted; SHBG adjusted for: body weight, free testosterone and estradiol; 3, Adjusted for: season, diseases of reproductive organs, smoking, maternal smoking or alcohol intake during pregnancy, time of blood sampling; 4, Adjusted for: age, BMI, season, alcohol intake and smoking; 5, Adjusted for: age; 6, Adjusted for: age, race/ethnicity, % body fat, diabetes, alcohol intake, smoking, physical activity, serum calcium and creatinine; 7, Adjusted for: age, race/ethnicity, season; 8, Adjusted for: age, race/ethnicity, % body fat, smoking; 9, Adjusted for: age, BMI, smoking; 10, Adjusted for: age, BMI, residence area, economic status, smoking, HOMA-IR, diabetes and systolic pressure; 11, Adjusted for: age, BMI, study site, season, alcohol intake, smoking, physical activity, physical function, heart conditions, hypertension, diabetes and depression; 12, Adjusted for: age, BMI, season, geographical region, smoking, physical activity, time of blood collection. Total testosterone further adjusted for SHBG.; 13, Adjusted for: age, BMI, alcohol intake, smoking, beta-blocker use, statin use and diabetes; 14, Adjusted for: Total testosterone adjusted for age, BMI, waist circumference, % body fat, fasting plasma glucose, diabetes, dyslipidemia. Free testosterone adjusted for age, muscle mass, total cholesterol, diabetes, dyslipidemia, alcohol intake; 15, Adjusted for: age, BMI, season, alcohol intake, smoking, number of chronic diseases, physical function, serum creatinine; 16, Adjusted for: age, BMI, race/ethnicity, study site, smoking, education, self-reported good health status, physical activity, diabetes, systolic blood pressure, use of antihypertensive medications, eGFR, total cholesterol, HDL cholesterol, use of lipid lowering medication, C-reactive protein; 17, placebo-controlled; 18, compared to baseline.

determine the definitive role of VitD on testis hormone production, in both fertile and, particularly, infertile men, in order to address the discrepancies observed in clinical studies, and to clarify the underlying molecular mechanisms. A summary of clinical observational and interventional studies in humans is provided in Table 4. A schematic overview of the proposed mechanisms driving the effects of VitD on hormone production by the testis in humans is provided in Table 3.

5.2 Vitamin D and testis function: Semen quality

The effects of VitD on semen quality and fertility have been investigated in experimental *in vivo* studies in animals. In humans, the effect of VitD on semen quality and/or fertility has been investigated in three different types of studies: 1) clinical observational studies evaluated the relationship between vitamin D status and semen quality

and/ or fertility; 2) one clinical interventional study evaluated the effect of vitamin D supplementation on semen quality; and 3) experimental studies on human spermatozoa investigated on the molecular mechanisms underlying VitD effect on semen quality.

5.2.1 Animal studies

The effects of vitamin D status, determined by the assessment of both VitD and the surrogate marker 25-hydroxy-vitamin D3 circulating levels, on semen quality and fertility have been investigated in *in vivo* studies in animal models. Studies in male rats showed that successful mating was significantly reduced in vitamin D-depleted animals, and insemination of female rats with spermatozoa from these rats resulted in significantly decreased pregnancy rate, compared to vitamin D-repleted rats [133]. A more recent *in vivo* study, however, demonstrated that restoring normal circulating calcium levels in hypocalcaemic vitamin D-depleted rats improved pregnancy rate, although this rate was still reduced compared to normocalcaemic vitamin D-repleted rats; these results suggested that the detrimental effects of vitamin D deficiency on male rats might be at least partially related to an impairement of calcium homeostasis, and to the consequent reduced calcium concentrations in the reproductive system [57, 134]. The effects of vitamin D deficiency on male fertility might be justified by an impairment of spermatogenesis, and an impairment of semen quality in animals [135, 136]. Indeed, *in vivo* studies showed that in vitamin D-depleted rats, Sertoli cell function was compromised, as assessed by glutamyl transpeptidase activity, and testicular and epididymal sperm count were reduced, as compared to vitamin D-repleted rats, and accompained by degenerative changes in the germinal epithelium [135]; these effects were reversed by supplementing an adequate dose of vitamin D3 [136]. In VDR-null mice, and in mice with 1α-hydroxylase deletion, resulting in vitamin D deficiency and reduced circulating calcium levels, histological abnormalities of the testis, with a decrease of proliferation and an increase of apoptosis in germ cells, were observed, and impairment of spermatogenesis was reported, toghether with a significant reduction of sperm count and motility, compared to wild-type group [94, 137]. Normalization of circulating calcium levels were shown to reverse the pathological changes affecting testis histology, spermatogenesis, and semen quality [137], thus reinforcing the hypothesis that vitamin D deficiency might exert detrimental effects on semen quality and fertility by interfering with calcium homeostasis, and, consequently, calcium concentrations in the reproductive system, and by modulating the balance between cell proliferation and apoptosis in germ cells [137]. A summary of the effects of VitD on semen quality and male fertility, in animal models, is provided in Table 2. A schematic overview of the proposed mechanisms driving the effects of VitD on semen quality, in animal models, is provided in Table 3.

5.2.2 Human studies

The relationship between vitamin D status, determined by the assessment of the surrogate marker 25-hydroxy-vitamin D3 circulating levels, and semen quality and fertility, has been investigated by observational studies, but the results are controversial for some seminal parameters; the effects of supplementation with vitamin D3, and the molecular mechanisms underlying the effects of VitD on semen quality, have been investigated by one interventional study, and several experimental studies on human spermatozoa, respectively. The observational studies investigating the relationship between vitamin D status and semen quality and fertility show a great heterogeneity in study design, particularly as concerns the subject cohort characteristics and the methodology and cut off values used for the assessment of vitamin D status. The difference in the statistical analysis among the studies also contributes to challenge the interpretation of data, particularly in relation to the presence or absence of adjustment of eventual confounding factors; when adjustment has been performed, exclusively the data that persisted after adjustments for confounders in the different studies has been considered in the current review. The observational studies suggested that VitD might be favourable to human male fertility potential; indeed, a seasonal variation in the conception rate was reported in the northern hemisphere, with a peak of fertility during the summer, coinciding with the highest circulating levels of 25-hydroxy-vitamin D3 [138]. Consistently, a recent study reported that men with vitamin D deficiency had longer history of infertility, compared to men with adequate vitamin D status [103]; moreover, in infertile couples subjected to gonadotropin-induced mono-ovulation and timed intercourses, infertile couples whose male partner had optimal circulating levels of 25-hydroxy-vitamin D3, showed a higher pregnancy rate, compared to couples in which male partner had vitamin D deficiency [139]. The observed associations between vitamin D status and male fertility potential might be mediated by the beneficial effects of vitamin D status on semen quality. A consistent positive association was found between circulating 25-hydroxy-vitamin D3 levels and sperm total motility and/or progressive motility in men from the general population [98, 140], men with a fertility profile [141, 142], and men with a subfertility or infertility profile [11, 103, 141–143]; only one study on the general population was not able to show any association between circulating 25-hydroxy-vitamin D3 levels and sperm total motility [97]. Moreover, several studies showed that both vitamin D deficiency or excess might be detrimental to sperm motility [78, 97, 98, 103, 140, 141]. These results strongly support the hypothesis that VitD might have a role in modulating sperm

motility. The relationship between vitamin D status and sperm count and morphology is highly controversial. Indeed, circulating levels of 25-hydroxy-vitamin D3 were reported to be either unrelated or positively associated to sperm count in men from the general population [97, 98, 140], fertile [142], or subfertile and infertile [103, 142, 143] men. The majority of studies showed that circulating levels of 25-hydroxy-vitamin D3 were not associated with sperm morphology in men from the general population [97, 98], fertile [143], or subfertile and infertile [11, 103, 143] men; conversely, one study in men from the general population [140], and one study in fertile and infertile men [141] showed a positive association. Lastly, two observational studies evaluated the prevalence of vitamin D deficiency in men with infertility associated with impairment of semen quality, due to different abnormalities of semen parameters; one study reported that infertile men with oligozoospermia and/or asthenozoospermia, or azoospermia had lower circulating levels of 25-hydroxy-vitamin D3, compared to fertile men, and that circulating 25-hydroxy-vitamin D3 levels were positively associated with sperm count and sperm progressive motility [142]. These findings were confirmed by a different study on oligo-astheno-teratozoospermic male partners of infertile couples, showing that circulating 25-hydroxy-vitamin D3 levels were positively associated to sperm motility [143]. Only one interventional study evaluated the effects of vitamin D3 supplementation for 3 months in men with oligo-asthenozoospermia, and showed a significant increase in both sperm progressive motility and pregnancy rate, compared to men not receiving supplementation [144]. The experimental studies demonstrated that a direct non-genomic action of VitD on human spermatozoa might be the driver of the beneficial effects of VitD. Indeed, VitD was shown to increase sperm motility and intracellular calcium concentration in both capacitated and uncapacitated spermatozoa, through the activation of inositol trisphosphate-receptor-gated intracellular calcium reservoir, located in the neck of spermatozoa [15, 55, 56, 140, 145], and to increase cholesterol efflux and tyrosine/threonine protein phosphorylation [55, 56, 145]; these molecular pathways have a key role in sperm motility and in the processes of capacitation and acrosome reaction, which are necessary for proper spermatozoa function. Lastly, VitD was shown to increase lipid metabolism, which is considered an important source of energy for spermatozoa capacitation [55, 56, 145]. In conclusion, data from observational and interventional studies investigating the relationship between vitamin D status and semen quality and fertility, suggest that VitD might have a beneficial effect on male reproductive health, which seems to be, at least partially, mediated by a strong causal relationship with improved sperm motility in men from the general population, as well as in fertile and infertile men. This hypothesis is further corroborated by experimental studies on human spermatozoa, showing that VitD modulates several molecular pathways involved in spermatozoa functions, and increases sperm motility, by a direct non-genomic action. Conversely, the relationship between vitamin D status and sperm count or sperm morphology is controversial. A summary of clinical observational and interventional studies, in humans, is provided in Table 5. A schematic overview of the proposed mechanisms driving the effects of VitD on semen quality, in humans, is provided in Table 3.

Table 5 Summary of Clinical Observational and Interventional Studies - Relationship Between Vitamin D and Semen Quality

REF.	Type of study	*Age*	*Cohort*	SEMINAL PARAMETERS			*Notes*
				Sperm count	*Sperm motility*	*Sperm morphology*	
Ramlau-Hansen C.H. 2010	*OBS*	18–21	*Healthy Subjects*	=	= (T)	=	*1*
Hammoud A.O. 2012	*OBS*	18–67	*Healthy Subjects*	↑	↑ (P)	=	*2*
Blomberg Jensen M. 2016	*OBS*	28–38	*Infertile Subjects*	↑	↑ (T)	=	*3*
Blomberg Jensen M. 2011	*OBS*	18–21	*Healthy Subjects*	=	↑(T, P)	↑	*4*
Yang B. 2012	*OBS*	20–40	*Fertile and Infertile Subjects*	NA	↑ (P)	↑	*5*
Tirabassi G. 2016	*OBS*	Mean 33	*Subfertile/Infertile Subjects**	NA	↑(T, P)	=	*6*
Zhu C.L. 2016	*OBS*	Mean 28	*Fertile and Infertile Subjects*	↑	↑ (P)	NA	*Unadjusted*
Abbasihormozi S. 2016	*OBS*	20–50	*Subfertile Subjects*	=	↑ (T)	=	*7*
Deng X.L. 2014	*INT*	Na	*Infertile subjects*	Na	↑ (P)	Na	*8*

↑, increase or positive association; ↓, decrease or negative association; =, no change or no association; *Subjects referring for fertility issues. Abbreviations: OBS, observational study; INT, interventional study; T, total motility; P, progressive motility; Na, Not available; NA, Not Assessed. 1, Adjusted for: season, diseases of reproductive organs, smoking, maternal smoking or alcohol intake during pregnancy, abstinence, spillage during semen collection, time from collection; 2, Adjusted for: age, BMI, season, alcohol intake and smoking; 3, Adjusted for: age, BMI, smoking, season, abstinence, time from collection, free testosterone and estradiol, total estradiol, testosterone/estradiol ratio, SHBG; 4, Adjusted for: season, medications, fever, abstinence, serum calcium, FSH; 5, Adjusted for: season, abstinence, time from collection; 6, Adjusted for: age, BMI, varicocele, total testosterone; 7, Adjusted for: age, BMI, season; 8, compared to baseline.

6 Vitamin D and testis cancer

VitD has been shown to modulate a wide number of cancer-related molecular pathways, by interfering with cancer cell proliferation, differentiation, and apoptosis, as well as angiogenesis and epithelial-to-mesenchymal transition, which are events involved in cancer progression [146]. Testis cancer can arise from the entire series of cell populations of the testis, but the majority originate from a pre-invasive precursor lesion, derived from germ cells, and named carcinoma in situ (CIS), [147], and are referred to as germ cell tumors (GCT), which most commonly affect young men and represent a risk for fertility [148]. Few studies evaluated the potential effects of VitD in testis cancer, mainly focusing on GCT, but epidemiological evidences linking potential VitD actions to testis cancer are lacking. VDR and VitD metabolizing enzymes are highly expressed in human testis CIS, and show a reduced expression in more aggressive tumors [58, 59, 149, 150], suggesting that VitD might inhibit neoplastic cell proliferation [149]. Moreover, VitD was shown to induce osteogenic differentiation *in vitro*, in human seminoma (TCam-2) and human embryonal carcinoma pulmonary metastasis (NTera2) cell lines, and in *in vivo* NTera2 mouse xenografts [149]. Lastly, VitD was shown to potentiate the antiproliferative effects of cisplatin by down-regulation of pluripotency genes and up-regulation of oncosuppressor genes *in vitro*, in NTera2 cells, and *in vivo*, in NTera2 mouse xenografts [151–153]. These experimental findings demonstrated that VitD might exert relevant actions on several cancer-related features, including cell proliferation, differentiation, and progression, in testis cancer models, by means of the observed effects on the expression of selected genes, and suggest that VitD might potentially serve as adjuvant treatment in combination with chemotherapy, by possibly encouraging chemotherapy regimen reduction and fertility preservation in these young patients.

7 Conclusions

VitD has been suggested to play a role in male fertility, as highlighted by clinical and experimental studies in animals and humans, mainly exerting a beneficial effect on semen quality. The co-expression of VDR and VitD metabolizing enzymes in animal and human reproductive system, particularly in the testis, suggests that VitD might be synthesized in the testis and might exert paracrine or autocrine actions, by potentially modulating testis hormone production and spermatogenesis. In animals, the relationship between vitamin D status and testosterone production is not completely consistent in experimental studies, showing either reduced or unchanged circulating testosterone levels in vitamin D-deficient or VDR-null animals. In humans, the relationship between vitamin D status and testosterone production or prevalence of hypogonadism is even more controversial. The majority of clinical studies in men from the general population did not find association between vitamin D status and circulating testosterone levels, but some studies showed a positive association between vitamin D status and circulating SHBG levels, supporting the hypothesis that VitD might contribute to the modulation of the bioavailable testosterone. Moreover, some clinical studies demonstrated the presence of vitamin D deficiency in men with hypogonadism, and an increased prevalence or risk for hypogonadism in men with vitamin D deficiency, although other studies failed to demonstrate these findings. Additionally, the effect of vitamin D supplementation on circulating levels of testosterone showed conflicting results, with the majority of studies reporting lack of an effect. Nevertheless, animal and human experimental studies suggest both an indirect and a direct effect of VitD on testosterone production, mediated by genomic VitD actions. Conversely, vitamin D status was not associated to testis estradiol production in humans, although in animals a direct genomic and non-genomic action of VitD was demonstrated on aromatase expression and activity. On the other hand, testis AMH production was found to be not associated in animals, and positively associated in humans, to vitamin D status, although the potential mechanisms underlying this association have not been specifically investigated. Animal and human studies demonstrated that VitD exerts a beneficial effect on semen quality, particularly on sperm motility, which is probably driven by the modulation of factors involved in spermatozoa function, namely, calcium homeostasis, in animals, and by direct non-genomic VitD actions on spermatozoa, in humans. In particular, in human spermatozoa, non-genomic actions of VitD included the modulation of intracellular calcium levels and of spermatozoa lipid metabolism, with consequent improvement of sperm motility, sperm capacitation, and acrosome reaction. Taken together, available studies in animals and humans suggest that VitD exerts the most consistent beneficial effect on semen quality, and suggest that vitamin D supplementation might improve sperm motility in men with vitamin D deficiency displaying asthenozoospermia, and, possibly, subfertile men from couples enrolled in assisted reproductive programs. Lastly, vitamin D supplementation might be considered in men with hypogonadism and suboptimal vitamin D status, in order to normalize VitD levels, although further investigation is needed to definitely clarify the effects of vitamin D supplementation on testosterone production. In addition, dedicated experimental studies aimed at better characterizing the definitive role of vitamin D in male fertility are encouraged, in order to fulfill the need of shedding new light in this extremely challenging field.

AMH, anti-Müllerian hormone; Bax, bcl-2-like protein 4; Bcl-xl, B-cell lymphoma-extra large; CDK2, cyclin-dependent kinase 2; CIS, carcinoma in situ; DBP, vitamin D-binding

protein; FGF23, fibroblast growth factor 23; GCT, germ cell tumors; hCG, human chorionic gonadotropin; INH-B, inhibin-B; INSL3, insulin-like 3; PTH, parathyroid hormone; PTH-rP, PTH-related peptide; RXR, retinoid X receptor; VDR, VitD receptor; VDRE, VitD response element; VitD, 1α,25-dihydroxy-vitamin D3; SHBG, sex hormone-binding globulin.

Authors' contributions CdA and MG conceived and developed the manuscript in all its aspects, performed the literature search, wrote the manuscript, conceived and prepared tables and figs. CP substantially contributed to the writing of the section on the expression of VDR and VitD metabolizing enzymes in the male reproductive system. FG, DM and CS substantially contributed to the writing of the sections on clinical studies. GG substantially contributed to the writing of the sections on the relationship between VitD and semen quality. AV substantially contributed to the preparation of tables and figs. AC critically reviewed and revised the manuscript. RP is the principal investigator, helped conceive and supervised the manuscript drafting, critically reviewed and revised it for important intellectual content. FC was added as author in the revised version of the manuscript since she provided a significant contribution to the scientific content of the manuscript during the revision process; in particular, she substantially contributed to the writing of the section on testis function. MP was added as author in the revised version of the manuscript since she provided a significant contribution to the graphic content of the manuscript during the revision process; in particular, she substantially contributed to schematization of data and to the preparation of tables and figures. All authors, including those listed on the first submitted version of the manuscript and those added to the revised version, read and approved the final manuscript. All authors, including those listed on the first submitted version of the manuscript and those added to the revised version, agree to the proposed new authorship.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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