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TITLE: Metabolomic Footprints of Lethal Versus Indolent Prostate Cancer

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The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
The current study proposed to use a targeted, LC-MS-based metabolite profiling platform to measure and compare metabolic profiles of prediagnostic blood samples collected from men subsequently diagnosed with PC and sample of men who remained cancer-free in Physicians’ Health Study (PHS). And test whether these relationships are independent of the known metabolic risk factors (overweight/obese, insulin marker C-peptide, insulin-like growth factor I (IGF-I), IGF binding protein 3, (IGFBP-3), and adiponectin) as well as the clinical characteristics defined as the D’Amico risk. In the original protocol, we planned to measure samples of PC cases from both HPFS and PHS. However, the HPFS team has received separate grant for metabolomics measurement. Therefore, we amended our study population to exclude HPFS data but obtained the HPFS PI’s agreement to be able to access to their data for cross validation. Based on the available samples, we utilized matched case control design to select the blood samples to be measured. The selected blood samples are currently being analyzed in the lab and we expect to receive the lab data by early 2015. While we are waiting for the lab results, we have been working on analyzing the related metabolic biomarkers and writing up manuscripts.
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1. Introduction
A major challenge in prostate cancer (PCa) research is to distinguish aggressive from indolent disease. Although the D’Amico risk stratification is helpful and widely used to guide PCa treatment, it relies on a few standard clinical parameters (prostate specific antigen (PSA), stage, and grade) and cannot always reliably distinguish patients who will die from PCa from those who do not, leading to over-treatment and unnecessary side effects in many men with “low-risk” disease, preventing PCa-specific mortality only in a small minority. On the other hand, some cancers may be destined to recur despite aggressive multi-modality therapy. There is an urgent need for additional biologically relevant markers to improve prognostication beyond algorithms based solely on PSA, stage, and grade. Ideally, such biomarkers could also provide clinical guidance for alternative or novel treatments. Our prospective studies of adiposity, physical activity, and several individual biomarkers in two large Harvard cohorts demonstrate that markers of energy metabolism such as insulin, adipokines, and de novo fatty acid synthesis may play important roles in risk of lethal PCa. Recent development of a metabolite profiling platform by Dr. Clish’s laboratory at the Broad Institute of MIT/Harvard further showed promising potential along this line of research. This technology has identified in vitro an aberrant activation of the PI3K downstream target as a common molecular event in cancer pathology and obesity; revealed significant associations of several amino acids and lipid metabolites in human plasma as signatures of insulin resistance and diabetes risk; and identified signatures of exercise performance and cardiovascular disease susceptibility, proving its validity of metabolic profiling. In addition, the methods have also passed our own rigorous reproducibility assessments. All these provide important ground work for the current proposal. The current study is using a targeted, LC-MS-based metabolite profiling platform to measure and compare metabolic profiles of prediagnostic blood samples collected from men subsequently diagnosed with PC and sample of men who remained cancer-free in Physicians’ Health Study (PHS). And test whether these relationships are independent of the known metabolic risk factors (overweight/obese, insulin marker C-peptide, insulin-like growth factor I (IGF-I), IGF binding protein 3, (IGFBP-3), and adiponectin) as well as the clinical characteristics defined as the D’Amico risk.

2. Keywords
Prostate cancer survivorship, metabolomic profiling, metabolic biomarkers

3. Accomplishments

What were the major goals of the project?
The three original aims were:
Aim 1: Explore and validate the metabolomic footprints for normal controls (n=50 x 2 cohorts) vs. three groups of cases (metastatic PC at diagnosis, initially localized PC and long-time survivors, and initially localized PC but died of PC; n=50 in each of the three groups, total n=150 cases x 2 cohorts);
Aim 2: Among men with initial localized PCa, explore and validate the metabolomic footprints for long-term survivors vs. men who subsequently died of PCa;
Aim 3: Test and validate whether these associations are independent of the known metabolic risk factors (overweight/obese, insulin marker C-peptide, insulin-like growth factor I (IGF-I), IGF...
binding protein 3 (IGFBP-3), and adiponectin), as well as the clinical characteristics defined as
the D’Amico risk.

In the original protocol, we plan to measure samples of PCa cases from both HPFS and PHS. However, the HPFS team has received separate grant for metabolomics measurement. Therefore, we amended our study population to exclude HPFS data, instead we use the fund to increase the sample size from 50 to 100 for each of the proposed aims so that the total sample size remain unchanged.

**Modified aims:**
Aim 1: 1a. Compare to 100 healthy men without cancer (at least at the time when the cases were diagnosed), metabolomic profiling for 100 men with "high risk" (T1-3 and Gleason 8+) or metastasis at diagnosis; 1b. Among men with "high risk" (T1-3 and Gleason 8+) or metastasis at diagnosis, metabolomic profiling between men who died of the cancer vs. those who were still alive by 2012;
Aim 2: Compare the metabolomic profiles between 100 men with "low-intermediate risk" (T1-3 and Gleason 2-7) PCa and died of the cancer with those (n=100) who survived at least 10 years after diagnosis.
Aim 3: Test whether these associations are independent of the known metabolic risk factors (overweight/obese, insulin marker C-peptide, insulin-like growth factor I (IGF-I), IGF binding protein 3 (IGFBP-3), and adiponectin), as well as the clinical characteristics defined as the D’Amico risk.

**What was accomplished under these goals?**
Based on the available samples, we utilized matched case control design to select the blood samples to be measured. The selected blood samples are currently being analyzed in the lab and we expect to receive the lab data by early 2015. While we are waiting for the lab results, we have been working on analyzing the related metabolic biomarkers and writing up manuscripts.

**I. Sample selection**
Based on the available samples, we utilized matched case control design to select the blood samples to be measured. The following part showed the matching method for each specific aim.

**Aim 1: Study Population:**
1) All population in the cohort;
2) Blood volume >100 ml;
   Cases:
   1) Incidence PCa cases 2) Localized and high grade cases or mets at diagnosis; 3) status in mortality file: died of PCa or alive;
   Control Matching criteria:
   1) Same age group at baseline: 40-<50 50-<60 6-<70 70+years
   2) Same fasting >=8, 0<8 hrs
   3) Controls have no cancer, or have cancer, but diagnosed after the last PCa diagnosis date in the age and fasting group.( we only have cancer information, no other disease info)
4) Frequency matching: get same percentile at each age group and total controls are 100.

Program: SAS proc surveyselect

Aim 2: Study Population:
1) Incidence PCa cases with blood collected in 1982;
2) Localized and low grade cases;
3) Blood volume >100 ml;

Cases:
1) Status in mortality file: 1)PCa death; 2) Survived less than 10 years

Control Matching criteria:
1) Same age at diagnosis
2) Same fasting >=8, 0<8 hrs
3) Same Gleason category: 2-6,7
4) Controls are alive now, and alive more than 10 years.
5) 1:1 match", program: proc sql and hash table

In summary, for aim 1, we selected 100 cases who have localized, high grade PCa (clinical stage T1-T3, Gleason grade 8-10) or who have metastatic PCa (clinical stage T4N1M1, Gleason grade 2-10). We matched 100 controls from participants in PHS who were cancer free or have cancer, but diagnosed after the same group of cases. For aim 2, 48 eligible cases were identified from PHS, those cases have localized low grade (clinical stage T1-T3, Gleason grade 2-7) PCa who died of PCa within 10 years after diagnosis. Among them, 43 got matched with controls, who have localized low grade (clinical stage T1-T3, Gleason grade 2-7) PCa, alive at the end of this study or who have been alive more for than 10 years (Table 1).

<table>
<thead>
<tr>
<th>Classification (N)</th>
<th>Sample size</th>
<th>Matched controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aim 1 (All Population)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized T1-T3 &amp; Gleason 8-10</td>
<td>Died of PCa</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Alive</td>
<td>57</td>
</tr>
<tr>
<td>Metastatic PCa (T4N1M1)</td>
<td>Died of PCa</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Alive</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td><strong>181</strong></td>
</tr>
<tr>
<td>Healthy controls</td>
<td></td>
<td><strong>100</strong></td>
</tr>
<tr>
<td><strong>Aim 2 (Incident PCa with 82 blood)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Localized T1-T3 &amp; Gleason 2-6,7</td>
<td>died of PCa</td>
<td><strong>48</strong></td>
</tr>
<tr>
<td></td>
<td>Long-term survivor 10 yr+ controls</td>
<td>487</td>
</tr>
<tr>
<td></td>
<td><strong>43</strong></td>
<td></td>
</tr>
</tbody>
</table>

Following this matching procedure, we selected a total of 372 (Aim1: 181 cases and 100 matched controls; Aim 2: 48 cases and 43 matched controls) blood samples for analyses. 28 QC samples were also included. After checking with the blood lab staff, we have only successfully identified 329 out of 372 eligible samples, and 40 quality control samples from lab. We then decided to add 31 additional samples from PCa patients who have been died from other cancers. We sent a final number of 400 samples to Dr. Clish’s lab at November, 2014.
Power consideration:
The data generated in Aims 1a, 1b, and 2 are all from matched/paired design. Hence, we use the same power calculation formula (Cohen, 1988) to estimate the powers given the sample size and type I error rate. We set the type I error rate as 0.05. The differences among the 3 aims are the effect size, which is unknown until we obtain the data. Hence, we tried a sequence value of effect size. In our original plan, we have n=50 subjects for each of the 2 groups. In the current plan, we have n=100 subjects per group. The power curves shown in the following Figure shows that the power is significantly improved when the sample size is increased from n=50 per group to n=100 subjects per group. For n=50 per group, as long as the effect size > 0.41, the power would be > 0.8. For n=50 per group, as long as the effect size > 0.41, the power would be > 0.8. For n=100 per group, as long as the effect size > 0.29, the power would be > 0.8.

![Power comparison between nPerGroup=50 and nPerGroup=100](image)

Some concerns regarding the PI’s access to the HPFS data for validation:
Because the HPFS have separate funding to measure metabolomics for their PCa cases and controls, we decided to focus this grant solely on the PHS to avoid redundant work but we will validate the model with the HPFS data even though the DOD is not paying for the analysis. To reassuring the DOD that the PI could do so, we have a letter of agreement from Dr. Mucci, the co-leader of the HPFS SPORE project, to confirm that the PI Dr. Ma will be granted access to the HPFS data so that we can complete the validation.

II. Completed/ongoing studies & results
a. Insulin-like growth factor (IGF) pathway genetic polymorphisms, circulating IGF1 and IGFBP3 levels and prostate cancer survival
We conducted kernel machine pathway analysis to evaluate whether 530 tagging single-nucleotide polymorphism (SNP) in 26 IGF pathway-related genes were collectively associated with prostate cancer mortality among 5,887 prostate cancer patients (704 prostate cancer deaths) from 7 cohorts in the NCI Breast and Prostate Cancer Cohort Consortium (BPCa3).

IGF signaling pathway was associated with prostate cancer mortality (P=0.03), and SNP sets of IGF2-AS and SSTR2 were the main contributors (both P=0.04) (Table 5). In SNP-specific analysis, 36 SNPs were associated with prostate cancer mortality with Ptrend<0.05 but only 3 SNPs in the IGF2-AS remained significant after gene-based corrections. Two of the three SNPs
were in perfect linkage disequilibrium ($r^2=1$ for rs1004446 and rs3741211) whereas the third rs4366464 was independent ($r^2=0.03$). The hazard ratios (HRs) per each additional risk allele were 1.19 (95% CI 1.06-1.34; $P_{\text{trend}}=0.003$) for rs3741211 and 1.44 (1.20-1.73; $P_{\text{trend}}=0.0001$) for rs4366464. Rs4366464 remained significant after correction for all the SNPs tested ($P_{\text{trend,corr}}=0.04$, $M_{\text{eff}}=424$). Pre-diagnostic circulating levels of IGF1 (HR$_{\text{highest vs lowest quartile}}$ 0.71; 95%CI 0.48-1.04) and IGFBP3 (HR 0.93; 95%CI 0.65-1.34) were not associated with prostate cancer mortality.

The manuscript has been published by JNCI (June, 2014).

b. Characterization of energy-related biomarkers measured before and after PCa diagnosis in predicting all-cause and PCa-specific mortality.
In the PHS, we defined “high energetic risk” as BMI $>25$ kg/m$^2$ and elevated C-peptide levels (in the highest quartile). We found that this “energetic risk” significantly predicted PCa mortality among men with localized disease at diagnosis independent of clinical characteristics. We replicated this association in an independent cohort, the Health Professionals Follow-up Study (HPFS).

In both cohorts, we found that incorporating this “energetic risk” to the D’Amico risk score (defined by three clinical perimeters: PSA, clinical stage, and Gleason score) significantly improved the predictability of PCa-specific mortality and all-cause mortality in men with initial diagnosis of localized cancer; the C-statistic for PCa-specific mortality was improved from 0.72 to 0.78 ($P<0.001$). Moreover, “energetic risk” identified ~20% of patients who are at high risk of disease specific mortality but are classified as low risk according to clinical characteristics. The resulting paper is undergoing peer-review. One major concern raised was the potential confounding by comorbidity and treatments. We therefore carefully evaluated the impact of these two factors from both cohorts and found little changes of the overall results.

c. Pre-diagnostic Obesity, Smoking and PCa survival
Although obesity and smoking has not been strongly associated with prostate cancer (PCa) incidence, merging evidence linked them to increased PCa-specific mortality. We investigated the
associations of pre-diagnostic BMI and smoking status with risk of progression from time of PCa diagnosis to fatal outcome among 10,106 PCa patients from the NCI Breast and Prostate Cancer Cohort Consortium (BPC3).

Figure 1. Age-adjusted cumulative incidences of a) PCa-specific mortality; and b) Total mortality stratified by BMI categories and smoking status among PCa men from BPC3 study.

Cumulative incidence curves show the probability of prostate cancer-specific mortality or total mortality after diagnosis according to baseline smoking and BMI categories, controlling for age at diagnosis.

The cumulative PCa-specific and overall mortality was much higher in current smokers as compared with never or former smokers. In contrast, the difference according to BMI categories among non-current smokers is much smaller, but still apparent for total mortality. This study provides further evidence that overweight/obesity and smoking history prior to diagnosis are related to poor survival among patients with PCa. The manuscript has been developed and is now circulating among coauthors.

d. Type 2 Diabetes before and after PCa diagnosis with PCa-specific and all-cause mortality. Utilizing the same cohorts data from BPC3, we also observed that New T2D cases after PCa diagnosis was linked to improved survival among PCa cases (Table 2). We plan to look into this together with T2D related SNPs and C-peptide information.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Prostate cancer specific mortality</th>
<th>Other mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR 95%CI p value</td>
<td>HR 95%CI p value</td>
</tr>
<tr>
<td>Diabetes status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Before PCa diagnosis</td>
<td>1.02 0.95 1.09 0.583</td>
<td>1.25 1.08 1.46 0.004</td>
</tr>
<tr>
<td>After PCa diagnosis</td>
<td><strong>0.72 0.61 0.84 &lt;.001</strong></td>
<td>0.62 0.41 0.93 0.021</td>
</tr>
</tbody>
</table>

Multivariate model adjusted for age at diagnosis (continuous), smoking status (never, former, current), BMI (18-22.9, 23-24.9, 25-27.9, 28-29.9, 30-34.9 kg/m2), drinking status (never, <15g/day, ≥15&<30g/day, ≥30g/day), diabetes status (never, baseline, new), cohort (ATBC, CPS2, EPIC, HPFS, MCCS, MEC, PHS, PLCO), duration between baseline and PCa diagnosis (continuous);

e. GWAS-identified type 2 diabetes SNPs and risk of progression to fatal prostate cancer
This study will be based on our recently published study using the BPCa3 genome-wide association study of 2,782 advanced PCa cases and 4,458 controls to evaluate whether 36 T2D susceptibility loci and PCa incident risk (M Machiela et al. Am J Epidemiol 2012). Ten T2D markers near 9 loci (NOTCH2, ADCY5, JAZF1, CDKN2A/B, TCF7L2, KCNQ1, MTNR1B, FTO, and HNF1B) were nominally associated with PCa risk (P < 0.05); the association for rs757210 at the HNF1B locus was significant when multiple comparisons were accounted for (adjusted P = 0.001). Genetic risk scores weighted by the T2D log odds ratio and multilocus kernel tests also indicated a significant relation between T2D variants and PCa risk. These T2D risk variants have not been fully investigated for PCa progression to fatal outcome. Also, few studies have T2D phenotypes or sufficient power to assess whether T2D status mediates the relationship between T2D risk variants and PCa risk. We will fully evaluate these genes and mediation through or interaction with T2D for fatal PCa. We will also explore the association between T2D risk variants and risk of specific type of PCa cases (advanced PCa and died, advanced PCa and long term survivors 10+, localized PCa cases) For our analysis, all 36 T2D SNPs have been pulled out for 7,240 participants including both cases and controls from the imputed data files for BPCa3 Adv Prostate Cancer GWAS. The imputation was done using HapMap 2 Rel 22 CEU phased data as the reference panel.

f. **Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development.**

The pancreatic cancer group from Danna Farber Cancer Institute has also been working on the metabolomics of pancreatic cancer development. We are also working closely with this group on metabolomics data analysis.

This study utilized profiled metabolites in prediagnostic plasma from individuals with pancreatic cancer (cases) and matched controls from four prospective cohort studies. And find that elevated plasma levels of branched-chain amino acids (BCAAs) are associated with a greater than twofold increased risk of future pancreatic cancer diagnosis. This elevated risk was independent of known predisposing factors, with the strongest association observed among subjects with samples collected 2 to 5 years before diagnosis, when occult disease is probably present. We show that plasma BCAAs are also elevated in mice with early-stage pancreatic cancers driven by mutant *Kras* expression but not in mice with *Kras*-driven tumors in other tissues, and that breakdown of tissue protein accounts for the increase in plasma BCAAs that accompanies early-stage disease. Together, these findings suggest that increased whole-body protein breakdown is an early event in development of pancreatic ductal adenocarcinoma (PDAC).

This manuscript has been published in Nature Medicine (Sep, 2014).

**What opportunities for training and professional development has the project provided?**

This provided has provided funding and research opportunities for several doctoral and post-doctoral students from Harvard T.H. Chan School of Public Health.

Yin Cao, graduated from the doctoral of science program from Epidemiology department, and a current post-doc student at Nutrition Department. One of her thesis paper was based on and supported by the current project.
Changzheng Yuan, doctoral candidate at Nutrition and Epidemiology Department. She is now working on three research topics related to this project, mainly focusing on obesity, T2DM and genetic variants related to prostate cancer development.

Meng Yang, postdoc fellow at Nutrition Department. She currently working on studying the BMI trajectory, dietary factors, metabolic biomarkers and prostate cancer survivorship.

CY and MY also work closely with the project leader and statisticians to discuss the study design and sample selections.

**How were the results disseminated to communities of interest?**
Nothing yet to report.

**What do you plan to do during the next reporting period to accomplish the goals?**
We plan to conduct the analyses based on the proposed aims of metabolomic analysis after receiving the lab analyses results.

**4. Impact**

**What was the impact on the development of the principal discipline(s) of the project?**
The most notable strength of our proposal is the use of unbiased metabolomic profiling to distinguish lethal from indolent disease, a major challenge in prostate cancer research. Prostate cancer accounts for 25% of all newly diagnosed cancers and 9% of all cancer deaths in men, making it the most commonly diagnosed and second most lethal cancer for men in the United States. Wide spread use of PSA screening has changed the stage and grade distribution of disease at diagnosis but appears to have only modest effects on prostate cancer mortality. In the United States, 80 to 90% of prostate cancer cases are confined to the prostate and two-thirds of the cases are localized or regional disease and low- to moderate- grade at diagnosis. Current use of clinical features cannot always reliably distinguish patients who will die from prostate cancer from those who do not. Thus, it is important to identify novel markers specifically associated with lethal prostate cancer and lifestyle factors that influence disease progression.

The short-term (1-3 years) impact of this prospective study will be to provide a deeper understanding of the mechanisms of lethal PC phenotype so that relevant biological pathways can be revealed and new biomarkers can be developed. Findings from this study will help better understand the mechanisms of action of energy balance in tumor growth and metastasis and reveal novel biomarkers and pathway effects. This line of research is especially important in the context of highly prevalent obesity and hyperinsulinimia among nondiabetic U.S. adults in recent decades. Findings of this study could then provide biological rationale that risk of lethal PC could be reduced before or at early stage of the disease by modifying metabolic risk through physical activity, healthy diets, and other innovative approaches.

The long-term (3-8 years) goal of our biomarker study is to provide targeted patient identification and stratification to link patients with common biomarkers to the appropriate therapy. Our research could be extended to larger validation studies using novel biomarkers for better stratification methods than the current clinical parameters (e.g., D’Amico risk) to link
patients with common biomarkers to the appropriate personalized prevention and therapeutic strategies. The new risk stratifications could then help to identify candidates for randomized trials of novel agents targeting metabolic dysregulation and pathways. Ultimately, these novel biomarkers could also be candidates for response to such interventions. The overarching challenges and focus areas of this proposal are discovery and validation of biomarkers for the detection and prediction of lethal prostate cancer. This study addresses the overarching challenge and one of the PY11PCRP focus areas: discovery and validation of biomarkers for the detection and prediction of lethal prostate cancer from indolent disease, so that men with indolent disease could be spared from over-treatment, whereas those with high risk potential for lethal phenotype could receive appropriate personalized interventions at an early stage.

What was the impact on other disciplines?  
Nothing yet to report.

What was the impact on technology transfer?  
Nothing yet to report.

What was the impact on society beyond science and technology?  
Nothing yet to report.

5. Changes/Problems  
Changes in approach and reasons for change  
As mentioned above, we now only focus on the PHS cohort.

Actual or anticipated problems or delays and actions or plans to resolve them  
Nothing yet to report.

Changes that had a significant impact on expenditures  
Nothing yet to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents  
Nothing yet to report.

6. Products  
Journal Publications:  


**Other publications, conference papers, and presentations:**

### 7. Participants & Other Collaborating Organizations

**What individuals have worked on the project?**

<table>
<thead>
<tr>
<th>Name</th>
<th>Jing Ma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Role:</td>
<td>PI</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>0000-0002-9132-0741</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>2.52</td>
</tr>
<tr>
<td>Contribution to Project</td>
<td>As the project PI, Dr. Ma has leaded the weekly meetings for project team members. She direct and is responsible for the overall study design and performance, report and manuscript preparation</td>
</tr>
</tbody>
</table>
| Funding Support  | R01CA141298 (Stampfer) – 0.6 Calendar Month  
R01CA137178 (Chan) – 0.24 Calendar Month  
W81XWH-11-1-0529 (Chavarro)- 0.24 Calendar Month  
U45 CA10006 (Hu)- 7.08 Calendar Month  
U01CA155340 (Han-Sub)- 0.12 Calendar Month |

<table>
<thead>
<tr>
<th>Name</th>
<th>Jorge Chavarro</th>
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<tbody>
<tr>
<td>Project Role:</td>
<td>Other Significant Contributor</td>
</tr>
<tr>
<td>Researcher Identifier</td>
<td>N/A</td>
</tr>
<tr>
<td>Nearest person month worked</td>
<td>No Measurable Effort</td>
</tr>
<tr>
<td>Contribution to Project</td>
<td>Dr. Chavarro works closely with Drs. Ma, Clish, and Qiu on data analysis, interpretation, and manuscript preparation</td>
</tr>
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| Funding Support  | HHSN275201000020C (Hu)- 2.40 Calendar Month  
CA-10-006 (Hu)- 2.28 Calendar Month  
W81XWH-11-1-0529 (Chavarro)- 4.20 Calendar Month |
<table>
<thead>
<tr>
<th>Name</th>
<th>HayHaiyan Zhang</th>
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<tr>
<td>Project Role:</td>
<td>Data Manager</td>
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<tr>
<td>Researcher Identifier</td>
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<tr>
<td>Nearest person month worked</td>
<td>3</td>
</tr>
<tr>
<td>Contribution to Project</td>
<td>Zhang is responsible for managing the database for Physicians’ Health Study biobank, case-control selection and preparation for the biospecimen pulling list preparation.</td>
</tr>
</tbody>
</table>

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
Nothing to Report.

**What other organizations were involved as partners?**
Clary Clish, sub-contract PI, conducting metabolic analysis, the Metabolite Profiling Platform, Broad Institute of MIT/Harvard. Dr. Clish is an expert in metabolic profiling assay development and validations, and oversee the assay development, measurement, and data annotation at his laboratory.
8. Special Reporting Requirements
COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to https://ers.amedd.army.mil for each unique award.
QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

No.

9. Appendices


BACKGROUND

- Meta-analysis linked elevated BMI with increased risk of PSA recurrence or prostate cancer (PC)-specific mortality. However, short follow-up and lack of control for smoking are major limitations in many of the clinical studies.

- Few prospective studies have sufficient power to investigate the relationship between obesity and lethal PC by time of BMI measurement before PC diagnosis and by smoking status.

- This study aimed to investigate the associations of pre-diagnostic BMI with risk of progression from time of PC diagnosis to fatal outcome, to study whether the relationship differs by time of BMI measurement before PC diagnosis and to assess effect modification by smoking status on BMI status and PC survival.

METHODS

- The study included 10,106 PC cases from the NCI Breast and Prostate Cancer Cohort Consortium (BPC3).
- BMI and smoking status were estimated at baseline before PC diagnosis. Deaths among PC patients were categorized into deaths from PC and other causes.
- We conducted the analysis in 3 parts according to exposures: BMI (18-22.9 kg/m², 23-24.9 kg/m², 25-27.9 kg/m², 28-29.9 kg/m², 30-34.9 kg/m², 35 kg/m²+), smoking (Never, former, current) and their joint effect, respectively.
- Competing-risks regression model was used to take into account of other causes of death. Each analysis was performed on prostate cancer specific mortality and other mortality separately.

RESULTS

Table 1. Characteristics of subjects according to death status

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total prostate cancer cases (n=10,106)</th>
<th>Prostate cancer death (n=7,213)</th>
<th>Survivors (n=2893)</th>
<th>p for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (mean±SD, yr)</td>
<td>68.7±6.8</td>
<td>71.8±5.9</td>
<td>65.8±7.0</td>
<td>0.009</td>
</tr>
<tr>
<td>BMI at diagnosis (mean±SD, kg/m²)</td>
<td>28.3±4.3</td>
<td>28.1±3.5</td>
<td>28.7±3.3</td>
<td>0.010</td>
</tr>
<tr>
<td>Follow-up time (mean±SD, yr)</td>
<td>7.5±4.7</td>
<td>7.4±3.5</td>
<td>7.8±4.7</td>
<td>0.010</td>
</tr>
<tr>
<td>Cohort (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATBC</td>
<td>10.2</td>
<td>28.6</td>
<td>24.7</td>
<td>0.440</td>
</tr>
<tr>
<td>CPS2</td>
<td>22.2</td>
<td>12.3</td>
<td>18.2</td>
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<tr>
<td>HPFS</td>
<td>15.3</td>
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</tr>
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<td>MEC</td>
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<td>6.7</td>
<td>11.5</td>
<td></td>
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<tr>
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<td>MRC</td>
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<td>8.6</td>
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<tr>
<td>PLCO</td>
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<td>17.2</td>
<td>14.7</td>
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<tr>
<td>PLCO</td>
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<td>8.5</td>
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<td>BMI category (%)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>18-22.9</td>
<td>36.9</td>
<td>37.8</td>
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</tr>
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<td>23-24.9</td>
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<td>45.6</td>
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<tr>
<td>25-27.9</td>
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<td>12.9</td>
<td>12.9</td>
<td>0.123</td>
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<tr>
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<td>23.3</td>
<td>24.7</td>
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<tr>
<td>30-34.9</td>
<td>44.8</td>
<td>37.1</td>
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<tr>
<td>35+</td>
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<td>54.2</td>
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<tr>
<td>Drinking status (%)</td>
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<td>11.6</td>
<td>15.4</td>
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<td>&lt;15 g/day</td>
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<td>≥15 &amp; &lt;30 g/day</td>
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<td>≥30 g/day</td>
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<td>16.9</td>
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<tr>
<td>Diabetes status (%)</td>
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<td>84.5</td>
<td>83.9</td>
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<td>6.1</td>
<td>5.8</td>
<td>0.811</td>
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<tr>
<td>Glucose status (%)</td>
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</tr>
<tr>
<td>eGlu (mg/dL)</td>
<td>40.4</td>
<td>11.7</td>
<td>33.7</td>
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<tr>
<td>G2T (mg/dL)</td>
<td>26.4</td>
<td>19.2</td>
<td>20.8</td>
<td>0.289</td>
</tr>
<tr>
<td>G2T (mg/dL)</td>
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<td>12.2</td>
<td>25.5</td>
<td>0.966</td>
</tr>
<tr>
<td>Triglyceride status (%)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>ATR (mg/dL)</td>
<td>85.1</td>
<td>67.1</td>
<td>79.1</td>
<td>0.849</td>
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<tr>
<td>C25T</td>
<td>10.8</td>
<td>12.5</td>
<td>13.9</td>
<td>0.105</td>
</tr>
<tr>
<td>TRG</td>
<td>7.8</td>
<td>10.4</td>
<td>15.8</td>
<td>2.0</td>
</tr>
</tbody>
</table>

![Figure 1. Effect of BMI (smoking) on PC mortality and other mortality (overall and by measurement time before PC diagnosis)](image1)

![Figure 2. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image2)

![Figure 3. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image3)

![Figure 4. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image4)

![Figure 5. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image5)

![Figure 6. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image6)

![Figure 7. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image7)

![Figure 8. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image8)

![Figure 9. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image9)

![Figure 10. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image10)

![Figure 11. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image11)

![Figure 12. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image12)

![Figure 13. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image13)

![Figure 14. BMI and Smoking Joint Effects on a) Prostate Cancer Specific Mortality and b) Cancer survivorship)](image14)
Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development

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Most patients with pancreatic ductal adenocarcinoma (PDAC) are diagnosed with advanced disease and survive less than 12 months1. PDAC has been linked with obesity and glucose intolerance2–4, but whether changes in circulating metabolites are associated with early cancer progression is unknown. To better understand metabolic derangements associated with early disease, we profiled metabolites in prediagnostic plasma from individuals with pancreatic cancer (cases) and matched controls from four prospective cohort studies. We find that elevated plasma levels of branched-chain amino acids (BCAAs) are associated with a greater than twofold increased risk of future pancreatic cancer diagnosis. This elevated risk was independent of known predisposing factors, with the strongest association observed among subjects with samples collected 2 to 5 years before diagnosis, when occult disease is probably present. We show that plasma BCAAs are also elevated in mice with early-stage pancreatic cancers driven by mutant Kras expression but not in mice with Kras-driven tumors in other tissues, and that breakdown of tissue protein accounts for the increase in plasma BCAAs that accompanies early-stage disease. Together, these findings suggest that increased whole-body protein breakdown is an early event in development of PDAC.

PDAC is a leading cause of cancer-related death worldwide, and most patients have incurable disease at diagnosis1. The best-characterized predisposing factors, current tobacco use and a first-degree relative with PDAC, both impart an approximate 1.8-fold increased risk for the disease5,6. These risk factors, however, have thus far provided limited insight into the biology of early disease progression of sporadic tumors. Development and progression of PDAC is also associated with altered systemic metabolism including obesity2, glucose intolerance3,4 and cancer-induced cachexia7. Nevertheless, no systematic examination of circulating metabolites has been performed to determine whether altered metabolism may indicate subclinical pancreatic cancer or inform understanding of early disease progression when interventions might improve patient outcomes.

Prior efforts to identify changes in circulating metabolites related to cancer have employed a cross-sectional design, comparing cancer-free subjects to affected individuals with blood samples collected at diagnosis8–10. This approach is problematic for discovery of changes related to early cancer progression, as consequences of advanced disease are likely to have an impact on circulating metabolite profiles. This is particularly true for patients with pancreatic cancer, who commonly have significant anorexia, weight loss and pancreatic insufficiency at the time of diagnosis1. To investigate how altered metabolism might contribute to pancreatic malignancy, we profiled...
plasma metabolites in cases with PDAC and matched controls drawn from four prospective cohort studies with blood collected at least 2 years before cancer diagnosis (Supplementary Table 1). The median time between blood collection and PDAC diagnosis was 8.7 years. In conditional logistic regression models, levels of 15 metabolites were associated with future diagnosis of PDAC to \( P < 0.05 \); three metabolites, the BCAAs isoleucine, leucine and valine were significant to \( P \approx 0.0006 \), the predefined significance threshold after correction.

**Figure 1** Plasma metabolites and risk of future pancreatic cancer diagnosis. \( P \) values of the log-transformed, continuous metabolite levels from human plasma comparing pancreatic cancer cases and controls in conditional logistical regression models conditioned on matching factors and adjusted for age at blood draw (years, continuous), fasting time (<4 h, 4–8 h, 8–12 h, ≥12 h, missing) and race or ethnicity (white, black, other, missing). The dashed green line indicates the statistically significant \( P \) value threshold after Bonferroni correction for multiple-hypothesis testing, \( P \approx 0.0006 \) (0.05/83). The dashed blue line indicates \( P \) of 0.05. The number of cases and controls analyzed for each metabolite is provided in Supplementary Table 2.
Table 1 Odds ratios for pancreatic cancer by prediagnostic plasma levels of BCAAs

<table>
<thead>
<tr>
<th>Model</th>
<th>Isoleucine</th>
<th>Leucine</th>
<th>Valine</th>
<th>Total BCAAs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extreme quintiles</td>
<td>Per s.d.</td>
<td>Extreme quintiles</td>
<td>Per s.d.</td>
</tr>
<tr>
<td>Base model</td>
<td>2.11</td>
<td>1.30</td>
<td>2.08</td>
<td>1.31</td>
</tr>
<tr>
<td>(1.40–3.18)</td>
<td>(1.15–1.48)</td>
<td>(1.38–3.13)</td>
<td>(1.14–1.50)</td>
<td>(1.37–2.92)</td>
</tr>
<tr>
<td>+ BMI and physical activity</td>
<td>2.05</td>
<td>1.29</td>
<td>2.01</td>
<td>1.29</td>
</tr>
<tr>
<td>(1.34–3.12)</td>
<td>(1.14–1.48)</td>
<td>(1.32–3.06)</td>
<td>(1.12–1.49)</td>
<td>(1.31–2.86)</td>
</tr>
<tr>
<td>+ BMI, physical activity and reported diabetes at blood collection</td>
<td>2.00</td>
<td>1.28</td>
<td>1.97</td>
<td>1.28</td>
</tr>
<tr>
<td>(1.31–3.05)</td>
<td>(1.13–1.46)</td>
<td>(1.29–2.99)</td>
<td>(1.11–1.48)</td>
<td>(1.28–2.81)</td>
</tr>
<tr>
<td>+ BMI, physical activity, reported diabetes, HbA1c, plasma insulin, proinsulin and C-peptide</td>
<td>1.86</td>
<td>1.24</td>
<td>1.81</td>
<td>1.24</td>
</tr>
<tr>
<td>(1.13–3.03)</td>
<td>(1.06–1.45)</td>
<td>(1.11–2.96)</td>
<td>(1.04–1.47)</td>
<td>(1.06–2.63)</td>
</tr>
<tr>
<td>Exclude subjects with reported diabetes or HbA1c ≥6.5% at blood collection</td>
<td>2.12</td>
<td>1.33</td>
<td>2.16</td>
<td>1.32</td>
</tr>
<tr>
<td>(1.37–3.27)</td>
<td>(1.16–1.52)</td>
<td>(1.39–3.35)</td>
<td>(1.14–1.54)</td>
<td>(1.28–2.85)</td>
</tr>
<tr>
<td>≥6.5% at blood collection and those with reported diabetes after blood collection</td>
<td>2.18</td>
<td>1.32</td>
<td>2.21</td>
<td>1.33</td>
</tr>
<tr>
<td>(1.39–3.43)</td>
<td>(1.15–1.53)</td>
<td>(1.40–3.49)</td>
<td>(1.13–1.55)</td>
<td>(1.27–2.96)</td>
</tr>
</tbody>
</table>

*Odds ratio (95% CI) from conditional logistic regression models conditioned on matching factors and adjusted for age at blood draw (years, continuous), fasting time (<4 h, 4–8 h, 8–12 h, ≥12 h, missing) and race or ethnicity (white, black, other, missing). Subsequent models also adjusted for the indicated measure of energy balance, hyperglycemia or insulin resistance: body-mass index (kg/m², continuous), physical activity (metabolic equivalent task-hours per week (MET-h/week), continuous), reported diabetes at blood collection (yes or no), hemoglobin A1c (HbA1c) (%), continuous), plasma insulin (>1U/ml, continuous), plasma proinsulin (pM, continuous) and plasma C-peptide (ng/ml, continuous). *Odds ratios (95% CI) for the comparison of the fifth quintile to the first quintile (referred) for the circulating BCAAs. *Sum of the concentrations of the three circulating BCAAs, isoleucine, leucine and valine.

for multiple-hypothesis testing (Fig. 1 and Supplementary Table 2). To evaluate the magnitude of risk for PDAC diagnosis, we divided participants into quintiles of increasing BCAA levels. Compared to the bottom quintile, subjects in the top quintile had at least a two-fold increased risk of developing PDAC (Table 1, Supplementary Table 3 and Supplementary Fig. 1). As noted previously, circulating levels

Figure 2 Plasma BCAA levels are elevated during subclinical disease. (a) Graph of odds ratio (error bars indicate 95% confidence interval (CI)) for future pancreatic cancer diagnosis among cohort cases and matched controls comparing highest versus lowest quintiles of circulating BCAA levels stratified by time from blood collection to the case’s cancer diagnosis. Odds ratio was determined from conditional logistic regression models conditioned on matching factors and adjusted for age at blood draw (years, continuous), fasting time (<4 h, 4–8 h, 8–12 h, ≥12 h, missing) and race or ethnicity (white, black, other, missing). Red horizontal line marks an odds ratio of 1.0. The number of cases and controls in each time period and association results for the individual BCAAs are provided in Supplementary Table 6. (b) Graph of mean (±s.e.m.) total plasma BCAA concentration in LSL-KrasG12D;Lsl-Trp53R172H;Pdx1-cre (KPC) mice over time and in littermate controls lacking either LSL-KrasG12D or Pdx1-cre or both. Each control data point is an average for one mouse over the course of the study (Supplementary Fig. 4b), and values for KPC mice living longer than 19 weeks are averaged for the >19-weeks time point. For weeks 15–17, n = 6 KPC and n = 9 control, Student’s t-test, P = 0.001. For >19 weeks, n = 4; 11–13 weeks, n = 6; 7–9 weeks, n = 7; 3–5 weeks, n = 9. (d) H&E staining pancreatic tissue obtained from KPC−/− mice and littermate controls at 3–4 weeks of age. Tissues are from a control mouse with histologically normal pancreas (left) a KPC−/− mouse with areas of PDAC adjacent to areas of normal pancreas (middle) and a KPC−/− mouse with areas of PDAC and pancreatic intraepithelial neoplasia (arrows; right). Scale bars, 50 μm. (d) Mean (±s.e.m.) body weights at 3–4 weeks of age for KPC−/− mice and littermate controls (n = 7 KPC−/−, n = 11 control). (e) Mean (±s.e.m.) total plasma BCAAs levels from KPC−/− mice and littermate controls at 3–4 weeks of age (n = 10 KPC−/−, n = 14 control, Student’s t-test, P = 0.002). (f) P values for comparison of circulating amino acid levels in KPC−/− mice and littermate controls at 3–4 weeks of age (n = 10 KPC−/−. n = 14 control). Red points indicate BCAAs. The dashed red line indicates P value of 0.05. (g) Top, glucose tolerance test in KPC−/− mice and littermate controls at the time of weaning (n = 7 KPC−/−, n = 11 control). Bottom, insulin tolerance test in KPC−/− mice and littermate controls at 4 weeks of age (n = 7 KPC−/−, n = 15 control). Error bars indicate s.e.m. (h) Mean (±s.e.m.) fasting plasma insulin levels from KPC−/− mice and littermate controls at 4 weeks of age (n = 7 KPC−/−, n = 11 control).
of the three BCAAs were highly correlated (Supplementary Table 4), reflecting their common pathways of metabolism\(^1\) and leading to similar results for the sum total of BCAAs (Table 1 and Supplementary Table 3).

Circulating BCAAs are elevated in obese individuals and those with insulin resistance\(^1\). In study participants, plasma BCAA levels modestly correlated with markers of energy balance, obesity and glucose intolerance (Supplementary Table 4). To evaluate the independent effect of BCAAs on PDAC risk, we assessed models that included these markers and found that the odds ratios for PDAC remained largely unchanged (Table 1). Elevated circulating levels of BCAAs are also associated with future risk of diabetes\(^1\)\(^1\)\(^,\)\(^4\). As type 2 diabetes is a predisposing factor for PDAC\(^5\)\(^,\)\(^6\), we questioned whether the intermediate development of diabetes underlined the association of BCAAs with future PDAC diagnosis. Exclusion of subjects with diabetes at blood collection did not change our results (Table 1), indicating that we had not identified a signature of prevalent diabetes associated with later PDAC diagnosis. To determine whether increased circulating BCAAs identify a population at risk for diabetes, who are then at elevated risk of PDAC, we excluded subjects who developed diabetes between the time of blood collection and cancer diagnosis and found the results unchanged (Table 1). These data suggest that the association of circulating BCAAs with future PDAC diagnosis is not dependent on intermediate development of diabetes.

To examine the contribution of circulating BCAAs to risk stratification models for PDAC, we evaluated the area under the curve (AUC) of receiver-operating-characteristic (ROC) curves\(^6\)\(^,\)\(^7\) and net reclassification improvement (NRI)\(^7\) with low-risk and high-risk categories. Compared to the base model, including circulating BCAAs led to a significant increase in AUC (Supplementary Table 5a and Supplementary Fig. 2) and a net 8.2% of cases moving to the high-risk category with an NRI of 5% (Supplementary Table 5b). Thus, in our population, inclusion of circulating BCAAs in risk stratification models improved the ability to identify future PDAC cases.

In stratified analyses, we noted no significant differences in the association of BCAAs with PDAC by cohort, sex, smoking status, body-mass index (BMI) or fasting status at blood collection (Supplementary Fig. 3, all interaction \(P \geq 0.14\)). To examine when circulating BCAAs were most associated with PDAC, we stratified cases and matched controls by time interval between blood collection and PDAC diagnosis (<2 years, 2 to <5 years, 5 to <10 years and ≥10 years). These analyses demonstrated particularly strong associations between elevated BCAAs at 2–5 years before diagnosis and future PDAC diagnosis (Fig. 2a and Supplementary Table 6).

Experimental studies indicate years elapse between formation of the initial malignant clone and cancer diagnosis\(^8\),\(^9\), suggesting that occult PDAC might have been present at the time points showing the strongest associations with elevated BCAAs. We therefore hypothesized that elevated circulating BCAAs are a marker of early PDAC. To test this possibility, we conducted a prospective serial blood sampling study using lox-stop-lox (LSL)-Kras\(^{G12D}\)\(^{−/−}\); LSL-Trp53\(^{R172H}\)\(^{−/−}\); Pdx1-cre (KPC) mice, which develop PDAC with variable latency\(^9\). KPC mice progress through all histological stages of disease, from normal pancreata to invasive adenocarcinoma, with a median survival of approximately 21 weeks (Supplementary Fig. 4a). KPC mice initially displayed similar BCAA levels to littermate controls, but they developed significant elevations from 15–17 weeks before death (Fig. 2b and Supplementary Fig. 4b). These data suggest circulating BCAA elevations accompany early PDAC.

\(\text{LSL-Kras}^{G12D}\)\(^{−/−}\); Trp53\(^{R172H}\)\(^{−/−}\); Pdx1-cre (KPC) mice develop PDAC with more consistent kinetics, displaying precursor lesions with limited invasive cancer by 3–4 weeks of age (Fig. 2c) and a median lifespan of 10–12 weeks\(^9\). In mice at 3–4 weeks of age, we observed no difference in body weight or food consumption between KP\(^−/−\) mice and littermate controls lacking either Pdx1-cre or LSL-Kras\(^{G12D}\) or both, suggesting animals with early PDAC had not yet developed overt constitutional symptoms (Fig. 2d and Supplementary Fig. 4c). Consistent with findings in patients and KPC mice, circulating BCAA levels were higher in KP\(^−/−\) animals with subclinical PDAC when compared with those in littermate control mice (Fig. 2e), a pattern not observed for most other amino acids (Fig. 2f and Supplementary Fig. 4d, e). We observed no significant differences in fasting blood amino acid pools to the BCAAs present in plasma (\(n \geq 22\) KP\(^−/−\), \(n = 24\) control, two-sample t-test, \(P = 0.02\) for leucine, \(P = 0.01\) for valine).

\(\text{Gastrocnemius muscle from fasted KP}^{−/−}\) mice and control littermates (\(n = 8\) KP\(^−/−\), \(n = 6\) control). (d) Mean (±s.e.m.) fractional labeling of plasma amino acids in KP\(^−/−\) and control mice in the fast state (\(n = 3\) KP\(^−/−\), \(n = 4\) control). (e) The calculated contribution (±s.e.m.) of the short- and long-term BCAA pools to the BCAAs present in plasma (\(n = 22\) KP\(^−/−\), \(n = 24\) control, two-sample t-test, \(P = 0.02\) for leucine, \(P = 0.01\) for valine). (f) Mean (±s.e.m.) gastrocnemius (a predominantly fast-twitch muscle) weight (left, Student’s t-test, \(P = 0.01\)), and heart weight (right) normalized to body weight of KP\(^−/−\) mice and littermate controls at 4 weeks of age (\(n = 6\) KP\(^−/−\), \(n = 10\) control).

Figure 3 BCAA elevations are derived from a long-term pool of amino acids. (a) Plasma levels (mean ± s.e.m.) of 1\(^3\)C-labeled leucine (M+6) and 1\(^3\)C-labeled valine (M+5) normalized to food intake over time following a two-hour exposure to diets containing 1\(^3\)C\(^−/−\)C-labeled leucine and 1\(^3\)C-labeled valine (\(n = 8\) KP\(^−/−\), \(n = 6\) control). The time points correspond to the red arrowheads in the diagram. (b) Diagram of experiment using labeled diets to investigate contributions to plasma BCAA levels from long-term pools. Two cohorts of mice were used for these experiments, one killed in the fed state and another in the fasted state.

The calculated contribution (±s.e.m.) of the short- and long-term BCAA pools to the BCAAs present in plasma (\(n \geq 22\) KP\(^−/−\), \(n = 24\) control, two-sample t-test, \(P = 0.02\) for leucine, \(P = 0.01\) for valine). (f) Mean (±s.e.m.) gastrocnemius (a predominantly fast-twitch muscle) weight (left, Student’s t-test, \(P = 0.01\)), and heart weight (right) normalized to body weight of KP\(^−/−\) mice and littermate controls at 4 weeks of age (\(n = 6\) KP\(^−/−\), \(n = 10\) control).
These data suggest that an early consequence of PDAC is enhanced breakdown of tissue proteins leading to elevated plasma BCAA levels. Consistent with this hypothesis, KP−/− mice with early PDAC had smaller fast-twitch muscles with no changes in slow-twitch and cardiac muscle weight (Fig. 3f and Supplementary Fig. 9). Notably, muscle atrophy associated with prolonged fasting and late-stage malignancy exhibits a similar pattern30−32.

Increased muscle catabolism represents one aspect of cancer-associated cachexia, a wasting syndrome frequently affecting patients with advanced PDAC and contributing to worse outcomes33−36. Our findings, however, suggest that protein breakdown begins much earlier than previously appreciated and predates onset of clinical cachexia. Inflammatory cytokines produced by immune and/or tumor cells have been implicated in cachexia11,37, and the low disease burden at the time of BCAA elevation suggests hormonal factors may be involved in early PDAC to cause these elevations as well. Liberation of tissue amino acids could support the elevated amino acid requirements of pancreatic cancer cells38,39, with BCAAs and/or other amino acids derived from tissue breakdown contributing to disease progression. Because hepatic metabolism maintains relatively constant plasma levels of all amino acids except BCAAs27,29,40, increased liberation of tissue amino acids would be expected raise BCAA concentrations in blood. The association between elevated BCAA levels and other metabolic disease states11,13,14,41 suggests that high plasma BCAA concentrations could be a general marker of increased protein turnover, and elevated BCAA levels could contribute to the peridiagnostic hyperglycemia commonly found in patients with PDAC42.

In participants from four large prospective cohorts, circulating BCAAs were associated with future diagnosis of PDAC. We observed similar BCAA elevations in two mouse models of PDAC and demonstrated that these elevations result from breakdown of peripheral protein stores. These findings provide new insight into how early disease affects whole-body metabolism and suggest that muscle protein loss occurs much earlier in disease progression than previously appreciated.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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glucose, response to glucose load, response to insulin challenge or fasting insulin levels during intraperitoneal glucose and insulin tolerance tests in 4-week-old KP−/− and control mice (Fig. 2g,h and Supplementary 4f−i). These findings argue that BCAA elevations are not reflective of hyperglycemia or insulin resistance and instead represent an early consequence of subclinical PDAC.

We examined whether malignancies in other tissues induced by the same genetic lesions could cause elevated plasma BCAA levels. Cre recombinase introduction into lung or muscle of mice with the LSL-KrasG12D and Tprp53floxt/loxa alleles from the KP−/− model leads to non–small-cell lung cancer and sarcoma, respectively21–23. Neither model displayed the BCAA alterations seen with early PDAC (Supplementary Fig. 5). Subcutaneous and orthotopic implantation of cancer cell lines derived from the KP−/− model into immunocompetent syngeneic hosts both also failed to cause elevated BCAA levels (Supplementary Fig. 6). These data argue that elevations in BCAAs are associated with early-stage autochthonous tumors arising in the pancreas and are not a general feature of Kras-driven cancer. They also suggest that implantation of cells from end-stage disease does not model the early disease state that results in BCAAs elevations.

Chronic pancreatitis is a risk factor for human PDAC24, and pancreatic inflammation can promote PDAC development and progression in mice25,26. Therefore, we examined whether BCAA elevations might be a cause or consequence of pancreatic inflammation in early disease in mice. Mild, chronic pancreatitis induced by caerulein in the absence of tumorigenesis failed to cause elevations in BCAAs (Supplementary Fig. 7a−h), and prolonged increases in plasma BCAA levels caused by dietary interventions did not cause pancreatic inflammation or pancreatitis (Supplementary Fig. 7i−o). Nevertheless, further studies are needed to understand the relationship between BCAAs and more severe pancreatitis.

Unlike levels of other amino acids, plasma BCAA levels are not regulated by the liver27,28; instead, levels are determined by dietary uptake, tissue utilization and breakdown of muscle and other body protein stores27,29. Therefore, plasma BCAAs may originate from short-term pools related to dietary uptake and disposal or long-term pools related to breakdown of tissue proteins. To determine the involvement of the short-term pool, we fed 4-week-old KP−/− mice and littermate control mice lacking either Pdx1-Cre or LSL-KrasG12D or both a defined amino acid diet, in which 20% of leucine and valine were 13C labeled. KP−/− and control mice consumed similar amounts of food when exposed to labeled diet for 2 h (Supplementary Fig. 8a), and we observed no difference in appearance and disappearance of plasma 13C label (Fig. 3a and Supplementary Fig. 8b), arguing that gut uptake and peripheral disposal of BCAAs are similar in mice with or without PDAC.

To determine the contribution of long-term BCAA pools to plasma levels, we exposed mice to labeled diet during a period of rapid growth in early life and then switched them to unlabeled diet for 3 d to chase label from the short-term pool (Fig. 3b). Despite similar peripheral tissue protein labeling (Fig. 3c and Supplementary Fig. 8c), the fraction of labeled BCAAs in plasma was elevated in 4-week-old KP−/− mice relative to that in littermate controls (Fig. 3d). Furthermore, by comparing the amount of label in plasma under fed conditions, encompassing both labeled long-term and unlabeled short-term pools, to that under fasted conditions, in which only labeled long-term pools contribute, we calculated that increased liberation of BCAAs from long-term body stores was solely responsible for the elevations in BCAAs in KP−/− mice (Fig. 3d,e and Supplementary Fig. 8d). These data suggest that an early consequence of PDAC is enhanced
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AUTHOR CONTRIBUTIONS


COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Study population. Our study population included pancreatic cancer cases and controls from four prospective cohort studies: the Health Professionals Follow-Up Study (HPFS), the Nurses’ Health Study (NHS), the Physicians’ Health Study 1 (PHS) and the Women’s Health Initiative-Observational Study (WHI-OS). HPFS was initiated in 1986 when 51,529 US men 40–75 years of age working in health professions completed a mailed biennial questionnaire. NHS was established in 1976 when 121,700 female nurses aged 30–55 years completed a mailed biennial questionnaire. PHS is a completed trial initiated in 1982 of aspirin and β-carotene among 22,071 male physicians, aged 40–84 years. After trial completion, study participants were followed as an observational cohort. WHI-OS consists of 93,676 postmenopausal women, aged 50–79 years, enrolled from 1993–1998 at 40 US clinical centers. Participants completed a baseline clinic visit and annual mailed questionnaire. The study was approved by the Human Research Committee at Brigham and Women’s Hospital (Boston, MA), and participants provided written informed consent.

We included incident pancreatic adenocarcinoma cases diagnosed after blood collection through 2010 with available plasma and no prior history of cancer. Cases were identified by self-report or follow-up of deaths. Deaths were ascertained from next-of-kin, postal service or National Death Index; this method captures >98% of deaths. Medical records were reviewed by physicians blinded to exposure data to confirm pancreatic cancer diagnoses. Similar to prior studies in these cohorts, we based on a predefined analysis plan, we included only cases diagnosed ≥2 years after blood collection, as the weight loss and insulin resistance that develop due to pancreatic cancer manifest in the 2 years before diagnosis. For each case, we randomly selected two controls, matching on cohort (also matches on sex), year of birth (±5 years), smoking status (never, past, current, missing), fasting status (<8 h, ≥8 h), and month/year of blood collection (±3 months in HPFS, ±3 months in NHS, ±6 months in PHS, and exact matching in WHI). Controls were alive without cancer at the case’s diagnosis date and provided a blood sample. Covariate data were obtained from baseline questionnaires in PHS and WHI and questionnaires before blood collection in HPFS and NHS, as described previously.

In HPFS, NHS, and PHS, cancer stage among cases was directly classified based on medical record review as local disease amenable to surgical resection, locally advanced disease that is unresectable but without distant metastases, or distant metastatic disease. In WHI, medical records were coded using Surveillance Epidemiology End Results summary staging, which classifies tumors as localized, regional, or distant. These stages were then classified in the same manner as in HPFS, NHS and PHS, as local, locally advanced, and metastatic disease, respectively.

We previously measured hemoglobin A1c (HbA1c) in 389 cases and 757 controls in the laboratory of N. Rifai (Children’s Hospital, Boston, MA) using reagents from Roche Diagnostics (Indianapolis, IN). We measured plasma insulin in 386 cases and 743 controls, plasma proinsulin in 388 cases and 746 controls, and plasma C-peptide in 408 cases and 785 controls using reagents from Diagnostic Systems Laboratory (Webster, TX) and Millipore Corporation (Billerica, MA). Randomly inserted samples from quality control (QC) plasma pools had mean intra-assay coefficients of variance (CVs) of 2.0% for HbA1c, 5.4% for insulin, 3.1% for proinsulin, and 4.9% for C-peptide.

Metabolite profiling. Profiles of endogenous polar metabolites were obtained using liquid chromatography–tandem mass spectrometry (LC-MS) at the Broad Institute of the Massachusetts Institute of Technology and Harvard University (Cambridge, MA). The LC-MS methods were designed to enable broad measurement of metabolic markers and intermediates, including metabolites from central metabolism and amino acid metabolism, using low plasma sample volumes. LC-MS parameters for targeted analyses, including chromatographic retention times and mass spectrometry multiple reaction monitoring (MRM) settings (declustering potentials, collision energies, and lens voltages), were determined using over 300 commercially available reference compounds. A subset of 133 polar metabolites were measurable in plasma using a combination of two distinct hydrophilic interaction liquid chromatography (HILIC) methods, one operated under acidic mobile phase conditions with positive-ion-mode MS detection and the other under basic elution conditions with negative-ion-mode MS detection. The acidic HILIC method used positive-ionization-mode MS analyses was similar to the method described by Wang et al. Briefly, the LC-MS system consisted of a 4000 QTRAP triple quadrupole mass spectrometer (AB SCIEX) coupled to an 1100 Series pump (Agilent) and an HTS PAL autosampler (Leap Technologies). Plasma samples (10 μL) were extracted using nine volumes of 74.9:24.9:0.2 (v/v/v) acetonitrile/methanol/formic acid containing stable
isoazole-labeled internal standards (valine-d8, Isotec; and phenylalanine-d8, Cambridge Isotope Laboratories). The samples were centrifuged (10 min, 9,000g, 4 °C), and the supernatants (10 μL) were injected onto an Atlantis HILIC column (150 × 2.1 mm, 5 μm particle size; Waters Inc.). The column was eluted isocratically at a flow rate of 250 μL/min with 5% mobile phase A (10 mM ammonium formate and 0.1% formic acid in water) for 1 min followed by a linear gradient to 40% mobile phase B (acetonitrile with 0.1% formic acid) over 10 min. The ion spray voltage was 4.5 kV and the source temperature was 450 °C.

A second method using basic HILIC separation and negative ionization mode MS detection was established on an LC-MS system consisting of an ACQUITY UPLC (Waters Inc.) coupled to a 5500 QTRAP triple quadrupole mass spectrometer (AB SCIEX). Plasma samples (30 μL) were extracted using 120 μL of 80% methanol (VWR) containing the internal standards inosine-15N4, thymine-d4, and glycocholate-d4 (Cambridge Isotope Laboratories). The samples were centrifuged (10 min, 9,000g, 4 °C), and the supernatants were injected directly onto a Luna NH2 column (150 × 2.0 mm, 5 μm particle size; Phenomenex) that was eluted at a flow rate of 400 μL/min with initial conditions of 10% mobile phase A (20 mM ammonium acetate and 20 mM ammonium hydroxide (Sigma-Aldrich) in water (VWR)) and 90% mobile phase B (10 mM ammonium hydroxide in 75:25 v/v acetonitrile/methanol (VWR)) followed by a 10-min linear gradient to 100% mobile phase A. The ion spray voltage was −4.5 kV and the source temperature was 500 °C.

Raw data were processed using MultiQuant 1.2 software (AB SCIEX) for automated LC-MS peak integration. All chromatographic peaks were manually reviewed for quality of integration and compared against a known standard for each metabolite to confirm compound identities. Internal standard peak areas were monitored for quality control, to assess system performance over time, and to identify any outlier samples requiring re-analysis. A pooled plasma reference sample was also analyzed after sets of 20 study samples as an additional quality control. Control measure of analytical performance and to serve as a reference for scaling raw LC-MS peak areas across sample batches. Metabolites with a signal-to-noise ratio <10 were considered unquantifiable. Metabolite signals were analyzed in relation to pancreatic cancer risk as LC-MS peak areas, which are proportional to metabolite concentration and appropriate for metabolite clustering and correlational analyses.

Of the 133 metabolites measured (Supplementary Note, Fig. S1), 83 were included in the analyses of our nested pancreatic cancer case-control population (Supplementary Note, Table S3). In pilot work,50 we determined that 32 metabolites had poor reproducibility in samples with delayed processing, so these metabolites were excluded as they could not be reliably measured in two of the participating cohorts. In the current study, three heparin plasma pools (57 total QC samples) and three EDTA plasma pools (128 total QC samples) were randomly interspersed among participant samples as blinded QC samples. The samples were centrifuged (10 min, 9,000g, 4 °C), and the supernatants were injected directly onto a Luna NH2 column (150 × 2.0 mm, 5 μm particle size; Waters Inc.) coupled to a 5500 QTRAP triple quadrupole mass spectrometer (AB SCIEX).

Raw data were processed using MultiQuant 1.2 software (AB SCIEX) for automated LC-MS peak integration. All chromatographic peaks were manually reviewed for quality of integration and compared against a known standard for each metabolite to confirm compound identities. Internal standard peak areas were monitored for quality control, to assess system performance over time, and to identify any outlier samples requiring re-analysis. A pooled plasma reference sample was also analyzed after sets of 20 study samples as an additional quality control measure of analytical performance and to serve as a reference for scaling raw LC-MS peak areas across sample batches. Metabolites with a signal-to-noise ratio <10 were considered unquantifiable. Metabolite signals were analyzed in relation to pancreatic cancer risk as LC-MS peak areas, which are proportional to metabolite concentration and appropriate for metabolite clustering and correlational analyses.

Three separate plasma samples were prepared at each concentration and were analyzed using the acidic HILIC LC-MS method described above. The median concentrations of endogenous isoleucine, leucine and valine in the reference pooled plasma were calculated, and the concentration of each metabolite in each study sample was determined from the response ratio relative to the nearest reference pooled plasma sample in the analysis queue.

Statistical analyses for human studies. To compare baseline characteristics, we used conditional logistic regression conditioned on the matching factors and including the covariate of interest. Partial Spearman correlation coefficients were calculated for metabolites and covariates. Metabolites were log-transformed to improve normality and included as continuous variables in conditional logistic regression models conditioned on matching factors and adjusted for age at blood draw (years, continuous), fasting time (<4 h, 4–8 h, 8–12 h, ≥12 h, missing) and race or ethnicity (white, black, other, missing). Using a conservative Bonferroni correction for multiple-hypothesis testing,50 metabolites with $P < 0.0006$ (0.05/83) were considered statistically significant.

To provide estimates of effect magnitude, significant metabolites were examined in conditional logistic regression models after categorization into quintiles based on log-transformed metabolite levels in controls. Separate quintiles were generated for fasting (≥8 h since last meal) and nonfasting (<8 h since last meal) participants, given the possible effect of fasting time on metabolite levels. Quintiles were generated from the population of selected controls, which may not exactly reflect the characteristics of the full cohort population. Odds ratios (ORs) and 95% confidence intervals (CIs) were also calculated per s.d. change in log-transformed metabolite levels. To control for possible confounding, we evaluated regression models adjusted for body-mass index (BMI), physical activity, history of diabetes mellitus, Hba1c, plasma insulin, plasma proinsulin, and plasma C-peptide. We also evaluated regression models after excluding subjects with diabetes by self-report or Hba1c ≥6.5% at blood collection (prevalent diabetes). We further evaluated models that excluded subjects who developed diabetes after blood collection but ≥2 years before cancer diagnosis (incident diabetes not thought to be recent onset from pancreatic cancer)82,47. Metabolite values were considered missing when an LC-MS peak was below the limit of detection. In the primary analysis, any case or control with missing data for a metabolite was excluded from the analysis of that metabolite. However, we also conducted sensitivity analyses, in which participants with missing values were assigned the lower limit of detection or half of the lower limit of detection, and our results were unchanged.

We assessed heterogeneity of metabolite associations with pancreatic cancer risk across cohorts using Cochran’s Q-statistic51. We examined associations in predefined subgroups by sex, smoking status, BMI, and fasting status. Statistical interactions were assessed by entering into models the main effect terms and cross-product terms of metabolites and stratification variables, evaluating likelihood ratio tests. We also examined associations by time between blood collection and the case’s cancer diagnosis. In these time-based analyses, one stratum included 40 pancreatic cancer cases with blood collected within 2 years of diagnosis and their matched controls. These cases and controls were not part of the primary analysis population, but were included in the stratified analyses by time to more fully delineate the association of metabolites with pancreatic development by time before diagnosis. Associations were also examined for circulating BCAAs with previously explored risk factors for pancreatic cancer in our cohorts (Supplementary Note, Table S4) and with cancer stage at diagnosis (Supplementary Note, Table S5).

Since association of a marker with disease does not indicate the suitability of the marker to serve as a screening test for the disease, we examined two approaches to quantify the value of metabolites in a multifactor risk discrimination tool for pancreatic cancer. Discrimination quantifies the ability of one or more disease markers to separate cases (individuals with the disease) from controls (individuals without the disease). We investigated the discrimination of risk models for predicting pancreatic cancer diagnosis in the 10 years after measurement of circulating BCAAs, i.e., the 10-year risk of pancreatic cancer.

In the first approach, we investigated receiver-operating-characteristic (ROC) curve analysis and calculated of the area under the ROC curve (AUC), also known as the concordance (C) statistic.51 The base model included age at blood collection (continuous), cohort (HPFS, NHS, PHS, WHI), which also
accounts for sex), race/ethnicity (white, black, other/missing), smoking status (never, past, current, missing) and fasting time (<4 h, 4–8 h, 8–12 h, ≥12 h, missing). Three subsequent models mirrored the base model but additionally included (1) body mass index, physical activity, and history of diabetes, (2) circulating BCAAs, or (3) body-mass index, physical activity, history of diabetes, and circulating BCAAs. Each point on the ROC curve shows the effect of a rule for turning a risk estimate into a prediction of the development of an event. The y axis of the ROC curve is the true positive rate or sensitivity (i.e., the proportion of individuals with pancreatic cancer who were correctly predicted to have the disease). The x axis shows the false positive rate, which is the complement of specificity (i.e., the proportion of individuals without pancreatic cancer who were incorrectly predicted to have pancreatic cancer). The area under the ROC curve, the AUC, measures how well the model discriminates between case subjects and control subjects. An ROC curve that corresponds to a random classification of case subjects and control subjects is a straight line with an AUC of 50%. An ROC curve that corresponds to perfect classification has an AUC of 100%. The improvement in AUC for a model containing a new marker is defined as the difference in AUCs calculated using a model with and without the new marker of interest.

For context, the Breast Cancer Risk Assessment Tool, commonly referred to as the Gail model, estimates a woman’s risk for breast cancer using clinically available information including current age, age at menarche, age of first live birth, number of first-degree relatives with breast cancer, number of previous breast biopsies, breast biopsies that show atypical hyperplasia, and race/ethnicity. The Gail model is used to counsel women on appropriate screening tests for breast cancer, for determining whether tamoxifen will be useful as a chemopreventative agent, and for determining sample size calculations in randomized clinical trials of prevention strategies. Several studies have evaluated the discrimination of the Gail model using ROC curve analysis and calculated the AUC to be 0.58 to 0.63. Follow-up studies have described an AUC of 0.62 to 0.66 when breast density is added as an additional predictor in the original Gail model.

Although ROC curves are commonly used, they have a number of limitations and may underestimate the ability of a new marker to contribute to risk prediction when added to previously defined predictors. Another approach to evaluating model discrimination is to evaluate the ability of a new marker to shift an individual’s risk up or down between predefined risk categories. This is known as the prediction increment of a marker and has been codified in an approach known as net reclassification improvement (NRI). The NRI (sometimes referred to as the net reclassification index) constructs reclassification tables separately for participants with and without events and quantifies the correct movement between categories of risk, namely, to higher risk categories for participants with events and to lower risk categories for those without events. Furthermore, incorrect movement in categories of risk (downwards for events and upwards for non-events) reduces the net correct reclassification of individuals within the study population.

The NRI calculation is represented by the following formula:

\[
NRI = \frac{[P(\text{up}|D = 1) - P(\text{down}|D = 1)] - [P(\text{up}|D = 0) - P(\text{down}|D = 0)]}{\text{up} - \text{down} - \text{events}}
\]

Upward movement (up) is defined as a change into a higher risk category based on the new model and downward movement (down) as a change into a lower risk category based on the new model, where \(P\) indicates probability and \(D\) denotes the event indicator (1, event; 0, non-event).

Using the NRI, we evaluated the ability of the prediction model including circulating BCAAs to appropriately reclassify individuals into risk groups compared to the base model. The base model was calculated using conditional logistic regression conditioned on matching factors and adjusted for race/ethnicity, body-mass index, physical activity and history of diabetes. The subsequent model included the covariates in the base model with the addition of circulating metabolites. As in prior studies, we defined the high-risk group as those individuals with risk for pancreatic cancer at least twofold greater than an individual with average risk.

For context, the Emerging Risk Factors Collaboration has examined the integration of novel risk factors into risk prediction models for cardiovascular disease. In these studies, additional potential risk predictors were added to a model of known risk predictors for cardiovascular disease, including age, sex, smoking status, blood pressure, history of diabetes, and cholesterol. The net reclassification improvement was then calculated for three 10-year risk categories for cardiovascular disease. C-reactive protein (CRP) is a marker of systemic inflammation, and elevated CRP has been associated with an increased risk for cardiovascular events in numerous studies.

In an analysis of nearly 250,000 individuals, the addition of CRP to know cardiovascular disease risk factors was associated with a statistically significant improvement in the area under the ROC curve and a NRI of 1.52% for 10-year risk of cardiovascular disease. In contrast, additional analyses demonstrated a <1% improvement in the NRI for body-mass index, waist circumference, waist-to-hip ratio, plasma fibrinogen, and circulating apolipoproteins. Such that the clinical utility of these additional predictors remains unclear.

All analyses were performed with SAS 9.2 statistical package. All \(P\) values were two-sided.

**Experimental mice.** All studies were approved by the MIT Committee on Animal Care (IACUC). All experimental groups were assigned based on genotype. All animals were numbered and experiments conducted blinded. After data collection, genotypes were revealed and animals assigned to groups for analysis. The experiments were not randomized.

**KPC** Experimental KPC mice were male mice on a mixed background, heterozygous for the conditionallox-stop-loxKrasG12D allele, heterozygous for the conditionallox-stop-loxTrp53R172H allele and expressing Cre recombinase under control of the Pdx1 promoter (Tygiflif-pre-Try239). Littermate controls lacked either the LSL-KrasG12D allele, the Cre allele or both. Control mice were killed at the same time as their tumor-bearing littermates.

**KP*P*C** Experimental KP*P*C mice were male mice on a mixed background, heterozygous for the conditionallox-stop-loxKrasG12D allele, homozygous forloxP sites flanking exons 2–10 ofTrp53 and expressing Cre recombinase under control of the Pdx1 promoter (Tygiflif-pre-Try239). Littermate controls lacked either the Cre-recombinase allele, LSL-KrasG12D allele or both (controls were non–tumor-bearing mice of all genotypes). Inbred C57BL/6J male mice containing the same alleles were also examined where indicated, and cancer cell lines derived from these mice (established in culture from tumors prior to the described implantation studies) were used for syngeneic implantation studies.

**Non–small-cell lung cancer.** Six-month-old male mice on a pure 129 background, heterozygous for the conditionallox-stop-loxKrasG12D allele and homozygous forloxP sites flanking exons 2–10 ofTrp53, were administered 2.5 × 10^7 PFU of Cre-expressing adenovirus intratracheally as previously described. High-titer adenovirus was obtained from the Gene Transfer Vector Core (University of Iowa).

**Hindlimb sarcoma.** Four-week-old male mice on a mixed background, heterozygous for the conditionallox-stop-loxKrasG12D allele and homozygous forloxP sites flanking exons 2–10 ofTrp53, were administered 2.5 × 10^7 PFU of Cre-expressing adenovirus intratracheally as previously described. High-titer adenovirus was obtained from the Gene Transfer Vector Core (University of Iowa).

**Implantation, pancreatitis and BCAA diet studies.** Male C57BL/6J mice aged 4–6 weeks at the start of the study were used for these experiments.

**Diet.** Standard chow diet was RMH 3000 (ProLab). For amino acid–defined diets, 1× BCAA (TD.110839) and 2× BCAA (TD.110843) were designed in consultation with and subsequently obtained from Harlan Teklad. 20% 1C-leucine– and 20% 13C-valine–labeled diets were based on diet TD.110839 and produced by Cambridge Isotopes and Harlan Teklad.

**Plasma for metabolomics.** Plasma was collected for each experiment at the time points indicated. Mice were anesthetized under 2% isoflurane-oxygen mixture and plasma for metabolomics. Mice were anesthetized under 2% isoflurane-oxygen mixture and harvested. Mice were anesthetized under 2% isoflurane-oxygen mixture and harvested.

For context, the Emerging Risk Factors Collaboration has examined the integration of novel risk factors into risk prediction models for cardiovascular disease. In these studies, additional potential risk predictors were added to a model of known risk predictors for cardiovascular disease, including age, sex, smoking status, blood pressure, history of diabetes, and cholesterol. The net reclassification improvement was then calculated for three 10-year risk categories for cardiovascular disease. C-reactive protein (CRP) is a marker of systemic inflammation, and elevated CRP has been associated with an increased risk for cardiovascular events in numerous studies.

In an analysis of nearly 250,000 individuals, the addition of CRP to know cardiovascular disease risk factors was associated with a statistically significant improvement in the area under the ROC curve and a NRI of 1.52% for 10-year risk of cardiovascular disease. In contrast, additional analyses demonstrated a <1% improvement in the NRI for body-mass index, waist circumference, waist-to-hip ratio, plasma fibrinogen, and circulating apolipoproteins, such that the clinical utility of these additional predictors remains unclear.

All analyses were performed with SAS 9.2 statistical package. All \(P\) values were two-sided.
Fasting blood samples were harvested in the same manner first thing in the morning after a 16-hour overnight fast.

**Food consumption.** Mice were housed individually for 48 h, and remaining food pellets weighed at 0, 24 and 48 h. A two-day average was then calculated for each mouse. Body weight was determined on the second day. To assess consumption of BCAA defined diets, mice were housed individually and fed diets for 5 d. Food was weighed after 2 d of feeding and again on day 5, and the average consumption per 24 h over the 72-h period was calculated.

**Blood glucose, plasma insulin, glucose tolerance test and insulin tolerance test.** Four-week-old KP−/− mice were fasted overnight and blood glucose measured using a One Touch Ultra handheld glucometer. 25 μL of plasma from the same mice was harvested in heparinized tubes, aliquoted, and frozen at −80 °C for further analysis. Plasma insulin levels were determined using an ultrasensitive mouse insulin ELISA kit (Crystal Chem, #90080). After measuring fasting parameters, a glucose tolerance test was performed in accordance with published protocols81. Briefly, conscious mice received an intraperitoneal injection of 2 g/kg glucose at time 0. Blood glucose was subsequently measured at 15, 30, 60, 90 and 120 min post-injection as described above. For insulin tolerance test, 4-week-old KP−/− mice were fasted for 6 h during daytime hours. Following initial blood glucose measurement, conscious mice received an intraperitoneal injection of 0.75 IU/kg recombinant human insulin (Novolin, Novo Nordisk). Blood glucose was subsequently measured at 15, 30, 60 and 90 min post-injection as described above.

**KP−/− cell line and implantation studies.** End-stage tumors were dissected from C57BL/6J KP−/− mice and mechanically chopped before trypsin disaggregation, with tumor cells then propagated for three to five passages in DMEM with 10% FBS, 4 mM glutamine and penicillin/streptomycin to obtain enough cells for implantation. Cell lines were negative for mycoplasma. For subcutaneous implantation studies, recipient mice were anesthetized with inhalated 2% isoflurane-oxygen mixture, a vertical incision made in the abdomen at the left mid-calvicular line, the spleen mobilized, and 25 μL of either PBS or PBS containing 1.0 × 10⁵ cells (passage 3 for each line) was injected into the flank of syngeneic mice. For orthotopic implantation studies, recipient mice were anesthetized with inhaled 2% isoflurane-oxygen mixture, a vertical incision made in the abdomen at the left mid-calvicular line, the spleen mobilized, and 25 μL of either PBS or PBS containing 1.0 × 10⁵ cells (passage 3 for each line) was injected into the tail of the pancreas.

**Caerulein-induced chronic pancreatitis.** Mice were treated with either USP-grade saline or 5 × g caerulein (Sigma) via intraperitoneal injection daily, 5 d per week for 10 weeks as previously described25. Blood was obtained and the mice killed on the final day. Tissues were fixed in 10% formalin for subsequent histological analysis.

**Plasma markers of pancreatitis.** Plasma amylase and lipase measurements were performed by IDEXX BioResearch Laboratory (North Grafton, MA).

**BCAA diet studies.** Mice were fed either 1× or 2× BCAA diets for 10 weeks. Blood was obtained and mice killed on the final day of the experiment. Tissues were fixed in 10% formalin for subsequent histological analysis.

**Studies to determine source of BCAA elevations.** Acute uptake and disposal. Following a 16-h overnight fast, mice were fed 20% 13C-leucine and valine containing diet for 2 h before removal of food, and food consumption during this period quantified as described above. At the time points indicated by the red arrowheads in Figure 3a, 10–25 μL of plasma was harvested from the tail vein of conscious mice in a heparinized tube and centrifuged to separate plasma. Plasma was aliquoted and frozen at −80 °C for subsequent GC-MS analysis. Total ion counts from GC-MS analysis of leucine and valine were then normalized to norvaline internal standard and multiplied by fractional labeling to determine the amount of label present. This number for each animal was then normalized to that animal’s food intake to control for interanimal variation in labeled food consumption.

**Long-term pool contribution of BCAA to plasma.** Mice were exposed to 20% 13C-leucine– and 13C-valine–labeled diets from 7 d of age to 24 d of age followed by 3 d of unlabeled diet (according to the protocol depicted in Fig. 3b). Two cohorts of mice were used in this study. One cohort of mice was killed on day 27 in the fed state, and a second cohort of mice was killed on day 28 after a 16-h overnight fast (the points indicated by the red arrowheads in Fig. 3b). At time of killing, anesthetized mice were terminally bled and tissues harvested within 5 min, snap frozen in liquid nitrogen using Biosqueezer (BioSpec Products), and stored at −80 °C for subsequent GC-MS analysis. Plasma was aliquoted and frozen at −80 °C for subsequent GC-MS analysis.

Total contributions from short and long-term pools were calculated according to the following equations:

\[
\text{Long Term Pool} = \left( \frac{\text{Fed % Labeled}}{\text{Fasted % Labeled}} \right) \times \text{Relative Fed Pool Size}
\]

\[
\text{Short Term Pool} = \text{Relative Fed Pool Size} \times \text{Long Term Pool}
\]

Raw data are summarized in Supplementary Note, Table S6.

**Tissue and body weights.** For measurement of tissue weights, mice were weighed before killing, then gastrocnemius, tibialis anterior, soleus and heart were subsequently dissected and weighed. Muscle weights for each individual mouse were normalized to the body weight of that mouse.

**LC-MS plasma amino acid measurements.** Plasma amino acids were measured by LC-MS at the Koch Institute of the Massachusetts Institute of Technology (Cambridge, MA) using similar methods used for assessment of metabolites in human plasma. Raw data were analyzed as peak area tops using the open-access MAVEN software tool82.

**GC-MS assessment of 13C-leucine and 13C-valine labeling.** Plasma polar metabolites were extracted in ice-cold 4:1 methanol/water with norvaline internal standard (5 μL plasma in 200 μL extraction solution). Extracts were clarified by centrifugation and the supernatant evaporated under nitrogen and frozen at −80 °C for subsequent derivitization. Dried polar metabolites were dissolved in 20 μL of 2% methoxyamine hydrochloride in pyridine (Thermo) and held at 37 °C for 1.5 h. After dissolution and reaction, tert-butylidemethylsilyl derivatization was initiated by adding 25 μL N-methyl-N-(tert-butylidemethylsilyl)tri fluorooctoacetamide + 1% tert-butylidemethylchlorosilane (Sigma) and incubating at 37 °C for 1 h. The acid hydrolysis protocol was adapted from Antoniewicz et al.83. Briefly, acid hydrolysis of tissue proteins was performed on snap-frozen tissues by boiling 1–5 mg tissue in 1 mL 18% hydrochloric acid overnight at 100 °C. 50 μL supernatant was evaporated under nitrogen and frozen at −80 °C for subsequent derivitization. Dried hydrolysates were re-dissolved in pyridine (10 μL/1 mg tissue) before tert-butylidemethylsilatization, which was initiated by adding N-methyl-N-(tert-butylidemethylsilyl)trifluoroacetamide + 1% tert-butylidemethylchlorosilane (12.5 μL/1 mg tissue, Sigma) and incubating at 37 °C for 1 h.

**GC-MS analysis** was performed using an Agilent 7890 GC equipped with a 30-m DB-35MS capillary column connected to an Agilent 5975B MS operating under electron impact ionization at 70 eV. One microliter of sample was injected in splitless mode at 270 °C, using helium as the carrier gas at a flow rate of 1 mL min⁻¹. For measurement of amino acids, the GC oven temperature was held at 100 °C for 3 min and increased to 300 °C at 3.5 °C min⁻¹. The MS source and quadrupole were held at 230 °C and 150 °C, respectively, and the detector was run in scanning mode, recording ion abundance in the range of 100–605 m/z. MIDs were determined by integrating the appropriate ion fragments83 listed in Supplementary Note, Table S7 and corrected for natural isotope abundance using an algorithm adapted from Fernandez et al.84.

### Statistical analyses for mouse studies

Appropriate statistical tests were performed where required. Two-sided unpaired Student’s t-tests were performed for all statistical analyses unless otherwise specified using Microsoft Excel for Mac 2011 (Microsoft) or GraphPad Prism 6 (GraphPad Software). Two-sided
repeated-measures analysis of variance was performed to compare mean plasma glucose levels in the glucose tolerance test and insulin tolerance tests\textsuperscript{45}, using SAS 9.2 statistical package. No statistical method was used to predetermine sample size.


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insulin-like Growth Factor Pathway Genetic Polymorphisms, circulating iGF1 and iGFBP3, and Prostate cancer Survival


Background

The insulin-like growth factor (IGF) signaling pathway has been implicated in prostate cancer (PCa) initiation, but its role in progression remains unknown.

Methods

Among 5887 PCa patients (704 PCa deaths) of European ancestry from seven cohorts in the National Cancer Institute Breast and Prostate Cancer Cohort Consortium, we conducted Cox kernel machine pathway analysis to evaluate whether 530 tagging single nucleotide polymorphisms (SNPs) in 26 IGF pathway-related genes were collectively associated with PCa mortality. We also conducted SNP-specific analysis using stratified Cox models adjusting for multiple testing. In 2424 patients (313 PCa deaths), we evaluated the association of prediagnostic circulating IGF1 and IGF binding protein 3 (IGFBP3) levels and PCa mortality. All statistical tests were two-sided.

Results

The IGF signaling pathway was associated with PCa mortality ($P = .03$), and IGF2-AS and SST2 were the main contributors (both $P = .04$). In SNP-specific analysis, 36 SNPs were associated with PCa mortality with $P_{\text{trend}}$ less than .05, but only three SNPs in the IGF2-AS remained statistically significant after gene-based corrections. Two were in linkage disequilibrium ($r^2 = 1$ for rs1004446 and rs3741211), whereas the third, rs4366464, was independent ($r^2 = 0.03$). The hazard ratios (HRs) per each additional risk allele were 1.19 (95% confidence interval [CI] = 1.06 to 1.34; $P_{\text{trend}} = .003$) for rs3741211 and 1.44 (95% CI = 1.20 to 1.73; $P_{\text{trend}} < .001$) for rs4366464. Rs4366464 remained statistically significant after correction for all SNPs ($P_{\text{trend,corr}} = .04$). Prediagnostic IGF1 (HR$_{\text{highest vs lowest quintile}} = 0.71$; 95% CI = 0.48 to 1.04) and IGFBP3 (HR = 0.93; 95% CI = 0.65 to 1.34) levels were not associated with PCa mortality.

Conclusions

The IGF signaling pathway, primarily IGF2-AS and SST2 genes, may be important in PCa survival.


Abundant experimental evidence indicates that the insulin-like growth factor (IGF) signaling pathway is important for cell survival and tumorigenesis (1,2). Epidemiological research, focused primarily on IGF1 and IGF binding protein 3 (IGFBP3) and risk of incident prostate cancer, suggests that higher circulating IGF1 were associated with increased risk of prostate cancer (3), with mixed findings for IGFBP3 levels (4). However, little is known about the role of prediagnostic circulating levels of IGF1 and/or IGFBP3 in prostate cancer survival.

Data on genetic variations in IGF-related genes and prostate cancer survival are sparse, limited by relatively small number of fatal outcomes and assessment of only a handful of single nucleotide polymorphisms (SNPs) related to risk of prostate cancer, as identified by tagging SNPs or from genome-wide association studies (5,6). To the best of our knowledge, a systematic evaluation of genetic variants of IGF pathway–related genes and progression to fatal prostate cancer is lacking.

The National Cancer Institute Breast and Prostate Cancer Cohort Consortium (BPC3), pooled data from multiple large cohort studies, was designed to examine associations of variations in genes that mediate the steroid hormone and the IGF signaling pathway with breast and prostate cancer risk (7). With an average 8.9 years of follow-up among 5887 prostate cancer patients of European ancestry in BPC3, we aimed to 1) use a novel kernel machine pathway analysis and SNP-specific analysis to evaluate whether common variations among 26 genes involved in the synthesis, metabolism, and regulation of IGFs were associated with prostate cancer mortality; and 2) investigate the associations of prediagnostic circulating IGF1 and IGFBP3 levels with prostate cancer mortality in a subset of 2424 patients.
Methods

Study Population
The BPC3 consists of seven nested case–control studies of prostate cancer from prospective cohort studies in the United States and Europe: Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC), American Cancer Society Cancer Prevention Study II (CPS-II), European Prospective Investigation into Cancer and Nutrition (EPIC), Health Professionals Follow-up Study (HPFS), Multiethnic Cohort Study (MEC), Physicians’ Health Study (PHS), and Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial (7). Prostate cancer case patients were ascertained through population-based registries, self-report, or death certificates and verified by medical records. Height, body weight, and family history of prostate cancer were obtained by self-report. Data on disease stage (Jewett–Whitmore classification) and grade (Gleason score) were collected from each cohort. Written informed consent was obtained from all subjects, and each study was approved by the institutional review boards at their respective institutions. Details of vital status follow-up and determination of cause of death are described in the Supplementary Methods (available online).

SNP Selection and Genotyping
A total of 590 SNPs in 26 genes involved in the synthesis, metabolism, and regulation of insulin-like growth factors were genotyped (Figure 1). After restricting to self-reported European ancestry, a total of 5887 prostate cancer patients were included in this analysis. Two approaches were taken to evaluate linkage disequilibrium (LD) patterns and select the SNPs for this analysis as described elsewhere (7,8). Genotyping was performed in six laboratories: National Cancer Institute Core Genotyping Facility (Gaithersburg, MD), University of Southern California (Los Angeles, CA), University of Hawaii (Honolulu, HI), Harvard School of Public Health (Boston, MA), Imperial College (London, UK), and Cambridge University (Cambridge, UK). A total of 40 SNPs from GNRH1, GNRHR, IGF1, IGFBP1, and IGFBP3 were genotyped using TaqMan (Applied Biosystems, Foster City, CA). The remaining SNPs were genotyped by Illumina Golden Gate platform (San Diego, CA). Interlaboratory concordance was evaluated by genotyping 94 samples from the SNP 500 cancer panel (9) for the TaqMan SNPs and 30 HapMap CEU (Utah residents with ancestry from northern and western Europe) trios for the Illumina panel, with concordance rates greater than 99% between laboratories.

Figure 1. IGF signaling pathway. Genes included in this analysis were SST, SSTR1-5, GHRH, GHRHR, GHR, IGF1, IGF1R, IGFBP1-6, IGF2-AS, IGF2R, IGFALS, INS, IRS1, IRS2 (shown in Figure 1) and POU1F1, GNRH1, and GNRHR (not shown); the insulin receptor is encoded by a single gene, INSR, from which alternate splicing during transcription results in either IRA or IRB isoforms; the insulin gene (INS) was not genotyped, and genes in PI3k/Akt/mTOR and Ras-MAPK pathway were not included in this analysis.
Genotype data from the Taqman and Illumina platforms were filtered separately. Any sample in which more than 25% of the SNPs attempted on a given platform failed was removed from the dataset. Within each study, any SNP that failed in 25% or more of the samples, exhibited a statistically significant ($P < 10^{-5}$) deviation from Hardy–Weinberg proportions among European-ancestry controls, or had a minor allele frequency less than 1% was removed from the dataset. SNPs that were missing in more than 25% of the prostate cancer patients or showed large differences in allele frequency among subjects with European ancestry across studies (fixation index $F_{st} > 0.02$) were also excluded from analysis. For each gene region, SNPs that were polymorphic in any of the HapMap reference panels were imputed using MACH (10). Genotypes were imputed by cohort using the CEPH (Utah residents with ancestry from northern and western Europe) European (CEU) reference panel for subjects of European ancestry (release No. 21). Imputed data was filtered by study, and poorly imputed SNPs ($r^2 < 0.3$) were removed from analysis.

Circulating IGF1 and IGFBP3 levels
Prediagnostic measurements of IGF1 and IGFBP3 were available for five of the seven cohorts (ATBC, EPIC, HPFS, PHS, and PLCO; n = 2445) (11–15). Details of sample collection and storage were described previously. Samples from ATBC, HPFS, and PHS were measured in the Pollak laboratory (McGill University, Montreal, QC, Canada), and the remaining studies were measured in the laboratory of the Hormones and Cancer Team at International Agency for Research on Cancer (IARC) with enzyme-linked immunosorbent assays (Diagnostic System Laboratories, Webster, TX). We excluded cohort and assay batch-specific statistical outliers (n = 21) based on the generalized extreme studentized deviate many-outlier detection approach, setting alpha to 0.05 for both IGF1 and IGFBP3 blood levels (16).

Statistical Analysis
IGF Gene Pathway. The kernel machine Cox regression framework (17,18), a novel and comprehensive approach for pathway analysis of censored survival outcomes, was used to assess associations with deaths from prostate cancer and other causes for SNP sets defined by all 26 genes in the IGF pathway and each gene individually after adjusting for continuous age and study cohort. Because genotyped SNPs may be imperfect surrogates for the true casual SNP, their individual relative risks are likely to be modest, and a multimarker global test will more effectively capture the true effect. The kernel machine accounts for LD in an SNP set, leading to a powerful test with reduced degrees of freedom. More attractive, it can also capture potential nonlinear SNP effects, SNP–SNP interactions (epistasis), and the joint effects of multiple causal variants without requiring a priori knowledge of directionality. The kernel machine tests whether an SNP set is associated with event time of interest after adjusting for covariates, and the test statistic under the null follows a mixture of $\chi^2$ distributions, which can be approximated by resampling methods. Logistic kernel machines have been applied in a variety of traits and diseases (19,20).

SNP-specific analyses were conducted by stratified Cox proportional hazards models under a log-additive hazards assumption and stratified by study cohort, allowing different baseline hazards for each study. Follow-up was defined from the date of prostate cancer diagnosis to the date of any death or last follow-up. The assumption of proportionality was verified by testing each SNP and time since diagnosis, and no violation was identified. All analyses were adjusted for age at diagnosis and further adjusted for stage and Gleason score at diagnosis. To correct for multiple testing with possible presence of LD, the number of effective SNPs, $M_{eff}$, was calculated for each gene using a spectral decomposition approach (21). For gene-based $P$ value correction, nominal $P$ values for each SNP were multiplied by the $M_{eff}$ for the gene. For the pathway-based correction, the $M_{eff}$ values for all 26 genes were summed to correct the $P$ values.

Cumulative incidence of prostate cancer death by years since diagnosis were plotted for statistically significant SNPs after gene-level–based correction using competing-risks regression by the method of Fine and Gray (22).

Stratified analysis of statistically significant SNPs and prostate cancer mortality association by age at diagnosis (<65 or ≥65 years) and BMI (<25, 25–30, or ≥30 kg/m²), Gleason score (2–6, 7, or 8–10) and stage (A/B or C/D) were conducted under a dominant model as a result of limited sample size. To assess effect modification, we added a product term of statistically significant SNPs with the variables above and computed $P$ values from log likelihood ratio test.

Circulating IGF1 and IGFBP3 Levels. We created batch-specific (n = 10) quartiles for IGF1 and IGFBP3 and assessed their associations with prostate cancer mortality simultaneously by stratified Cox proportional hazards models adjusting for age at diagnosis. Models were also additionally adjusted for BMI assessed at the baseline of each study to assess possible confounding or stage and Gleason score at diagnosis to evaluate possible mediation. Tests for trend were done by treating the median concentration for each quartile as a continuous variable. Stratified analysis by stage and Gleason score at diagnosis were also performed. To account for the possibility of reverse causation in which an undiagnosed tumor could affect biomarker levels, sensitivity analyses were conducted by excluding cases diagnosed within 2 years of blood draw.

Analyses were conducted using SAS 9.2 (SAS Institute, Cary, NC), R (The R Foundation for Statistical Computing; http://www.r-project.org.Foundation), and Stata 12 (StataCorp, College Station, TX). All statistical tests were two-sided. A $P$ value of less than .05 was considered statistically significant.

**results**
During an average follow-up of 8.9 years among the 5887 case patients, 1,999 patients died, 704 of whom had prostate cancer as the underlying cause of death. Among the 2424 men in the subgroup of biomarker analysis, 313 of the 810 deaths were due to prostate cancer. Compared with those who were either alive at last follow-up or had died from other causes, patients who died from prostate cancer had higher Gleason score and clinical stage at diagnosis but similar BMI (Table 1; Supplementary Table 1, available online).

IGF Gene Pathway and Prostate Cancer Mortality
Pathway Analysis. A total of 530 SNPs were included in the genetic analysis. Kernel machine pathway analysis suggests that this set of SNPs covering all 26 genes in the IGF signaling pathway
was associated with prostate cancer mortality \((P = .03)\) (Table 2). When testing the SNP set of each gene, IGF2-AS (9 SNPs; \(P = .04\)) and SSTR2 (14 SNPs; \(P = .04\)) showed statistically significant associations with prostate cancer mortality. The overall pathway \(P\) values were .05 without either IGF2-AS or SSTR2 and .08 without both IGF2-AS and SSTR2, suggesting both IGF2-AS and SSTR2 may contribute to the progression to fatal prostate cancer. Neither the overall pathway nor IGF2-AS or SSTR2 were associated with risk of dying from causes other than prostate cancer.

**SNP-Specific Analysis.** A total of 36 SNPs were associated with prostate cancer mortality with \(P_{\text{trend}} < .05\) (Supplementary Table 2, available online). After correcting for multiple testing at gene level, three SNPs, all in IGF2 antisense gene (IGF2-AS, 11p15.5), were statistically significantly associated with prostate cancer–specific mortality. Two of these SNPs, rs1004446 (intron) and rs3741211 (3’-UTR), were in LD with each other \((r^2 = 1\) in 1000 Genome CEU population) but independent with the third SNP rs4366464 (intron) \((r^2 = 0.03)\). For rs3741211, each additional A allele was associated with a 19% (hazard ratio \([HR] = 1.19\); 95% confidence interval \([CI] = 1.06\) to 1.34; \(P_{\text{trend}} = .003\)) increased risk of prostate cancer–specific mortality. For rs4366464, each additional minor allele G was associated with a 44% (HR = 1.44; 95% CI = 1.20 to 1.73) increased risk of prostate cancer mortality \((P_{\text{trend}} < .001)\) (Table 3; Supplementary Figure 1, available online). The association for rs4366464 remained statistically significant after further correcting for multiple testing of all SNPs \((P_{\text{trend,corr}} = .04; M_{\text{corr}} = 424)\). When mutually adjusted for each other, the hazard ratios remained similar for rs3741211 (HR = 1.15; 95% CI = 1.03 to 1.30) and rs4366464 (HR = 1.37; 95% CI = 1.13 to 1.67), suggesting independent additive effects of the two SNPs on prostate cancer progression. Cohort-specific associations (Figure 2) also indicated the robustness of these associations, and minimal heterogeneities were observed \((rs3741211: F < 0.05\%, P_{\text{heterogeneity}} = .44; rs4366464: F < 0.05\%, P_{\text{heterogeneity}} = .55)\).

SNP rs4366464 or rs3741211 was not statistically significantly associated with either Gleason score or stage (data not shown). After additionally adjusting for these clinical parameters, the association between rs3741211 and prostate cancer death remained unchanged, whereas the hazard ratio for rs4366464 was slightly attenuated. Neither rs3741211 nor rs4366464 was associated with risk of dying from other causes (Table 3). These data suggest that the association between the two SNPs in IGF2-AS and prostate cancer mortality were independent of tumor characteristics and specific to death from prostate cancer.

Joint effect analysis suggests that for rs3741211, the association with prostate cancer mortality tended to be stronger among men with cancer diagnosed at younger age or patients with BMI less than 25 kg/m2 (Supplementary Figure 2, available online). For rs4366464, the association was stronger among men diagnosed at younger age. For both SNPs, the associations were somewhat stronger among patients with higher stage (C or D) or higher Gleason score \((\geq 7)\). However, only interaction between rs3741211 and stage was statistically significant \((P = .02)\).

**Circulating IGF1 and IGFBP3 and Prostate Cancer Mortality**

IGF1 levels were statistically significantly correlated with IGFBP3 \((r = 0.52; P < .001)\). Prediagnostic circulating levels of IGF1 (HR\(_{\text{highest}}\) vs lowest quartile = 0.71; 95% CI = 0.48 to 1.04) and IGFBP3 (HR = 0.93; 95% CI = 0.65 to 1.34) were not associated with prostate cancer.

### Table 1. Characteristics of prostate cancer patients in the National Cancer Institute Breast and Prostate Cancer Cohort Consortium*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PCa death (n = 704)</th>
<th>Censored (n = 5183)</th>
<th>Total (n = 5887)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis, y, mean (SD)</td>
<td>69.1 (7.1)</td>
<td>68.3 (6.4)</td>
<td>68.4 (6.5)</td>
</tr>
<tr>
<td>Diagnosis to prostate cancer death/ censoring, y, mean (SD)</td>
<td>5.3 (3.8)</td>
<td>9.4 (3.9)</td>
<td>8.9 (4.1)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18–24.9</td>
<td>265 (38)</td>
<td>2030 (39)</td>
<td>2295 (39)</td>
</tr>
<tr>
<td>25–29.9</td>
<td>342 (49)</td>
<td>2393 (46)</td>
<td>2735 (46)</td>
</tr>
<tr>
<td>≥30</td>
<td>78 (11)</td>
<td>572 (11)</td>
<td>650 (11)</td>
</tr>
<tr>
<td>Missing</td>
<td>19 (2)</td>
<td>188 (4)</td>
<td>207 (4)</td>
</tr>
<tr>
<td>Family history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>39 (6)</td>
<td>576 (11)</td>
<td>615 (10)</td>
</tr>
<tr>
<td>No</td>
<td>358 (51)</td>
<td>3038 (59)</td>
<td>3396 (58)</td>
</tr>
<tr>
<td>Missing</td>
<td>307 (44)</td>
<td>1569 (30)</td>
<td>1876 (32)</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2–6</td>
<td>115 (16)</td>
<td>2567 (50)</td>
<td>2682 (46)</td>
</tr>
<tr>
<td>7</td>
<td>225 (32)</td>
<td>1465 (28)</td>
<td>1690 (29)</td>
</tr>
<tr>
<td>8–10</td>
<td>217 (31)</td>
<td>485 (9)</td>
<td>702 (12)</td>
</tr>
<tr>
<td>Missing</td>
<td>147 (21)</td>
<td>666 (13)</td>
<td>813 (14)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A or B</td>
<td>259 (37)</td>
<td>3801 (73)</td>
<td>4060 (69)</td>
</tr>
<tr>
<td>C or D</td>
<td>343 (49)</td>
<td>702 (14)</td>
<td>1045 (18)</td>
</tr>
<tr>
<td>Missing</td>
<td>102 (14)</td>
<td>680 (13)</td>
<td>782 (13)</td>
</tr>
<tr>
<td>Biomarker subcohort</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of patients</td>
<td>313</td>
<td>2111</td>
<td>2424</td>
</tr>
<tr>
<td>Age at blood draw, y, mean (SD)</td>
<td>64.0 (78)</td>
<td>63.0 (6.9)</td>
<td>63.1 (71)</td>
</tr>
<tr>
<td>Circulating IGF1, ng/mL, median (IQR)</td>
<td>161 (124–212)</td>
<td>182 (142–228)</td>
<td>179 (139–227)</td>
</tr>
<tr>
<td>Circulating IGFBP3, ng/mL, median (IQR)</td>
<td>3110 (2544–3753)</td>
<td>3613 (2597–4333)</td>
<td>3544 (2899–4290)</td>
</tr>
</tbody>
</table>

* Data are No. (%) unless otherwise specified.
mortality in the model mutually adjusted for each other and age at diagnosis (Table 4). The hazard ratios were similar after additionally adjusting for stage and Gleason score at diagnosis in the model, or BMI at baseline, or excluding IGF1 and IGFBP3 measurements within 2 years of prostate cancer diagnosis (data not shown). In subgroup analysis, higher IGF1 levels were statistically significantly associated with lower prostate cancer mortality (Padj = .02) among men diagnosed with more advanced tumors (stage C or D).

### Discussion

To the best of our knowledge, this analysis of IGF pathway genes in relation to prostate cancer mortality among prostate cancer patients is the largest study to date. Using the kernel machine pathway analysis, a powerful test allowing assessment of the joint associations of variants in a predefined pathway, we demonstrated that the IGF pathway was statistically significantly associated with prostate cancer mortality and two genes, **IGF2-AS** and **SSTR2**, may play important roles in prostate cancer progression. Using SNP-specific association analysis, we further identified two SNPs, rs3741211 and rs4366464 in **IGF2-AS**, that were statistically significantly associated with prostate cancer mortality.

Additionally, among a subset of 2424 patients, we found no overall associations between prediagnostic circulating levels of IGF1 and IGFBP3 and prostate cancer mortality. The null associations between **IGF1** and **IGFBP3** genes and prostate cancer mortality suggest that their roles in the progression of prostate cancer were limited. In previous analyses of BPC3 patients, genetic variations in **IGF1** and **SSTR5** were associated with circulating levels of IGF1, and **IGFBP3** and **IGFALS** genes were associated with IGFBP3 levels (8,23). However, none of the SNPs in these genes were associated with prostate cancer mortality in our analysis, which is in line with the null findings between circulating levels of IGF1 and IGFBP3 and prostate cancer mortality. Although these findings should be interpreted with caution given the heterogeneities in blood collection, sample storage, and assay variation across the cohorts, the findings are not surprising because recent prospective studies did not support stronger associations of IGF1 levels with risk of advanced prostate cancer, favoring the hypothesis that common germline variations or circulating levels of IGF1 may contribute to early growth of prostate carcinogenesis (4), but not during progression.

The role of **IGF2-AS** and **IGF2** in prostate cancer initiation and progression is largely underexplored. A previous genome-wide
Table 3. Single nucleotide polymorphisms in *IGF2-AS* associated with prostate cancer–specific mortality after gene-based *P* value correction

<table>
<thead>
<tr>
<th>SNP</th>
<th>Risk allele</th>
<th>RAF</th>
<th>Chromosomal region</th>
<th>Position</th>
<th>Genotype</th>
<th>Person-years</th>
<th>PCa death</th>
<th>Other death</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3741211</td>
<td>A</td>
<td>0.626</td>
<td>11p15.5</td>
<td>2169110</td>
<td>GG</td>
<td>6945</td>
<td>1.00 (referent)</td>
<td>1.00 (referent)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GA</td>
<td>312</td>
<td>1.40 (1.07 to 1.83)</td>
<td>1.43 (1.09 to 1.87)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>295</td>
<td>1.55 (1.18 to 2.03)</td>
<td>1.50 (1.15 to 1.97)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AA/GA</td>
<td>607</td>
<td>1.47 (1.13 to 1.90)</td>
<td>1.46 (1.13 to 1.90)</td>
</tr>
<tr>
<td>per allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.19 (1.06 to 1.34)</td>
<td>1.16 (1.04 to 1.30)</td>
</tr>
<tr>
<td></td>
<td><em>P</em>&lt;sub&gt;treld&lt;/sub&gt;§</td>
<td></td>
<td></td>
<td></td>
<td>—</td>
<td>—</td>
<td>0.03</td>
<td>.01</td>
</tr>
<tr>
<td></td>
<td><em>P</em>&lt;sub&gt;treld,corr&lt;/sub&gt;§</td>
<td></td>
<td></td>
<td></td>
<td>—</td>
<td>—</td>
<td>.02</td>
<td>.08</td>
</tr>
<tr>
<td>rs4366464</td>
<td>G</td>
<td>0.066</td>
<td>11p15.5</td>
<td>2164799</td>
<td>CC</td>
<td>44133</td>
<td>1.00 (referent)</td>
<td>1.00 (referent)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GC</td>
<td>117</td>
<td>1.39 (1.14 to 1.70)</td>
<td>1.32 (1.08 to 1.62)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>169</td>
<td>2.87 (1.28 to 6.44)</td>
<td>2.34 (1.04 to 5.25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GG/GC</td>
<td>6764</td>
<td>1.43 (1.18 to 1.74)</td>
<td>1.35 (1.11 to 1.65)</td>
</tr>
<tr>
<td>per allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.44 (1.20 to 1.73)</td>
<td>1.36 (1.13 to 1.63)</td>
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<td></td>
<td><em>P</em>&lt;sub&gt;treld&lt;/sub&gt;§</td>
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<td><em>P</em>&lt;sub&gt;treld,corr&lt;/sub&gt;§</td>
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<td>—</td>
<td>—</td>
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* CI = confidence interval; HR = hazard ratio; PCa = prostate cancer; SNP = single nucleotide polymorphism; RAF = risk allele frequency in patients who did not die from prostate cancer.
† The Cox model was stratified by study cohort and adjusted for age at diagnosis.
‡ The model was additionally adjusted for Gleason score and stage at diagnosis. Because adding body mass index to the multivariable model did not alter the hazard ratios, we decided not to present results adjusted for body mass index.
§ *P*<sub>treld</sub> were calculated using stratified Cox proportional hazards models under a log-additive hazards assumption and were two-sided. *P*<sub>treld,corr</sub> were *P*<sub>treld</sub> after gene-based correction for multiple testing (*M*<sub>eff</sub> = 8).
Figure 2. Association of IGF2-AS single nucleotide polymorphism rs3741211 and rs4366464 with prostate cancer–specific mortality by study cohort. Hazard ratios (HRs; diamonds) and 95% confidence intervals (CIs; error bars) calculated for the association for the individual studies and the pooled analysis for rs3741211 (A) and rs4366464 (B) are shown. Size of gray square represents percentage weight of each study. RAF = risk allele frequency. ATBC = Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study; CI = confidence interval; CPS-II = American Cancer Society Cancer Prevention Study II; EPIC = European Prospective Investigation into Cancer and Nutrition; HPFS = Health Professionals Follow-up Study; HR = hazard ratio; MEC = Multiethnic Cohort Study; PHS = Physicians’ Health Study; PLCO = Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial.

association study identified SNP rs7127900 in IGF2-AS as associated with risk of incident prostate cancer (24) but not with prostate cancer mortality (5). This SNP was not in LD with the two SNPs we identified ($r^2 = 0.01$ for rs3741211 and $r^2 = 0.003$ for rs4366464 in 1000 Genome CEU population).

IGF2 is a peptide growth factor that is homologous to both IGF1 and insulin; interaction of IGF2 with insulin receptor subtype A (IRA) may play a role both in fetal growth and cancer biology (25). IGF2-AS expresses a paternally imprinted antisense transcript of the IGF2 gene. It is transcribed in the opposite
direction to the IGF2 transcripts, with some genomic regions shared with IGF2 (Figure 3) (26). IGF2-AS and IGF2 were overexpressed in Wilms’ tumor through loss of imprinting (26,27). Loss of imprinting of IGF2 is generally manifested by the activation of the normally silenced maternal allele with the subsequent expression of both gene copies. Evidence from Wilms’ tumor, colorectal cancer, and ovarian cancer suggests that the biallelic IGF2 expression also correlates with aberrant IGF2/H19 methylation (28,29). IGF2 levels were increased in prostate tumor–associated tissues, and a widespread IGF2 loss of imprinting throughout the peripheral prostate in men with prostate cancer was observed but not in samples of benign prostatic hyperplasia or other adult tissues, suggesting that epigenetic modification may play an important role in prostate cancer carcinogenesis (30). Overexpression of IGF2 and/or IRA has been proposed as a potential mechanism of resistance to IGF1R-directed therapies (31).

SSTR2 has been documented in experimental and clinical prostate cancer research but not in population studies. Somatostatin exerts inhibitory effects on cancer cells, including prostate, through five specific G-protein-coupled membrane receptors, SSTR1–5, with SSTR2 being predominant in human cancers (32,33). Its analogs, octreotide and lanreotide, which have high affinity for SSTR2, have been used to treat hormone-refractory prostate cancers (34,35) but are still under development.

The major strength of this study is the use of a large cohort consortium to study genetic predispositions, which are less likely to be affected by screening and treatment. Another strength is our
comprehensive evaluations of genetic variants in the IGF pathway using pathway, SNP-specific, and study cohort–specific analysis. However, additional genotyping to narrow down the region harboring the causal allele, followed by functional work on the identified variants and validations in other independent studies and/or races/ethnicities are necessary. Lack of patient treatment information was another limitation. However, associations of IGF genetic polymorphisms or biomarkers with prostate cancer mortality were unlikely to be affected by treatment because the two SNPs we identified, rs3741211 and rs4366464, were not associated with tumor characteristics (stage and Gleason score), the major determinants of treatment.

In summary, in this large consortium analysis of prostate cancer, both pathway and SNP-specific analyses showed that germline variations in IGF2-AS gene were associated with prostate cancer mortality, independent of stage and Gleason score and specific to prostate cancer. In contrast, neither genetic polymorphisms nor prediagnostic circulating levels of IGF1 and IGFBP3 were associated with prostate cancer mortality. Pathway analysis suggests that SSTR2 may also play a role in prostate cancer progression, but SNP-specific analysis failed to show any statistically significant SNP in this gene after gene-level correction. Further research on the role of IGF2/IGF-2-AS and SSTR2 is needed.

references

Funding
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Notes
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Whole Milk Intake Is Associated with Prostate Cancer-Specific Mortality among U.S. Male Physicians1–4

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Abstract

Previous studies have associated higher milk intake with greater prostate cancer (PCa) incidence, but little data are available concerning milk types and the relation between milk intake and risk of fatal PCa. We investigated the association between intake of dairy products and the incidence and survival of PCa during a 28-y follow-up. We conducted a cohort study in the Physicians’ Health Study (n = 21,660) and a survival analysis among the incident PCa cases (n = 2806). Information on dairy product consumption was collected at baseline. PCa cases and deaths (n = 305) were confirmed during follow-up. The intake of total dairy products was associated with increased PCa incidence [HR = 1.12 (95% CI: 0.93, 1.35); ≥2.5 servings/d vs. #0.5 servings/d]. Skim/low-fat milk intake was positively associated with risk of low-grade, early stage, and screen-detected cancers, whereas whole milk intake was associated only with fatal PCa [HR = 1.49 (95% CI: 0.97, 2.28); $237 mL/d (1 serving/d) vs. rarely consumed]. In the survival analysis, whole milk intake remained associated with risk of progression to fatal disease after diagnosis [HR = 2.17 (95% CI: 1.34, 3.51)]. In this prospective cohort, higher intake of skim/low-fat milk was associated with a greater risk of nonaggressive PCa. Most importantly, only whole milk was consistently associated with higher incidence of fatal PCa in the entire cohort and higher PCa-specific mortality among cases. These findings add further evidence to suggest the potential role of dairy products in the development and prognosis of PCa.


Introduction

Prostate cancer (PCa)13 is one of the most common cancers among elderly men (1,2). Dairy product intake has been associated with higher risk of PCa in many (3–9) but not all (10–12) studies. In the Physicians’ Health Study (PHS), we previously reported that higher intake of dairy products and dairy-derived calcium were associated with a greater risk of developing incident PCa, based on 11 y of follow-up (9). Compared with men consuming #0.5 servings/d of dairy products, those consuming ≥2.5 servings/d had a 34% increase in risk of developing PCa (95% CI: 4%, 71%). In 2 meta-analyses of the relation between dairy product intake and PCa incidence, one showed a significant positive association (13), whereas the other reported an overall null association (14). Part of the reason for this inconsistency could be that most cohort studies (including our previous report in the PHS) and the 2 meta-analyses did not separately evaluate whole milk and skim/low-fat milk. In addition, most studies did not consider advanced disease or PCa-specific death as a major outcome, partly due to the variable duration of follow-up.

In the present study, we assessed the relation between intakes of types of dairy products and PCa risk, with a special emphasis on cases that were high grade and in advanced stages at diagnosis as well as the occurrence of fatal PCa during a 28-y follow-up.
Participants and Methods

Study population. The PHS was a randomized, blinded, and placebo-controlled trial of aspirin and β-carotene in the prevention of heart disease and cancer among 22,071 U.S. male physicians aged 40–84 y in 1982 (15,16). At enrollment, participants provided information in the enrollment questionnaires on medical history and several lifestyle factors. All the physicians who were eligible and willing to participate were enrolled in a run-in phase. After 18 wk, participants were sent a questionnaire asking about their health status, side effects of treatment, compliance, and willingness to continue in the trial. Follow-up questionnaires were mailed at 6 and 12 mo after randomization and annually thereafter. Participants were asked to report newly diagnosed diseases, including PCa. For this study, we limited the study population to men who returned the run-in questionnaires with relevant abbreviated dietary information. To reduce the potential for undiagnosed PCa to influence diet and to utilize the dietary data collected on the 12-mo questionnaire, we excluded PCa cases diagnosed during the first year in the study, men with BMI <18.5 kg/m² at baseline, and men without baseline BMI information. These exclusions resulted in a study population of 21,660 men for analysis. The study design and methods used in this investigation were reviewed and approved by the Institutional Review Board of Partners Healthcare.

Dietary assessment. The run-in and 12-mo questionnaires in the PHS included abbreviated FFQs. The run-in questionnaire asked about the consumption of whole milk, skim/low-fat milk, and cold breakfast cereal (categories: <2 servings/d, daily, 5–6 servings/wk, 2–4 servings/wk, 1 serving/wk, 1–3 servings/wk, rarely/never) in the past year. The 12-mo questionnaire asked about the intake during the previous year of hard cheese (e.g., American, Cheddar) and ice cream. We considered these 5 foods to be the main contributors to dairy product intake and combined those responses by servings to estimate total daily dairy product intake (9). Because the potential effects of dairy calcium on PCa risk were of interest, we also calculated total daily calcium intake from each dairy product. Calcium content was obtained from the nutrient composition database of the USDA (17). The calcium content per serving (as weights in the total calcium consumption) is as follows: whole milk (1 serving = 237 mL), 276 mg; skim/low-fat milk (1 serving = 237 mL), 299 mg; ice cream (1 serving = 214 g, as in vanilla savor), 169 mg; and hard cheese (1 serving = 28 g, as an average of American cheese and Cheddar cheese), 173 mg. Two questions about red meat intake were also included in the 12-mo questionnaire, which asked about the intake of beef, pork or lamb as a sandwich or mixed dish (hamburger, stew, casserole, lasagna, etc.) and those as a main dish (steak, roast, ham, etc.). Daily intake of red meat was calculated as the sum of the servings (1 serving = 227 g) for each of these 2 items.

Ascertainment of PCa outcomes. For the PCa incidence analyses, men were followed from the date when the 12-mo questionnaire was returned until the date of PCa diagnosis, date of death, or the end of follow-up (March 9, 2010), whichever came first. For the PCa-specific analyses, men were followed from the date of PCa diagnosis until the date of death from PCa, date of death from other causes, or March 9, 2010, whichever came first. We learned of deaths in the cohort through notification by family members and postal authorities and through periodic systematic searches of the National Death Index. Cause of death was determined by an endpoint committee of 3 physicians based on all available information, including medical records and death certificates. Follow-up for mortality was at least 97.7% complete and for morbidity, 95.3% (18). Whenever a participant reported a new diagnosis of PCa, we requested hospital records and pathology reports to confirm the diagnosis and determine tumor stage, grade, and other clinical characteristics at diagnosis. Histological grade was recorded following the Gleason scoring system from the pathology reports. Low-grade tumors were defined as Gleason ≤7 and high-grade was defined as Gleason >7. Clinical stage was determined using the TNM staging system. Tumors of stage T3 or higher (T3/T4/N1/M1) were categorized as advanced-stage tumors and tumors of stage T1 or T2 were defined as early-stage tumors. Cases without pathologic staging were classified as undetermined stage unless there was clinical evidence of distant metastases. Because prostate-specific antigen (PSA) screening has dramatically changed the clinical presentation of the cancer, we also categorized the cases into 3 groups: pre-PSA era cases (diagnosed before 1990), post-PSA era cases (diagnosed 1990 or thereafter) who presented with prostatic or metastatic symptoms, and post-PSA era cases detected by PSA or digital rectal examination screening.

Statistical analyses. To examine the association of dairy products and calcium consumption with PCa risk, we used Cox proportional hazards regression models to calculate the HR and 95% CI, with the lowest intake category as the reference group. We categorized the intake of each dairy food into 4 groups (rarely, ≤1 serving/wk, 2–6 servings/wk, and ≥7 servings/wk). Calcium intake from dairy products was categorized into 5 groups by quintiles. Tests for linear trend were performed using the median intake values in each category as a continuous variable. Beyond age-adjusted models, multivariable models additionally included terms for baseline (time when 12-mo questionnaire was returned) cigarette smoking (never, past, or current smoker), vigorous exercise (exercise vigorously to sweat more than twice per week or not), alcohol intake (drink alcoholic beverages every day or not), race (Caucasian or non-Caucasian), BMI (<25.0, 25.0–29.9, or ≥30.0 kg/m²), diabetes status (yes or no), red meat consumption (servings/week), and assignment in the original trial (active treatment or placebo for aspirin and β-carotene). In addition, the models for whole milk and skim/low-fat milk were mutually adjusted for each other.

The abbreviated FFQs in the PHS were not comprehensive; thus, we were unable to calculate and adjust for total energy intake directly. To minimize the potential confounding due to total energy intake, we calculated total energy intake using only the food items that were recorded in the run-in and 12-mo questionnaires. These food items included 13 types of fruits and vegetables, 5 types of dairy foods investigated in this study, eggs, chicken, beef, 4 types of fish and seafood, cookies, chips, nuts, and fried foods. Under similar situations, previous studies used food scores by summing up servings of all recorded food items (9,19). In this study, we weighted the servings of recorded food items with total calorie per serving of each individual item to better emulate total energy intake calculated from comprehensive FFQs.

Separate multivariable models for PCa incidence were fit for subgroups of cancer according to Gleason grade, clinical stage, and disease presentation at diagnosis, and disease fatality during follow-up. We then modeled the relation between dairy product and PCa-specific mortality among cases using the Cox proportional hazard regression model. Besides the age- and multivariable-adjusted model [including the same set of covariates as in the incidence model and stage of tumor (T3/T4/N1/M1 or T1/T2) and Gleason score (<7 or ≥7)], we further stratified the analyses by disease presentation at diagnosis (pre-PSA era presented, post-PSA era presented by symptom, and post-PSA era presented by screening). To account for potential false positives due to multiple comparisons, we calculated the false-discovery rate (FDR) by incorporating all P values from multiple tests performed for the linear trends. The FDR statistics were obtained for each P value, and FDR statistics with q < 0.05 were considered significant (20). All analyses were performed in SAS version 9.3 (SAS Institute). All P values are 2-sided.

Results

We confirmed 2806 incident cases of PCa diagnosed among 21,660 men in 470,612 person-years through 2010. The baseline characteristics of the study population by categories of dairy product intake are presented in Table 1. Men who consumed more dairy products tended to be older, smoked less, drank less alcohol, exercised more, and were more likely to be Caucasian and diabetic. When stratified by type of milk, the data showed that men who consumed more skim/low-fat milk tended to smoke less, drink less alcohol, and exercise more and were more likely to be Caucasian, whereas men who consumed more...
Whole milk tended to be current smokers, exercise less, and less likely to be Caucasian.

Total dairy food intake was marginally associated with overall PCa risk. In multivariable-adjusted analyses, men in the highest category of total dairy foods had a 12% (95% CI: 27%, 35%) higher risk to develop PCa than men in the lowest intake category (P-trend = 0.06) (Table 2). For individual dairy foods, skim/low-fat milk had the strongest association with PCa incidence: the multivariable-adjusted HR was 1.19 (95% CI: 1.06, 1.33; P-trend = 0.001), comparing the highest [927 mL/d (1 serving/d)] with the lowest (rarely consumed) intake category. In contrast, whole milk, hard cheese, ice cream, and cold breakfast cereal intakes were not significantly associated with overall risk of PCa incidence. Calcium from dairy foods was marginally associated with PCa incidence (P-trend = 0.07).

We next examined the association of total dairy products, whole milk, and skim/low-fat milk with special attention to cancer subtypes and the timing of diagnosis (i.e., 1982–1989, pre-PSA era vs. 1990–2010, post-PSA era) (Table 3). We found that higher intake of skim/low-fat milk was mainly associated with a higher risk of low-grade, early-stage, and screen-detected disease; comparing the highest with the lowest intake category, the HR were 1.20 for low-grade cases (95% CI: 1.06, 1.37), 1.19 for early-stage cases (95% CI: 1.04, 1.35), and 1.21 for post-PSA era cases detected by screening (95% CI: 1.02, 1.43) (P-trend # 0.01 for all the subgroup analyses). In contrast, for risk of fatal PCa, whole milk was the only dairy food that had a positive association [HR = 1.49 (95% CI: 0.97, 2.28); P-trend = 0.01]. This association was independent of age, cigarette smoking status, BMI, alcohol intake, vigorous physical activity, diabetes status, red meat consumption, and total energy intake from recorded food items.

Finally, among all the PCa cases, we conducted a survival analysis to evaluate the associations of prediagnostic dairy food intake with risk of progression to fatal PCa after initial diagnosis and found that whole milk was the only dairy food that was significantly associated with an increased risk of PCa-specific mortality (Table 4). Compared with nondrinkers of whole milk, the multivariable-adjusted HR was 2.17 (95% CI: 1.34, 3.51; P-trend < 0.001) for those who consumed $237 mL/d (1 serving/d). A stratified analysis on age at diagnosis showed that high intake of whole milk was significantly associated with risk of progression to fatal PCa in both old and young age groups, except that there tended to be a J-shaped relation in the older group (data not shown). In a stratified analysis on the presentation of disease, we found that, among post-PSA era cases presented by screening, whole milk intake was associated with PCa deaths, although the q value was not significant [HR = 1.82 (95% CI: 0.69, 4.84); P-trend = 0.07]. The associations with skim/low-fat milk, however, were not significant in any of the substrata by PSA era and screening.

### Discussion

In this study, we confirmed and extended our previous findings that total dairy product intake and calcium from dairy foods were positively associated with overall risk of PCa. Admittedly, the dairy variables in our study did not capture all dairy product intake (did not include information on intakes of yogurt, cream, butter, etc.). However, according to data from the NHANES, milk and cheese intakes can account for ≈98% of total dairy product intake (21). Thus, our data on available dairy food items sufficiently represented the total dairy product intake in our population. The magnitude of the overall association between total dairy product intake and the risk of incident PCa [HR = 1.12 (95% CI: 0.93, 1.35)] in this study, however, was weaker than in our previous report [RR = 1.34 (95% CI: 1.04, 1.71)]. Because the current analysis had a much larger sample size (2806 cases vs. 1012 cases) and an additional 15 y of follow-up, these allowed us to specifically evaluate subtypes of dairy products and by subtypes of PCa, cancer diagnosed before vs. in the PSA era, mode of diagnosis, and cancer-specific mortality (9). We found that skim/low-fat milk intake were
related to a higher risk of nonaggressive disease (low-grade, early-stage, and screen-detected cases), whereas whole milk intake was associated with a higher risk of fatal PCa and, among all the cases, with a higher risk of progression to fatal PCa.

The positive association between dairy product intake and PCa has been reported in several studies, including the European Prospective Investigation into Cancer and Nutrition (22) and studies from Canada (23) and Japan (4). These data raised concerns regarding whether dairy should be recommended as part of a healthy diet for aging men (24,25). However, the results of 2 meta-analyses of the relation between dairy product intake and PCa provided conflicting conclusions: one showed a significant positive association (13) and the other (supported by the National Dairy Council) showed an overall null association (14). Part of the reason for this inconsistency could be a lack of detailed data for the effect of whole compared with skim/low-fat milk and their impact on high-risk disease or PCa-specific death.

Our finding that the strongest association with total dairy products was in the pre-PSA era was consistent with findings of Rodriguez et al. (26). We observed a significant positive association of skim/low-fat milk with overall PCa risk. These results are consistent with previous studies (6,27). Few studies specifically evaluated high-risk PCa. Park et al. (28) observed that skim milk, but not other dairy foods, was associated with a nonsignificantly increased risk of advanced PCa. The null effect of whole milk on overall PCa risk is likely due to the fact that the whole milk drinkers accounted for only a small portion of all milk drinkers. Thus, the associations of whole milk with the nonfatal cases, if any, were not large enough to be detected with a limited number of cases, which may have driven the overall effect.

The commonly accepted risk factors for incident PCa are older age, a family history of PCa, and being African American (29). However, there is no consensus about risk factors for fatal PCa beyond clinical characteristics such as PSA at diagnosis, Gleason grade, and clinical stage. Identifying modifiable risk factors for fatal PCa and the nonfatal cases may therefore provide an opportunity for a reduction in prostate cancer mortality.

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<td>0.96</td>
<td>1.02</td>
<td>1.11</td>
<td>1.11</td>
</tr>
<tr>
<td>Multivariable-adjusted6</td>
<td>1.00</td>
<td>0.95</td>
<td>1.00</td>
<td>1.06</td>
<td>1.06</td>
</tr>
<tr>
<td>Calcium from dairy food6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases/person-years</td>
<td>487/95,147</td>
<td>516/95,489</td>
<td>578/93,334</td>
<td>598/92,688</td>
<td>609/91,575</td>
</tr>
<tr>
<td>Age-adjusted</td>
<td>1.00</td>
<td>1.04</td>
<td>1.15</td>
<td>1.16</td>
<td>1.17</td>
</tr>
<tr>
<td>Multivariable-adjusted6</td>
<td>1.00</td>
<td>1.01</td>
<td>1.12</td>
<td>1.12</td>
<td>1.14</td>
</tr>
</tbody>
</table>

1 Values are HR (95% CI). FDR, false-discovery rate; PCa, prostate cancer; PHS, Physicians' Health Study.
2 Calculated in a separate regression model with the median intake levels in each category as a continuous variable.
3 Adjusted for baseline measures of age (y), cigarette smoking (never, past, current), vigorous exercise (exercise vigorously to a sweat more than twice per week or not), alcohol intake (drink alcoholic beverages every day or not), race (Caucasian, non-Caucasian), BMI (normal weight, overweight, obese), baseline diabetes status (yes, no), red meat consumption (servings/wk), total energy intake from recorded food items (kcal), assignment in the original aspirin trial (treatment, placebo), and assignment in the original b-carotene trial (treatment, placebo).
4 FDR < 0.05.
5 Adjusted for baseline measures of age (y), cigarette smoking (never, past, current), vigorous exercise (exercise vigorously to a sweat more than twice per week or not), alcohol intake (drink alcoholic beverages every day or not), race (Caucasian, non-Caucasian), BMI (normal weight, overweight, obese), baseline diabetes status (yes, no), red meat consumption (servings/wk), total energy intake from recorded food items (kcal), assignment in the original aspirin trial (treatment, placebo), and assignment in the original b-carotene trial (treatment, placebo).
6 The 4 intake level groups were: rarely, #1 serving/wk, 2–6 servings/wk, and $1 serving/d.
7 The 5 intake level groups were categorized according to quintiles.
TABLE 3 Multivariable-adjusted HR estimates for categories of PCa cases by intake of dairy product in the PHS (n = 21,660)\(^1\)\(^2\)

<table>
<thead>
<tr>
<th>Dairy product(^3)</th>
<th>Category 1</th>
<th>Category 2</th>
<th>Category 3</th>
<th>Category 4</th>
<th>Category 5</th>
<th>P-trend(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High grade</td>
<td>1.00</td>
<td>1.04 (0.69, 1.58)</td>
<td>0.77 (0.50, 1.20)</td>
<td>1.09 (0.71, 1.68)</td>
<td>1.04 (0.60, 1.80)</td>
<td>0.64</td>
</tr>
<tr>
<td>Low grade</td>
<td>1.00</td>
<td>0.95 (0.81, 1.12)</td>
<td>1.11 (0.95, 1.30)</td>
<td>1.13 (0.95, 1.33)</td>
<td>1.10 (0.91, 1.39)</td>
<td>0.12</td>
</tr>
<tr>
<td>Advanced</td>
<td>1.00</td>
<td>0.92 (0.59, 1.46)</td>
<td>0.79 (0.50, 1.27)</td>
<td>0.92 (0.57, 1.48)</td>
<td>0.68 (0.36, 1.27)</td>
<td>0.35</td>
</tr>
<tr>
<td>Localized</td>
<td>1.00</td>
<td>0.94 (0.80, 1.11)</td>
<td>1.09 (0.93, 1.29)</td>
<td>1.11 (0.94, 1.32)</td>
<td>1.13 (0.91, 1.39)</td>
<td>0.13</td>
</tr>
<tr>
<td>Fatal</td>
<td>1.00</td>
<td>1.19 (0.68, 2.06)</td>
<td>1.81 (1.08, 3.02)</td>
<td>2.14 (1.26, 3.64)</td>
<td>1.75 (0.90, 3.35)</td>
<td>0.05</td>
</tr>
<tr>
<td>Pre-PSA</td>
<td>1.00</td>
<td>1.70 (0.95, 3.05)</td>
<td>1.77 (1.00, 3.33)</td>
<td>1.82 (1.01, 3.27)</td>
<td>2.12 (1.07, 4.19)</td>
<td>0.10</td>
</tr>
<tr>
<td>Post-PSA (symptom)</td>
<td>1.00</td>
<td>1.44 (0.78, 2.68)</td>
<td>1.25 (0.66, 2.34)</td>
<td>1.83 (0.99, 3.40)</td>
<td>1.61 (0.76, 3.40)</td>
<td>0.19</td>
</tr>
<tr>
<td>Post-PSA (screening)</td>
<td>1.00</td>
<td>0.83 (0.67, 1.03)</td>
<td>1.10 (0.90, 1.34)</td>
<td>1.04 (0.84, 1.28)</td>
<td>0.99 (0.75, 1.30)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

Whole milk\(^5\)

| High grade          | 1.00        | 0.69 (0.48, 1.00) | 1.29 (0.91, 1.84) | 0.78 (0.49, 1.25) | 0.81 |
| Low grade           | 1.00        | 1.09 (0.97, 1.23) | 0.86 (0.73, 1.01) | 0.91 (0.76, 1.10) | 0.10 |
| Advanced            | 1.00        | 0.89 (0.61, 1.29) | 1.04 (0.86, 1.61) | 0.83 (0.69, 1.41) | 0.63 |
| Localized           | 1.00        | 1.06 (0.94, 1.19) | 0.87 (0.74, 1.03) | 0.89 (0.74, 1.07) | 0.08 |
| Fatal               | 1.00        | 0.89 (0.60, 1.31) | 1.77 (1.23, 2.54) | 1.49 (0.97, 2.28) | 0.01\(^7\) |
| Pre-PSA             | 1.00        | 1.29 (0.89, 1.86) | 1.51 (1.00, 2.27) | 1.35 (0.85, 2.15) | 0.15 |
| Post-PSA (symptom)  | 1.00        | 1.22 (0.80, 1.86) | 1.19 (0.71, 1.99) | 1.29 (0.76, 2.21) | 0.38 |
| Post-PSA (screening)| 1.00        | 1.00 (0.86, 1.17) | 0.74 (0.59, 0.93) | 0.73 (0.57, 0.94) | 0.002\(^7\) |

Skim/low-fat milk\(^6\)

| High grade          | 1.00        | 1.11 (0.79, 1.56) | 1.07 (0.76, 1.51) | 1.19 (0.85, 1.67) | 0.39 |
| Low grade           | 1.00        | 0.99 (0.87, 1.13) | 1.18 (1.04, 1.35) | 1.20 (1.06, 1.37) | 0.001\(^7\) |
| Advanced            | 1.00        | 0.94 (0.64, 1.38) | 1.02 (0.69, 1.49) | 0.99 (0.67, 1.45) | 0.96 |
| Localized           | 1.00        | 0.99 (0.86, 1.13) | 1.18 (1.04, 1.35) | 1.19 (1.04, 1.35) | 0.004\(^7\) |
| Fatal               | 1.00        | 1.04 (0.72, 1.51) | 1.01 (0.69, 1.47) | 1.04 (0.71, 1.51) | 0.91 |
| Pre-PSA             | 1.00        | 1.38 (0.92, 2.07) | 1.69 (1.15, 2.48) | 1.43 (0.97, 2.12) | 0.11 |
| Post-PSA (symptom)  | 1.00        | 0.84 (0.52, 1.35) | 1.02 (0.64, 1.62) | 1.22 (0.79, 1.88) | 0.23 |
| Post-PSA (screening)| 1.00        | 0.98 (0.63, 1.17) | 1.20 (1.01, 1.42) | 1.21 (1.02, 1.43) | 0.01\(^7\) |

1 Values are HR (95% CI). FDR, false-discovery rate; PCa, prostate cancer; PHS, Physicians’ Health Study; PSA, prostate-specific antigen.
2 Adjusted for baseline measures of age (y), cigarette smoking (never, past, current), vigorous exercise (exercise vigorously to a sweat more than twice per week or not), alcohol intake (drink alcoholic beverages every day or not), race (Caucasian, non-Caucasian), BMI (normal weight, overweight, obese), baseline diabetes status (yes, no), and red meat consumption (servings/wk), total energy intake from recorded food items (kcal), assignment in the original aspirin trial (treatment, placebo), and assignment in the original b-carotene trial (treatment, placebo). In addition, the models for whole milk and skim/low-fat milk were mutually adjusted for each other (rarely, #1 serving/wk, 2–6 servings/wk, and $1 serving/d).
3 High grade (n = 317): Gleason .7; low grade (n = 2105): Gleason #7; advanced (n = 272): T3/T4/N1/M1; localized (n = 2016): T1/T2; fatal (n = 305): died of PCa; pre-PSA era (n = 274): diagnosed before 1990; post-PSA era: diagnosed after 1990; presented by symptom (n = 192); presented by prostate-related symptoms or metastases; presented by screening (n = 1233); presented by PSA test screening or digital rectal examination;
4 Calculated in a separate regression model with the median intake in each category as a continuous variable.
5 Based on baseline consumption of 5 major dairy foods (whole milk, skim/low-fat milk, hard cheese, ice cream, and cold breakfast cereal).
6 The 4 intake level groups were: rarely, #1 serving/wk, 2–6 servings/wk, and $1 serving/d.
7 FDR < 0.05.
TABLE 4 HR estimates of PCa death by prediagnostic intake of dairy product and dairy calcium in PCa cases in the PHS (n = 2806)1

<table>
<thead>
<tr>
<th>Dairy Food</th>
<th>Category 1</th>
<th>Category 2</th>
<th>Category 3</th>
<th>Category 4</th>
<th>Category 5</th>
<th>P-trend2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deaths/pers-yr</td>
<td>27/3601</td>
<td>45/4012</td>
<td>74/5222</td>
<td>115/8503</td>
<td>43/4416</td>
<td></td>
</tr>
<tr>
<td>Age-adjusted</td>
<td>1.00</td>
<td>1.50 (0.93, 2.41)</td>
<td>1.83 (1.18, 2.85)</td>
<td>1.73 (1.14, 2.63)</td>
<td>1.22 (0.75, 1.97)</td>
<td>0.75</td>
</tr>
<tr>
<td>Multivariable-adjust4,5</td>
<td>1.00</td>
<td>0.97 (0.53, 1.78)</td>
<td>2.23 (1.26, 3.92)</td>
<td>1.87 (1.04, 3.37)</td>
<td>1.71 (0.82, 3.58)</td>
<td>0.16</td>
</tr>
<tr>
<td>Pre-PSA</td>
<td>1.00</td>
<td>1.16 (0.33, 4.50)</td>
<td>2.20 (0.66, 7.29)</td>
<td>1.02 (0.29, 3.51)</td>
<td>2.15 (0.53, 8.79)</td>
<td>0.76</td>
</tr>
<tr>
<td>Post-PSA (screening)</td>
<td>1.00</td>
<td>0.74 (0.27, 2.03)</td>
<td>1.09 (0.45, 2.64)</td>
<td>1.24 (0.50, 3.06)</td>
<td>0.93 (0.26, 3.36)</td>
<td>0.80</td>
</tr>
<tr>
<td>Skim/low-fat milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deaths/pers-yr</td>
<td>115/8106</td>
<td>58/4856</td>
<td>53/5493</td>
<td>68/6789</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age-adjusted</td>
<td>1.00</td>
<td>0.89 (0.65, 1.22)</td>
<td>0.71 (0.51, 0.98)</td>
<td>0.70 (0.52, 0.94)</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>Multivariable-adjust4,5</td>
<td>1.00</td>
<td>1.01 (0.67, 1.52)</td>
<td>0.87 (0.56, 1.36)</td>
<td>1.02 (0.67, 1.56)</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>Pre-PSA</td>
<td>1.00</td>
<td>1.18 (0.53, 2.59)</td>
<td>0.61 (0.24, 1.56)</td>
<td>0.77 (0.34, 1.76)</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>Post-PSA (screening)</td>
<td>1.00</td>
<td>1.48 (0.71, 3.11)</td>
<td>1.34 (0.66, 2.73)</td>
<td>1.22 (0.54, 2.73)</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>Calcium from dairy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deaths/pers-yr</td>
<td>41/4412</td>
<td>52/4755</td>
<td>73/5174</td>
<td>74/5535</td>
<td>64/5879</td>
<td></td>
</tr>
<tr>
<td>Age-adjusted</td>
<td>1.00</td>
<td>1.18 (0.79, 1.78)</td>
<td>1.45 (0.99, 2.13)</td>
<td>1.36 (0.93, 1.99)</td>
<td>1.11 (0.75, 1.64)</td>
<td>0.75</td>
</tr>
<tr>
<td>Multivariable-adjust4,5</td>
<td>1.00</td>
<td>1.06 (0.63, 1.79)</td>
<td>1.70 (1.00, 2.89)</td>
<td>1.64 (0.94, 2.89)</td>
<td>1.71 (0.91, 3.21)</td>
<td>0.09</td>
</tr>
<tr>
<td>Pre-PSA</td>
<td>1.00</td>
<td>0.83 (0.33, 2.08)</td>
<td>1.30 (0.49, 3.48)</td>
<td>0.63 (0.23, 1.77)</td>
<td>1.30 (0.41, 4.19)</td>
<td>0.99</td>
</tr>
<tr>
<td>Post-PSA (screening)</td>
<td>1.00</td>
<td>1.09 (0.44, 2.69)</td>
<td>1.08 (0.45, 2.62)</td>
<td>1.63 (0.65, 4.10)</td>
<td>1.22 (0.41, 3.65)</td>
<td>0.59</td>
</tr>
</tbody>
</table>

1 Values are HR (95% CI). FDR, false-discovery rate; PCa, prostate cancer; PHS, Physicians’ Health Study; PSA, prostate-specific antigen.
2 Calculated in a separate regression model with the median intake levels in each category as a continuous variable.
3 Based on the consumption of 5 major dairy foods (whole milk, skim/low-fat milk, hard cheese, ice cream, and cold breakfast cereal) assessed from 1982 to 1984. The 5 intake level groups are: ≤0.5 servings/d, 0.5–1.0 servings/d, 1.0–1.5 servings/d, 1.5–2.5 servings/d, and ≥2.5 servings/d. One serving of whole milk, skim/low-fat milk, or cold breakfast cereal = 237 mL; 1 serving of ice cream = 214 g.
4 Adjusted for baseline measures of age at diagnosis (y), cigarette smoking (never, past, current), vigorous exercise (exercise vigorously to a sweat more than twice per week or not), alcohol intake (drink alcoholic beverages every day or not), race (Caucasian, non-Caucasian), BMI (normal weight, overweight, obese), baseline diabetes status (yes, no), red meat consumption (servings/wk), Gleason score (.7, ≥7), stage of tumor (T3/T4/N1/MI, T1/T2), total energy intake from recorded food items (kcal), assignment in the original aspirin trial (treatment, placebo), and assignment in the original b-carotene trial (treatment, placebo). In addition, the models for whole milk and skim/low-fat milk were mutually adjusted for each other (rarely, 1 serving/wk, 2–6 servings/wk, and ≥1 serving/d).
5 Pre-PSA era (n = 274): diagnosed before 1990; post-PSA era: diagnosed after 1990; presented by symptom (n = 192): presented by prostate-related symptoms or metastases (results not presented because of very low statistical power); presented by screening (n = 1233): presented by PSA test screening or digital rectal examination.
6 The 4 intake level groups were: rarely, 1 serving/wk, 2–6 servings/wk, and ≥1 serving/d.
7 FDR = 0.05.
8 The 5 intake level groups were categorized according to quintiles.

### Discussion

Several potential mechanisms could explain the observed associations of dairy food (primarily skim/low-fat milk) with overall PCa risk. First, skim/low-fat milk is the major source of dairy calcium and higher intake might lower intra-cellular 1,25-dihydroxycholecalciferol concentrations and induce prostate carcinogenesis (8,34–36). Second, the association could be mediated via phytic acid, which may upregulate expression of α-methylacyl-CoA racemase (37,38). The involvement of α-methylacyl-CoA racemase in PCa is implicated by a recent observation (39). Third, the relation could be through the effect of phosphate. Newmark et al. (40) suggested that the high dietary phosphate content of dairy products might explain the risk of PCa induced by dairy products, because the plasma phosphate concentration can appreciably infl 1,25-dihydroxycholecalciferol concentrations. Fourth, the ability of dairy products to raise concentrations of insulin-like growth factor 1 have also been suggested as a possible explanation for the association (41–43). The association of whole milk with fatal PCa and their diet after diagnosis. We evaluated correlations among nutrients between the 2000 and 2004 FFQs, comparing men diagnosed with PCa in that interval with those who remained free of PCa. We found that the correlations ranged between 0.5 and 0.7 for all nutrients assessed, including dairy products. There were no obvious trends in the absolute levels of intake between cases and non-cases. These observations suggest that men tended to keep their dietary habits after PCa diagnosis. One advantage of using prediagnostic dietary information is to avoid confounding by recall bias, change of diet due to disease severity or treatments, or other reasons. Recently, Pettersson et al. (32) found that in the Health Professionals Follow-up Study, post-diagnostic milk and dairy product intake was not significantly associated with increased risk of fatal PCa, whereas Torfadottir et al. (33) found that milk intake during adolescence, rather than in midlife or currently, was associated with advanced PCa. One possibility is that dairy product intake in earlier life may be more relevant to the progression and mortality of PCa in later life.
PCa-specific mortality may be via the effects of dairy fat (primarily saturated fat) or other factors (including obesity and hyperinsulinemia). Whole milk has an ;40 times higher content of saturated fat compared with skim milk and the difference of the saturated fat content between 237 mL of whole milk and skim milk is ;20% of its average daily intake (17). High-fat dairy has been positively correlated with higher C-peptide concentrations, which were positively related to risk of aggressive PCa (44).

In summary, the results from the present study confi a potential role of dairy products in PCa risk and survival. Skim/low-fat milk dairy products have been suggested as being benefi for several disease outcomes, including colorectal cancer; so future research is warranted to investigate the optimal intake of skim/low-fat dairy products. However, our results add further evidence to suggest that the intake of whole-fat dairy products is associated with the risk of developing advanced or fatal PCa in elderly men and worse survival in PCa cases. Thus, minimal intake of whole-fat dairy products may be benefi for elderly men, particularly PCa survivors. However, these results still need to be confi in other male populations.

Acknowledgments
Y.S. analyzed data and wrote the manuscript; J.M. supervised the analysis and edited the manuscript; and J.E.C., Y.C., W.Q., L.M., H.D.S., M.J.S., E.G., M.P., and S.L. revised the article for intellectual content. All authors read and approved the fi nal manuscript.

Literature Cited

Whole milk and prostate cancer

195


Original Contribution

Association of Type 2 Diabetes Susceptibility Variants With Advanced Prostate Cancer Risk in the Breast and Prostate Cancer Cohort Consortium


*Correspondence to Dr. Peter Kraft, Department of Epidemiology, Harvard School of Public Health, 655 Huntington Avenue, Building II, Room 207, Boston, MA 02115 (e-mail: pkraft@hsph.harvard.edu).

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Observational studies have found an inverse association between type 2 diabetes (T2D) and prostate cancer (PCa), and genome-wide association studies have found common variants near 3 loci associated with both diseases. The authors examined whether a genetic background that favors T2D is associated with risk of advanced PCa. Data from the National Cancer Institute’s Breast and Prostate Cancer Cohort Consortium, a genome-wide association study of 2,782 advanced PCa cases and 4,458 controls, were used to evaluate whether individual single nucleotide polymorphisms or aggregations of these 36 T2D susceptibility loci are associated with PCa. Ten T2D markers near 9 loci (NOTCH2, ADCY5, JAZF1, CDKN2A/B, TCF7L2, KCNQ1, MTNR1B, FTO, and HNF1B) were nominally associated with PCa (P < 0.05); the association for single nucleotide polymorphism rs757210 at the HNF1B locus was significant when multiple comparisons were accounted for (adjusted P = 0.001). Genetic risk scores weighted by the T2D log odds ratio and multilocus kernel tests also indicated a significant relation between T2D variants and PCa risk. A mediation analysis of 9,065 PCa cases and 9,526 controls failed to produce evidence that diabetes mediates the association of the HNF1B locus with PCa risk.

These data suggest a shared genetic component between T2D and PCa and add to the evidence for an interrelation between these diseases.

cancer; diabetes mellitus, type 2; genetic predisposition to disease; genetics; genome-wide association study; humans; polymorphism, single nucleotide; prostatic neoplasms

Abbreviations: BPC3, Breast and Prostate Cancer Cohort Consortium; CI, confidence interval; GRS, genetic risk score; OR, odds ratio; PCa, prostate cancer; SNP, single nucleotide polymorphism; T2D, type 2 diabetes.

Prostate cancer (PCa) and type 2 diabetes (T2D) are two of the most common chronic diseases afflicting the US aging male population (1, 2). Observational studies have consistently shown an apparent inverse association between T2D and risk of PCa, with meta-analysis risk ratios ranging from 0.84 to 0.91 (3, 4). The reduction in PCa risk has been reported to increase with years since T2D diagnosis, with men who have had T2D for more than 15 years being at a 22% reduced hazard of PCa (5). The association is poorly understood, with one hypothesis suggesting that the metabolic status of men with T2D could move gradually from hyperinsulinemia to endogenous insulin deficiency, which could mitigate the oncogenic action of insulin in the prostate (6, 7). Recently, 3 shared genomic regions for T2D and PCa have been highlighted. The first region, located on chromosome 17, is in intron 2 of HNF1B, formerly known as TCF2. The major allele A of rs4430796 is positively...
associated with PCA risk (odds ratio (OR) = 1.22) and inversely associated with risk of T2D (OR = 0.91) (8–10). The second region is located on chromosome 7 near the JAZF1 locus, where the major allele G of rs10486567 is inversely associated with risk of PCa (aggressive PCa: OR = 0.89; nonaggressive PCa: OR = 0.74) (11), whereas the minor allele G of rs864745 is positively associated with T2D (OR = 1.10) (12). THADA is the third region, located on chromosome 2, with the minor allele A of rs1465618 being associated with PCa (OR = 1.08) (13) and the major allele T of rs7578597 associated with T2D (OR = 1.15) (12). However, the single nucleotide polymorphisms (SNPs) for T2D and PCa in the JAZF1 and THADA regions are weakly linked, with $R^2$ values of 0.03 and 0.02, respectively. It is not clear that these associations are driven by the same haplotype (14, 15).

Stevens et al. (16) investigated the T2D-PCa relation further and concluded that diabetic status did not mediate the observed relation between the HNF1B and JAZF1 gene variants and PCa risk. In the Atherosclerosis Risk in Communities cohort, Meyer et al. (17) examined the relation of T2D-associated variants with risk of PCa and found that 4 of 13 T2D SNPs were nominally associated with PCa, which provides additional evidence that some of the T2D-PCa association could be driven by shared genetic factors. Another study by Pierce et al. (18) evaluated the ability of risk scores, consisting of 18 replicated T2D risk variants, to predict PCa risk and concluded that persons with increased genetic susceptibility to T2D have a reduced risk of PCa. However, in a recent study of 5 racial/ethnic groups in the Multiethnic Cohort and PAGE (Population Architecture using Genomics and Epidemiology), Waters et al. (19) found no association between T2D risk variants, either individually or in risk scores, and PCa risk.

With a large sample size and an expanded set of recently published T2D susceptibility loci, we aimed to investigate whether and to what extent individual T2D risk variants and aggregations of T2D replicated risk variants are associated with PCa risk. We used novel approaches to test both whether these risk variants are inversely associated with PCa risk in accordance with the inverse relation observed between T2D and PCa in observational studies and, more generally, whether these T2D loci are associated with PCa risk without regard to directionality of association. Additionally, using causal inference methods, our study attempted to more definitively investigate the potential for mediation of the effect of HNF1B on PCa risk through T2D phenotype.

MATERIALS AND METHODS

Genotyping data for PCa cases and controls came from the National Cancer Institute’s Breast and Prostate Cancer Cohort Consortium (BPC3). The BPC3 is a consortium of prospective cohort studies, with contributors including the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (20), the American Cancer Society Cancer Prevention Study II Nutrition Cohort (21), the European Prospective Investigation into Cancer and Nutrition (22), the Health Professionals Follow-up Study, the Melbourne Collaborative Cohort Study (23), the Multiethnic Cohort Study (24), the Physicians’ Health Study, and the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (25). In total, 9,065 PCa cases and 9,526 controls comprised the PCa nested case-control study. Diabetes phenotype was self-reported at study baseline, with data available for 96.7% of BPC3 participants. A genome-wide association scan was conducted on a subset of 2,782 European cases with advanced disease and 4,458 controls with European ancestry. Advanced PCa was defined as PCa cases that had either a high histologic grade (Gleason score ≥8) or extraprostatic extension (stage C/D). All controls were free of PCa at the time of selection and were sampled from the same cohort as the cases. Controls were age-matched to cases, and study indicator variables were used to adjust for sampling differences between studies. Informed consent was received from all study participants, and all study protocols were reviewed by the institutional review boards of the National Cancer Institute and each participating study center.

A literature search was conducted to find robustly replicated disease susceptibility loci that are associated with T2D at genome-wide significance levels ($P < 5 \times 10^{-8}$). In total, 36 independent autosomal loci associated with T2D were identified, and published T2D risk alleles and odds ratios were extracted (9, 10, 12, 26–36).

Individual association tests were carried out for each T2D SNP with PCa risk in the BPC3 genome-wide association study (37). Quality control filters were used to remove samples with heterozygosity, underperforming samples or markers, markers with genotype frequencies that significantly departed from Hardy-Weinberg equilibrium, and subjects with significant evidence of non-European ancestry or sample structure. Of the 36 T2D SNPs, 19 were not directly genotyped on the Illumina HumanHap610 Quad Arrays (Illumina, San Diego, California) and were therefore imputed with MACH (http://www.sph.umich.edu/csg/abecasis/MaCH/) (38). MACH references the HapMap (http://hapmap.ncbi.nlm.nih.gov/) CEU population (Utah residents with Northern and Western European ancestry from the Centre d’Etude du Polymorphisme Humain (CEPH) collection) to infer expected genotype counts for each marker locus. MACH quality scores and $R^2$ values were more than 0.85 and 0.75, respectively, for all imputed SNPs. Logistic regression models were used to test for T2D SNP associations with PCa risk. The number of T2D risk alleles was used as the exposure, and adjustment was made for cohort (indicator variables). A nominal association $P$ value of 0.05 was used to assess whether T2D markers exhibited more significant associations with PCa than would be expected by chance. Additional binomial and permutation tests (39) (10,000 permutations) were carried out to test for a relation in risk allele directionality and significant departures of the PCa association statistics from the null distribution, respectively.

The T2D SNPs were combined to form a genetic risk score (GRS) using the --score command in PLINK (40). The GRS was calculated in two ways. The first method, referred to here as the count method, involved summing the number of T2D risk alleles at each locus (0, 1, or 2) and then summing across all T2D loci. This count method is an additive model that weights each locus equally and assumes no gene-gene interactions. The second method, referred to
here as the weighted method, uses the log odds ratio of the
published T2D loci to weight the sum of T2D risk alleles at
each locus and then sums across all T2D loci. The weighted
method is an additive model that weights each locus in ac-
cordance with the T2D literature and assumes no gene-gene
interactions. The rationale for weighting is to create a score
that is the best GRS for T2D and therefore can be used as
an instrument for testing an association with PCa. For each
GRS method, we included the GRS as a predictor in a lo-
 gistic regression model with PCa case-control status as the
outcome, and we adjusted for cohort with an indicator vari-
able. Cohort-specific associations were also calculated.

Additionally, multilocus linear kernel tests were used to
assess the joint relation between the 36 T2D variants and
PCa risk. These linear models allow associations of multi-
ple genetic loci to be tested simultaneously with one test
statistic (41) and have been generalized for dichotomous
outcomes (42). Unlike the GRS methods, these tests
require no prespecification of risk allele directionality (i.e.,
that the risk allele is associated with increased risk of T2D
and decreased risk of PCa).

The HNF1B locus was the only T2D locus significantly
associated with PCa risk after adjustment for multiple com-
parisons, so it was carried forward for mediation analysis to
evaluate whether T2D phenotype is a potential mediator of
the relation between HNF1B and PCa. We used an expand-
ed set of data on 9,065 PCa cases (including nonaggressive
cases) and 9,526 controls from the BPC3 (43) with self-
reported information on diabetes phenotype. Data on
rs7501939 at HNF1B were generated as part of a previous
project characterizing known PCa loci; this SNP is in high
linkage disequilibrium with rs757210 ($R^2 = 0.81$). This was
the only T2D risk marker typed in the larger BPC3 data
set. To assess mediation, we used the mediation framework
proposed by Baron and Kenny (44), extended into the coun-
terfactual framework by VanderWeele and Vansteelandt
(45) as direct and indirect effects, and further generalized
for use with dichotomous intermediate and outcome. This
framework for mediation analysis is flexible to an interac-
tion between exposure and an intermediate factor, has a
causal interpretation, and can assess mediation on both the
multiplicative and additive scales. Assessing mediation in
this manner involved fitting both an outcome model and a
mediator model. The outcome model was a logistic regres-
sion model that modeled PCa cases and controls, who repre-
sent the study’s base popula-
tion, and assuming a rare outcome. Once both the outcome
and mediator models were fitted, parameter estimates were
used to calculate direct and indirect (mediated) effects by
which to assess mediation (45).

The PCa study was conducted between May and August of
2011. All statistical analyses were carried out in SAS 9.1
(SAS Institute Inc., Cary, North Carolina) and R 2.11.1 (R
Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Results from the individual association tests showed that
10 of the 36 T2D markers had a $P$ value less than $0.05$ for
association with PCa, significantly more than the 1.8
markers that would be expected by chance ($P = 7.5 \times 10^{-6}$)
(Table 1). These markers include the HNF1B and JAZF1
loci, as well as NOTCH2, ADCY5, CDKN2A/B, TCF7L2,
MTNR1B, FTO, and 2 independent loci at KCNQ1
(Table 1). After permutation adjustment for multiple com-
parisons, only HNF1B remained significant (adjusted
$P = 0.001$). Small fluctuations in effect estimates of $\leq 3\%$
were observed when adjustment for diabetes status was
made in the models, with overall conclusions remaining the
same (results not shown). We observed an inflation in the
observed $P$ values for these 36 SNPs ($\lambda_{GC} = 2.0$; Figure 1).
When the observed $\lambda_{GC}$ was compared with the distribu-
tion of permutation $\lambda_{GC}$ values, the observed $\lambda_{GC}$ was sig-
ificantly elevated ($P = 0.03$), which indicated that the distri-
bution of association $P$ values was significantly lower than
expected.

We used exact binomial tests to assess whether signifi-
cantly more T2D risk alleles were inversely associated with
PCa risk than would be expected by chance. By chance
alone, 1.8 of the 36 markers would be expected to be sig-
ificant, of which, under the null, 0.9 would be expected to be
significantly associated with increased risk of PCa and
0.9 would be expected to be significantly associated with
decreased risk of PCa. In our data, we observed 2 T2D loci
that were significantly associated with increased PCa risk,
which did not differ statistically from the 0.9 loci expected
by chance ($P = 0.23$). However, the 8 T2D loci we ob-
served to be significantly associated with reduced risk of
PCa were significantly more than the 0.9 that would be ex-
pected by chance ($P = 2.45 \times 10^{-6}$), which indicates that
more T2D risk alleles than expected are associated with
reduced risk of PCa.

Associations for GRS using both the unweighted count
and the weighted log odds method are shown in Table 2.
The risk score for the unweighted count did not show evi-
dence for an association of these genetic variants with PCa
risk. However, a significant association was observed for
the weighted log odds method when HNF1B was both in-
cluded in ($P = 0.002$) and excluded from ($P = 0.015$) the
GRS. No changes in results were observed when we adjust-
ed for diabetes status in the models (results not shown).
Study-specific analyses showed that the log odds-weighted
GRS was statistically significant only in the Prostate, Lung,
Colorectal, and Ovarian Cancer Screening Trial, although
the test for heterogeneity indicated no significant departures
from homogeneity ($P = 0.60$).

The multilocus kernel test that jointly tested for a PCa
association with all 36 T2D loci without specifying weight

Table 1. Individual Associations of 36 Independent Type 2 Diabetes Susceptibility Variants With Prostate Cancer Risk in the Breast and Prostate Cancer Cohort Consortium

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Reported Gene(s)</th>
<th>Single Nucleotide Polymorphism</th>
<th>Genotyped?</th>
<th>Type 2 Diabetes Risk Allele</th>
<th>Frequency of Risk Allele</th>
<th>Odds Ratio</th>
<th>95% Confidence Interval</th>
<th>P Value</th>
<th>Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NOTCH2</td>
<td>rs10923931</td>
<td>No</td>
<td>T</td>
<td>0.11</td>
<td>0.86</td>
<td>0.76, 0.96</td>
<td>0.008*</td>
<td>0.255</td>
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<tr>
<td>1</td>
<td>PROX1</td>
<td>rs340874</td>
<td>Yes</td>
<td>C</td>
<td>0.52</td>
<td>1.01</td>
<td>1.08, 0.94</td>
<td>0.845</td>
<td>1.000</td>
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<tr>
<td>2</td>
<td>GCKR</td>
<td>rs780094</td>
<td>Yes</td>
<td>C</td>
<td>0.61</td>
<td>0.98</td>
<td>1.05, 0.91</td>
<td>0.498</td>
<td>1.000</td>
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<td>2</td>
<td>THADA</td>
<td>rs7578597</td>
<td>Yes</td>
<td>T</td>
<td>0.91</td>
<td>1.03</td>
<td>1.16, 0.91</td>
<td>0.644</td>
<td>1.000</td>
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<td>2</td>
<td>BCL11A</td>
<td>rs243021</td>
<td>Yes</td>
<td>A</td>
<td>0.47</td>
<td>1.02</td>
<td>0.95, 1.10</td>
<td>0.511</td>
<td>1.000</td>
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<td>1</td>
<td>IRS1</td>
<td>rs2943641</td>
<td>Yes</td>
<td>C</td>
<td>0.64</td>
<td>0.95</td>
<td>1.02, 0.88</td>
<td>0.140</td>
<td>0.995</td>
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<td>3</td>
<td>PPARG</td>
<td>rs1801282</td>
<td>No</td>
<td>C</td>
<td>0.86</td>
<td>0.96</td>
<td>1.07, 0.87</td>
<td>0.465</td>
<td>1.000</td>
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<td>3</td>
<td>ADAMTS9</td>
<td>rs4607103</td>
<td>No</td>
<td>C</td>
<td>0.76</td>
<td>0.99</td>
<td>1.08, 0.91</td>
<td>0.853</td>
<td>1.000</td>
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<td>3</td>
<td>ADCY5</td>
<td>rs11708067</td>
<td>No</td>
<td>A</td>
<td>0.78</td>
<td>0.91</td>
<td>0.99, 0.84</td>
<td>0.028*</td>
<td>0.630</td>
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<td>4</td>
<td>IGF2BP2</td>
<td>rs4402960</td>
<td>Yes</td>
<td>T</td>
<td>0.32</td>
<td>1.03</td>
<td>0.95, 1.11</td>
<td>0.456</td>
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<td>5</td>
<td>WFSI</td>
<td>rs10010131</td>
<td>No</td>
<td>G</td>
<td>0.60</td>
<td>1.00</td>
<td>1.07, 0.93</td>
<td>0.924</td>
<td>1.000</td>
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<tr>
<td>5</td>
<td>ZBED3</td>
<td>rs4457053</td>
<td>No</td>
<td>G</td>
<td>0.29</td>
<td>1.02</td>
<td>0.94, 1.10</td>
<td>0.672</td>
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<td>6</td>
<td>CDKAL1</td>
<td>rs7754840</td>
<td>Yes</td>
<td>C</td>
<td>0.32</td>
<td>1.04</td>
<td>0.97, 1.13</td>
<td>0.270</td>
<td>1.000</td>
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<tr>
<td>7</td>
<td>DGKB</td>
<td>rs2191349</td>
<td>No</td>
<td>T</td>
<td>0.52</td>
<td>1.00</td>
<td>1.07, 0.93</td>
<td>0.945</td>
<td>1.000</td>
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<tr>
<td>7</td>
<td>JAZF1</td>
<td>rs864745</td>
<td>No</td>
<td>T</td>
<td>0.50</td>
<td>1.08</td>
<td>1.16, 1.01</td>
<td>0.033*</td>
<td>0.694</td>
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<td>7</td>
<td>GCK</td>
<td>rs4607517</td>
<td>Yes</td>
<td>A</td>
<td>0.15</td>
<td>1.06</td>
<td>0.96, 1.16</td>
<td>0.256</td>
<td>1.000</td>
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<td>7</td>
<td>KLFL4</td>
<td>rs972283</td>
<td>No</td>
<td>G</td>
<td>0.53</td>
<td>1.02</td>
<td>1.09, 0.95</td>
<td>0.627</td>
<td>1.000</td>
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<tr>
<td>8</td>
<td>TP53INP1</td>
<td>rs896854</td>
<td>Yes</td>
<td>T</td>
<td>0.51</td>
<td>1.02</td>
<td>1.09, 0.95</td>
<td>0.668</td>
<td>1.000</td>
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<td>8</td>
<td>SLC30A8</td>
<td>rs13266634</td>
<td>Yes</td>
<td>C</td>
<td>0.68</td>
<td>1.00</td>
<td>1.08, 0.93</td>
<td>0.963</td>
<td>1.000</td>
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<td>9</td>
<td>CDKN2A/B</td>
<td>rs10811661</td>
<td>No</td>
<td>T</td>
<td>0.82</td>
<td>0.91</td>
<td>1.00, 0.83</td>
<td>0.931</td>
<td>0.298</td>
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<td>10</td>
<td>TLE4</td>
<td>rs13292136</td>
<td>No</td>
<td>C</td>
<td>0.93</td>
<td>0.93</td>
<td>1.07, 0.81</td>
<td>0.312</td>
<td>1.000</td>
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<tr>
<td>10</td>
<td>CDC123/</td>
<td>rs12779790</td>
<td>No</td>
<td>G</td>
<td>0.18</td>
<td>1.06</td>
<td>0.97, 1.16</td>
<td>0.206</td>
<td>1.000</td>
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<td>10</td>
<td>CAMK1D</td>
<td>rs1111875</td>
<td>Yes</td>
<td>C</td>
<td>0.58</td>
<td>1.01</td>
<td>1.09, 0.94</td>
<td>0.713</td>
<td>1.000</td>
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<tr>
<td>10</td>
<td>HHEX/IDE</td>
<td>rs7903146</td>
<td>Yes</td>
<td>T</td>
<td>0.28</td>
<td>0.90</td>
<td>0.83, 0.97</td>
<td>0.009*</td>
<td>0.276</td>
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<td>11</td>
<td>KNCQ1</td>
<td>rs231362</td>
<td>No</td>
<td>G</td>
<td>0.50</td>
<td>0.92</td>
<td>0.86, 0.98</td>
<td>0.014*</td>
<td>0.393</td>
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<td>11</td>
<td>KNCQ1</td>
<td>rs2237892</td>
<td>Yes</td>
<td>C</td>
<td>0.94</td>
<td>0.85</td>
<td>0.98, 0.74</td>
<td>0.030*</td>
<td>0.659</td>
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<td>11</td>
<td>KCNJ11</td>
<td>rs5215</td>
<td>Yes</td>
<td>T</td>
<td>0.61</td>
<td>0.99</td>
<td>1.06, 0.92</td>
<td>0.719</td>
<td>1.000</td>
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<td>11</td>
<td>CENTD2</td>
<td>rs1552224</td>
<td>Yes</td>
<td>A</td>
<td>0.83</td>
<td>1.00</td>
<td>1.10, 0.91</td>
<td>0.963</td>
<td>1.000</td>
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<tr>
<td>11</td>
<td>MTNR1B</td>
<td>rs10830963</td>
<td>No</td>
<td>G</td>
<td>0.28</td>
<td>1.10</td>
<td>1.01, 1.19</td>
<td>0.023*</td>
<td>0.561</td>
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<td>12</td>
<td>HMGA2</td>
<td>rs1531343</td>
<td>No</td>
<td>C</td>
<td>0.10</td>
<td>0.98</td>
<td>0.88, 1.10</td>
<td>0.764</td>
<td>1.000</td>
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<td>12</td>
<td>TSPAN8/</td>
<td>rs7961581</td>
<td>No</td>
<td>C</td>
<td>0.26</td>
<td>1.05</td>
<td>0.97, 1.13</td>
<td>0.259</td>
<td>1.000</td>
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<td>12</td>
<td>LGR5</td>
<td>rs9761581</td>
<td>Yes</td>
<td>T</td>
<td>0.80</td>
<td>0.96</td>
<td>1.05, 0.88</td>
<td>0.346</td>
<td>1.000</td>
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<td>15</td>
<td>HNF1A/TCF1</td>
<td>rs9757197</td>
<td>No</td>
<td>T</td>
<td>0.80</td>
<td>0.96</td>
<td>1.05, 0.88</td>
<td>0.346</td>
<td>1.000</td>
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<td>15</td>
<td>ZFAND6</td>
<td>rs11634397</td>
<td>No</td>
<td>G</td>
<td>0.66</td>
<td>1.04</td>
<td>1.12, 0.96</td>
<td>0.346</td>
<td>1.000</td>
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<td>15</td>
<td>PRC1</td>
<td>rs8042680</td>
<td>Yes</td>
<td>A</td>
<td>0.32</td>
<td>1.04</td>
<td>0.97, 1.12</td>
<td>0.286</td>
<td>1.000</td>
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<tr>
<td>16</td>
<td>FTO</td>
<td>rs9939609</td>
<td>No</td>
<td>A</td>
<td>0.40</td>
<td>0.93</td>
<td>0.86, 1.00</td>
<td>0.041*</td>
<td>0.775</td>
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<tr>
<td>17</td>
<td>HNF1B/TCF2</td>
<td>rs757210</td>
<td>Yes</td>
<td>T</td>
<td>0.35</td>
<td>0.85</td>
<td>0.79, 0.92</td>
<td>3e−08*</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; OR, odds ratio; T2D, type 2 diabetes; RA, risk allele; SNP, single nucleotide polymorphism.

* P < 0.05.

a Association tests were carried out in the Breast and Prostate Cancer Cohort Consortium using a log-additive genetic model with adjustment made for cohort indicators.

b Indicates whether or not variants were genotyped. Variants that were not directly genotyped were imputed.

c Odds ratio for the increase in prostate cancer risk associated with a 1-unit increase in the number of type 2 diabetes risk alleles carried at each locus.

d Association for prostate cancer was in the inverse direction.

e Significant after permutation correction for multiple testing.
investigate whether the locus had effects that act directly on PCa risk or whether the effects of the locus were mediated between diabetes phenotype and PCa risk (OR = 0.76, 95% CI: 0.79, 0.86; Figure 1). The mediator model indicated that a substantial portion of the association of the HNF1B locus with PCa risk was mediated through diabetes phenotype.

DISCUSSION

Our study suggests that genetic variants associated with T2D are also associated with PCa risk. Ten of 36 T2D susceptibility markers were nominally associated with PCa risk at NOTCH2, ADCY5, CDKN2A/B, TCF7L2, KCNQ1, MTNR1B, FTO, and HNF1B, although only the HNF1B locus remained significantly associated with PCa risk after adjustment for multiple testing. However, log odds ratio-weighted GRS and kernel machine models also were associated with PCa risk both with and without inclusion of the HNF1B locus, which suggests that other genetic variants associated with T2D risk also contribute to PCa risk. Finally, mediation analysis provided insufficient evidence that the association of the HNF1B locus with PCa risk is mediated through diabetes phenotype.

Our study adds to the evidence that a genetic background favorable to the development of T2D is associated with PCa risk. The HNF1B locus was most strongly associated with PCa risk in this analysis and accounted for some but not all of the association between the T2D variants and PCa risk in the GRS and the kernel regression. The noted inflation in our association P values for other T2D SNPs is consistent with what others have observed (17, 18) and indicates that more germline variants are held in common between T2D and PCa than would be expected by chance.

Our study’s large sample size and recently published T2D susceptibility loci permitted us to detect potentially novel genetic relations between T2D and PCa that have not been reported previously. Seven loci (NOTCH2, ADCY5, CDKN2A/B, TCF7L2, KCNQ1, MTNR1B, and FTO) not previously associated with PCa at genome-wide significance levels were seen as nominally associated in our study, one of which (FTO) was also reported by Pierce et al. (18). Four of these loci (CDKN2A/B, TCF7L2, KCNQ1, and MTNR1B) are associated with altered beta cell dysfunction or impaired insulin release and could result in less insulin production, thus blunting insulin effects in increasing PCa risk (46). Additionally, our second most highly associated locus, the NOTCH2 locus (P = 0.008; permutation P = 0.26), is of interest. NOTCH2 is a member of the NOTCH family of receptors, which modulate cellular differentiation, proliferation, and apoptosis (47). The locus has been reported to be associated with both T2D and breast cancer (48, 49). Evidence from gene expression data indicates that NOTCH2 is expressed in developing prostate stroma and that NOTCH signaling affects stromal survival only in the presence of testosterone (50). Therefore, the regulatory ability of NOTCH2 and its sensitivity to the presence of testosterone might be important in prostate carcinogenesis, although additional studies are needed to investigate this further.

Our use of GRS and kernel machine models allowed us to investigate the cumulative effect of T2D susceptibility markers and the remaining 35 markers were fitted.

or directionality of risk alleles was statistically significant (P = 0.0001). When HNF1B was removed from the list of included markers and the remaining 35 markers were fitted, the P value was attenuated but remained significant (P = 0.01), which indicated that a substantial portion of the association was a result of the HNF1B locus but that other T2D loci were associated with PCa as well.

We conducted mediation analyses for the HNF1B locus to investigate whether the locus had effects that act directly on PCa risk or whether the effects of the locus were mediated through diabetes phenotype (Table 3). The outcome model produced significant evidence for an association between HNF1B and PCa risk (OR = 0.83, 95% confidence interval (CI): 0.79, 0.86; P = 6.37 × 10^-19) and an association between diabetes phenotype and PCa risk (OR = 0.76, 95% CI: 0.66, 0.87; P = 8.13 × 10^-5). The mediator model indicated that the minor allele of rs7501939 was not statistically significantly associated with an increased risk of diabetes among the 9,526 PCa controls (OR = 1.10, 95% CI: 0.97, 1.25; P = 0.14), although the per-allele odds ratio for association with T2D was consistent with previous reports (8–10). When these results were combined together, the estimated direct effect of HNF1B on PCa risk was statistically significant (OR = 0.83, 95% CI: 0.79, 0.86; P = 1.02 × 10^-18), but the mediated (indirect) effect through diabetes phenotype was nonsignificant (OR = 1.00, 95% CI: 1.00, 1.00; P = 0.71). These results are in agreement with the standard mediation analysis, which produced an insignificant 0.5% change in the parameter estimate for the effect of HNF1B when diabetes status was included as a covariate.
Table 2. Individual Cohort and Combined Results for Unweighted and Log Odds Ratio-Weighted Type 2 Diabetes Genetic Risk Score in the Breast and Prostate Cancer Cohort Consortium

<table>
<thead>
<tr>
<th>Cohort</th>
<th>No. in Cohort</th>
<th>No. of Cases</th>
<th>Total</th>
<th>Meanc</th>
<th>GRS</th>
<th>GRS (-HNF1B)d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OR</td>
<td>95% CI</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>245</td>
<td>72</td>
<td>36.48</td>
<td>36.44</td>
<td>1.00</td>
</tr>
<tr>
<td>CPSII</td>
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<td>636</td>
<td>72</td>
<td>37.48</td>
<td>37.55</td>
<td>1.00</td>
</tr>
<tr>
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<td>72</td>
<td>37.47</td>
<td>37.66</td>
<td>0.99</td>
</tr>
<tr>
<td>HPFS</td>
<td>418</td>
<td>214</td>
<td>72</td>
<td>37.70</td>
<td>37.47</td>
<td>1.02</td>
</tr>
<tr>
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</tr>
<tr>
<td>PHS</td>
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<td>298</td>
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<tr>
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<td>714</td>
<td>72</td>
<td>37.36</td>
<td>37.64</td>
<td>0.98</td>
</tr>
<tr>
<td>Combinedd</td>
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<td>2,782</td>
<td>72</td>
<td>37.42</td>
<td>37.31</td>
<td>0.99</td>
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<tr>
<td>Weighted log OR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATBC</td>
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<td>245</td>
<td>8.16</td>
<td>4.33</td>
<td>4.34</td>
<td>0.93</td>
</tr>
<tr>
<td>CPSII</td>
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<td>8.16</td>
<td>4.45</td>
<td>4.47</td>
<td>0.89</td>
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<tr>
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<td>8.16</td>
<td>4.45</td>
<td>4.47</td>
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<tr>
<td>HPFS</td>
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<td>4.49</td>
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<td>4.49</td>
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<td>PLCO</td>
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<td>8.16</td>
<td>4.43</td>
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<tr>
<td>Combinedd</td>
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<td>8.16</td>
<td>4.44</td>
<td>4.45</td>
<td>0.84</td>
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</table>

Abbreviations: ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study; CI, confidence interval; CPSII, American Cancer Society Cancer Prevention Study II Nutrition Cohort; EPIC, European Prospective Investigation into Cancer and Nutrition; GRS, genetic risk score; HPFS, Health Professionals Follow-up Study; MEC, Multiethnic Cohort Study; OR, odds ratio; PCa, prostate cancer; PHS, Physicians’ Health Study; PLCO, Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial; T2D, type 2 diabetes.

a Logistic regression models were used to regress GRS on risk of PCa.
b Total indicates the maximum bound for the respective GRS, with a value close to this total indicating high genetic predisposition for T2D.
c Mean GRS was calculated for PCa cases and PCa controls.
d HNF1B was excluded from the GRS and included as a separate covariate.
e For combined estimates, cohort indicators were added to adjust for cohort effects.

variants on PCa risk. Although another study was successful in showing an association between unweighted T2D risk scores and PCa. A potential explanation for our lack of association is that with the most recent T2D loci added to our risk score, including T2D variants found through meta-analyses with lower-than-average effect sizes, the number of SNPs doubled, and the range of effect estimates for each variant might have widened. Our study did find a significant association between the log odds-weighted T2D risk scores and PCa. This association was significant when the HNF1B locus was both included in and excluded from the GRS. Although one of the larger T2D-prostate cancer association, the test of heterogeneity indicated that there was no significant evidence for heterogeneity. The fact that the log odds ratio-weighted GRS was significant and the unweighted risk score was insignificant indicates that some T2D variants could have a stronger influence on PCa risk than others. The GRS approach makes the assumption that all T2D loci included in the GRS have T2D risk alleles that function in the same direction when PCa risk is considered. This might not be the case, with some T2D-associated loci

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Table 3. Mediation Analysis for the Association Between HNF1B (rs7501939) and Prostate Cancer With Diabetes Phenotype as a Potential Intermediate in the Breast and Prostate Cancer Cohort Consortium

<table>
<thead>
<tr>
<th>HNF1B-T2D association</th>
<th>Odds Ratio</th>
<th>95% Confidence Interval</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural indirect effect</td>
<td>1.00</td>
<td>1.00, 1.00</td>
<td>0.71</td>
</tr>
<tr>
<td>Natural direct effect</td>
<td>0.83</td>
<td>0.79, 0.86</td>
<td>1.02 × 10⁻¹⁸</td>
</tr>
<tr>
<td>Total effect</td>
<td>0.83</td>
<td>0.79, 0.86</td>
<td>6.37 × 10⁻¹⁹</td>
</tr>
</tbody>
</table>

Abbreviation: T2D, type 2 diabetes.

a All analyses were conducted in the Breast and Prostate Cancer Cohort Consortium and were adjusted for cohort indicator, age at baseline (years), and body mass index (weight (kg)/height (m)²).
possibly having the same rather than the (expected) opposite direction of effect on PCa. Multilocus kernel tests allowed us to assess the cumulative effect of these 36 T2D variants on PCa risk without requiring an assumption about risk allele directionality. Results from the multilocus kernel tests indicated that the 36 T2D variants were significantly associated with PCa risk when \textit{HNF1B} was both included in and excluded from the models, which suggests that common pathways could be involved in both T2D and PCa.

A potential limitation of this study is that information on diabetes phenotype was self-reported (43). However, previous studies have shown that self-reporting of diabetes has up to 97\% agreement with medical records (51, 52). Another limitation is that we could not differentiate between cases of type 1 diabetes and T2D, although the median age (62 years; interquartile range, 55–70) and ethnicity of our study population were such that the majority of diabetes cases were likely to be T2D (53). Furthermore, BPC3 data on T2D status were available only at baseline, and although this could have resulted in underestimation of the true prevalence of diabetes in our study population, it did guard against potential reverse causality.

Our study showed a highly significant inverse relation between T2D and PCa. The estimate was adjusted for body mass index, age at baseline, and cohort indicator and is unlikely to be due to chance or uncontrolled bias. To our knowledge, this is the largest case-control study in which this inverse association has been examined, and our estimate (OR = 0.76) is comparable to, albeit slightly stronger than, the point estimates reported in meta-analyses and other studies, including prior reports from 2 cohorts (i.e., relative risks ranged from 0.84 to 0.91) (3–5, 54).

We further assessed the potential for T2D phenotype to mediate the effect of \textit{HNF1B} with PCa risk. Results indicated a highly significant direct association between \textit{HNF1B} and PCa risk, but there was no significant evidence for an indirect association. Although other investigators have observed a significant relation between \textit{HNF1B} and T2D risk (8, 9), we did not, which indicates that our sample set might have lacked sufficient statistical power to detect this effect. The lack of a mediation role for diabetes phenotype in the \textit{HNF1B}-PCa association has been reported elsewhere in a smaller subset of the BPC3 data (16), although larger studies are needed to more definitively rule out the potential for mediation.

The majority of our analysis, excluding the mediation analysis, was conducted on data from a genome-wide association study of advanced PCa. Although there is concern that results from our study might not be generalizable to other subtypes of PCa, the overwhelming number of similarities between our analysis and others indicates that T2D risk variants have a similar effect on advanced PCa risk and on total PCa risk. This is in agreement with association studies comparing PCa germline variants that show very few examples of different effects by disease aggressiveness.

In conclusion, our data provide additional evidence for a relation between T2D and PCa. Current investigations of a shared genetic background that could underlie this observed association are still in their infancy but suggest that a genetic predisposition to T2D might also be associated with PCa risk. Future studies should further investigate the potential genetic factors that link these two common chronic diseases.

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