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Intratumoral sterol-27-hydroxylase (*CYP27A1*) expression in relation to cholesterol synthesis and vitamin D signaling and its association with lethal prostate cancer

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Abstract

Background: Higher intratumoral cholesterol synthesis is associated with a worse prognosis in prostate cancer. The vitamin D-regulated enzyme sterol-27-hydroxylase (CYP27A1) converts cholesterol to 27-hydroxycholesterol, a potential endogenous selective estrogen receptor modulator (SERM). We hypothesized that low *CYP27A1* expression is associated low vitamin D signaling, high cholesterol synthesis, and higher risk of lethal prostate cancer.

Methods: We studied 404 patients in the prospective prostate cancer cohorts within the Health Professionals Follow-up Study (HPFS) and the Physicians' Health Study (PHS). We quantified intratumoral *CYP27A1* expression and proxies of cholesterol synthesis and vitamin D signaling using transcriptome profiling and assessed prediagnostic plasma 25-hydroxyvitamin D (25(OH)D, n = 132) and intratumoral vitamin D receptor protein expression (VDR, n = 300). Lethal disease was defined as prostate cancer mortality or metastases, in contrast to non-lethal disease without metastases after >8 years of follow-up since diagnosis.

Results: CYP27A1 expression was lower in tumors with higher Gleason grade and higher expression of the second rate-limiting enzyme of cholesterol synthesis, *SQLE*. We did not detect consistent associations between *CYP27A1* and plasma 25(OH)D, *CYP24A1*, or intratumoral VDR expression. Lower *CYP27A1* was associated with higher risk of lethal cancer in both cohorts (adjusted odds ratio for lowest vs. highest quartile of expression, 3.04; 95% CI, 1.46–6.33); this association was attenuated when additionally adjusting for Gleason grade and *SQLE* (odds ratio, 1.76; 95% CI, 0.75–4.17).

Conclusion: Low *CYP27A1* expression is associated with higher cholesterol synthesis and a higher risk of lethal disease in prostate cancer. Whether the SERM-properties of 27-hydroxycholesterol underlie these associations requires further study.

Introduction

Prostate tissue has long been recognized to contain considerable amounts of cholesterol, particularly when undergoing carcinogenic transformation (Schaffner, 1981). More recently, several studies have suggested that higher serum cholesterol levels are associated with increased risk of advanced stage, higher-grade, or fatal prostate cancer (Batty *et al.*, 2011, Mondul *et al.*, 2011, Platz *et al.*, 2008), while others reported null associations (Jacobs *et al.*, 2012). Higher intratumoral synthesis of cholesterol, as assessed through expression of the second rate-limiting enzyme of cholesterol synthesis, squalene monooxygenase (*SQLE*), is associated with a higher risk of lethal prostate cancer (Stopsack *et al.*, 2016).

A key metabolite of cholesterol is 27-hydroxycholesterol. In preclinical breast cancer models, added 27-hydroxycholesterol stimulated tumor growth, acting partially as an endogenous selective estrogen receptor modulator (SERM) (Nelson *et al.*, 2013, Umetani *et al.*, 2007, Warner and Gustafsson, 2014). Intriguingly, high expression of the enzyme that synthesizes 27-hydroxycholesterol, 27-hydroxylase (*CYP27A1*), has been reported to be associated with higher tumor grade in breast cancer yet better prognosis (Kimbung *et al.*, 2017, Nelson *et al.*, 2013). In prostate cancer, a recent study reported *CYP27A1* expression to be strongly inversely related to Gleason grade (Alfaqih *et al.*, 2017). Moreover, *CYP27A1* catalyzes the 25-hydroxylation step of vitamin D, which might have a protective effect in various cancers including prostate cancer (Feldman *et al.*, 2014, Shui and Giovannucci, 2014).

We hypothesized that low *CYP27A1* expression, potentially resulting in cholesterol accumulation, occurs in prostate cancers that have higher expression of the cholesterol synthesis pathway. We also hypothesized that low *CYP27A1* expression is associated with low vitamin D signaling. To test these hypotheses, we conducted a cross-sectional analysis of two large, well characterized populations of patients with prostate cancer. In a longitudinal design, we tested our hypothesis that low *CYP27A1* would be associated with a higher risk of lethal prostate cancer over long-term follow-up.

Methods

Study populations

We studied patients who were diagnosed with prostate cancer during follow-up of two prospective cohort studies, the Health Professionals Follow-up Study (HPFS) and the Physicians' Health Study (PHS).

The HPFS enrolled 51,529 male health professionals, aged 40 to 75 years, in 1986 (Giovannucci *et al.*, 2007). Participants have been followed through biannual questionnaires since. The PHS enrolled 29,071 male physicians, aged ≥40 years, in 1982, initially for randomized-controlled trials of aspirin (Steering Committee of the Physicians' Health Study Research Group, 1989) and micronutrients (Christen *et al.*, 2000). Blood samples were collected from cancer-free participants in 1982 (PHS) and in 1993–95 (HPFS). Self-reported prostate cancer diagnoses in both cohorts are verified through review of medical records. Tissue from all patients included in this study also underwent centralized pathology review. Patients are followed prospectively for metastases and

prostate cancer-specific death (lethal cancer; with 98% completeness in HPFS and 99% in PHS for adjudication of death causes).

Within the prostate cancer biorepository from HPFS and PHS, we conducted a nested "extreme" case-control study, including of whole-transcriptome profiling of the tumor tissue, which oversampled patients with lethal outcome and those with available blood specimens from before cancer diagnosis (Sinnott *et al.*, 2017).

Participants provided written informed consent. The research was approved by the institutional review boards at Harvard T.H. Chan School of Public Health and Partners Healthcare.

Tumor profiling and plasma levels

For all patients included in this study, we retrieved tumor specimens from cancer diagnosis from the treating hospital. Expert genitourinary pathologists performed centralized histologic re-review, including Gleason grading (Stark (Rider) *et al.*, 2009), and selected high-density tumor areas (>80% tumor cell density). Transcriptome profiling included mRNA expressions of *CYP27A1*, *SQLE*, and *CYP24A1*. Tumor tissue and, if available, adjacent non-cancer prostate tissue, was measured on the Affymetrix GeneChip Human Gene 1.0 ST array (Gene Expression Omnibus: GSE62872), with post-processing as previously described (Penney *et al.*, 2016).

Plasma 25-hydroxy-vitamin D [25(OH)D] from blood samples before cancer diagnosis was measured as a part of a case-control study nested within HPFS. A radioimmunosorbent assay was used, as previously described (Shui *et al.*, 2012).

Vitamin D receptor (VDR) expression in the cytoplasm and membrane was stained via immunohistochemistry on tissue microarrays. Using a semiautomated quantitative image analysis system, the VDR score was generated as a combination of the relative area positively stained and the intensity of staining, as previously described (Hendrickson *et al.*, 2011). *TMPRSS2:ERG* status was determined using a genomically-validated ERG immunohistochemistry (Pettersson *et al.*, 2012).

Statistical analysis

Our analysis plan had two main parts. First, we assessed cross-sectionally how *CYP27A1* expression was associated with measures of vitamin D signaling and intratumoral cholesterol synthesis. Second, in a longitudinal analysis, we assessed the association between *CYP27A1* at cancer diagnosis and the risk of lethal disease over long-term follow-up. All tests were two-sided.

To assess the associations of 25(OH)D, VDR, *SQLE*, *CYP24A1*, ERG, and *CYP27A1*, we used linear regression. Between *CYP27A1* and *CYP24A1* expressions, we also calculated Pearson correlation coefficients *r*. Values for 25(OH)D were adjusted for season- and batch, as previously described (Shui *et al.*, 2012); VDR scores were adjusted for differences in mean values between tissue microarrays (Hendrickson *et al.*, 2011). We modeled the predictor in categories and inspected plots to assess for potential non-linear relationships, and we calculated tests for linear trend across quartiles by modeling the category medians (for 25(OH)D and VDR) or category indices (mRNA variables) as ordinal predictors. In a sensitivity analysis, we replaced *SQLE* as a proxy for cholesterol synthesis activity of the tumor by a summary score of all cholesterol synthesis genes (Stopsack *et*

al., 2016). This summary score was the first principal component from principal components analysis of all cholesterol synthesis genes. Higher levels indicated higher expression of the cholesterol synthesis pathway, as 20 of the 21 cholesterol synthesis genes were positively loaded on this principal component.

To assess the association of *CYP27A1* expression (modeled in quartiles) and lethal disease, we used logistic regression to estimate odds ratios (OR) and 95% confidence intervals. Models were additionally adjusted for age (linear), year of diagnosis (categorical: pre-prostate specific antigen [PSA] era, 1982–1988; peri-PSA era, 1989–1993; PSA era, 1994–2005), smoking status (binary: current smoker vs. never/prior smoking), family history of prostate cancer in father or brothers (binary: yes/no), body mass index (categorical: <25, 25–30, >30 kg/m^2), and hyperlipidemia (binary: self-report of hyperlipidemia by the health professionals vs. no such report). In separate models, we adjusted for Gleason grade (categorical: 5–6, 3+4, 4+3, 8, 9–10) and *SQLE* expression (categorical: quartiles). In an exploratory analysis, we assessed the association of *CYP27A1* within low and high strata of cholesterol synthesis activity defined by *SQLE* and the cholesterol signature. Given its strong association with lethal disease specifically in the highest quartile (Stopsack *et al.*, 2017), *SQLE* in the fourth quartile was considered high; the upper half of the signature was considered high.

Results

Study populations and tumor characteristics at cancer diagnosis

Baseline characteristics of 254 patients from HPFS and 150 patients from PHS are shown in Table 1. 59% of patients had pathologically organ-confined cancers (T1/T2 N0 M0), and 59% were diagnosed in the PSA screening era. 92% of tumor samples were from radical prostatectomy. Plasma concentrations of 25(OH)D before cancer diagnosis were available for a subset of 132 patients from HPFS. VDR protein expression had been quantified for 300 patients.

Notably, *CYP27A1* expression was lower in higher-grade, advanced stage, and ERG-positive cancers (Table 1). Compared to Gleason grade 5–6, tumors with Gleason grade 9–10 had on average 0.73 standard deviations (SD) lower *CYP27A1* expression (95% CI, 0.38 to 1.08 SD; $p_{trend} < 0.001$). ERG-positive tumors had 0.27 SD lower *CYP27A1* expression (95% CI, 0.06 to 0.47) than ERG-negative tumors.

Cross-sectional analysis: Vitamin D signaling, cholesterol synthesis, and CYP27A1 expression

We assessed the association of circulating and intratumoral indicators of vitamin D signaling and *CYP27A1* mRNA expression. Circulating plasma 25(OH)D was not associated with *CYP27A1*; the difference in *CYP27A1* expression between the lowest quartile of 25(OH)D and the highest quartile was 0.04 SD (95% CI, –0.55 to 0.46; $p_{trend} = 0.42$; Figure 1.A). *CYP27A1* expression was also not associated with VDR expression in the tumor; the difference in VDR expression score was 0.20 SD (95% CI, –0.25 to 0.66 SD) between the lowest and the highest quartile of *CYP27A1* ($p_{trend} = 0.09$; Figure 1.B). In contrast, we observed a weak positive correlation between *CYP27A1* and the expression of the VDR target gene *CYP24A1* ($r = 0.17$; 95% CI, 0.07 to 0.27; Figure 1.C).

To assess the association between intratumoral cholesterol synthesis and *CYP27A1*, we used the second rate-limiting enzyme of cholesterol synthesis, *SQLE*, and a score summarizing the

mRNA expression of all cholesterol synthesis enzymes as proxies. *CYP27A1* was lower in tumors with higher *SQLE* expression; the difference in *CYP27A1* between first and fourth quartile of *SQLE* was –0.42 SD (95% CI, –0.69 to –0.14; p_{trend} = 0.002 across quartiles of *SQLE*; Figure 1.D). Similar results were observed when we used the summary score instead of *SQLE* as a proxy for cholesterol synthesis in the tumor, observing a difference in *CYP27A1* between first and fourth quartile of the score of –0.49 SD (95% CI, –0.77 to –0.22; p_{trend} = 0.001).

In normal prostate tissue, we also did not observe associations between *CYP27A1* expression and plasma 25(OH)D and VDR expression (results not shown). In contrast to tumor tissue, *CYP27A1* expression and *CYP24A1* expression were not correlated in normal prostate tissue (r = 0.03; 95% CI, –0.11 to 0.17), and there was no statistically significant difference in *CYP27A1* expression between the lowest and highest quartiles of *SQLE* (difference, –0.15 SD; 95% CI, –0.54 to 0.25; p_{trend} = 0.44)

Longitudinal analysis: CYP27A1 and lethal disease

Patients were followed a median of 15.3 years for the development of metastases or death from prostate cancer (lethal disease). Lower intratumoral *CYP27A1* mRNA expression was associated with a higher risk of lethal disease over long-term follow-up in both cohorts (Table 2). In HPFS, patients with *CYP27A1* mRNA expression in the lowest quartile had a 2.64-fold higher risk of lethal disease (95% CI, 1.23 to 5.67), compared to patients with *CYP27A1* in the highest quartile. In PHS, the OR was 4.65 (95% CI, 0.92 to 23.5). Combining both cohorts and adjusting for additional baseline characteristics, the OR was 3.04 (95% CI, 1.46–6.33; p_{trend} = 0.007 across quartiles of *CYP27A1*). The association of *CYP27A1* and lethal disease remained significant but was attenuated somewhat when additionally adjusting for *SQLE* (OR for lowest vs. highest quartile of *CYP27A1*, 2.64; 95% CI, 1.24 to 5.62). Results were similar when adjusting for the summary score of cholesterol synthesis. Additional adjustment for Gleason grade considerably attenuated the association of *CYP27A1* and lethal disease (OR, 1.92; 95% CI, 0.83 to 4.46).

As expected, *CYP27A1* expression in tumor-adjacent normal prostate tissue was not associated with lethal disease (OR for lowest vs. highest quartile, 1.50; 95% CI, 0.66 to 3.44; p_{trend} = 0.24).

Finally, we assessed if the association of intratumoral *CYP27A1* with lethal disease differed within levels of cholesterol synthesis. The association between *CYP27A1* and lethal disease appeared to be slightly stronger in patients with low *SQLE* ($p_{interaction}$ = 0.17). However, we did not observe a similar pattern when stratifying by the summary score of cholesterol synthesis (Table 2; $p_{interaction}$ = 0.63).

Discussion

In this study, we assessed regulators of *CYP27A1*, which synthesizes 27-hydroxycholesterol from cholesterol, and its associations with long-term prognosis in patients with primary prostate cancer. We found *CYP27A1* expression to be low in tumors that had higher expression of cholesterol synthesis enzymes including *SQLE*. In contrast, we did not detect strong associations between several measures of vitamin D signaling and *CYP27A1*. Notably, low *CYP27A1* expression was

associated with a higher risk of lethal disease, beyond the elevated risk associated with higher expression of the cholesterol synthesis pathway. These findings advance our understanding of the complex role of cholesterol in prostate cancer and may help inform us about the role of *CYP27A1* in cancer more broadly.

We observed a strong inverse relationship between *CYP27A1* expression and two different measures of intratumoral cholesterol synthesis, the expression of the second rate-limiting enzyme *SQLE* as well as all enzymes constituting the cholesterol synthesis pathway. These obsevations suggest that in tumors with activated cholesterol synthesis, hydroxylation of cholesterol to 27-hydroxycholesterol is inhibited, perhaps in order to make cholesterol available for other purposes in rapidly dividing cells like cell membrane function. Concordantly, a preclinical study found the addition of 27-hydroxycholesterol to prostate cancer cell lines and xenografts attenuated their growth and decreased the expression of SREBP2, the main transcription factor regulating cholesterol synthesis (Alfaqih *et al.*, 2017). However, discordant experimental results, partially using the same cell line, have been reported as well (Raza *et al.*, 2017).

Despite assessing multiple proxies of vitamin D signaling activity, including plasma 25(OH)D concentrations, VDR protein expression in tumor tissue, and mRNA expression of the VDR target gene *CYP24A1*, we did not find consistent evidence that showed *CYP27A1* to be strongly related to vitamin D signaling. However, these measures may not have fully captured an effect of exogenous vitamin D on *CYP27A1* expression, particularly if vitamin D is 25-hydroxylated directly within prostate cells without changing plasma 25(OH)D concentrations. This 25-hydroxylation step of vitamin D has indeed been observed in a non-tumor prostate cell line, in which vitamin D also induced CYP27A1 expression (Tokar and Webber, 2005). However, the 25-hydroxylase function of CYP27A1 may not be physiologically relevant in peripheral tissues as the prostate, but rather fulfilled by the microsomal 25-hydroxylase *CYP2R1* (Jones *et al.*, 2014). Ultimately, our observations lend no additional support to *CYP27A1* expression in prostate cancer tissue being tightly controlled by vitamin D signaling. We also assessed if *TMPRSS2:ERG* status was associated with *CYP27A1* expression. Bidirectional influences between vitamin D signaling, including VDR and *CYP24A1*, and *TMPRSS2:ERG* have been reported in prostate cancer cell lines (Kim *et al.*, 2014, Washington and Weigel, 2010), and we previously observated that ERG-positive tumors have higher VDR expression (Hendrickson *et al.*, 2011). In the present study, we only observed a modest association between ERG status and *CYP27A1* expression, and *CYP24A1* expression did not differ by ERG status (data not shown).

In breast cancer, several studies found *CYP27A1* expression to be higher in high-grade compared to low-grade cancers (Kimbung *et al.*, 2017, Nelson *et al.*, 2013). In prostate cancer, a relatively strong, inverse relationship with Gleason grade has been reported previously (Alfaqih *et al.*, 2017) and was confirmed by our data. *CYP27A1* expression has also been reported to be lower in castration-resistant cancer tissue compared to tissue from castration-sensitive tumors (Tamura *et al.*, 2007). How strongly *CYP27A1* expression would be associated with risk of clinically relevant outcomes such as metastases or cancer death was unknown. Our data indicated an appropriately three-fold higher risk of lethal cancer among the 25% patients with the lowest *CYP27A1* expression (first quartile), compared to the 25% with the highest expression (fourth quartile; Table 2). Given the tight association of *CYP27A1* and Gleason grade, it is unsurprising that these estimates were

attenuated considerably when additionally adjusting for Gleason grade. Our results are supported by a previous study that included a larger set of prostate cancer patients from HPFS and found single nucleotide polymorphisms within *CYP27A1* to be associated with the risk of lethal disease (Shui *et al.*, 2012); however, we do not know if and how these single nucleotide polymorphisms influence *CYP27A1* mRNA expression.

While *CYP27A1* does not appear to be well suited as a prognostic marker, our results are informative for mechanistic studies. In particular, it has been suggested that a large proportion of the association of *CYP27A1* expression and prognosis in breast cancer is due to SERM effects of 27-hydroxycholesterol (Nelson, 2017). These associations were also most pronounced in premenopausal patients with estrogen receptor-positive tumors (Kimbung *et al.*, 2017). Upregulated cholesterol synthesis and production of 27-hydroxycholesterol in estrogen receptor-positive breast cancer under antiestrogen therapy has been suggested as a mechanism of therapy resistance (Nguyen *et al.*, 2015, Simigdala *et al.*, 2016). In our study, we found a moderately strong association of *CYP27A1* with lethal disease, which might suggest that at least in prostate cancer, CYP27A1 and 27-hydroxycholesterol would act to some extent through non-SERM mechanisms. Alternatively, 27-hydroxycholesterol might be important to consider as a potential ligand when assessing the role of estrogen receptor beta, which is expressed in at least a subset of prostate tumors (Nanni *et al.*, 2009).

We did not find that the association of *CYP27A1* expression and lethal disease differed across levels of cholesterol synthesis enzyme expression (Table 2). This could mean that CYP27A1, or tumor features associated with it, drive cancer progression in addition to cholesterol synthesis. Importantly, absence of a statistical interaction between cholesterol synthesis and *CYP27A1* on a multiplicative scale should not be interpreted as evidence against biological interaction between them, but rather as the opposite (Greenland *et al.*, 2008).

It should be noted that we measured mRNA levels of *CYP27A1* and not protein expression. In an ecologic analysis of a small number of breast tissue samples before and after atorvastatin exposure, CYP27A1 protein appeared to show changes in the opposite direction than *CYP27A1* mRNA (Kimbung *et al.*, 2017). In contrast, in prostate cancer tissue, CYP27A1 protein expression was lost in the tumor epithelium in contrast to normal glands, consistent with observations on the mRNA level (Alfaqih *et al.*, 2017). However, this comparison was also ecological, and we are unaware of a study directly comparing mRNA and protein levels within the same patients. An additional limitation of our study is that only a relatively small subset of patients had prediagnostic plasma samples, which may have contributed to the null results for plasma 25(OH)D.

In summary, we found low intratumoral *CYP27A1* mRNA expression to be associated with higher markers of intratumoral cholesterol synthesis, higher Gleason grade, and a higher risk of lethal disease over long-term follow-up. We did not find strong associations of *CYP27A1* and circulating 25(OH)D or with two measures of intratumoral vitamin D signaling. Future studies should consider estrogen receptor expression when evaluating CYP27A1 in prostate cancer and should ideally attempt to directly measure intratumoral or circulating 27-hydroxycholesterol. Interestingly, serum 27-hydroxycholesterol concentrations were decreased by atorvastatin treatment and by vitamin D supplementation in two small-scale clinical trials among patients with breast cancer (Going

et al., 2017, Kimbung *et al.*, 2017). It remains to be seen how such interventions might affect intratumoral cholesterol and 27-hydroxycholesterol levels as well as clinical outcomes for patients with prostate cancer.

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Table 1. Characteristics of prostate cancer patients from the Health Professionals Follow-up Study (HPFS) and the Physicians' Health Study (PHS), by *CYP27A1* mRNA expression in tumor tissue. Within each quartile, absolute counts (out of 101 patients) closely approximate percentages.

	Quartile of *CYP27A1* mRNA expression in tumor tissue			
	1st (lowest)	2nd	3rd	4th (highest)
n	101	101	101	101
Age at diagnosis, median (range)	67 (49–80)	66 (47–80)	66 (50–81)	65 (52–77)
Year of diagnosis, *n*				
Before 1993	31	47	43	37
After 1993	69	54	57	63
Gleason grade, *n*				
5–6	10	11	13	23
7 (3+4)	24	36	38	41
7 (4+3)	34	24	24	20
8	12	17	9	5
9–10	21	13	17	12
Stage, *n*				
T1/T2	50	56	65	68
T3	38	37	29	28
T4/N1/M1	13	8	7	5
Prostate-specific antigen [ng/dl], *n*				
<4	6	9	9	9
4-10	50	41	49	58
>10	27	32	31	20
Missing	18	19	12	14
Current smoking at diagnosis, *n*	9	6	2	7
Body mass index [kg/m^2], *n*				
<25	54	53	45	41
25–30	35	43	54	52
>30	12	5	2	8
Hypercholesterolemia, *n*	32	29	30	24
Statin use at diagnosis, *n*	9	11	10	13
Plasma 25(OH)D [ng/ml], median (interquartile range)	25 (21–32)	24 (19–29)	27 (22–35)	26 (19–28)
TMPRSS2:ERG status, *n*[a]				
ERG-positive	53	47	47	35
ERG-negative	39	47	41	56

[a] Based on immunohistochemistry for ERG protein. Missing for 39 patients in total.

Table 2. *CYP27A1* mRNA expression in tumor tissue and lethal prostate cancer.

	Quartile of *CYP27A1* mRNA expression in tumor tissue				
	1st (lowest)	2nd	3rd	4th (highest)	p_{trend}[a]
HPFS					
Cases: lethal, non-lethal, *n*	28, 34	20, 45	20, 44	15, 48	
OR (95% CI),[b] unadjusted	2.64 (1.23–5.67)	1.42 (0.65–3.11)	1.45 (0.66–3.19)	1 (reference)	0.018
PHS					
Cases: Lethal, non-lethal, *n*	8, 31	10, 26	10, 27	2,[c] 36	
OR (95% CI),[b] unadjusted	4.65 (0.92–23.5)	6.92 (1.40–34.3)	6.67 (1.35–33.9)	1 (reference)	0.12
Combined HPFS and PHS, OR (95% CI)[b]					
Model 1: Unadjusted	2.73 (1.41–5.30)	2.09 (1.06–4.10)	2.09 (1.06–4.10)	1 (reference)	0.005
Model 2: Adjusted[d]	3.04 (1.46–6.33)	2.17 (1.04–4.53)	2.40 (1.15–5.00)	1 (reference)	0.007
Model 3: Model 2 + *SQLE*[e]	2.64 (1.24–5.62)	2.17 (1.03–4.58)	2.30 (1.08–4.88)	1 (reference)	0.022
Model 4: Model 2 + chol. score[e]	2.86 (1.35–6.05)	2.02 (0.95–4.29)	2.40 (1.14–5.05)	1 (reference)	0.015
Model 5: Model 3 + Gleason	1.76 (0.75–4.17)	1.84 (0.77–4.41)	2.05 (0.87–4.86)	1 (reference)	0.31
By cholesterol score,[f] OR (95% CI)[b]					[g]
Score < median	2.95 (1.12–7.81)	1.89 (0.67–5.41)	1.77 (0.65–4.79)	1 (reference)	0.032
Score ≥ median	2.22 (0.89–5.57)	1.79 (0.72–4.48)	2.18 (0.85–5.60)	1 (reference)	0.17

[a] Test for linear trend across quartiles, modelled as ordinal indices
[b] Odds ratios for lethal disease with 95% confidence intervals
[c] Because of the few events in the reference category for PHS (*n* = 2), the odds ratios for PHS alone should be interpreted cautiously in light of probable sparse-data bias (Greenland *et al.*, 2016).
[d] Adjusted for age (linear), year of diagnosis (categorical), smoking status at cancer diagnosis (binary), body mass index (categorical), high serum cholesterol (binary)
[e] *SQLE* and summary score of expression levels for all cholesterol synthesis genes were in quartiles
[f] HPFS and PHS combined; unadjusted estimates within levels of the cholesterol summary score
[g] Test for multiplicative interaction between *CYP27A1* quartile indices (ordinal) and cholesterol summary score (binary)

Figure Labels

Figure 1. Regulation of *CYP27A1* mRNA expression in tumor issue. **A.** Plasma 25(OH)D and *CYP27A1* mRNA. **B.** *CYP27A1* mRNA and VDR expression in tumor tissue (in dimensionless units). **C.** *CYP27A1* and *CYP24A1* mRNA. **D.** *SQLE* mRNA and *CYP27A1* mRNA. All units of mRNA expression are standard deviations.

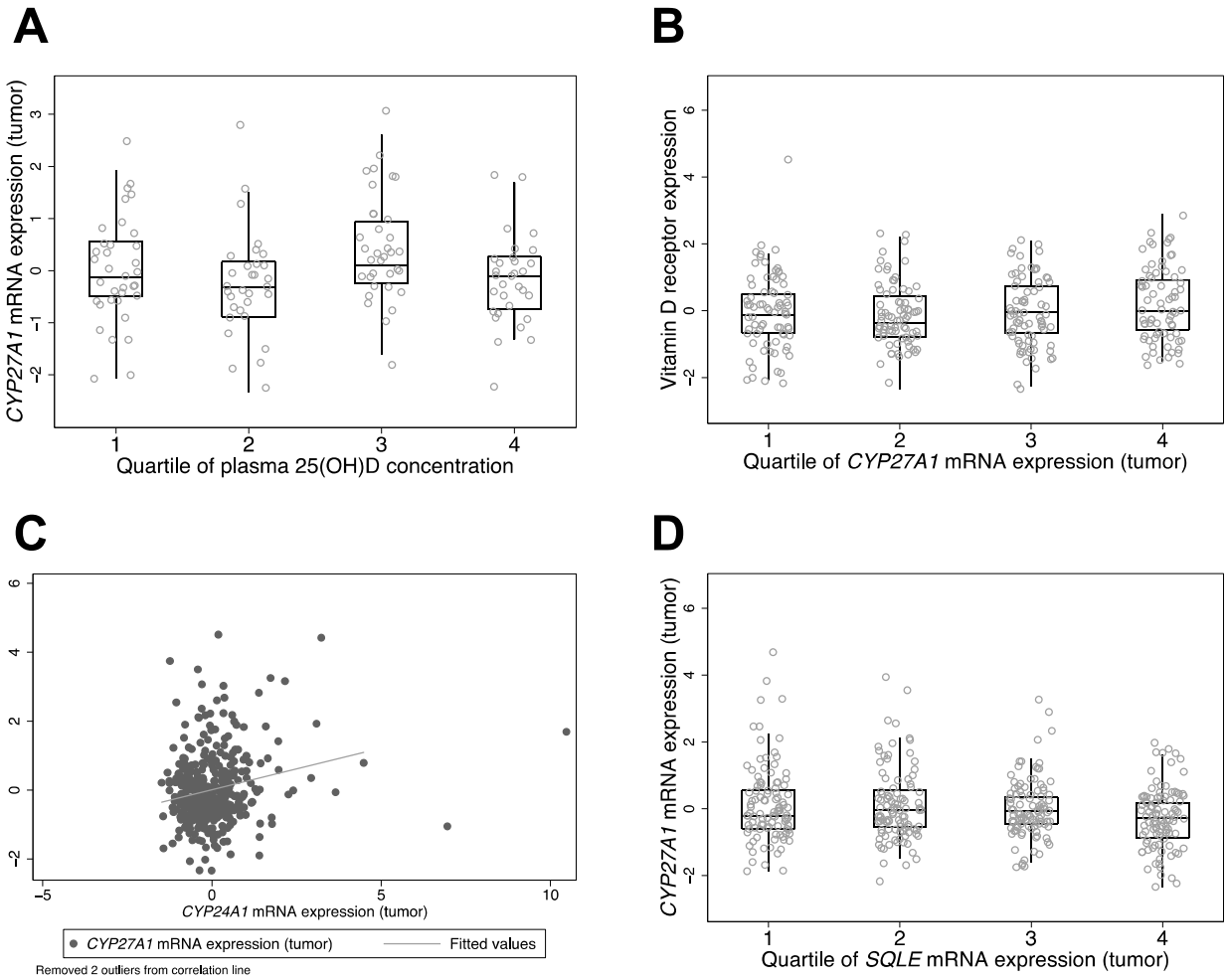

Figure 2. Causal diagram highlighting the complex interplay between cholesterol synthesis, vitamin D signaling, and CYP27A1 in prostate cancer. Note that the graph should be considered incomplete from causal inference standpoint (i.e., it does not list all confounders necessary to estimate causal effects).
[appropriate for readership?]