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Tumor Cholesterol Synthesis, Statin Use, and Lethal Prostate Cancer

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:
Flanagan, S., Lis, R.T., Huang, Y., Jehane, L., Vaselkiv, J.B., Allott, E.H., et al. (2025). Tumor Cholesterol Synthesis, Statin Use, and Lethal Prostate Cancer. MOLECULAR CANCER RESEARCH, 23(12), 1025-1033 [10.1158/1541-7786.MCR-24-0864].

Availability:
This version is available at: https://hdl.handle.net/11585/1032651 since: 2025-12-15

Published:
DOI: http://doi.org/10.1158/1541-7786.MCR-24-0864

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05 May 2026

Tumor Cholesterol Synthesis, Statin Use, and Lethal Prostate Cancer

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Running title: HMGCR Expression and Lethal Prostate Cancer

Keywords: Prostate cancer, HMGCR, cholesterol, atorvastatin

Counts: Abstract, 248 words; Main text, 4,122 words; 2 figures; 2 tables; 4 supplementary tables; 5 supplementary figures, 1 supplementary file.

Conflicts of Interest: PWK has investment interest in Convergent Therapeutics, Context Therapeutics LLC, Candel Therapeutics and ESSA Pharma. He is a company board member of Convergent Therapeutics, Context Therapeutics, and Essa Pharma. He is a consultant/scientific advisory board member for ImmunisAI, Candel Therapeutics and PrognomIQ. LAM reports research funding from Janssen and Astr Zeneca; serves on the SAB and holds equity interest in Convergent Therapeutics; and was a consultant to Bayer Pharmaceuticals. SPF has received honoraria from Pfizer, AstraZeneca, Astella, Servier, Amgen and Roche. RTL is a consultant for Janssen. GC has served as a scientific consultant for GuidePoint and received consultation fees. TLL has received research funding for other studies from AIRA Matrix, DeepBio, Roche, and Myriad Genetics. The remaining authors report no conflict of interest.

ABSTRACT

Prostate tumor cells produce cholesterol *de novo*, and statin therapy targets the initial rate-limiting enzyme in this process, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR). The extent to which the expression of HMGCR in prostate tumors could influence progression and predict the potential anti-neoplastic effects of statins remains unclear. In a prospective cohort study of 1098 men diagnosed with primary prostate cancer in 1982-2009 from the Health Professionals Follow-up Study and Physicians' Health Study, 16% of prostate tumors showed strong HMGCR staining intensity and 31% no staining. HMGCR expression was higher in tumors with PTEN loss but did not differ by statin use or long-term dietary cholesterol or saturated fat intake. Participants were followed for lethal events (distant metastases or prostate cancer-related death) over up to 32 years, and 96 lethal events occurred in those without metastases at diagnosis. Strong HMGCR expression was associated with higher rates of lethal prostate cancer (hazard ratio 2.2, 95% confidence interval 1.3-3.7), adjusting for age at diagnosis and Gleason score but without a linear dose response. *In vitro* in the LNCaP human prostate cancer cell line, atorvastatin impacted tumor cell viability in cells with experimentally lowered HMGCR expression. This study corroborates that high cholesterol synthesis in prostate tumor cells is associated with PTEN loss, aggressive tumor characteristics, and a greater risk of lethality.

Implications: High expression of HMGCR, the first rate-limited enzyme of cholesterol synthesis, is a feature of prostate tumors that are more likely to progress to metastatic disease or death from prostate cancer.

INTRODUCTION

Cholesterol is implicated in prostate cancer initiation and aggressiveness by multiple lines of evidence including animal models (1–4), prostate cancer cell lines (5,6), and cohort studies (7–10). Potential mechanisms for the role of cholesterol in prostate cancer progression include intra-tumoral steroidogenesis, cell proliferation, amplified inflammation, and changes in the cell membrane (1,11). Prostate cancer cells can take up cholesterol from the bloodstream or synthesize it *de novo* (3,11). Intra-tumoral cholesterol is produced via the mevalonate pathway, and the first rate-limiting enzyme of this pathway is 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) (12,13). Elevated expression of the HMGCR enzyme in prostate tumors may thus contribute to the progression of prostate cancer.

HMGCR inhibitors, statins, are proposed to have an anti-neoplastic effect in many cancers, including prostate (1,14). *In vitro* studies have demonstrated that statin therapy reduces the growth and proliferation of prostate cancer cells (15,16). Furthermore, epidemiological studies have observed a lower risk of advanced and fatal prostate cancer among statin users in prospective cohort studies (17–22). Nevertheless, prior studies on statins and cancer have also exhibited significant methodological limitations, such as immortal time bias (23). Moreover, the selection of populations in which randomized controlled trials would be justified remains unclear (24,25). Additional data on the varying effects of statins in relation to HMGCR expression in prostate tumors, both in humans and *in vitro*, could enhance the causality of associations and facilitate the identification of target populations for trials.

The primary aim of our study was to investigate the association between intra-tumoral HMGCR expression and the rates of progression to lethal prostate cancer in a primary prostate cancer cohort. In addition, we examined whether HMGCR expression is associated with specific clinical or pathological tumor features, given the regulation of cholesterol synthesis by the PI3K/PTEN pathway (2,26) and the impact of the *TMPRSS:ERG* fusion on fatty acid metabolism (27–29). Finally, we quantified the extent to which statin therapy could modify the association between HMGCR expression and lethal prostate cancer. *In vitro*, the impact of atorvastatin on viability of the LNCaP prostate cancer cell line at varying levels of HMGCR expression was assessed.

METHODS

Cohort Study

Study Population

The study population included men diagnosed with prostate cancer during prospective follow-up of the Physicians' Health Study (PHS) and the Health Professionals Follow-up Study (HPFS) between 1982 and 2009. The PHS were initially randomized, controlled trials of acetylsalicylic acid and micronutrients in primary prevention of cardiovascular disease and cancer among 29,071 healthy U.S. male physicians

initially aged 40 to 84 years (30,31). The HPFS is an ongoing prospective observational study of 51,529 U.S. male health professionals aged 40 to 75 years at enrollment in 1986 (32).

Dietary cholesterol and saturated fat intake were estimated using validated semiquantitative food frequency questionnaires administered every four years starting at enrollment linked to an updated nutrient database (33,34).

Incident prostate cancers were initially self-reported by the participating health professionals and confirmed through medical and pathology record review. Data on date of prostate cancer diagnosis, tumor characteristics and statin use were obtained via biennial questionnaires and systematic medical record review. A tumor tissue repository was established among the subset of participants with prostate cancer in these cohorts. The tissue diagnosis was subsequently re-reviewed by genitourinary pathologists (35). Participants for whom archival prostate tumor tissue was available either from radical prostatectomy or from transurethral resection of the prostate (TURP) were included in this study.

Cohort enrollment constituted written informed consent. This project was conducted in accordance with the U.S. Common Rule and approved by the institutional review boards of Mass General Brigham and Harvard T.H. Chan School of Public Health, and those of participating registries as required.

Tissue Microarrays

Formalin-fixed, paraffin-embedded prostate tumor tissue specimens and hematoxylin/eosin slides, from the time of prostate cancer diagnosis or surgery were retrieved from the relevant healthcare institution for participants of the HPFS and PHS. Hematoxylin/eosin slides were centrally re-reviewed by expert genitourinary pathologists for Gleason score and other histologic features (35) and to identify areas of high-density tumor for creation of tumor tissue microarrays. Fourteen tissue microarrays (TMAs) were constructed by sampling at least three 0.6 mm cores of tumor per case from the dominant nodule or nodule with the highest Gleason pattern.

Immunohistochemistry

A BOND RX Automated Staining system (Leica) was utilized. 5-µm tumor sections from each TMA were deparaffinized in xylene, followed by graded alcohol rehydration. Antigen retrieval was performed by heating the tissue in BOND epitope retrieval solution 1 (AR9961), a citrate-based pH 6 epitope retrieval solution, for 20 minutes. A polyclonal rabbit anti-HMGCR antibody (Cat. No HPA008338, Atlas Antibodies AB, Stockholm, Sweden) was applied at 1:100 for 1 hour. PC3 and 22RV prostate cancer cell lines were used as positive controls for immunohistochemistry.

HMGCR expression in tumor cells was scored by a genitourinary research pathologist (R.T.L.). Peripheral nerve fibers were used as a positive control for moderate/strong intensity of HMGCR expression within each TMA (**Supplementary Figure 1**). HMGCR expression was categorized based on the staining intensity in the cytoplasm as none, weak, moderate, or strong. If staining was visible in any

cell, it generally involved >50% of cells, therefore the proportion of positive cells was not recorded. Where HMGCR staining intensity differed between cores, median expression per case across cores was utilized.

TMAs were also stained for the proliferation marker Ki-67 and scored by quantitative image analysis as the number of Ki-67-positive nuclei over the total number of neoplastic nuclei, as described previously (36). A genetically validated immunohistochemistry assay for PTEN was also performed, as described previously (37). A tissue core was considered to have PTEN protein loss if the intensity of cytoplasmic and nuclear staining was markedly decreased or entirely lost (0+ intensity) or markedly decreased (1+ intensity) across more than 10% of tumor cells compared with surrounding benign glands and/or stroma. In addition, the presence of the *TMPRSS2:ERG* fusion was determined by a genetically validated immunohistochemistry assay for ERG, as described previously (38).

Lethal Prostate Cancer

The primary outcome was lethal prostate cancer, defined as the development of metastatic disease or prostate cancer-specific death. Dates of death were obtained from next-of-kin, the postal system, and the National Death Index. Cause of death was assigned by an endpoints committee of physicians by reviewing medical history, medical records, registry information, next-of-kin communication, and death certificates. Data on development of distant metastases was tracked by way of patient questionnaires and through review of medical records.

Cell Line Experiments

Materials

The LNCaP human prostate cancer cell line was obtained from the American Type Culture Collection. LNCaP cells had been derived from lymph node metastasis of a White patient with hormone-sensitive prostate cancer (39). LNCaP cells were maintained with 10% fetal bovine serum (Gibco), supplemented with L-glutamine and IX antibiotic/antimycotic (GeminiBio) in RPMI 1640 medium, and regularly tested for *Mycoplasma* infection.

HMGCR Expression and Knockdown

For western blotting, cells were washed and lysed, and equal amounts of protein were loaded on Tris-HCl gels (Invitrogen) and transferred via electrophoresis to Immobilon-PVDF membranes (Millipore). A HMGCR monoclonal mouse antibody was used (AMAb90619, Atlas Antibodies, Sweden) at a dilution of 1:1000 in 5% bovine serum albumin blocking buffer in Tris-buffered saline with 0.02% sodium azide (Alfa Aesar) (40), with GAPDH as a loading control (#sc-365062, Santa Cruz Biotechnology).

For lentiviral-based silencing of *HMGCR*, short-hairpin RNAs (shRNAs, **Supplementary Table 1**) were designed using the splash RNA algorithm (41,42), cloned into SGEP constitutive lentivectors, and viral supernatants were produced in HEK293T cells. After transfection using TransIT-X2 transfection reagents

(Mirus), 48-hour viral supernatants were used concentrated using Pierce Protein Concentrator (Thermo Scientific) and used for transducing LNCaP cells using Lentiblast reagents (OZ Bioscience) for 24 hours. Transduced LNCaP cells were selected with puromycin during all further steps.

Cell Viability Assay

Isogenic scramble control and *HMGCR* knodown LNCaP cells (5000 cells/well) were plated in 96-well plates under sterile conditions. Atorvastatin (PHR1422, Sigma-Aldrich) was administered at a concentration of 0 nM, 200 nM, and 1000 nM in dimethyl sulfoxide. Cell viability was evaluated after 0, 1, 3, and 5 days with the CellTiter-Glo Luminescent Cell Viability Assay (Promega), which quantifies cellular adenosine triphosphate (ATP) levels as a proxy for metabolically active, viable cells (43). Cell viability was quantified photometrically according to manufacturer instructions. All steps after transfection were repeated in 6 independent experiments with 6 wells per condition (Supplementary File 1.)

Statistical Analysis

Ratios of ERG positivity and PTEN loss prevalence (both binary) by HMGCR expression were estimated using semiparametric log-linear ("Poisson") models with robust standard errors. To model arithmetic means of Ki-67 positivity (continuous), generalized Gaussian log-linear models with robust standard errors were used. Potential batch effects between TMAs were quantified using linear mixed models (44), treating HMGCR intensity (from 0 for none to 3 for strong) as a Gaussian variable. Between-case reproducibility in HMGCR was estimated with the same models, including random effects for both TMA and case. To mitigate measurement error from batch effects, stratification-based approaches were used for all subsequent analyses of TMA data. For the relation of HMGCR to other TMA-based biomarkers (ERG, PTEN, Ki-67), models were fit by TMA and pooled by inverse-variance weighting; for Cox models, TMA was included as a stratification factor. Usual pre-diagnostic dietary cholesterol and saturated fat intake were based on the cumulative average of derived intake up until the year of cancer diagnosis, energy-adjusted using the residual method, and their correlation with HMGCR intensity (coded as above) was assessed using Pearson correlation.

For analyses of HMGCR expression and lethal prostate cancer using Cox proportional hazards models, person-time was calculated from the date of cancer diagnosis to the development of metastases or death due to prostate cancer (if metastasis or its date was unknown), with censoring at death from other causes or end of follow-up (December 2020 in HPFS, December 2014 in PHS). Adjusted models included age at diagnosis and Gleason score in grade groups (5–6, 3+4, 4+3, 8, 9–10; ordinal). Models were also stratified by statin use at cancer diagnosis (user vs. non-user, based on the two HPFS questionnaires cycles preceding diagnosis), time-varying current post-diagnosis statin use (updating until the date of metastasis at maximum), and PTEN status (PTEN intact vs. PTEN loss in all cores). In a sensitivity

analysis, maximum HMGCR expression across replicate cores per case was utilized as an alternative to median expression across cores.

Cell line viability data were analyzed after dividing intensity values within each experiment and day by intensity in wells containing empty medium. Linear mixed models for log-transformed intensity included terms for time, HMGCR concentration, shRNA type, and all interactions, as well as random intercepts and time slopes per experiment. Replicates within each experiment were included as individual data points, leveraging all measurements without exclusions. The three-way interaction term for time, HMGCR concentration, and shRNA type quantified the shRNA-dependent effect of HMGCR knockdown on cell viability over the entire five-day course, adjusting for baseline differences.

Data Availability

The data underlying this article are available through a project proposal for the Health Professionals Follow-up Study as described at https://hsph.harvard.edu/research/health-professionals/resources/for-external-collaborators.

RESULTS

Study Population

The cohort analysis included 1098 participants diagnosed with prostate cancer for whom tumor tissue was available and HMGCR expression was evaluable (**Supplementary Figure 2**). The majority of tissue was from prostatectomy (95%), with the remainder originated from TURP. The median participant age at cancer diagnosis was 66 years (interquartile range [IQR] 62 to 70) and 97% of participants self-identified as White. At diagnosis, 94% of participants had clinically localized prostate cancer (T1/T2). Patient tumors had a broad spectrum of Gleason scores; 5-6 (19%), 3+4 (36%), 4+3 (24%), 8 (8%) and 9-10 (14%). 23% of participants were statin users at prostate cancer diagnosis.

Clinicopathological Characteristics and HMGCR Expression

A median of 3 tumor cores (IQR 2–3) per participant were analyzed. The median HMGCR expression intensity per tumor was calculated across cores. HMGCR expression intensity was strong in 181 (16%), moderate in 277 (25%), weak in 301 (27%) and none in 339 (31%) in tumors (**Figure 1**). Of cases with exactly 3 cores, HMGCR expression varied across tumor cores in 47% of cases; in 87% of these, intensity differed by only one level.

HMGCR expression did not vary substantially across calendar year of cancer diagnosis, participant age, Gleason score, clinical TNM stage, or prostatectomy TNM stage (**Table 1**). In addition, no substantial differences in HMGCR expression were noted between statin users (8% strong HMGCR) and non-users (10% strong HMGCR) at prostate cancer diagnosis (**Supplementary Table 2**).

Potential batch effects in HMGCR expression between TMA slides were of notable magnitude, with 22% of the variance in expression being attributable to between-TMA differences (95% confidence interval [CI] 9-35%). Between-case (tumor) differences explained 59% (95% CI 49-68%) of variance in HMGCR intensity, indicating moderate biomarker reproducibility. Batch effects fully explained small between-cohort differences in HMGCR expression (**Supplementary Figure 3**). All subsequent analyses remove batch effects analytically.

Tumors with HMGCR expression were more likely to have PTEN loss (prevalence ratio 1.4, 95% CI 1.2-1.6), were slightly more likely to be ERG-positive (prevalence ratio per one-category higher HMGCR 1.2, 95% CI 1.1-1.3), and had a higher Ki-67 proliferative index (mean ratio 1.5, 95% CI 1.3-1.7).

In terms of dietary intake in the years before prostate cancer diagnosis (median 11 years, interquartile range 7-16), dietary cholesterol (Pearson *r* 0.04, 95% CI -0.03 to 0.11; **Supplementary Figure 4**) and saturated fat intake (Pearson *r* 0.04, 95% CI -0.03 to 0.12) were not associated with HMGCR expression.

HMGCR Expression and Lethal Prostate Cancer

Among 1082 participants with non-metastatic prostate cancer at diagnosis, 96 lethal events occurred over a follow-up of 32 years (median 17 years, IQR: 13-21), corresponding to 17,484 person-years.

Tumors with strong HMGCR expression had higher rates of progression to lethal prostate cancer (unadjusted hazard ratio [HR] 2.1, 95% CI 1.3-3.5) compared to tumors with no HMGCR expression. This association was essentially unchanged following stratification by TMA to address potential batch effects (HR 2.3, 95% CI 1.3-4.2) and after adjustment for age at diagnosis and Gleason score (HR 2.2, 95% CI 1.3-3.7). There was no clear linear trend across categories of HMGCR intensity (**Table 2**). The results were similar when using maximum instead of median expression per case as the case-level summary measure (**Supplementary Table 3**).

In joint models of HMGCR expression and statin use at cancer diagnosis or time-varying statin use after prostate cancer diagnosis, there was no suggestion of strong multiplicative effect modification (**Table 2**). However, these analyses were limited by sparse data. Similarly, the association between HMGCR expression and lethal disease did not appear to differ substantially between PTEN-intact tumors and those with PTEN loss. In addition, among those with known PTEN status, the association of strong HMGCR expression and higher rates of lethal disease was unchanged after adjusting for PTEN loss (**Supplementary Table 4**).

Effect of HMGCR Knockdown and Atorvastatin Treatment on LNCaP Cell Line Viability

HMGCR knockdown in LNCaP cells was tested with different shRNAs (**Supplementary Table 1**). One shRNA that moderately downregulated HMGCR protein and one shRNA that strongly downregulated HMGCR protein were selected for viability assays (**Supplementary Figure 5**).

Cell viability was little affected by treatment with atorvastatin for up to 5 days in absence of HMGCR knockdown (**Figure 2**), with growth ratios of 0.99-fold (95% CI 0.90-1.09, $p = 0.8$) for 200 nM atorvastatin and 0.93-fold (95% CI 0.84-1.02, $p = 0.10$) for 1000 nM atorvastatin over the five-day course. Similarly, HMGCR knockdown without atorvastatin treatment also had limited to no effect on viability, with ratios of 0.93-fold (95% CI 0.85-1.03, $p = 0.15$) for the shRNA that induced moderate HMGCR knockdown and 0.97-fold (95% CI 0.88-1.07, $p = 0.53$) for the shRNA that induced strong HMGCR knockdown. In contrast, 200 nM atorvastatin treatment after strong HMGCR knockdown reduced viability to 0.86-fold (95% CI 0.76-0.98, $p = 0.027$), compared to 200 nM atorvastatin treatment of cells without HMGCR knockdown. The effect of 200 nM atorvastatin treatment after moderate HMGCR knockdown was weaker (0.95-fold, 95% CI 0.83-1.08, $p = 0.4$). Similar patterns were observed for 1000 nM atorvastatin treatment after strong HMGCR knockdown (0.61-fold, 95% CI 0.54-0.70, $p < 0.001$) showing greater effect than moderate HMGCR knockdown (0.80-fold, 95% CI 0.70-0.91, $p < 0.001$; **Figure 2**).

DISCUSSION

The study investigated HMGCR, a biomarker for cholesterol synthesis, in a prospective cohort of patients with prostate cancer and in a widely used cell line model of prostate cancer. Two-thirds of prostate tumors had visible HMGCR expression, including 16% with strong staining. We show that tumors with PTEN loss had stronger HMGCR expression; in contrast, we found no evidence that statin use, dietary cholesterol or saturated fat intake would noticeably influence intratumoral HMGCR expression. Strong intratumoral HMGCR expression was associated with a higher risk of lethality in patients, an association that was not explained by PTEN loss alone. *In vitro*, atorvastatin had little impact on LNCaP cell line viability on its own, but a modest reduction in cell viability was observed with atorvastatin in cells that had experimentally lowered HMGCR expression.

The results of this study largely align with prior lines of evidence regarding intra-tumoral cholesterol synthesis and its association with prostate cancer progression. In a small hospital-based cohort, prostate tumors with strong HMGCR expression tended to have higher biochemical recurrence rates versus those with weak or no HMGCR expression (45). Similarly, studies examining squalene monooxygenase (*SQLE*), the second rate-limiting enzyme of cholesterol synthesis, via mRNA expression profiling in HPFS and PHS (10) and via immunohistochemistry in two hospital-based cohorts (46) have found strong associations with biochemical recurrence and metastasis in prostate cancer. These findings, combined with our current study, are evidence for a potential involvement of the mevalonate pathway in prostate tumor progression. In addition, comparable findings have been observed in breast cancer cohorts (47–49). While some individual studies examining breast cancer yielded statistically inconclusive results (48,49), their collective findings offer at least suggestive evidence that higher HMGCR expression is associated with worse outcomes following a cancer diagnosis. A related but separate question is how statins may

influence the initiation and progression of prostate cancer prior to diagnosis; the current study specifically only evaluated their effect on cancer progression after diagnosis and surgery. For comparison, a landmark study, also in the HPFS cohort, demonstrated that statin use was associated with a substantially lower risk of a diagnosis with advanced prostate cancer (21), and a second study in this population suggested that the association might primarily be driven by a lower risk of prostate cancer with PTEN loss (22).

HMGCR is the first, rate-limiting enzyme in a complex cholesterol synthesis pathway. Cholesterol plays a role in numerous diverse cellular processes, including steroid hormone signaling and cell membrane formation, which could be potential mechanisms for its role in prostate cancer progression. Notably, we found little evidence of HMGCR being associated with global measures like Gleason scores or pathological TNM stages. These findings align with those of a prior prostate cancer cohort (45), while another study did report a positive correlation between greater HMGCR expression and higher Gleason scores (50). In our study, HMGCR expression was associated with loss of the tumor suppressor PTEN, a strong risk factor for metastases and death from prostate cancer (37,51), and slightly higher Ki-67 indices, a biomarker of tumor proliferation that is prognostic for prostate cancer progression (52–55) and that has even served as an endpoint for short-term clinical trials of atorvastatin in prostate cancer (56,57).

The regulation of HMGCR expression and thus of intratumoral cholesterol synthesis via the mevalonate pathway is tight in normal cells (58) but becomes dysregulated in prostate cancer cells, which have long been known to accumulate cholesterol (4). The current study underscores that cholesterol synthesis in prostate cancer cells is (dys-)regulated by activation of the PI3K pathway via PTEN loss (2,26) while the impact of the *TMPRSS:ERG* fusion is smaller and likely primarily on fatty acid metabolism (27–29). In contrast, we did not observe evidence that long-term dietary cholesterol, saturated fat intake or regular statin use would have substantial effects on intratumoral HMGCR expression. The latter findings could suggest that HMGCR expression in prostate tumors is primarily dysregulated by tumor-specific signaling pathways. Of note, these are observations regarding regulation of expression levels, not regarding potential HMGCR-dependent efficacy of statins.

The extent to which HMGCR expression could modify potential anti-neoplastic effects of statins is not fully clear, in part because such analyses would require numbers of lethal events that would be about an order of magnitude larger than among statin users in our cohort and thus more than in existing cohorts known to us presently. In a prior prostate cancer cohort, non-users of statins with strong HMGCR expression tended to have higher biochemical recurrence rates than participants with weak or no HMGCR expression (45). In our experimental study, we observed increased viability readings presumably reflective of proliferation of the LNCaP cancer cell line in general, and atorvastatin slowed this increase specifically in tumors with experimentally reduced HMGCR expression. These findings might suggest that statins could have a direct anti-tumor effect in prostate tumors with low levels of HMGCR expression. Such an additive effect could mean that atorvastatin treatment may only be able to overcome

intratumoral cholesterol synthesis if it is not highly activated. However, direct translation to humans should be considered carefully, given that this experimental model considered a two-dimensional layer of genetically similar tumor cells, lacked a tumor microenvironment, and involved treatment with supraphysiologic drug concentrations for a maximum of five days. Our observation that treatment alone with nanomolar doses of atorvastatin lacked a strong tumoricidal effect aligns with previous cell line-based statin studies, which have shown that substantially higher doses in the micromolar range are required (15,16,45,59). The required amounts would be several orders of magnitude higher than those found in human prostate tissue after short-term statin therapy at doses otherwise considered clinically safe (56,60). In addition, the current study focused on HMGCR as the first rate-limiting enzyme of the mevalonate pathway. Beyond this primary drug target, statins exhibit pleiotropic effects, including inhibition of cell proliferation and migration, induction of apoptosis, and modulation of inflammatory responses that may also contribute to anticancer effects (61). Further corroboration of our *in vitro* findings through orthogonal experimental approaches, including overexpression and rescue assays, would be desirable. Additional model systems, including additional cell lines and organoids, would be desirable. It is also plausible that cholesterol-depleted cell culture media could yield different results, even if such levels may not model physiologic conditions,given that prostate tissue has long been shown to contain substantial amounts of cholesterol (4). In any case, the cell line model afforded this study with the opportunity to intervene in tumor-level HMGCR expression and explore its potential as a predictive biomarker.

Limitations of our study should be considered. Beyond a visual examination for non-specific background staining, we did not perform additional validation for the HMGCR antibody, such as utilizing isogenic models or conducting knockdown and overexpression experiments. As with all research-grade antibodies, specificity is a major concern (62). In addition, intra-tumoral heterogeneity in HMGCR expression was evident, with differences in scoring apparent across TMA cores of the same tumor. However, through our use of multiple cores per case, this source of measurement error was reduced, but likely still led to some bias toward the null in associations of HMGCR expression with lethality. In TMA-based studies, batch effects may arise (44), and if unaddressed, could influence the associations between HMGCR and other biomarkers measured on the same TMAs, leading to dependent measurement errors. Our stratification-based approach mitigated this issue, and there was no appreciable influence of potential batch effects on prognostic analyses. Furthermore, the dose and type of statin used varied among participants, but this information was unavailable. As the vast majority of the study participants were White, the generalizability of this study may be limited if there are racial differences in intra-tumoral cholesterol synthesis. In the cell line model, we measured ATP levels, which serve as a surrogate for cellular metabolic activity and viability (43). However, they do not distinguish between decreased proliferation or increased cell death.

In summary, strong expression of the statin target HMGCR was associated with PTEN loss, aggressive tumor characteristics, and a greater risk of lethality in this prospective, population-based cohort of patients with nonmetastatic prostate cancer. This study corroborates than high cholesterol synthesis is a feature of prostate cancer with PTEN loss, which tend to be more aggressive, and suggests that intra-tumoral cholesterol synthesis may contribute to the progression of prostate cancer. Atorvastatin had a modest but detectable impact on cell line viability *in vitro*, which was observed only in tumors where HMGCR expression had been experimentally reduced. It is possible that a low HMGCR expression level could identify people who would benefit from statin treatment in prostate cancer, but further study in humans would be required.

ACKNOWLEDGMENTS

We thank the participants and staff of the HPFS and the PHS for their valuable contributions. In particular, we would like to recognize the contributions of Siobhan Saint-Surin, Betsy Frost-Hawes, Ann Fisher, and Maggie Bristol. We appreciate the support from the Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA. This work was supported by the Department of Defense Prostate Cancer Research Program [W81XWH-18-1-0330 to KHS] and the Zhu Family Center for Global Cancer Prevention. Tumor tissue microarrays were created at the Dana-Farber/Harvard Cancer Center Tissue Microarray Core Facility [P30 CA006516]. GC is supported by the National institute of Health [R01 CA274967]. The Health Professionals Follow-up Study is supported by the National Cancer Institute [U01 CA167552]. The research was also supported by a Cancer Center Support Grant [P30 CA008748]. SF, SPF, TLL, GC, LAM, and KHS are Prostate Cancer Foundation Young Investigators.

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Table 1. Characteristics of patients diagnosed with prostate cancer 1982–2009 during prospective follow-up of the Health Professionals Follow-up Study and Physicians' Health Study, by intratumoral HMGCR expression intensity.

		HMGCR expression intensity			
	Overall	**None**	**Weak**	**Moderate**	**Strong**
Participants, N	1098	339 (31%)	301 (27%)	277 (25%)	181 (17%)
Cohort					
HPFS	772 (70%)	275 (81%)	235 (78%)	189 (68%)	73 (40%)
PHS	326 (30%)	64 (19%)	66 (22%)	88 (32%)	108 (60%)
Year of prostate cancer diagnosis	1997 (1993, 2001)	1997 (1992, 2001)	1998 (1994, 2002)	1997 (1993, 2001)	1997 (1993, 2000)
Age at diagnosis, years	66 (62, 70)	65 (61, 69)	66 (62, 70)	66 (61, 70)	66 (62, 70)
Dietary cholesterol, mg/d	257 (203, 316)	254 (199, 308)	262 (209, 331)	253 (207, 311)	256 (192, 304)
Unknown	334	67	70	88	109
Saturated fat intake, g/d	22 (17, 27)	21 (16, 26)	22 (17, 28)	22 (17, 29)	22 (16, 27)
Unknown	334	67	70	88	109
Gleason score					
5-6	203 (19%)	71 (21%)	51 (17%)	48 (17%)	33 (18%)
3+4	391 (36%)	109 (32%)	108 (36%)	110 (40%)	64 (36%)
4+3	259 (24%)	88 (26%)	68 (23%)	62 (22%)	41 (23%)
8	90 (8%)	30 (9%)	27 (9%)	17 (6%)	16 (9%)
9-10	153 (14%)	40 (12%)	47 (16%)	40 (14%)	26 (14%)
Unknown	2	1	0	0	1
Clinical TNM stage					
T1/T2	1015 (94%)	312 (94%)	280 (96%)	258 (95%)	165 (93%)
T3	35 (3%)	10 (3%)	8 (3%)	9 (3%)	8 (4%)
T4/N1	9 (1%)	5 (2%)	1 (0%)	0 (0%)	3 (2%)
M1	16 (1%)	6 (2%)	4 (1%)	4 (1%)	2 (1%)
Unknown	23	6	8	6	3
Prostatectomy TNM stage					
pT1/T2	721 (71%)	225 (72%)	189 (67%)	192 (75%)	115 (71%)
pT3/T3a	185 (18%)	58 (18%)	58 (21%)	41 (16%)	28 (17%)
pT3b	74 (7%)	22 (7%)	24 (9%)	16 (6%)	12 (7%)
pT4/N1	36 (4%)	9 (3%)	11 (4%)	8 (3%)	8 (5%)
Unknown	82	25	19	20	18
Statin use at cancer diagnosis					
Non-user	578 (77%)	202 (76%)	175 (76%)	144 (77%)	57 (80%)
User	174 (23%)	63 (24%)	55 (24%)	42 (23%)	14 (20%)
Unknown	346	74	71	91	110
ERG by immunohistochemistry					
ERG-negative	547 (51%)	203 (61%)	161 (54%)	116 (42%)	67 (38%)
ERG-positive	534 (49%)	129 (39%)	137 (46%)	158 (58%)	110 (62%)
Unknown	17	7	3	3	4
PTEN by immunohistochemistry					
PTEN intact	770 (84%)	245 (87%)	229 (84%)	192 (87%)	104 (76%)
PTEN loss	144 (16%)	38 (13%)	45 (16%)	28 (13%)	33 (24%)
Unknown	184	56	27	57	44
Ki67, % positive nuclei					
< 1%	588 (83%)	194 (89%)	148 (85%)	146 (84%)	100 (70%)
1 to < 5%	101 (14%)	20 (9%)	25 (14%)	23 (13%)	33 (23%)
≥ 5%	19 (3%)	4 (2%)	2 (1%)	4 (2%)	9 (6%)
Unknown	390	121	126	104	39

[1] Values are count (percent) or median (interquartile range). Dietary intake of cholesterol and saturated fat are cumulative averages until diagnosis, available in HPFS only.

Table 2. HMGCR expression intensity (median expression per case across cores) and progression to lethal prostate cancer (metastases/prostate cancer death) among men with primary, non-metastatic prostate cancer in the Health Professionals Follow-up Study (1986-2020) and Physicians' Health Study (1982-2014).

	HMGCR expression intensity				
	None	**Weak**	**Moderate**	**Strong**	**Trend**[1]
Overall					
Participants, N	334	297	273	178	
Events/p-yrs	29/5698	25/4619	12/4432	30/2735	
Rate per 1000 p-yrs	5.1	5.4	2.7	11.0	
Unadjusted HR (95% CI)	1 (ref.)	1.0 (0.6, 1.8)	0.5 (0.3, 1.0)	2.1 (1.3, 3.5)	1.2 (1.0, 1.4)
TMA-stratified HR (95% CI)[2]	1 (ref.)	1.2 (0.7, 2.1)	0.6 (0.3, 1.2)	2.3 (1.3, 4.2)	1.2 (1.0, 1.5)
Multivariable-adjusted HR (95% CI)[3]	1 (ref.)	1.1 (0.7, 1.9)	0.5 (0.3, 1.0)	2.0 (1.2, 3.4)	1.2 (1.0, 1.4)
By statin use at diagnosis					
Non-users (events/p-yrs)	22/3549	15/2856	9/2530	14/893	
Users (events/p-yrs)	1/1009	4/771	0/646	2/209	
Non-users, HR (95% CI)	1 (ref.)	0.8 (0.4, 1.6)	0.6 (0.3, 1.2)	2.5 (1.3, 4.8)	1.2 (0.9, 1.5)
Users, HR (95% CI)	0.2 (0.0, 1.2)	0.8 (0.3, 2.3)	–	1.5 (0.4, 6.5)	1.5 (0.7, 3.0)
By statin use after diagnosis					
Non-users (events/p-yrs)	18/2922	14/2348	8/1933	11/768	
Users (events/p-yrs)	6/1664	7/1274	1/1162	5/326	
Non-users, HR (95% CI)	1 (ref.)	1.0 (0.5, 2.0)	0.7 (0.3, 1.6)	2.3 (1.1, 4.9)	1.2 (0.9, 1.5)
Users, HR (95% CI)	0.6 (0.2, 1.4)	0.8 (0.4, 2.0)	0.1 (0.0, 1.0)	2.3 (0.9, 6.2)	1.3 (0.8, 2.0)
By PTEN status					
PTEN intact (events/p-yrs)	18/4159	16/3549	8/3136	10/1644	
PTEN loss (events/p-yrs)	6/587	8/630	3/406	7/510	
PTEN intact, HR (95% CI)	1 (ref.)	1.0 (0.5, 2.0)	0.6 (0.3, 1.4)	1.4 (0.6, 3.0)	1.0 (0.8, 1.3)
PTEN loss, HR (95% CI)	2.4 (1.0, 6.1)	3.0 (1.3, 6.9)	1.7 (0.5, 5.8)	3.1 (1.3, 7.5)	1.1 (0.8, 1.5)

[1] Linear slope across across HMGCR intensities, per one unit on the scoring scale from 0 (no staining) to 3 (strong).

[2] Addresses batch effects in HGMCR staining by forming risk sets among cases on each tissue microarray (TMA) in Cox regression, with a pooled estimate across TMAs.

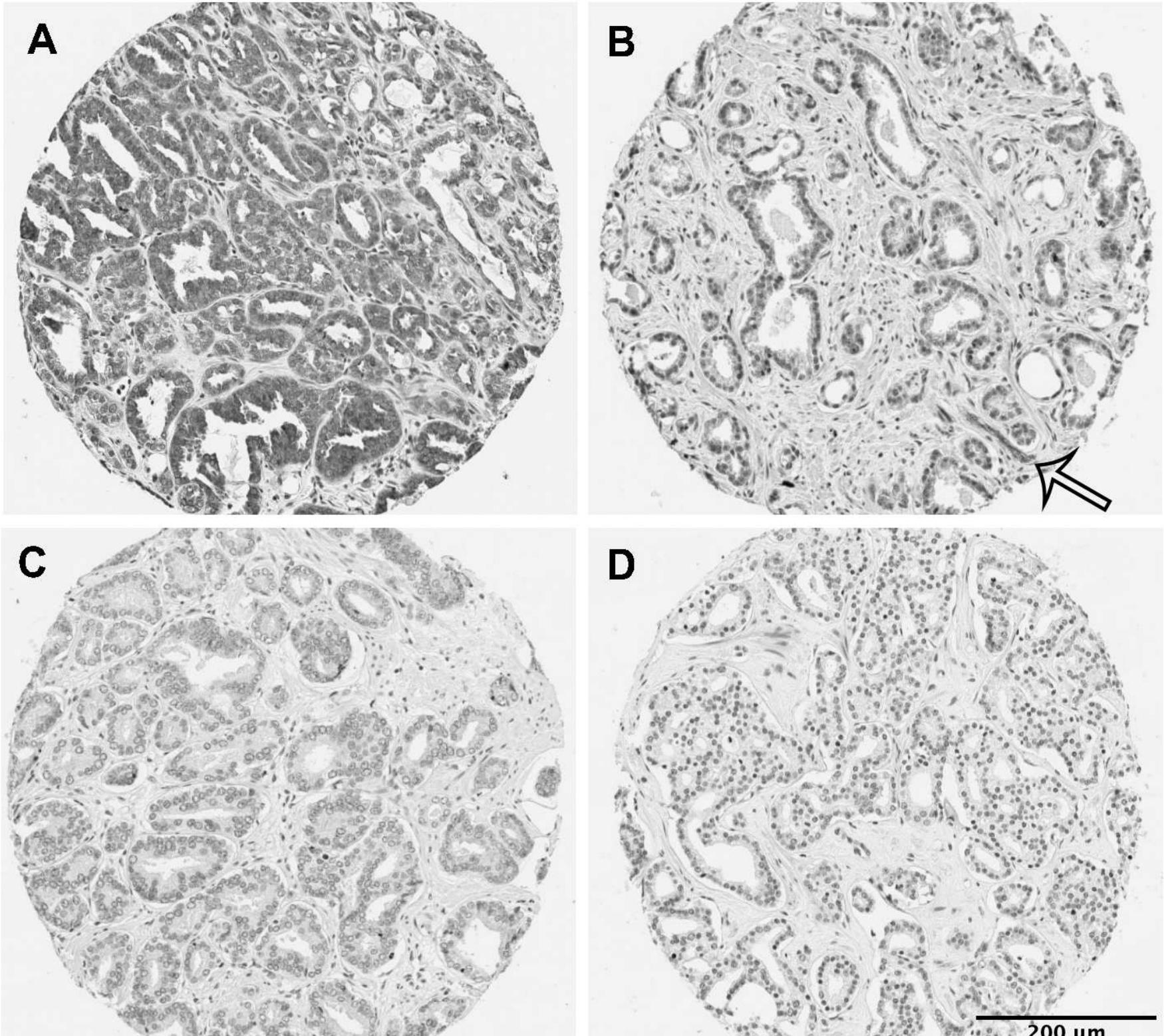
[3] Adjusted for cancer diagnosis (continuous), year of cancer diagnosis (continuous), and Gleason score (categorical as in Table 1).

Abbreviations: p-yrs: person years; HR: hazard ratio; CI: confidence interval; ref., reference.

Figure Legends

Figure 1. Representative photomicrographs (100×) of prostate tumor tissue microarray cores stained for HMGCR, with tumor staining intensity scored as strong (**A**, in a core with Gleason score 4 + 3), moderate (**B**, Gleason 3 + 3), weak (**C**, Gleason 3 + 4) and none (**D**, Gleason 4 + 3). The arrow in image **B** indicates a peripheral nerve used as a positive internal control.

Figure 2. LNCaP cell line viability after shRNA knockdown of *HMGCR* (none, modest, strong) and treatment with atorvastatin (0 nM/vector control, 200 nM, 1000 nM) over up to 5 days. Bars indicate means and 95% confidence intervals of relative estimated viable cells, compared to day 0. Data are from six independent experiments with six wells per condition in each experiment. Growth rate ratios from a linear mixed-effects model were 0.95-fold (95% CI 0.83-1.08, $p = 0.4$) for 200 nM atorvastatin after moderate HMGCR knockdown and 0.86-fold (95% CI 0.76-0.98, $p = 0.027$) after strong HMGCR knockdown; 0.80-fold (95% CI 0.70-0.91, $p < 0.001$) for 1000 nM atorvastatin after moderate HMGCR knockdown and 0.61-fold (95% CI 0.54-0.70, $p < 0.001$) after strong HMGCR knockdown.


A
B
C
D
200 µm

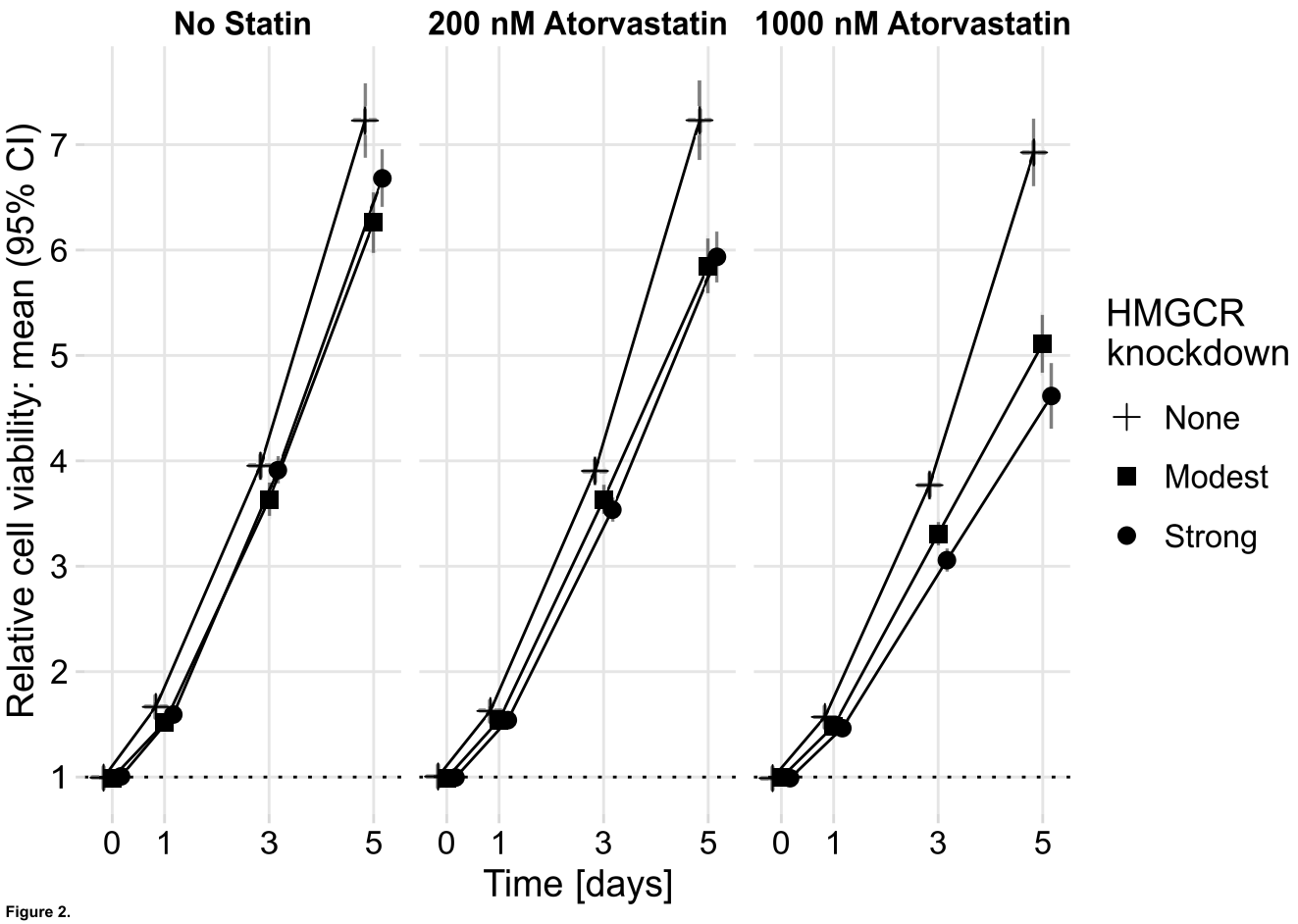

No Statin
200 nM Atorvastatin
1000 nM Atorvastatin
Relative cell viability: mean (95% CI)
1
2
3
4
5
6
7
0
1
3
5
Time [days]
HMGCR knockdown
None
Modest
Strong

Figure 2.