REPRODUCTION
REVIEW

Vitamin D metabolism, sex hormones, and male reproductive function

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Abstract

The spectrum of vitamin D (VD)-mediated effects has expanded in recent years, and VD is now recognized as a versatile signaling molecule rather than being solely a regulator of bone health and calcium homeostasis. One of the recently identified target areas of VD is male reproductive function. The VD receptor (VDR) and the VD metabolizing enzyme expression studies documented the presence of this system in the testes, mature spermatozoa, and ejaculatory tract, suggesting that both systemic and local VD metabolism may influence male reproductive function. However, it is still debated which cell is the main VD target in the testis and to what extent VD is important for sex hormone production and function of spermatozoa. This review summarizes descriptive studies on testicular VD metabolism and spatial distribution of VDR and the VD metabolizing enzymes in the mammalian testes and discusses mechanistic and association studies conducted in animals and humans. The reviewed evidence suggests some effects of VD on estrogen and testosterone biosynthesis and implicates involvement of both systemic and local VD metabolism in the regulation of male fertility potential.
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Introduction

The spectrum of vitamin D (VD)-mediated effects has broadened in recent years beyond the regulation of bone health and calcium homeostasis (Holick & Chen 2008). The main source of circulating VD is endogenous synthesis in the skin, where u.v.-B radiation from the sun initiates conversion of 7-dehydrocholesterol to inactive VD (cholecalciferol). Only small amounts of cholecalciferol are present in some dietary products, so in the absence of adequate sunlight, the supply of VD largely depends on fortification of dietary products or individual use of oral VD supplements (Holick & Chen 2008). Cholecalciferol undergoes two hydroxylation steps to form the active VD compound 1,25(OH)$_2$D$_3$ (Fig. 1; Ponchon *et al*. 1969, Gray *et al*. 1972, Prosser & Jones 2004). 25-Hydroxylation is mainly mediated by the hepatic enzyme CYP2R1 supported by CYP27A1. The newly formed 25-OHD$_3$ enters circulation and is subsequently hydroxylated by the renal 1α-hydroxylase (CYP27B1) to 1,25(OH)$_2$D$_3$ that binds and activates the VD receptor (VDR) in the target cell, until it is inactivated by CYP24A1 (Prosser & Jones 2004). Clinical VD status is determined by measuring serum level of 25-OHD$_3$, which is associated with bone mineral density (BMD), serum calcium level, and parathyroid hormone (PTH) secretion (Holick 2006, Lips 2006, Bouillon *et al*. 2008). Traditionally, four clinical subgroups are defined by serum levels of 25-OHD$_3$: VD deficiency <25 nmol/l (nM) (<10 ng/ml), VD insufficiency <50 nM, VD sufficiency ≥ 50 nM, and high VD status ≥ 75 nM (Dawson-Hughes *et al*. 2005, Ross 2011). In recent years, several studies suggested that 25-OHD$_3$ serum levels below 75 nM (30 ng/ml) should be considered insufficient, but the debate about optimal VD serum levels is ongoing (Heaney & Holick 2011, Holick *et al*. 2011).

Results from *Vdr* knockout mice demonstrated that *VDR* expression is necessary for most of the VD-mediated effects (Bouillon *et al*. 2008). VDR is a classical steroid receptor ubiquitously expressed in most tissues and organs. After substrate binding (normally 1,25(OH)$_2$D$_3$), VDR heterodimerizes with RXR and mediates genomic effects in the nucleus through binding to VD response elements (VDRE) in target genes and subsequent regulation of transcription (Fig. 2; Haussler *et al*. 2011). The subcellular location of the receptor is important as VDR also mediates rapid nongenomic effects when situated in the membrane or cytoplasm (Fig. 2; Haussler *et al*. 2011). Cellular VD responsiveness is complex and depends not only on *VDR* expression. The liver and kidney were previously thought to be the only organs with the ability to activate VD, but extra-renal expression of *CYP2R1*, *CYP27A1*, *CYP27B1*, and *CYP24A1* has been shown in many tissues initially thought only to be VD responsive (Nagakura *et al*. 1986,

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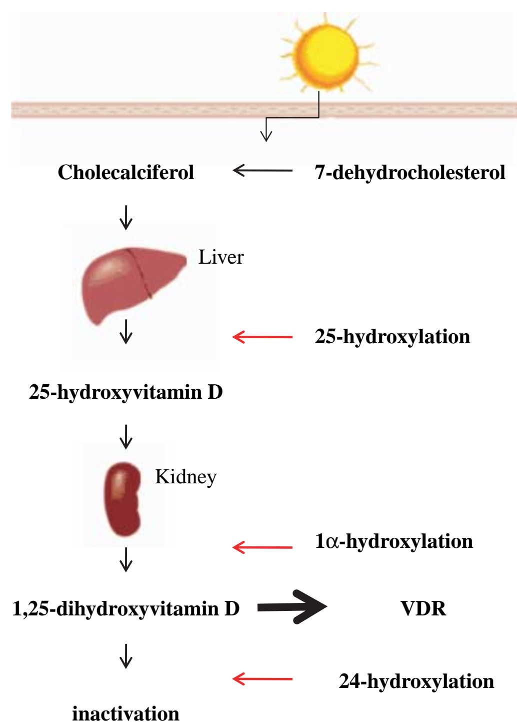

Gene name	Vitamin D metabolism	Protein function	Chromosomal location human	Chromosomal location mice	KO model
CYP2R1	Activation	25-hydroxylase	11p15.2	11	–
CYP27A1	Activation	25-hydroxylase	2q33-qter	1	–
CYP27B1	Activation	1α-hydroxylase	12q13.1-q13.3	10	+
VDR	Mediator	Receptor	12q13.11	15	+
CYP24A1	Inactivation	24-hydroxylase	20q13	2	+

Figure 1 Systemic vitamin D (VD) metabolism. VD metabolism normally starts in the skin, where u.v.-B radiation from the sun initiates conversion of 7-dihydrocholesterol to cholecalciferol. Cholecalciferol is not biologically active but has to undergo two enzymatic steps before active $1,25(OH)_2D_3$ is formed. Normally, 25-hydroxylation takes place in the liver by CYP2R1, while 1α-hydroxylation is conducted in the kidney following tubular reabsorption by CYP27B1. The activated form of VD $1,25(OH)_2D_3$ mediates its effect through the VD receptor (VDR) until inactivated by 24-hydroxylation. The table below indicates the factors involved in VD metabolism. KO refers to mice knockout model.

Choudhary *et al.* 2005). This implies that in addition to the mandatory presence of VDR, the cellular response to VD is influenced by the cells' uptake of substrate and metabolism of the circulating forms of VD (Fleet 2008). Moreover, cellular VD metabolism is not as tightly regulated as the systemic VD metabolism. Local VD metabolism is very sensitive to the intracellular concentration of $1,25(OH)_2D_3$ as the promoter of the *CYP24A1* gene contains activating VDREs and the transcription is upregulated by $1,25(OH)_2D_3$ to titrate an adequate cellular VD response and to balance activation of VDR in response to increased intracellular $1,25(OH)_2D_3$ (Haussler *et al.* 2011).

While the essential role of VD in bone turnover and function and for calcium transportation in intestine and kidney is well established knowledge, the involvement of VD in male reproductive function has only relatively recently been identified. This review summarizes and evaluates the expression of *VDR* and VD metabolizing enzymes in the testes of mammals supported by association and functional studies in order to clarify the role of serum and *in vitro* effects of VD on male reproductive function.

VDR expression and signaling in testes

Kream *et al.* (1977) showed that $1,25(OH)_2D_3$ has a specific high-affinity binding site to cytoplasmic homogenates of the rat testis. The testis-specific binding of $1,25(OH)_2D_3$ differed from the known binding site in plasma (supposedly VD binding protein) and was not influenced by competitive binding of 25-OHD_3 in the nanomolar range. This is in line with the later reported difference in dissociation constant (K_d) for the two VDR substrates. The following studies (predominantly using an autoradiographic approach) corroborated the presence of VDR in testes from both mice and rats, mainly in tissues obtained from young animals (Merke

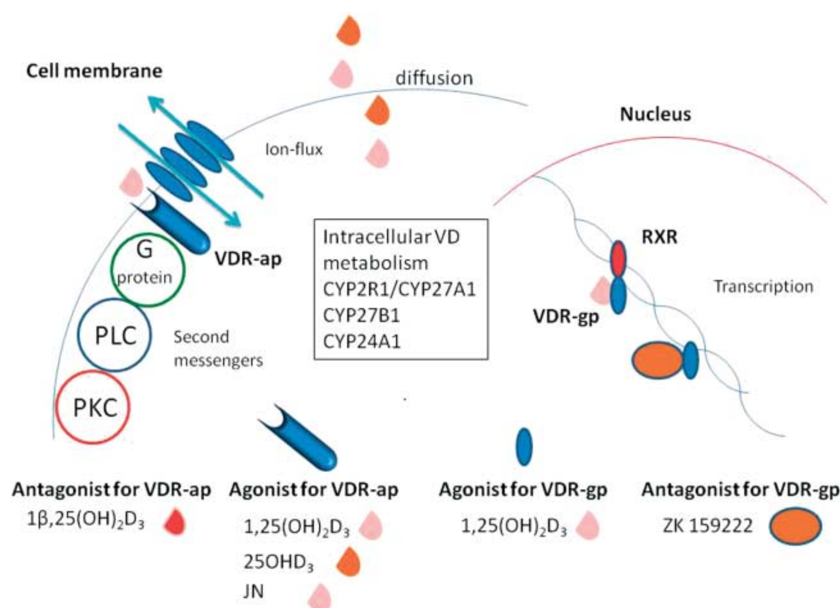

Figure 2 Schematic overview of the proposed cellular metabolism and actions of vitamin D (VD). The VD receptor (VDR) mediates rapid actions when situated at the membrane or in the cytoplasm through ion channel activation or modulation of second messengers. The VD progenitors diffuse freely across the plasma membrane and the intracellular concentration of VD progenitors is titrated by the presence of the VD metabolizing enzymes. VDR regulates transcription of ∼1000 genes in the nucleus. VDR heterodimerizes with RXR that subsequently binds to VDRE in target genes and regulates transcription. VDR also mediates rapid nongenomic effects through the alternative ligand-binding pocket (VDR-ap), while genomic effects are mediated through the genomic ligand-binding pocket (VDR-gp).

et al. 1983, 1985, Walters *et al.* 1983, Levy *et al.* 1985*a*, 1985*b*, Schleicher *et al.* 1989). It should be mentioned that receptors for 1,25$(OH)_2D_3$ were present in similar amounts in isolated seminiferous tubules and interstitial tissue of adult rats (Levy *et al.* 1985*a*). Hereafter, Sertoli cells were introduced as the main VD target cell in rodent testes, as an autoradiographic approach showed nuclear VDR binding exclusively in mice Sertoli cells, while the presence of VDR was sparse and predominantly cytoplasmic in germ cells (Schleicher *et al.* 1989). Subcellular expression of *VDR* determines the downstream signaling pathway (genomic or nongenomic) (Fig. 2). VDR is expressed in both nucleus and cytoplasm of primary cultures of immature Sertoli cells and in the immature mice Sertoli cell line TM4, and 1,25$(OH)_2D_3$ mediates fast nongenomic effects in both cell lines (Akerstrom & Walters 1992, Majumdar *et al.* 1994, Menegaz *et al.* 2010, 2011, Rosso *et al.* 2011, Zanatta *et al.* 2011*a*, 2011*b*). VDR is also expressed in spermatocytes and spermatids of both rodents and humans (Blomberg Jensen *et al.* 2010*a*, 2010*b*, Zanatta *et al.* 2011*c*), but Sertoli cells are still considered to be the main VD target in the adult testis (Zanatta *et al.* 2011*c*). This assumption may not be entirely correct as testicular *VDR* expression increases during puberty and the observed temporal correlation of increased VDR levels with testicular maturation suggests a better correlation to testicular function and spermatogenesis than to growth of the organ *in vivo*. If Sertoli cells were the main VD target, then the increased *VDR* expression during puberty would imply that mature Sertoli cells have a higher *VDR* expression than the immature form, because Sertoli cells do not proliferate after puberty (Sharpe *et al.* 2003). Sertoli cells mature following stimulation with FSH and testosterone and cease their production of anti-Müllerian hormone (AMH) and estrogen (Sharpe *et al.* 2003). VDR epitopes were found in spermatogonia, Sertoli cells, and spermatocytes in testes from 9-month-old rats (Johnson *et al.* 1996). Accordingly, *Vdr* expression was shown in spermatogonia and Sertoli cells of 8-week-old mice (Hirai *et al.* 2009). However, VDR is absent or at best weakly expressed in few mature Sertoli cells in adult human testis samples (Blomberg Jensen *et al.* 2010*b*). VDR is expressed predominantly in adult germ cells, consistent with a high postpubertal *VDR* expression (Walters 1984, Osmundsen *et al.* 1989, Blomberg Jensen *et al.* 2010*b*) and the immunohistochemical (IHC) and immunocytochemical (ICC) studies using different VDR antibodies (Fig. 3; Johnson *et al.* 1996, Nangia *et al.* 1998, Corbett *et al.* 2006, Blomberg Jensen *et al.* 2010*b*). VDR was also detected in spermatogonia, spermatocytes, and Sertoli cells in roosters (Oliveira *et al.* 2008). A functional role of VDR in adult germ cells is underpinned by the presence of classical VD-regulated genes such as calbindin subtypes, aromatase (*CYP19A1*), and the transient receptor potential cation channel subfamily V 5 (*TRPV5*) in both germ cells and spermatozoa (Kagi *et al.* 1988, Li *et al.* 2008, Carreau & Hess 2010). It remains to be investigated whether VD affects proliferation and/or maturation of the immature Sertoli cells (including AMH production) and whether the VD-regulated genes that are transcribed during spermatogenesis subsequently influence gamete quality and function.

Some controversy exists regarding the presence of VDR in Leydig cells. It is an important question to answer as *VDR* expression is a prerequisite for a direct effect of VD on systemic sex hormone production. Initially, VDR was found in similar amounts in isolated seminiferous tubules and interstitial tissue from adult rats,

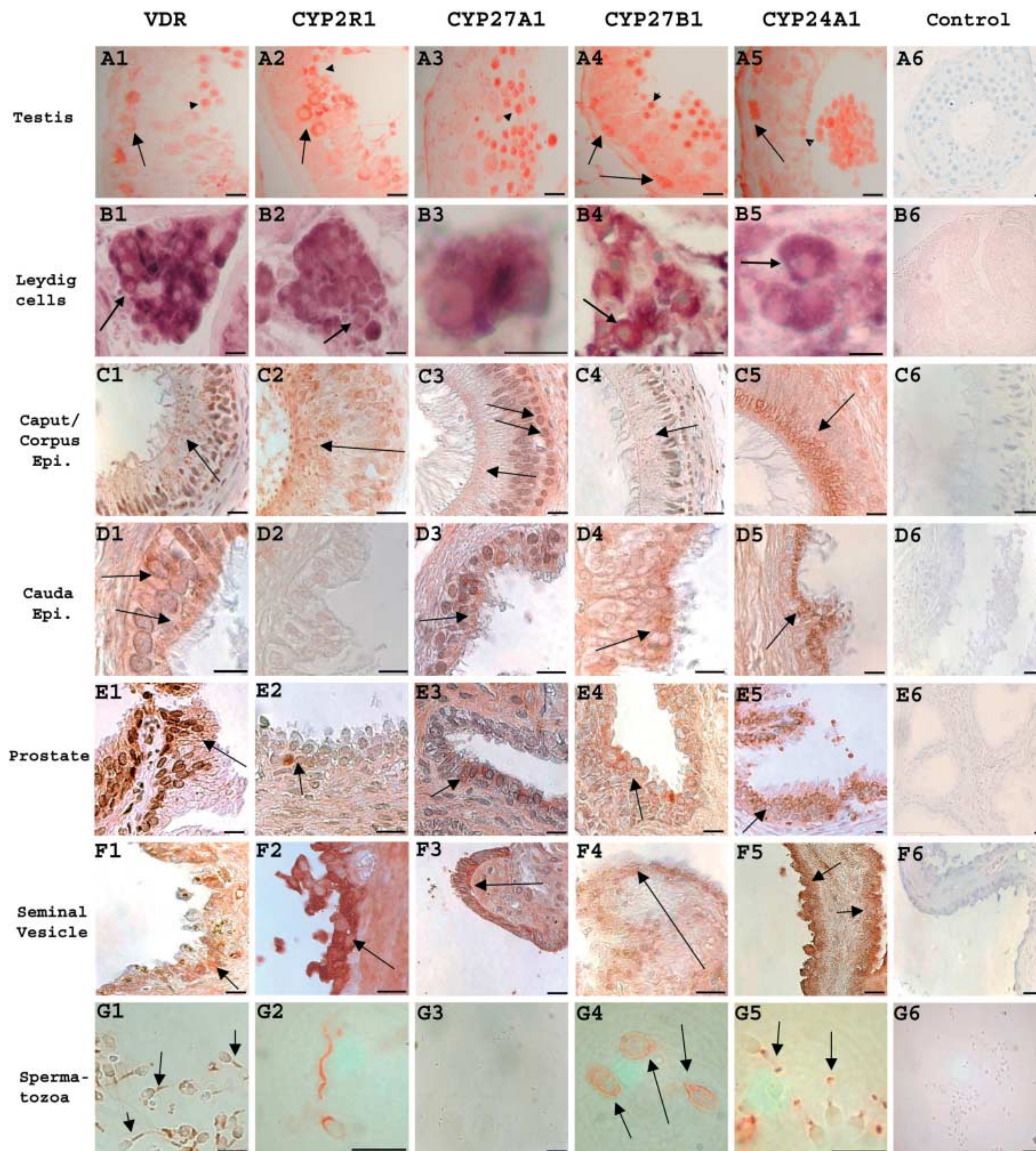

Figure 3 Expression of *VDR* and vitamin D (VD) metabolizing enzymes in the human testis and male reproductive tract. (A1) *VDR* expression in the nucleus and cytoplasm (black arrow) of spermatogonia and in round spermatids (arrowhead). (A2) *CYP2R1* expression in late spermatocytes (arrow) and round spermatids (arrowhead). (A3) *CYP27A1* expression in round spermatids (arrowhead). (A4 and A5) Expression of *CYP27B1* and *CYP24A1* in spermatogonia (arrows) and round spermatids (arrowheads). (B1–5) Cytoplasmic expression of *VDR* and all the metabolizing enzymes in Leydig cells. (C1–5) Black arrows indicate expression of *VDR* and all the enzymes in the luminal vesicles, while double arrow points on expression of *CYP27A1* in basal cells of caput/corpus epididymis. (D1–5) Abundant cytoplasmic expression of all investigated proteins except for *CYP2R1* (D2) in cauda epididymis. (E1) Nuclear and cytoplasmic staining (arrow) in the epithelium of the prostate. (E2 and E3) A few cells (arrow) with a marked cytoplasmic expression of *CYP2R1* and *CYP27A1* in the prostate gland. (E4 and E5) Abundant expression of *CYP27B1* and *CYP24A1* in the prostate. (F1–5) A strong cytoplasmic expression of VDR and all the enzymes in the epithelium of the seminal vesicle. (G1) Expression of *VDR* in the midpiece (arrows). (G2) *CYP2R1* expression in par, neck, and tail of ejaculated spermatozoa. (G3) No detectable expression of *CYP27A1* in sperm. (G4) Expression of *CYP27B1* in par and neck of spermatozoa. (G5) Detection of *CYP24A1* in the neck and at the annulus (arrows). (A6–G6) Negative controls, all except B6 and G6 counterstained with Mayer's. A6, B6, and G6 used monkey secondary antibody, while C6, D6, E6, and F6 used goat secondary antibody. Bar corresponds to 10 μm. Figure reproduced from **Blomberg Jensen M, Nielsen JE, Jorgensen A, Rajpert-de ME, Kristensen DM, Jorgensen N, Skakkebaek NE, Juul A & Leffers H** 2010*b* Vitamin D receptor and vitamin D metabolizing enzymes are expressed in the human male reproductive tract. *Human Reproduction* **25** 1303–1311. By permission of Oxford University Press.

which imply that interstitial Leydig cells could be a likely target of VD (Levy *et al*. 1985*a*). This observation was questioned by later studies showing no *VDR* expression in Leydig cells from younger rodents (Merke *et al*. 1985, Stumpf *et al*. 1987) concluding that Sertoli and germ cells were the only testicular targets of VD. This was supported by an IHC investigation in rats showing *VDR* expression in the intratubular cells but not in Leydig cells (Johnson *et al*. 1996). However, these results are not in accordance with more recent IHC studies that all show detectable *VDR* expression in Leydig cells from humans, roosters, and mice (Oliveira *et al*. 2008, Hirai *et al*. 2009, Blomberg Jensen *et al*. 2010*b*). A recent study showed presence of *Vdr* mRNA in Leydig cells from rats, and the abundance of *Vdr* in the Leydig cells was comparable with the observed expression of *Vdr* in immature Sertoli cells (Zanatta *et al*. 2011*a*). The observed discrepancies between species could imply that *VDR* expression is not conserved in the Leydig cells, although it is more likely to be due to differences in antibody specificity, method selection, age of the animals, etc. All IHC studies investigating protein expression in Leydig cells must be interpreted with caution as unspecific staining is a frequent finding when using commercially available antibodies and must be carefully optimized (Blomberg Jensen *et al*. 2010*b*). However, the presence of *VDR* mRNA in laser capture microdissected human Leydig cells revealed that *VDR* messenger was present in isolated human Leydig cells (unpublished data). Antibody selection is vital and may also be influencing the results in a recent study that found no *VDR* expression in 80 human testis tissue samples using a nonclassified polyclonal VDR antibody (Bremmer *et al*. 2012). Only two VDR antibodies have been extensively validated and should be preferred for detection of *VDR* expression (Blomberg Jensen *et al*. 2010*a*, Wang *et al*. 2010). In conclusion, accumulating evidence suggest that VDR may be expressed in Leydig cells: i) co-localization of the VD metabolizing enzymes in human Leydig cells (Blomberg Jensen *et al*. 2010*a*), ii) expression of VD-regulated genes such as Calbindin and Calretinin, and iii) the observed transcriptional changes of these genes in Leydig cells in response to VD deficiency in chickens (Kagi *et al*. 1988, Strauss *et al*. 1994, Inpanbutr *et al*. 1996).

VDR is also expressed in human spermatozoa, and the subcellular location and function of VDR in human spermatozoa were recently reviewed (Blomberg Jensen & Dissing 2012). The presence of VDR in human sperm supported the observed *VDR* expression during earlier stages of spermatogenesis as spermatozoa are virtually transcriptionally silent (Habib *et al*. 1990, Nangia *et al*. 1998, 2007, Corbett *et al*. 2006, Aquila *et al*. 2008, Blomberg Jensen *et al*. 2010*b*). *VDR* expression was observed in only a fraction of human spermatogonia and spermatocytes, while it was expressed in most spermatids (Blomberg Jensen *et al*. 2010*b*). The presence of VDR indicates that germ cells are exposed to VD progenitors and receptor binding can be achieved with $1,25(OH)_2D_3$ concentrations below 1 nM. The observed binding affinity is in line with a previous study showing that K_d for VDR in the rat testis was 50 pM (Gensure *et al*. 1991). Later *in vitro* studies also showed rapid effects when using similar $1,25(OH)_2D_3$ concentrations (50 pM–1 nM) in both immature rodent Sertoli cells and ejaculated human spermatozoa (Blomberg Jensen *et al*. 2011, 2012, Menegaz *et al*. 2011, Zanatta *et al*. 2011*c*). Spermatozoa are highly compartmentalized cells and each compartment has different functions reflected by the spatial expression of several ion channels, transporters, etc., that are confined to a particular compartment (Publicover *et al*. 2007). All studies investigating *VDR* expression in mature human spermatozoa showed *VDR* expression in the post-acrosomal part of the head, midpiece, and in the neck region (Corbett *et al*. 2006, Aquila *et al*. 2008, Blomberg Jensen *et al*. 2010*b*). Moreover, the reported pattern of *VDR* expression was comparable with the expression of VD-binding protein in the postacrosomal region, neck, and midpiece of human spermatozoa (Yu *et al*. 1994). These findings were corroborated by a recent investigation of VD metabolizing enzymes in human spermatozoa, which showed co-localization of VDR with the VD metabolizing enzymes. Interestingly, *VDR* expression not only differed among men but also among spermatozoa from the same individual, and the receptor was mainly detected in the neck and head region of morphologically normal spermatozoa (Blomberg Jensen *et al*. 2010*b*). Human spermatozoa are a heterogeneous population of cells, and even in fertile men, a large proportion ($>80\%$) of spermatozoa are morphologically abnormal and only few are able to reach and fertilize the egg (Skakkebaek *et al*. 1994, Ikawa *et al*. 2010). The presence of VDR in a distinct subcellular location may thus depend on both optimal spermatogenesis and maturation as incomplete maturation could lead to aberrant localization and potentially malfunction of the receptor (Jimenez-Gonzalez *et al*. 2006, Blomberg Jensen *et al*. 2012). The presence of VDR in human spermatozoa supports that germ cells is the main site for VDR-mediated effects in adult testis and that VDR mediates nongenomic effects in ejaculated spermatozoa.

Expression of the VD metabolizing enzymes

VD is metabolized by the microsomal (CYP) enzymes situated in the endoplasmatic reticulum or mitochondria (Table 1). Gene mutations in humans and knockout mouse models have clarified which enzymes are involved in VD metabolism (Ponchon *et al*. 1969, Gray *et al*. 1972, Ohyama & Yamasaki 2004, Prosser & Jones 2004, Jones *et al*. 2011). The specificity of the CYP enzymes varies; some are very substrate specific, while others have multiple functions and substrates. Several

Table 1 Vitamin D and serum levels of testosterone in men.

No.	Design	*n*	Age	Cohort	BMI	TT	FAI	LH	Comment	Reference
1	Intervention	8	NA	Hemodialysis patients	NA	↑	NA	NA	$1,25(OH)_2D_3$ treatment 2–4 months	Blumberg *et al.* (1980)
2	Intervention	3	<18	VD resistant rickets	NA	NA	NA	NA	Normal l hCG test	Hochberg *et al.* (1985)
3	Case–control	14	>50	Spinal-stenosis	NA	→	→	→		Jackson *et al.* (1987)
4	Intervention	9	30	Healthy	NA	→	NA	→	$1,25(OH)_2D_3$ i.v. sensitizes FSH secretion to GNRH	Zofkova *et al.* (1996)
5	Association	187	46	Healthy	NA	→	NA	NA		Livshits *et al.* (1999)
6	Association	204	20	Healthy	22.6	→	→	NA		Valimaki *et al.* (2004)
7	Association	55	59	Healthy	29	→	→	NA		Chen *et al.* (2008)
8	Association	2229	67	Coronary angiography	27	↑	↑	↓		Wehr *et al.* (2010)
9	Association	307	20	Healthy	23	→	↓	→	Few men with VD deficiency	Ramlau-Hansen *et al.* (2011)
10	RCT	54	48	Obese	33	→	↑	NA	Conclusion limited by study design	Pilz *et al.* (2011)
11	Association	1115	48	Healthy	28	→	→	NA		Ceglia *et al.* (2011)
12	Association	1362	66	Healthy	26	↑	↑	NA		Nimptsch *et al.* (2012)
13	Association	3369	59	Healthy	27	↑	↑	↓	Associations not significant after adjustment for confounders	Lee *et al.* (2012)

Thirteen human studies investigating the relationship between serum testosterone and vitamin D were evaluated. This table summarizes study design, cohort size and characteristics, main results, and important additional information. N, cohort size; BMI, body mass index; TT, total testosterone; FAI, free androgen index; →, no change; ↑, positively associated; ↓, negatively associated; NA, not available.

CYP enzymes are capable of mediating 25-hydroxylation of cholecalciferol, but CYP2R1 is recognized as the most important 25-hydroxylase probably supported by CYP27A1 (Ohyama & Yamasaki 2004). This was convincingly demonstrated *in vitro*, where cellular overexpression of *CYP2R1* caused transcriptional activation of *VDR* following addition of cholecalciferol. The resultant secosteroid product of CYP2R1 activation proved to be $25\text{-}OHD_3$ and co-expression of CYP2R1 with 1α-hydroxylase (*CYP27B1*) elicited additive activation, whereas co-expression with 24-hydroxylase (*CYP24A1*) caused inactivation (Cheng *et al.* 2003). Moreover, a patient homozygous for a transition mutation in exon 2 of the *CYP2R1* gene had low serum levels of $25\text{-}OHD_3$ thereby supporting *CYP2R1* candidacy as the microsomal VD 25-hydroxylase. Conversely, mice with a disrupted *Cyp27a1* gene showed no significant change in serum $25\text{-}OHD_3$ and $1,25(OH)_2D_3$ (Rosen *et al.* 1998). *CYP2R1* mRNA is expressed in various tissues, but it is predominantly found in the liver and testes of both mice and humans, indicating a role in activation of cholecalciferol not only in the liver but also in the male gonad (Choudhary *et al.* 2003, 2005, Bieche *et al.* 2007). *CYP27B1* is the only known 1α-hydroxylase (Gray *et al.* 1972, Ohyama & Yamasaki 2004) and strong evidence that it is mandatory for the formation of $1,25(OH)_2D_3$ came from observations in patients with kidney failure, children with inactivating mutations in the *CYP27B1* gene, and *Cyp27b1* knockout mice (Ritz *et al.* 1979, Panda *et al.* 2001). On the other hand, CYP24A1 was confirmed as the VD inactivating enzyme by inactivating mutations that caused infantile hypercalcemia due to lack of $1,25(OH)_2D_3$ degradation and further supported by knockout models (St-Arnaud 1999, St-Arnaud *et al.* 2000, Dauber *et al.* 2011, Ji & Shen 2011, Streeten *et al.* 2011).

Expression of VD metabolizing enzymes has been reported in several tissues of both animals and humans, with the highest concentrations of *CYP2R1*, *CYP27B1*, and *CYP24A1* mRNA in kidney specimens followed by testis (Akeno *et al.* 1997, Cheng *et al.* 2003). Our own studies on human testis samples revealed that VDR and all the VD metabolizing enzymes were co-expressed in a subset of spermatocytes, all spermatids, most Leydig cells, and a fraction of ejaculated spermatozoa, while only VDR, CYP27B1, and CYP24A1 were expressed in spermatogonia (Fig. 3; Blomberg Jensen *et al.* 2010*b*). Interestingly, VDR and all the VD metabolizing enzymes co-localized in the postacrosomal region of the head and in the neck of mature human spermatozoa, but CYP24A1 was distinctly expressed at the sperm annulus, together with VDR (Blomberg Jensen *et al.* 2010*b*, 2012). VDR and the VD metabolizing enzymes, including CYP24A1, were expressed in a higher fraction of spermatozoa from normal men compared with sperm from infertile men, suggesting a possible relationship between VD and high semen quality (Blomberg Jensen *et al.* 2012). In particular, CYP24A1 may be suitable marker for semen quality, because this enzyme is a strong indicator for the presence of activated *VDR* and the expression at the sperm annulus is easy to recognize (Fig. 4; Blomberg Jensen *et al.* 2012). CYP24A1 may also be a marker of adequate VD stimulation during spermatogenesis as its presence in transcriptionally inactive spermatozoa depends on the concentration of $1,25(OH)_2D_3$ that induces transcription through binding to VDRE's in the *CYP24A1* promoter region (Haussler *et al.* 2011). *CYP24A1* expression not only distinguished

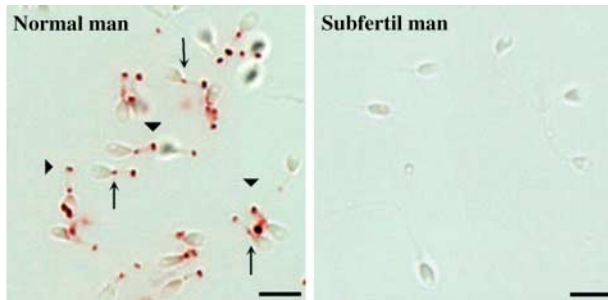

Figure 4 *CYP24A1* expression in human spermatozoa. Semen sample from a man from the general population with sperm concentration: 123 million/ml and 87% motile sperm. 85% of his spermatozoa are expressing *CYP24A1* at the annulus (left). Semen sample from a man part of an infertile couple with sperm concentration: 6 million/ml and 50% motile sperm. This man has 0% expressing CYP24A1 at the annulus (right). Arrowhead indicates annulus and arrows indicate expression at the neck. Bar corresponds to 10 μm. Figure reproduced from **Blomberg Jensen M, Jørgensen A, Nielsen JE, Leffers H, Andersen AN, Skakkebaek NE, Juul A, Rajpert-De-Meyts E & Jørgensen N** 2012 Expression of the vitamin D metabolizing enzyme CYP24A1 at the annulus of human spermatozoa may serve as a novel marker of semen quality. *International Journal of Andrology*. By permission of John Wiley and Sons.

spermatozoa from normal and infertile men with high specificity but was also positively associated with a number of semen quality variables (sperm count, concentration, motility, and morphology), thus supporting the use of CYP24A1 as a marker of semen quality (Blomberg Jensen *et al*. 2012).

The presence of VDR and the VD metabolizing enzymes in the testis and epididymis suggests that VD is metabolized locally (Fig. 3), which is in line with earlier studies that have shown metabolism of VD in the male reproductive organs following s.c. injections of tritiated 25-OHD_3 in mice. The highest concentration of the VD progenitor was found in the kidney, followed by epididymis, prostate, testis, and seminal vesicle, and the concentrations were augmented by intravenous route of dosing (Kidroni *et al*. 1983). A recent paper reported that CYP2R1 was exclusively expressed in the Leydig cells and suggested that testes were important not only for local VD metabolism but also for systemic activation of VD (Foresta *et al*. 2010, 2011). These authors observed that both serum levels of 25-OHD_3 and testicular expression levels of CYP2R1 were lower in men with oligozoospermia or Sertoli-cell-only syndrome (SCO) compared with normal men and explained this by low expression of *CYP2R1* in the Leydig cells (Foresta *et al*. 2011). However, from the pictures presented in the manuscript (Foresta *et al*. 2011), it is evident that *CYP2R1* is also expressed in the germ cells, in accordance with our previous study using the same CYP2R1 antibody (Blomberg Jensen *et al*. 2010*a*). Thus, the low CYP2R1 content in the testis from oligozoospermic or SCO men could be due to few or no germ cells and not due to an aberrant *CYP2R1* expression in the Leydig cells.

VD regulates biosynthesis of estradiol and testosterone

Numerous studies investigated the putative physiological relationship between circulating levels of $25OHD_3$/1,25$(OH)_2D$, the activity of VDR and VD metabolizing enzymes, and serum testosterone and estradiol levels in both animals and humans (Tanaka *et al*. 1976, Hyldstrup *et al*. 1984, Small *et al*. 1984, Hochberg *et al*. 1985, Krabbe *et al*. 1986, Sonnenberg *et al*. 1986, Hagenfeldt *et al*. 1992, Morley *et al*. 1993, Inpanbutr *et al*. 1996, Zofkova & Kancheva 1996, Otremski *et al*. 1997, Rapado *et al*. 1999, Kinuta *et al*. 2000, van Abel *et al*. 2002, Braga *et al*. 2002, Van Cromphaut *et al*. 2003, Echchgadda *et al*. 2004, Valimaki *et al*. 2004, Kastelan *et al*. 2009, Fleet & Schoch 2010, Krishnan *et al*. 2010*a*, 2010*b*, Meng *et al*. 2010, Pilz *et al*. 2011, Ramlau-Hansen *et al*. 2010, Wehr *et al*. 2010, Ceglia *et al*. 2011, Foresta *et al*. 2011, Lundqvist *et al*. 2011, Lee *et al*. 2012). It has been shown that estrogen promotes the two-step activation of cholecalciferol to 1,25$(OH)_2D_3$ and reinforces a positive effect on calcium homeostasis through a direct stimulatory effect on intestinal calcium absorption. Testosterone is also a stimulator of calcium absorption in prepubertal boys, but studies in mice show that androgens increase calcium excretion by inhibiting expression of renal calcium transport proteins (Fleet & Schoch 2010, Hsu *et al*. 2010).

Regulation of aromatase (*CYP19A1*) and estrogen production by VD has been extensively studied in tissue- and cell lines from breast, adipose tissue, bone, and gonads (Krishnan *et al*. 2010*a*, 2010*b*). 1,25$(OH)_2D_3$ is a known regulator of aromatase expression (Bouillon *et al*. 2008, Haussler *et al*. 2011), but the effect is tissue specific due to activity of different promoters (1.3,1.4,II) in various tissues. 1,25$(OH)_2D_3$ mediates transcriptional repression of aromatase expression in breast, in contrast to a modest gonadal induction (ovary) and a high induction in bone (Krishnan *et al*. 2010*b*). The proposed tissue-specific regulation is in line with an earlier study showing lower aromatase expression in testis and epididymis concomitant with lower serum estrogen and elevated gonadotropins in *Vdr* KO mice compared with wild-type animals (Kinuta *et al*. 2000). Interestingly, the reduced serum estrogen level was reversible following calcium supplementation, indicating that at least part of the impaired gonadal aromatase expression was due to hypocalcemia. In contrast, the elevated LH level persisted and may therefore depend on additional factors besides calcium imbalance (Kinuta *et al*. 2000). A recent study supported the tissue-specific 1,25$(OH)_2D_3$-mediated regulation of aromatase expression using three different cell lines. Besides tissue-specific regulation of aromatase, they also found that 1,25$(OH)_2D_3$ induced androgen production in

breast and prostate but caused an opposite effect in adrenal cells (Lundqvist *et al*. 2011).

It is noteworthy that the observed *in vitro* effects seemed less pronounced *in vivo* as VD-deficient chickens had higher (although not significantly) serum testosterone levels than controls, despite that VD deficiency caused low calbindin expression in the Leydig cells (Inpanbutr *et al*. 1996). However, another study found low serum testosterone in VD-deficient rats, which increased to normal values following supplementation of $1,25(OH)_2D_3$ (Sonnenberg *et al*. 1986). Importantly, a small human study conducted on boys younger than 18 years with VD-resistant rickets (no functional VDR) showed that the sensitivity to LH was not affected by VD, as serum testosterone concentration after hCG stimulation (hCG test) was normal (Hochberg *et al*. 1985). Several human studies (Table 1) have investigated the relationship between 25-OHD_3 or $1,25(OH)_2D_3$ and testosterone production. A direct comparison of these selected studies is not advisable because the cohorts differ greatly in size, age, comorbidities, median 25-OHD_3 level, and the use of relevant confounders such as time of blood sampling, serum LH, calcium, etc. The positive associations between serum 25-OHD_3 and testosterone/free androgen index (FAI) are mainly reported in men above 40 years of age, with a median BMI above 25 and presence of comorbidities such as metabolic syndrome, diabetes, or cardiovascular disease. By contrast, in younger healthy men, serum levels of 25-OHD_3 were either not associated or negatively associated with testosterone and FAI, while serum 25-OHD_3 was positively associated with SHBG (Valimaki *et al*. 2004, Ramlau-Hansen *et al*. 2011). Other studies have shown that both serum 25-OHD_3 and testosterone levels decline with age, while gonadotropins and SHBG increase. The age-related alterations may be of clinical importance and probably partly responsible for the increased risk of osteoporosis with age (van Abel *et al*. 2006, Giovannucci *et al*. 2006, Kaplan *et al*. 2006, Bischoff-Ferrari 2008, van Schoor *et al*. 2008, Ross 2011, Lee *et al*. 2012). Simple associations do not prove causality but may rather reflect other imbalances as indicated in a large study examining 1340 men aged 76 years or older. Here, total testosterone inversely correlated with serum phosphorous, while calcium levels were positively correlated with PTH (Meng *et al*. 2010).

Accumulating evidence suggests a complex interplay between bone, gonadal function, calcium absorption and excretion, glucose metabolism, and pituitary function (Fukumoto & Martin 2009, Hwang *et al*. 2011, Oury *et al*. 2011, Pi *et al*. 2011): sex hormones regulate calcium absorption, bone formation, and insulin production; VD stimulates calcium absorption, bone formation, estrogen and insulin production; and bone markers such as osteocalcin stimulate testosterone and insulin production. The complicated relationship must be taken into account when investigating associations between VD and sex hormones, and it is not advisable to extrapolate from simple associations between two factors, because the disturbance or imbalance in a selected organ may be fully or partly compensated by various mechanisms; for instance, low serum VD levels by calcium mobilization due to elevated PTH secretion. Such compensation may be decreasing with age, which could explain the positive associations reported in older men with comorbidities, but further studies are needed to clarify this. New association studies should therefore address the relationship between sex hormones and VD levels by including additional factors such as calcium, PTH, phosphate, LH, estrogen, and optimally also BMD, osteocalcin, IGF1, FGF23, body fat percentage, and glucose metabolism to determine causal associations that can be subsequently used as endpoints for validation in randomized clinical trials (RCT).

Most of the association studies investigating VD and testosterone stratified their cohort in tertiles or quartiles. This can be appropriate, but the conclusions drawn may be limited, in particular when 25-OHD_3 serum level is the explanatory variable. VD has the highest impact on most organs in the concentration range from undetectable to 50 nM (20 ng/ml), while most of the effects are stable when serum 25-OHD_3 levels exceed 75–100 nM (Dawson-Hughes *et al*. 2005, Lips 2006, Lips *et al*. 2010). Thus, the clinical relevance is reduced when the median VD concentration in the cohort is high, as an increase in serum 25-OHD_3 level from 80 to 90 nM is unlikely to have any major physiological effect. Recently, a large association study showed a dose–response relationship between 25-OHD_3 and testosterone. It was comparable to the effects observed between VD and PTH secretion or calcium absorption, because it was strong in the lower range of the serum 25-OHD_3 scale and leveled off above 50–80 nM (Nimptsch *et al*. 2012). This could be due to a direct effect of VD progenitors on the Leydig cells, although it could also be secondary to changes in calcium homeostasis caused by VD deficiency (Bouillon *et al*. 2008). Unfortunately, this study was limited by the low proportion of VD-deficient men, thus precluding investigation of associations at the very low end of 25-OHD_3 levels (<25 nmol/l), where the effect presumably would be larger. Moreover, most of the studies reporting a positive association between VD and total testosterone/FAI are limited by an age effect (Table 1). Men with high 25-OHD_3 serum concentrations are generally younger and have a higher testosterone level than men with low 25-OHD_3. The age difference between each quartile is more than 1 year in the large association study investigating more than 2000 men referred for coronary angiography, and a similar age trend was found from the lowest to the highest VD quintile in a recent association study (Wehr *et al*. 2010, Nimptsch *et al*. 2012). Although most studies

adjust their analyses for age, they are not adjusted for the frequency of comorbidities such as impaired bone health, glucose metabolism, kidney function, calcium absorption, etc., that follows with increasing age. This concern was corroborated by a recent association study including more than 3000 elderly men (Lee *et al*. 2012). The authors found the same positive associations between 25-OHD_3 serum level and testosterone/FAI, but after adjusting for health and lifestyle factors, no significant associations were observed between 25-OHD_3 and any of the reproductive hormones. However, the putative positive effect of VD on testosterone production was supported by a small RCT, which showed increasing testosterone following VD supplementation (average 48 years). The clinical value is limited by the design of the study as they mainly tested the effect of weight loss. Moreover, the causal factor is not obvious despite that the increase in testosterone was 2.7 nM in cholecalciferol-treated men vs 0.9 nM in the control group (Pilz *et al*. 2011). In conclusion, sufficient data exist to claim that VD is a strong regulator of aromatase expression and may be involved in regulation of steroidogenesis in human Leydig cells, but so far, there is not enough evidence for a stimulatory effect of VD on testicular testosterone production.

VD and reproductive function: lessons from knockout mice

Dent & Harris (1956) suggested that hereditary rickets (either no functional VDR or CYP27B1) in humans results in diminished fertility. Still, it has not yet been proven that VD is necessary for male reproductive function. Evaluating the reproductive effects of VD is complicated as impaired performance due to low serum 25-OHD_3 or diminished testicular VD metabolism is at least partly compensated by several other factors. Especially, the strong association between VD deficiency and hypocalcemia should always be considered when evaluating data from animal models or human studies (Sood *et al*. 1995, Lips 2006, Bouillon *et al*. 2008). Studies on KO mice provide strong evidence for beneficial effects of VD in male reproduction (Bouillon *et al*. 2008). Yoshizawa *et al*. (1997) showed that both male and female mice with homozygous *Vdr* deletion (Tokyo strain) were infertile. Intriguingly, two other *Vdr* KO strains were not infertile but presented with impaired fertility and low litter size (Johnson & DeLuca 2001, Kovacs *et al*. 2005, Bouillon *et al*. 2008). In the *Vdr* KO mice Tokyo strain, testes histology appeared grossly normal unlike the female counterpart, which is in accordance with the gonadal phenotype in humans with VD-dependent rickets type II (Malloy *et al*. 1990, Hawa *et al*. 1996). The initial normal testis histology of *Vdr* KO mice was also corroborated by the phenotype observed following targeted ablation of the 25-hydroxyvitamin-D-1α-hydroxylase (*Cyp27b1*), which not only caused infertility but also resulted in grossly normal testis histology (Panda *et al*. 2001). *Cyp24a1* KO mice develop hypercalcemia, and 50% of the homozygous mutants die before 3 weeks of age probably due to severe hypercalcemia. The animals surviving to adulthood are fertile despite deposition of mineral in several organs of the mutant mice (Masuda *et al*. 2005). A more detailed evaluation of testis histology and reproductive phenotype were also performed in males from the Tokyo *Vdr* KO strain, revealing an increased luminal diameter and shortened epithelial width in some tubules from 10-week-old *Vdr* KO mice compared with controls (Kinuta *et al*. 2000). The increase in luminal size was explained by fluid accumulation caused by diminished water reabsorption in rete testis and the proximal part of epididymis. Thereby suggesting imbalance in estrogen, as water reabsorption in this part of the male reproductive tract is mainly regulated by estrogen (Hess *et al*. 1997). Indeed, aromatase (*CYP19A1*) expression and activity were reduced in epididymis and testis from *Vdr* KO mice concomitant with a 40% decline in serum estrogen levels leading to hypergonadotropic hypogonadism with elevated FSH (twofold) and LH (eightfold) compared with wild type (Kinuta *et al*. 2000). Interestingly, FSH and estradiol but not LH levels normalized following calcium supplementation. This indicates a direct effect of VD and not solely impairment of reproductive performance due to hypocalcemia (Kinuta *et al*. 2000). VD regulates aromatase expression in a tissue-selective manner, and it is possible that elevated LH is due to diminished transformation of testosterone to estrogen in the hypothalamic area/pituitary gland. Testosterone concentration is 1000 times higher in males compared with females, and thus mainly responsible for feedback inhibition despite its dependency on local aromatization in the hypothalamic region/pituitary gland (Pitteloud *et al*. 2008).

The *Vdr* KO mice presented with a 50% reduction in sperm concentration in addition to a marked inhibitory effect on sperm motility that fell from 50 to 60% in wild type to 15% in the *Vdr* KO. The decline in sperm motility was aggravated over time and only 1% motile sperm was found after 10 weeks, while spermatogenesis was rarely detected in 15-week-old *Vdr* KO mice (Kinuta *et al*. 2000). Unfortunately, there exists no information about testosterone concentration in *Vdr* KO mice. However, estradiol supplementation corrected the reproductive phenotype, and it is therefore unlikely that testosterone level is severely impaired (Kinuta *et al*. 2000). Other studies have associated VD with reproductive performance in females, and female *Vdr* KO mice regained fertility following calcium supplements (Johnson & DeLuca 2001). Even though *Vdr* KO mice regained the ability to conceive (hard endpoint) following

calcium supplementation, several important reproductive questions remain such as the effect on time to pregnancy (TTP), semen quality variables, litter size, healthy pregnancy rate, rate of abortions, etc., in order to determine whether VD is important for male reproductive function.

VD deficiency and reproductive function

Some of the reproductive issues were addressed several years ago by two studies investigating the effect of VD deficiency on reproductive function in rodents. Kwiecinski *et al.* (1989) showed that VD-deficient males were capable of reproduction, but successful mating (evaluated by the presence of sperm in the female vaginal tract) between a healthy female and a VD-deficient male was reduced by 45% compared with VD replete males. Moreover, fertility (determined as successful pregnancies in sperm-positive females) was reduced by 73% in litters from VD-deficient male inseminations compared with litters from females inseminated by VD replete males (Kwiecinski *et al.* 1989). Three years later, the same group questioned the direct effect of VD on male reproduction and concluded that the impaired fertility could be restored by calcium supplements alone (Uhland *et al.* 1992). This follow-up study was well conducted but compromised by some methodological problems. When re-analyzing their data with an andrological perspective, the data can be interpreted differently and changing the original conclusions made by the authors (Tables 2 and 3; Uhland *et al.* 1992). The chance of successful pregnancy in mammals depends on several factors besides 25-OHD_3 and serum calcium level. In the study by Uhland *et al.* (1992), each male rat was not always mated with only one female rat, and the mating period varied from 1 to

Table 2 Effect of intervention on reproductive variables in VD-deficient rats.

	Pretreatment		Posttreatment		
Variable	VD replete	VD deficiency	VD replete	VD deficient treated for 3 weeks	
1. Intervention	2 μg D_3 i.p./week	None	UA μg D_3 orally	2 μg D_3 i.p./week	100 ng 1,25D_3 i.p./day
2. Start s-calcium (mM)	2.5	1.3	2.5	1.8	1.8
3. End s-calcium (mM)	ND	ND	2.5	2.6	2.7
4. Males (N)	40	66	15	17	17
5. Male:female ratio	0.82	0.78	0.79	0.68	0.71
6. Female index	1.00	1.05	1.00	1.16	1.11
7. Days mated	212	499	74	93	109
8. Sperm-positive smears	42	55	15	22	24
9. Positive sperm smear/day	0.20	0.11	0.20	0.24	0.22
10. Male activity	0.90	0.73	0.87	0.94	1.00
11. Females giving birth to normal litters (N)	32	20	8	11	8
12. Females giving birth to normal litters adjusted female index	32	19	8	10	7
13. Pregnancies per day	0.15	0.04	0.11	0.12	0.07
14. Pregnancies per day adjusted for female index	0.15	0.04	0.11	0.10	0.07
15. Pregnancies per day adjusted for female index and (N) males	0.15	0.02	0.11	0.09	0.06
16. TTP for healthy pregnancy	7	25	9	8	14
17. TTP for healthy pregnancy adjusted male:female ratio	7	26	9	10	15
18. TTP for healthy pregnancy adjusted male:female ratio and (N) males	7	42.9	9	11.3	17
19. Fraction healthy pregnancies/sperm positive	0.76	0.36	0.53	0.51	0.33
20. Fraction healthy pregnancies/sperm positive adjusted M:F ratio	0.76	0.35	0.53	0.44	0.30
21. Fraction healthy pregnancies/sperm positive per day	0.0036	0.0017	0.0025	0.0024	0.0016
22. Fraction healthy pregnancies/sperm positive adjusted M:F ratio per day	0.0036	0.0016	0.0025	0.0021	0.0014
23. Mean litter size	12.6	10.4	12.6	10.7	10.4

Data obtained and recalculated from Uhland *et al.* (1992). 1. Supplementation with either with D_3 or 1,25$(OH)_2D_3$ (1,25D_3). 2 and 3. Serum levels of calcium. 4. Number of male animals in each group. 5. Number of females mated with each male. 6. Number of females per male, VD replete animals set as reference. 7. Total days of allowed mating. 8. Presence of sperm in the vagina is considered successful mating. 9. Proportion of successful matings per day. 10. Percentage of active males. 11. Number of females giving birth to healthy litters. 12. Number of females giving birth to healthy litters adjusted for female index. 13. Number of pregnancies for each group per day of allowed mating. 14. Number of pregnancies per day of allowed mating adjusted for female index. 15. Proportion of pregnancies per day of allowed mating adjusted for number of animals. 16. TTP: time to pregnancy for the whole group (dependency on N). 17. TTP adjusted for female index. 18. TTP adjusted for female index and number of males (VD replete males = 1) as the number of pregnancies in the group depends on the total number of couples. 19–21. Proportion of healthy pregnancies after successful mating is an indicator of semen quality for the whole group (dependent on duration of allowed mating). This variable may serve as an indicator for semen quality after the adjustment for time of allowed mating (fertility potential). UA, unavailable, ND, not determined.

Table 3 Reproductive outcome after calcium supplementation of vitamin D-deficient male rats.

Variable	VD deficiency hypocalcemic	VD deficiency normocalcemic	VD replete
1. Cholecalciferol orally three times weekly (μg)	None	None	1.875
2. Start s-calcium (mM)	1.3	2.2	ND
3. End s-calcium (mM)	1.4	2.2	3.0
4. s-25-OHD$_3$ (nM)	ND	ND	21–26
5. s-1,25(OH)$_2$D$_3$ (pM)	1.0	17.0	53–75
6. Diet	Purified	High calcium	High calcium
7. Animals (N)	43	37	16
8. Male:female ratio	1	1	1
9. Days mated	121	102	44
10. Days mated/animal	2.88	2.76	2.75
11. Sperm-positive smears (successful mating)	42	36	16
12. Male activity	98%	97%	100%
13. Sperm-positive smears/day	0.347	0.353	0.364
14. Females giving birth to normal litters (N)	21	30	10
15. Pregnancies per day	0.17	0.29	0.23
16. Proportion of males responsible for pregnancy/day	0.4%	0.8%	1.4%
17. TTP healthy pregnancies (days)	5.8	3.4	4.4
18. TTP healthy pregnancies (days) adjusted (N) males	15.6	7.7	4.4
19. Fraction healthy pregnancies/sperm-positive smear	0.5	0.8	0.6
20. Fraction healthy pregnancies/sperm positive/day	0.4%	0.8%	1.4%
21. Mean litter size	13.0	13.5	15.1

Data obtained and recalculated from Uhland *et al.* (1992). 1. Only VD replete animals received oral supplements with D$_3$. 2–5. Serum levels of VD progenitors and calcium. 6. Type of diet. 7. Number of animals in each group. 8. Number of females mated with each male. 9. Total days of allowed mating. 10. Days each male is allowed to mate. 11. Presence of sperm in the vagina is considered successful mating. 12. Proportion of males able to produce a positive smear during the days of allowed mating. 13. Successful mating per day. 14. Number of females giving birth to healthy litters. 15. Number of pregnancies for each group per day of allowed mating. 16. Proportion of pregnancies per day of allowed mating adjusted for number of animals. 17. TTP: time to pregnancy for the whole group (dependency on N). 18. TTP adjusted to number of males (VD replete males = 1) as the number of pregnancies in the group depends on the total number of couples. 19. Proportion of healthy pregnancies after successful mating is an indicator of semen quality for the whole group (dependent on the duration of allowed mating). 20. This variable may serve as an indicator for semen quality after the adjustment for time of allowed mating (fertility potential).

10 days. This will obviously influence the results and the data should be corrected for number of female per male (male/female ratio) and the number of days they were allowed to mate as longer mating period increases the chances for pregnancy. The frequency of mating is also important because the chance of spermatozoa being present in the female reproductive tract at the time of ovulation increases with frequent mating. Moreover, infertility in humans is not defined as a definite inability to conceive but it is usually defined as an inability to conceive during 12 months of contraceptive-free intercourse (WHO 2010). Twelve months is the lower reference limit for TTP, and TTP is a good indicator for fertility potential because it depends on semen quality variables, oocyte quality, and regular ovulation (Bonde *et al.* 1998).

Based on these assumptions, the data published by Uhland *et al.* (1992) were re-calculated to compare the different treatments with the above-mentioned factors taken into account. From Table 2, it is obvious that VD-deficient animals are responsible for fewer sperm-positive smears per day of allowed mating and exposed to fewer females than the VD replete animals. TTP was longer in the VD-deficient groups especially after adjusting to the number of animals in each group. The presence of sperm in the vagina is not sufficient to secure pregnancy. After mating, the spermatozoa must swim up, bind, and fertilize the oocyte in the fallopian tube before fertilization occur (Ikawa *et al.* 2010), and the frequency of healthy pregnancies/successful mating would be an indicator of sperm function. None of the VD-deficient males achieved a reproductive level that was comparable with the male rats with high VD status from the start regardless to the choice of treatment. The VD replete group had the highest chance of healthy pregnancies and logically thereof the highest litter size. This indicates that the length and type of VD supplementation may be important and should be considered before treatment of VD deficiency. Supplementation with 1,25(OH)$_2$D$_3$ did not improve male fertility markedly compared with the untreated VD-deficient males despite the increase in serum calcium. The diminished effect of 1,25(OH)$_2$D$_3$ supplementation indicates that local VD metabolism is important and that testicular VD metabolism may be regulated by different factors besides circulating progenitors of VD (Blomberg Jensen *et al.* 2012). Table 3 shows the fertility potential of VD-deficient male rats with hypocalcemia and normocalcemia. Surprisingly, TTP and the fraction of healthy pregnancies/mating per day (healthy pregnancy) were not fully reversible after normalization of serum calcium in the VD-deficient rats. VD replete males had a threefold higher chance of healthy pregnancies compared with VD-deficient rats with hypocalcemia and an almost twofold higher chance than VD-deficient

rats with normocalcemia. This indicates that VD is important for semen quality, and the diminished effect caused by VD deficiency cannot be reversed by calcium supplements. This observation is supported by the difference in TTP and litter size. VD-deficient normocalcemic rats resembled the hypocalcemic VD-deficient rats and litter size remained lower in both groups compared with the VD replete rats (only borderline significant). In accordance, other animal studies conducted on jaguars, boars, and mice showed positive (borderline significant) effects of VD supplements on quality variables such as sperm morphology and sperm motility (Audet *et al.* 2004, Da Paz 2006, Hirai *et al.* 2009).

Only two studies have investigated associations between serum 25-OHD_3 and semen quality/reproductive hormones in humans (Blomberg Jensen *et al.* 2011, Ramlau-Hansen *et al.* 2011). Ramlau-Hansen *et al.* (2011) presented positive correlations between serum 25-OHD_3 and sperm motility in healthy young men (Ramlau-Hansen *et al.* 2011), but the association was not significant after adjustment for several confounders without reporting indication or influence. They found no association with sperm counts, morphology, or inhibin B level despite an unadjusted positive correlation with FSH. The conclusions obtained from this study are limited by some methodological problems, but especially the low frequency of men with VD deficiency and a remarkable high median serum 25-OHD_3 compared with other Danish studies makes it difficult to establish whether VD deficiency is associated with impaired semen variables (Mosekilde *et al.* 2005, Frost *et al.* 2010, Blomberg Jensen *et al.* 2011, Thuesen *et al.* 2011). They stratified their cohort into tertiles, which resulted in a comparison between various degrees of VD sufficiency. An extensive analysis conducted in a comparable cohort size of Danish men found the same positive association between serum 25-OHD_3 and sperm motility in addition to a positive association with the percentage of sperm with normal morphology and progressive sperm motility. Interestingly, the association was even stronger with progressive sperm motility, and the positive associations with total and progressive sperm motility persisted after adjustment of relevant confounders such as season, time from ejaculation to motility assessment, and serum calcium. The positive association between serum 25-OHD_3 and sperm motility was corroborated in a small mixed cohort of normal and infertile men (Blomberg Jensen *et al.* 2012). These observations indicate that VD deficiency in humans results in the same phenotype as reported in both VD-deficient animals and *Vdr* KO mice presenting with a low fraction of motile spermatozoa (Bouillon *et al.* 2008). This may be mediated directly by VD as $1,25(OH)_2D_3$ induced sperm motility in human spermatozoa *in vitro* and the associations between serum VD levels and sperm motility were not influenced by adjustment for serum calcium or albumin-corrected calcium levels. However, cross-sectional studies are warranted to determine whether the associations persist in infertile men, and RCTs are required to determine the clinical potential of cholecalciferol supplements to infertile men.

Regulation of testicular VD metabolism and putative downstream mediators

Testicular VD metabolism seems to be regulated differently compared with the tight systemic regulation of VD (Lips 2006, Fleet 2008). An animal study comparing VD-deficient (−VD) and control-treated rats raised on a normal VD-sufficient (+VD) diet showed threefold elevated *Vdr* levels in kidney but not in testis or in other extra-renal tissues. Upregulation of *Vdr* also occurred in kidney of +VD rats 1 day after a single 100 ng dose of $1,25(OH)_2D_3$, but no changes were seen in intestine, testis, or lung. Following infusion of $1,25(OH)_2D_3$ into −VD rats that were kept normocalcemic, the renal *Vdr* was upregulated, but testicular *Vdr* levels were again unaffected (Gensure *et al.* 1998). This shows that *Vdr* expression is regulated differently in the kidney and testis. This may be important, because ICC studies using human semen samples reported that a higher fraction of spermatozoa from fertile men express VDR than sperm from infertile men (Aquila *et al.* 2008, 2009, Blomberg Jensen *et al.* 2012). Likewise, $1,25(OH)_2D_3$ is a known inducer of testicular aromatase expression, and both aromatase level and activity are lower in the immotile rather than the motile fraction of sperm from the same semen sample. In addition, a lower abundance of aromatase mRNA was found in spermatozoa from asthenospermic (low motility), teratospermic (abnormal morphology), and asthenoteratospermic men compared with controls (Carreau *et al.* 2009), which is comparable to the reported expression pattern for VDR and the VD metabolizing enzymes (Blomberg Jensen *et al.* 2012). However, as indicated by *Vdr* KO mice, it is plausible that the main site for the VD-mediated reproductive effects is the male reproductive tract (epididymis) rather than the testis. Estrogen regulates fluid reabsorption in the reproductive tract and the estrogen receptor (*Er*) α KO male is infertile, although testes histology appears normal until puberty, when the tubules begin to degenerate. Sperm recovered from the cauda epididymis of these mice exhibit reduced motility and failed to fertilize eggs *in vitro* (Hess *et al.* 1997, Carreau & Hess 2010, Joseph *et al.* 2010). Moreover, male mice with deficient aromatase do not present with a severe phenotype and were initially fertile but developed progressive infertility and disrupted spermatogenesis after 4.5 months to 1 year. They presented with spermatogenic arrest and Leydig cell hyperplasia/hypertrophy despite no increase in gonadotropins or androgens (Robertson *et al.* 1999). Moreover, *Trpv6* KO mice

have severely impaired fertility due to a very low fraction of motile spermatozoa. *TRPV6* (VD-regulated gene) is expressed in the epididymis, but not in the testis and supports that the site of the VD-mediated effects on male reproduction is in the epididymis (Weissgerber *et al.* 2011). Immature Sertoli cells express VDR, aromatase, and AMH, while mature Sertoli cells do not express any of these proteins. The proposed actions of VD on immature Sertoli cells have recently been reviewed and will not be discussed further here, but it is likely that VD may be a regulator of AMH and GGT in immature Sertoli cells (Zanatta *et al.* 2011*c*). The findings described earlier indicate that most of the VD-mediated effects in the testis and male reproductive tract are mediated through estrogen synthesis or aberrant expression of ER. However, VD regulates transcription of more than 1000 genes though binding to the genomic pocket of the VDR (Haussler *et al.* 2011). The nuclear VDR expression in human spermatogonia indicates that VD exerts genomic actions there (Nangia *et al.* 2007, Blomberg Jensen *et al.* 2010*b*). Few have addressed the nuclear effects in male reproduction, but some of the known VD-regulated genes besides *CYP19A1* could also be of importance for male reproductive function: calcium transport and homeostasis (*NCX*, *PMCA*, *TRPV5–6*, *Calbindin 9k*, *28k*, *PTHrP*, *Calretinin*, and *CaSR*), endocrine (*IGFBP3*, *MIS(AMH)*, *CYP19A1*, and *VEGF*), and cell cycle control (*p21*, *p63*, *FOXO1*, and *RUNX2*) (Haussler *et al.* 2011).

The expression of *VDR* in human spermatozoa indicates that $1,25(OH)_2D_3$ mediates a direct effect in mature spermatozoa. Human spermatozoa are virtually transcriptionally silent, but activation of the alternative ligand binding pocket of VDR mediates several non-genomic effects, which may be of importance for sperm function as recently reviewed (Blomberg Jensen & Dissing 2012). Briefly, 1 nM $1,25(OH)_2D_3$ induce a rapid increase in intracellular calcium concentration $[Ca^{2+}]_i$ in human spermatozoa *in vitro* (Fig. 5). The increase in $[Ca^{2+}]_i$ was abrogated by the nongenomic VDR antagonist $1\beta,25(OH)_2D_3$, while the specific agonist for VDR-ap (JN) increased $[Ca^{2+}]_i$ with similar kinetics as $1,25(OH)_2D_3$, thereby confirming that this ionotropic effect was mediated by VDR activation (Blomberg Jensen *et al.* 2011, 2012). The rise in $[Ca^{2+}]_i$ originated from Ca^{2+}-release from intracellular stores as inhibition of phospholipase C diminished the $1,25(OH)_2D_3$-mediated Ca^{2+} response, while suspending spermatozoa in a nominally Ca^{2+}-free medium did not abrogate the VD-mediated Ca^{2+} rise (Blomberg

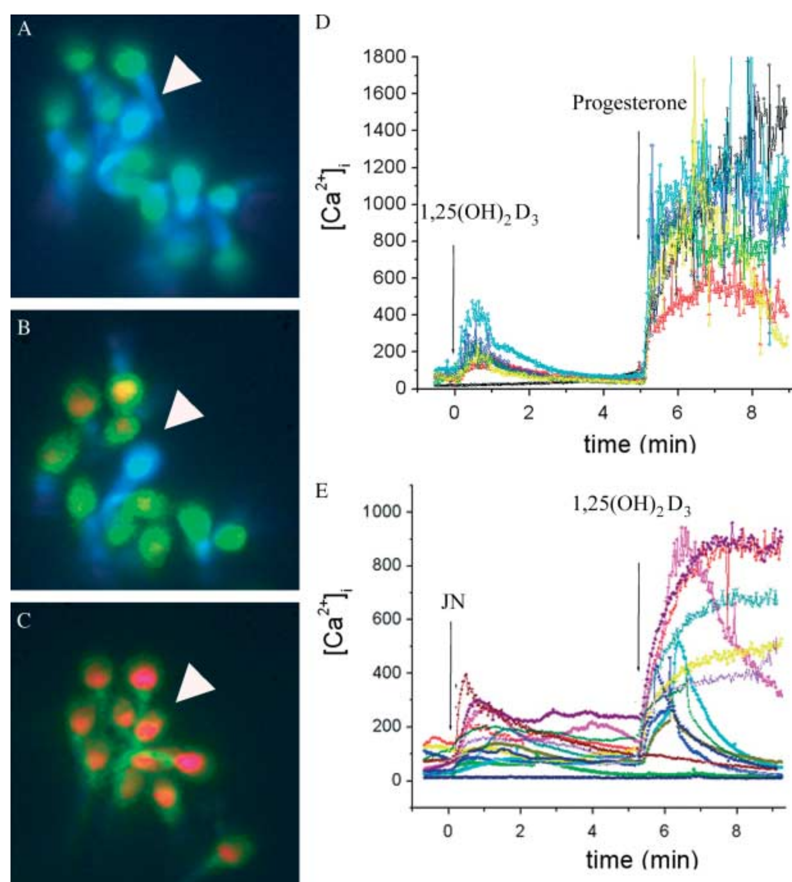

Figure 5 Vitamin D (VD) and intracellular calcium concentration $[Ca^{2+}]_i$ in human spermatozoa. (A) Fura-2-loaded spermatozoa. Color indicates intracellular calcium concentration (low to high: blue, green, yellow, and red). (B) Rapid change in intracellular calcium levels after addition of 1 nM $1,25(OH)_2D_3$, arrowhead indicates one unresponsive spermatozoa. (C) Corresponding changes in $[Ca^{2+}]_i$ after addition of 10 μM progesterone, arrowhead indicates the spermatozoa being unresponsive to 1 nM $1,25(OH)_2D_3$. (D) Increase in $[Ca^{2+}]_i$ following treatment with 1 nM $1,25(OH)_2D_3$ and 10 μM progesterone, each trace represents calcium levels in a single spermatozoa and arrows indicate the spermatozoa being unresponsive to $1,25(OH)_2D_3$ (black trace). (E) Increase in $[Ca^{2+}]_i$ following treatment with 1 nM $1,25(OH)_2$ $Lumisterol_3$ (JN) and 1 nM $1,25(OH)_2D_3$, each trace represents $[Ca^{2+}]_i$ in single spermatozoa. Note different gradations. Figure reproduced from **Blomberg Jensen M, Jørgensen A, Nielsen JE, Leffers H, Andersen AN, Skakkebaek NE, Juul A, Rajpert-De-Meyts E & Jørgensen N** 2012 Expression of the vitamin D metabolizing enzyme CYP24A1 at the annulus of human spermatozoa may serve as a novel marker of semen quality. *International Journal of Andrology*. By permission of John Wiley and Sons.

Jensen *et al*. 2011). The spatiotemporal kinetics of the VD response differed from the progesterone-mediated increase in $[Ca^{2+}]_i$ as the VD-mediated Ca^{2+} rise was not observed in the tail region and was independent of extracellular Ca^{2+} and therefore not mediated by CatSper (Blomberg Jensen *et al*. 2012). Moreover, pretreatment with $1,25(OH)_2D_3$ and the use of VDR antagonist did not abrogate the progesterone response, which supports different mechanisms of action (Blomberg Jensen *et al*. 2011). A functional role of the VD-mediated Ca^{2+} increase was supported by the $1,25(OH)_2D_3$-mediated increase in sperm motility and induction of the acrosome reaction *in vitro* (Blomberg Jensen *et al*. 2011). However, the functional importance of these findings depends on the concentration of VD progenitors in the seminal fluid and in the vicinity of the cumulus–oophorous complex in the female reproductive tract.

Perspectives

From animal and human studies, it is evident that VD is important for optimal male reproductive function. Some of the VD effects are presumably mediated locally by the presence of VDR and the VD metabolizing enzymes in adult male germ cells, Leydig cells, and male reproductive tract, while other actions may be influenced by the systemic effects of VD serum levels. The low expression of *VDR* and VD metabolizing enzymes in spermatozoa from infertile men compared with normal men supports a role for the local VD metabolism and may have functional consequences. Especially, as $1,25(OH)_2D_3$ mediates a nongenomic increase in intracellular calcium concentration, which seems to be important for sperm function especially following capacitation in the female reproductive tract. Most of the VD effects in the testes appear to be mediated through estrogen biosynthesis, but other candidate genes involved in calcium homeostasis, endocrine function, and cell cycle control may be involved. An association between serum 25-OHD_3 and testosterone production remains to be proven, although it is plausible that the declining serum VD levels with age may be involved in the age-related loss of function of both gonads and bone, but further studies are needed to determine causality. Moreover, both local VD metabolism and serum levels of 25-OHD_3 may have a direct effect on sperm motility in both animal and humans even after considering the indirect effect mediated by calcium homeostasis. The effects on sperm motility may be mediated mainly in epididymis rather than the testes, but further studies are needed to determine the exact site and mechanisms of action and to investigate whether supplementation of cholecalciferol improves semen quality in VD-deficient men.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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