AWARD NUMBER: W81XWH-14-1-0146

TITLE: Comprehensive Evaluation of Altered Systemic Metabolism and Pancreatic Cancer Risk

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Boston, MA 02115

REPORT DATE: October 2015

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2015	Annual	30 Sep 2014 - 29 Sep 2015

4. TITLE AND SUBTITLE
Comprehensive Evaluation of Altered Systemic Metabolism and Pancreatic Cancer Risk

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0146

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
.
Dana-Farber Cancer Institute
Boston, MA 02115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
An increased risk of pancreatic cancer and reduced survival is seen among patients with altered systemic metabolism, including obesity, hyperglycemia, and hyperinsulinemia. However, the mechanisms by which altered metabolism may promote pancreatic cancer development and growth remain to be identified. The primary purpose of the funded research is to understand the metabolic alterations that promote development of early pancreatic cancers and impact their proliferation. To explore altered metabolism is patients with pancreatic cancer, we are conducting studies in participants from four large prospective cohorts, where individuals provide extensive data on metabolic phenotypes, such as obesity and diabetes, and banked plasma samples for interrogation. The potential impact of understanding the mechanisms underlying early pancreatic cancer growth is substantial, with an improved ability to: (a) diagnose pancreatic cancer at an earlier stage when cure is possible, (b) formulate preventative recommendations based on an individual's metabolic phenotype, and (c) identify novel treatment strategies that disrupt pancreatic tumor metabolism.

15. SUBJECT TERMS-

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU		19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION:
An increased risk of pancreatic cancer and reduced survival is seen among patients with altered systemic metabolism, including obesity, hyperglycemia, and hyperinsulinemia. However, the mechanisms by which altered metabolism may promote pancreatic cancer development and growth remain to be identified. The primary purpose of the funded research is to understand the metabolic alterations that promote development of early pancreatic cancers and impact their proliferation. To explore altered metabolism is patients with pancreatic cancer, we are conducting studies in participants from four large prospective cohorts, where individuals provide extensive data on metabolic phenotypes, such as obesity and diabetes, and banked plasma samples for interrogation. The potential impact of understanding the mechanisms underlying early pancreatic cancer growth is substantial, with an improved ability to: (a) diagnose pancreatic cancer at an earlier stage when cure is possible, (b) formulate preventative recommendations based on an individual's metabolic phenotype, and (c) identify novel treatment strategies that disrupt pancreatic tumor metabolism.

KEYWORDS:
Pancreatic cancer; Metabolism; Obesity, Diabetes, Early detection, Cancer survival

ACCOMPLISHMENTS:

What were the major goals of the project?

Four major tasks were described in the approved Statement of Work.

Major Task 1: For all three Specific Aims, local IRB and HRPO approval will be obtained to use the previously collected plasma samples from subjects in four large prospective cohorts.
Major Task 1 included local IRB and HRPO approvals to be completed in the first 5 months of the award to allow evaluation of metabolism in human subjects. This task was completed in the described timeframe.

Major Task 2: Identify appropriate matched case-control set from four cohorts and send plasma samples to the Broad Institute at MIT and Harvard University.
Major Task 2 included generation of a human pancreatic cancer case-control population and aliquotting and shipment of plasma samples for metabolite profiling to the Broad Institute of MIT and Harvard University in months 6-9 of the award. This task was completed in the described timeframe.

Major Task 3: Generate and analyze metabolomics dataset.
Major Task 3 included the analysis by LC-MS of plasma samples for circulating metabolites (months 10-15), quality control of the metabolite data (months 16-17), and analysis of the metabolites related to development of early pancreatic cancer (months 18-24). We have completed LC-MS measurement of circulating metabolites and have just initiated our quality control analyses to identify those metabolites appropriate for inclusion in our studies related to the pancreatic cancer development. Thus, we are proceeding with this Task as per the timeframe described in our Statement of Work.

Major Task 4: Generate and analyze metabolites related to branched chain amino acid (BCAA) metabolism.

Major Task 4 included analyses of branched chain amino acid (BCAA) catabolic products (months 10-15), quality control of data (months 16-17), and analysis of BCAA catabolic products with pancreatic cancer development (months 18-23). We have completed measurement of BCAA catabolic products in our plasma samples and have begun the quality control processing of the resultant data. Thus, we are proceeding with this Task as per the timeframe described in our Statement of Work.

What was accomplished under these goals?

1) Major activities: The primary activities conducted during the first 12 months of this award were to obtain necessary regulatory approvals to allow the described human studies, identify eligible pancreatic cancer cases and controls with banked plasma samples from four large cohort studies, prepare and ship plasma samples to the Broad Institute, and evaluate circulating plasma metabolites using liquid chromatography-mass spectroscopy. Each of these activities was completed and we are now initiating our quality control procedures to identify those metabolites measured with sufficient reproducibility to warrant evaluation in our pancreatic cancer study.

2) Specific objectives: The primary objective in the first year of this award was to generate a high-quality, thoroughly-vetted dataset of pancreatic cancer cases and controls with extensive clinical characterization and >4,000 measured plasma metabolites. This dataset forms the basis for defining metabolic determinant of early pancreatic cancer development and progression.

3) Significant results: The important result from this first year of the award is the successful measurement of >4,000 plasma metabolites using our non-targeted LC-MS platforms in 1,500 pancreatic cancer cases and controls, as proposed in our award application. We have initiated our quality control procedures ahead of schedule and within the next 1-2 months should have a finalized dataset with which to initiate our analyses.

4) Other achievements: During the first year of this award, we conducted two analyses using our identified cases related to circulating determinants of pancreatic cancer survival. In the first of these analyses, we evaluated the association of a limited initial set of circulating metabolites with patient survival, noting that circulating levels of two TCA cycle intermediates were associated with pancreatic cancer survival.

Background: Pancreatic tumors cause changes in whole-body metabolism, but whether prediagnostic circulating metabolites predict survival is unknown. **Methods**: We measured 82 metabolites by liquid chromatography-mass spectrometry in prediagnostic plasma from 484 pancreatic cancer cases enrolled in four prospective cohort studies. Association of metabolites with survival was evaluated using Cox proportional hazards models adjusted for age, cohort, race/ethnicity, cancer stage, fasting time, and diagnosis year. After multiple-hypothesis testing correction, $P \leq .0006$ (.05/82) was considered statistical significant. Based on the results, we evaluated 33 tagging single nucleotide polymorphisms (SNPs) in the *ACO1* gene, requiring $P < .002$ (.05/33) for statistical significance. **Results**: Two metabolites in the tricarboxylic acid (TCA) cycle – isocitrate and aconitate – were statistically significantly associated with survival (see Figure 1 of attached manuscript). Participants in the highest versus lowest quintile had hazard ratios (HR) for death of 1.89 (95% CI, 1.06-3.35; *P*-trend<.001) for isocitrate and 2.54 (95% CI, 1.42-4.54; *P*-trend<.001) for aconitate (see Table 2 of attached manuscript). Isocitrate is inter-converted with citrate via the intermediate aconitate in a reaction catalyzed by the enzyme aconitase 1 (*ACO1*). Therefore, we investigated the citrate to aconitate plus isocitrate ratio and SNPs in the *ACO1* gene. The ratio was strongly associated with survival (*P*-

trend<.001; see Table 2 of attached manuscript), as was the SNP rs7874815 in the *ACO1* gene (HR for death per minor allele, 1.37; 95% CI, 1.16-1.61; *P*<.001; see Figure 2 of attached manuscript). Patients had an approximate 3-fold hazard for death when possessing ≥1 minor allele at rs7874851 and high aconitate or isocitrate (see Table 4 of the attached manuscript). **Conclusions**: Prediagnostic circulating levels of TCA cycle intermediates and inherited *ACO1* genotypes were associated with survival among patients with pancreatic cancer.

In the second analysis, we examined the association of plasma levels of 25-hydroxyvitamin D (25[OH]D) with patient survival, demonstrating that low levels of 25(OH) are associated with lower survival time. This manuscript is nearly ready for submission for publication and the abstract is included below for reference.

Purpose: Although vitamin D inhibits pancreatic cancer proliferation in laboratory models, the association of plasma 25-hydroxyvitamin D (25[OH]D) with patient survival is largely unexplored. **Patients and Methods**: We analyzed survival among 493 patients from five prospective U.S. cohorts diagnosed with pancreatic cancer in 1984-2008. We estimated hazard ratios (HRs) for death by plasma level of 25(OH)D (insufficient, <20 ng/ml; relative insufficiency, 20-<30 ng/ml; sufficient ≥30 ng/ml) using Cox proportional hazards models adjusted for age, cohort, race/ethnicity, smoking, diagnosis year, stage, and blood collection month. We also evaluated 33 tagging single nucleotide polymorphisms (SNPs) in the vitamin D receptor gene (*VDR*), requiring *P*<0.002 (0.05/33) for statistical significance. **Results**: The mean prediagnostic plasma level of 25(OH)D was 24.6 ng/ml, and 165 (33%) patients were vitamin D insufficient. Compared to patients with insufficient levels, multivariable-adjusted HRs for death were 0.79 (95% CI, 0.48-1.29) for patients with relative insufficiency and 0.66 (95% CI, 0.49-0.90) for patients with sufficient levels (*P*-trend=0.01; see Table 2 of attached manuscript). These results were unchanged after further adjustment for body-mass index and history of diabetes (*P*-trend=0.02; see Table 2 of attached manuscript). The association was strongest among patients with blood collected within 5 years of diagnosis, with a HR of 0.58 (95% CI, 0.35-0.98) comparing patients with sufficient to patients with insufficient 25(OH)D levels (see Table 4 of attached manuscript). No SNP at *VDR* met our corrected significance threshold of *P*<0.002; rs7299460 was mostly strongly associated with survival (HR per minor allele, 0.80; 95% CI, 0.68-0.95; *P*=0.01; see Table 5 of attached manuscript). **Conclusion**: We observed longer overall survival in patients with pancreatic cancer who had sufficient prediagnostic plasma levels of 25(OH)D.

What opportunities for training and professional development has the project provided?
During the first year of this award, Dr. Wolpin has met regularly with Dr. Clary Clish at the Broad Institute of MIT and Harvard University and Dr. Peter Kraft of the Harvard School of Public Health. These meeting have entailed detailed review of study design, analysis plans, and the analytic characteristics of the LC-MS platforms. The close proximity of Dana-Faber Cancer Institute, Harvard School of Public Health and Broad Institute enables these regular, in-person meetings, which will continue during the second year of the award. Dr. Wolpin meets regularly with the Designated Collaborator for the award, Dr. Charles Fuchs. Dr. Fuchs' office is adjacent to Dr. Wolpin's allowing for both regularly scheduled meetings and numerous impromptu discussions regarding design and analysis considerations. Furthermore, Dr. Wolpin has a graduate student and instructor in his group that accompany him to these meetings, allowing for further training and professional development opportunities for more junior investigators. Dr. Wolpin continues attend a number of relevant conferences at Dana-Farber and other nearby Harvard institutions. He presents his work at the Channing Laboratory cohort study meetings, obtaining real-time feedback and mentoring from senior faculty. He attends the DFCI Seminars in Oncology series and had the opportunity to present his work at this Institute-wide conference.

He also traveled to several conference and delivered talks on his work, including the American Association for Cancer Research national meeting (Philadelphia, PA), Gastrointestinal/Pancreas Cancer SPORE Workshop (Rockville, MD), American Association for Cancer Research (AACR) Integrative Molecular Epidemiology Workshop (Boston, MA), Cold Spring Harbor Laboratory Workshop on Pancreatic Cancer (Cold Spring Harbor, NY), NCI Cohort Consortium annual meeting (Rockville, MD), and National Transdisciplinary Research in Energetics and Cancer (TREC) Scientific Meeting (Boston, MA). Thus, Dr. Wolpin has had ample opportunities to expand his training and professional development during the first year of the award.

How were the results disseminated to communities of interest?
Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
During the second year of the award, we will first complete the quality control analyses of our non-targeted metabolite data set. As noted in our Statement of Work, we anticipate that this will require 2 months to complete, given the complexity of the dataset, containing > 4,000 measured metabolites. Once we are confident in the fidelity of our data, we will pursue individual metabolite associations, including evaluating BCAA catabolic products. Subsequently, we will use several pathway analysis modules developed at the Broad Institute to investigate how groups of metabolites in particular metabolic pathways are associated to pancreatic cancer development and progression. Thus, at the end of the second year of the award, we anticipate completing all the tasks described in our statement of work.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
The initiated work will advance our understanding of how pancreatic cancers use nutrients to promote their growth. This has important applications to early detection of pancreatic cancer and identifying new treatments that cut off the nutrient supply to tumors.

What was the impact on other disciplines?
The work performed within this award will advance the field technically by demonstrating how to pursue large-scale metabolism studies using banked blood samples from thousands of individuals. This has relevance to many disease states, beyond cancer.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
This work has the potential to increase awareness around pancreatic cancer, which is the fourth leading cause of cancer-related death in the United States. Furthermore, this work has the potential to highlight the link between obesity, diabetes, and cancer, which has important implications for lifestyle recommendations to the general public.

CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report

Changes that had a significant impact on expenditures
Nothing to Report

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

Significant changes in use or care of human subjects
Nothing to Report

Significant changes in use or care of vertebrate animals.
Nothing to Report

Significant changes in use of biohazards and/or select agents
Nothing to Report

PRODUCTS:

Publications, conference papers, and presentations

Journal publications.

1. Yuan C, Clish CB, Wu C, Mayers JR, Kraft P, Townsend MK, Zhang M, Tworoger SS, Bao Y, Qian ZR, Rubinson DA, Ng K, Giovannucci EL, Ogino S, Stampfer MJ, Gaziano JM, Ma J, Sesso HD, Anderson GL, Cochrane BB, Manson JE, Torrence ME, Kimmelman AC, Amundadottir LT, Vander Heiden MG, Fuchs CS, Wolpin BM. Circulating Metabolites and Survival among Patients with Pancreatic Cancer. *J Natl Cancer Inst.* 2016; Epub ahead of print. PMID: 26755275.
2. Yuan C, Qian ZR, Babic A, Morales-Oyarvide V, Rubinson DA, Kraft P, Ng K, Bao Y, Giovannucci EL, Ogino S, Stampfer MJ, Gaziano JM, Sesso HD, Buring JE, Cochrane BB, Chlebowski RT, Snetelaar L, Manson JE, Fuchs CS, Wolpin BM. Prediagnostic Plasma 25-Hydroxyvitamin D and Pancreatic Cancer Survival. *J Clin Oncol.* 2016; Submitted.

Books or other non-periodical, one-time publications.
Nothing to Report.

Other publications, conference papers, and presentations.

Local presentations:

2015	Identifying New Screening and Treatment Approaches for Pancreatic Adenocarcinoma	Executive Committee for Clinical Research

Dana-Farber Cancer Institute

2015	Pancreatic Cancer	Cancer Epidemiology Update Series

Dana-Farber/Harvard Cancer Center Cancer

2015	Understanding Host Metabolism in Pancreatic Cancer	Joint Seminar on Energetics and Cancer
Dana-Farber Cancer Institute / Yonsei University (South Korea)		
2015	Pancreatic Cancer and Altered Systemic Metabolism	Krakoff Family Lecture
Dana-Farber Cancer Institute		

National presentations:

2014	Leveraging Metabolomics to Understand Early Pancreatic Cancer	Invited Speaker
National Institutes of Health Think Tank on Metabolomics		Rockville, MD
2014	Host Systemic Metabolism and Early Pancreatic Adenocarcinoma	Invited Speaker
National Transdisciplinary Research in Energetics and Cancer (TREC) Scientific Meeting		Boston, MA
2014	Pancreatic Adenocarcinoma: Studying Early Detection in a Disease Found Too Late	Invited Speaker
Memorial Sloan Kettering Cancer Center		New York, NY
2014	Systemic Metabolism and Pancreatic Adenocarcinoma	Invited Speaker
National Cancer Institute Cohort Consortium Annual Meeting		Rockville, MD
2015	Branched Chain Amino Acid Metabolism and Pancreatic Cancer Development	Invited Speaker
American Association for Cancer Research (AACR) Annual Meeting		Philadelphia, PA
2015	Leveraging Patient Biospecimens to Identify New Screening and Treatment Approaches for Pancreatic Adenocarcinoma	Invited Speaker
Celgene Corporation Outside Speaker Conference		Summit, NJ
2015	Identifying New Screening and Treatment Approaches for Pancreatic Adenocarcinoma	R. Sant Memorial Lecture
James P. Wilmot Cancer Institute, University of Rochester		Rochester, NY
2015	Pancreatic Adenocarcinoma and Host Systemic Metabolism	Banbury Seminar Speaker

Cold Spring Harbor Laboratory Workshop on Pancreatic Cancer		Cold Spring Harbor, NY
2015	Pancreatic Adenocarcinoma and Host Systemic Metabolism	Invited Speaker
Gastrointestinal/Pancreas Cancer SPORE Workshop		Rockville, MD
2015	Metabolomics and Cancer	Invited Speaker
American Association for Cancer Research (AACR) Integrative Molecular Epidemiology Workshop		Boston, MA
2015	Pancreatic Adenocarcinoma: Studying Early Detection in a Disease Found Too Late	Invited Speaker
Medical College of Wisconsin Fall Pancreatic Cancer Symposium		Milwaukee, WI

In the above presentations, metabolism changes due to pancreatic cancer were discussed, including how these changes can inform early detection and prognosis of pancreatic cancer.

Website(s) or other Internet site(s)
Nothing to Report.

Technologies or techniques
Nothing to Report.

Inventions, patent applications, and/or licenses
Nothing to Report.

Other Products
Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Brian Wolpin. No change.
Chen Yuan. No change.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
See attached Other Support page.

What other organizations were involved as partners?
Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
Not applicable.

QUAD CHARTS:
Not applicable.

APPENDICES:

Two manuscripts:

1. Yuan C, Clish CB, Wu C, Mayers JR, Kraft P, Townsend MK, Zhang M, Tworoger SS, Bao Y, Qian ZR, Rubinson DA, Ng K, Giovannucci EL, Ogino S, Stampfer MJ, Gaziano JM, Ma J, Sesso HD, Anderson GL, Cochrane BB, Manson JE, Torrence ME, Kimmelman AC, Amundadottir LT, Vander Heiden MG, Fuchs CS, Wolpin BM. Circulating Metabolites and Survival among Patients with Pancreatic Cancer. *J Natl Cancer Inst.* 2016; Epub ahead of print. PMID: 26755275.
2. Yuan C, Qian ZR, Babic A, Morales-Oyarvide V, Rubinson DA, Kraft P, Ng K, Bao Y, Giovannucci EL, Ogino S, Stampfer MJ, Gaziano JM, Sesso HD, Buring JE, Cochrane BB, Chlebowski RT, Snetelaar L, Manson JE, Fuchs CS, Wolpin BM. Prediagnostic Plasma 25-Hydroxyvitamin D and Pancreatic Cancer Survival. *J Clin Oncol.* 2016; Submitted.

OXFORD

JNCI J Natl Cancer Inst (2016) 108(6): djv409

doi:10.1093/jnci/djv409
First published online January 11, 2016
Article

ARTICLE

Circulating Metabolites and Survival Among Patients With Pancreatic Cancer

Chen Yuan, Clary B. Clish, Chen Wu, Jared R. Mayers, Peter Kraft, Mary K. Townsend, Mingfeng Zhang, Shelley S. Tworoger, Ying Bao, Zhi Rong Qian, Douglas A. Rubinson, Kimmie Ng, Edward L. Giovannucci, Shuji Ogino, Meir J. Stampfer, John Michael Gaziano, Jing Ma, Howard D. Sesso, Garnet L. Anderson, Barbara B. Cochrane, JoAnn E. Manson, Margaret E. Torrence, Alec C. Kimmelman, Laufey T. Amundadottir, Matthew G. Vander Heiden, Charles S. Fuchs, Brian M. Wolpin

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Abstract

Background: Pancreatic tumors cause changes in whole-body metabolism, but whether prediagnostic circulating metabolites predict survival is unknown.

Methods: We measured 82 metabolites by liquid chromatography–mass spectrometry in prediagnostic plasma from 484 pancreatic cancer case patients enrolled in four prospective cohort studies. Association of metabolites with survival was evaluated using Cox proportional hazards models adjusted for age, cohort, race/ethnicity, cancer stage, fasting time, and diagnosis year. After multiple-hypothesis testing correction, a *P* value of .0006 or less (.05/82) was considered statistically significant. Based on the results, we evaluated 33 tagging single-nucleotide polymorphisms (SNPs) in the *ACO1* gene, requiring a *P* value of less than .002 (.05/33) for statistical significance. All statistical tests were two-sided.

Results: Two metabolites in the tricarboxylic acid (TCA) cycle—isocitrate and aconitate—were statistically significantly associated with survival. Participants in the highest vs lowest quintile had hazard ratios (HRs) for death of 1.89 (95% confidence interval [CI] = 1.06 to 3.35, $P_{trend} < .001$) for isocitrate and 2.54 (95% CI = 1.42 to 4.54, $P_{trend} < .001$) for aconitate. Isocitrate is interconverted with citrate via the intermediate aconitate in a reaction catalyzed by the enzyme aconitase 1 (*ACO1*). Therefore, we investigated the citrate to aconitate plus isocitrate ratio and SNPs in the *ACO1* gene. The ratio was strongly associated with survival ($P_{trend} < .001$) as was the SNP rs7874815 in the *ACO1* gene (hazard ratio for death per minor

Received: May 15, 2015; **Revised:** August 21, 2015; **Accepted:** December 7, 2015

allele = 1.37, 95% CI = 1.16 to 1.61, $P < .001$). Patients had an approximately three-fold hazard for death when possessing one or more minor alleles at rs7874851 and high aconitate or isocitrate.

Conclusions: Prediagnostic circulating levels of TCA cycle intermediates and inherited *ACO1* genotypes were associated with survival among patients with pancreatic cancer.

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States (1). Among patients with pancreatic adenocarcinoma, less than 5% will survive five years after diagnosis, and most patients live fewer than 12 months (2). The length of patient survival is associated with disease stage, but few other prognostic markers have been characterized.

Recent studies indicate that prediagnostic obesity (3,4) and diabetes (5–7) adversely impact survival in patients with pancreatic cancer, but the biologic mechanisms underlying these survival differences are unclear. Furthermore, cancer cells have different metabolic needs than normally differentiated cells, exhibiting altered metabolism to support inappropriate cell division (8). As a consequence of these metabolic changes, specific metabolites have been identified that support cancer growth, and some cancer cells demonstrate dependencies upon particular metabolic pathways (9–11).

Pancreatic cancer cells exist in a harsh local environment, surrounded by dense connective tissue and poor vascularization. This hypoxic and nutrient-depleted microenvironment, along with the hallmark *KRAS* oncogene mutation, reprograms pancreatic cancer cell metabolism and promotes tumor growth (11–15). Nevertheless, circulating markers of metabolism that may predict patient outcomes have not been systemically explored. We measured prediagnostic circulating metabolites using liquid chromatography–mass spectroscopy (LC-MS) to identify markers of altered metabolism associated with survival among patients with pancreatic cancer.

Methods

Study Population

Participants with pancreatic cancer and banked prediagnostic blood were included from four prospective cohort studies: Health Professionals Follow-up Study (HPFS), Nurses' Health Study (NHS), Physicians' Health Study (PHS), and Women's Health Initiative–Observational Study (WHI). HPFS was initiated in 1986 when 51 529 US men age 40 to 75 years working in health professions completed a mailed biennial questionnaire (16). NHS was established in 1976 when 121 700 female nurses age 30 to 55 years completed a mailed biennial questionnaire (17). PHS is a completed trial initiated in 1982 of aspirin and β-carotene among 22 071 male physicians, age 40 to 84 years. After trial completion in 1995, participants were followed as an observational cohort (18). WHI consists of 93 676 postmenopausal women age 50 to 79 years enrolled from 1994 to 1998 at 40 US clinical centers (19). Participants completed a baseline clinic visit and annual mailed questionnaires. This study was approved by Human Research Committee at Brigham and Women's Hospital (Boston, MA), and participants provided informed consent.

We identified 488 incident pancreatic adenocarcinoma case patients diagnosed through 2010 with available plasma. Deaths were ascertained from next-of-kin, the postal service, and the National Death Index, which captures more than 98% of deaths (20). Records were reviewed by study physicians blinded to exposure data, and diagnoses were confirmed by medical records, death certificates, and/or tumor registry data. Four case patients with unclear date of diagnosis were excluded.

Blood Collection and Metabolite Profiling

Blood samples in EDTA tubes were collected from 18 225 men in HPFS (1993–1995), 14 916 men in PHS (1982–1984), and 93 676 women in WHI (1994–1998), and in heparin tubes from 32 826 women in NHS (1989–1990). Samples in HPFS and NHS were collected by participants, mailed overnight on cold-packs, and spun to collect plasma (delayed processing) while PHS and WHI participants' whole blood was separated immediately into plasma and stored. Plasma metabolites were measured as peak areas by targeted LC-MS at the Broad Institute of MIT and Harvard University (Cambridge, MA). Blood processing and metabolite profiling methods have been described previously (21).

We measured 133 metabolites, and 82 were included in survival analyses (Supplementary Figure 1, available online). In pilot studies (22), 32 metabolites had poor reproducibility in samples with delayed processing, so were excluded. Three heparin quality control (QC) plasma pools (57 total QC samples) and three EDTA QC plasma pools (128 total QC samples) were randomly interspersed among participant samples. We calculated mean coefficients of variation (CVs) for each metabolite across QC plasma pools and set an a priori threshold of 25% or less for satisfactory reproducibility; 13 metabolites with a mean CV greater than 25% were excluded. Six metabolites were excluded for undetectable levels in greater than 10% of case patients. We evaluated 10 volunteers with plasma collected simultaneously in heparin and EDTA tubes. Spearman correlation coefficients between Heparin and EDTA samples were 0.70 for isocitrate, 0.84 for aconitate, and 0.89 for citrate.

Single-Nucleotide Polymorphism Selection and Genotyping

We selected 33 tagging single-nucleotide polymorphisms (SNPs) in the *ACO1* gene +/-20 kb using the tagger algorithm in Haploview with cutoffs at an r^2 of 0.8 and a minor allele frequency (MAF) of 5% or higher in whites from the HapMap Project database, forcing in two SNPs related to melanoma risk (rs7855483 and rs10813813) (23). From 387 of the case patients, DNA was extracted from archived buffy coat with QIAGEN QIAmp and whole-genome-amplified with GE Healthcare Genomiphi. All genotyping was performed at Partners HealthCare Center for Personalized Genetic Medicine using a custom-designed Illumina Golden Gate genotyping assay. Two tagging SNPs were not supported by the Golden Gate platform and could not be genotyped. Replicate samples included for QC (n = 44 sample groups) had mean genotype concordance of 97.7% across the 33 SNPs. No SNPs deviated from Hardy-Weinberg Equilibrium at a P value of less than .01. Most case patients from the cohorts (n = 371) were evaluated in a recently completed genome-wide association study (GWAS) of pancreatic cancer (PanScan) (24). To obtain a denser map of SNPs at *ACO1* (+/-20 kb) and investigate SNPs at mitochondrial aconitase *ACO2* (+/-20 kb), we investigated

genotyped or imputed SNPs from the HPFS, NHS, PHS, and WHI case patients included in PanScan with MAFs of 5% or greater (Supplementary Table 1, available online). Genotyping and imputation methods have been described previously (24). For more details, see the Supplementary Methods (available online).

Covariate Data

Age, race/ethnicity, smoking status, physical activity, body mass index (BMI), alcohol intake, and history of diabetes were obtained from baseline questionnaires in PHS and WHI and questionnaires prior to blood collection in HPFS and NHS (25). Date of cancer diagnosis and pancreatic cancer stage at diagnosis were obtained from medical record review, as described previously (21). For more details, see the Supplementary Methods (available online).

Statistical Analysis

Overall survival time was calculated from date of pancreatic cancer diagnosis to date of death or date of last follow-up, whichever came first. Metabolites were log-transformed to improve normality and included as continuous variables in Cox proportional hazards regression models adjusted for age at diagnosis (years, continuous), cohort (HPFS, NHS, PHS, WHI; which also adjusts for sex), race/ethnicity (white, black, other, missing), stage at diagnosis (localized, locally advanced, metastatic, unknown), fasting time (<4, 4–8, 8–12, ≥12 hours, missing), and year of diagnosis (1984–1995, 1996–2005, 2006–2010). Using a conservative Bonferroni correction for multiple hypothesis testing (26), metabolites with a P_{trend} of .0006 or less (.05/82) were considered statistically significant. In secondary analyses, we also adjusted for time between blood collection and cancer diagnosis (0-<5, 5-<10, ≥10 years), BMI (World Health Organization categories), and history of diabetes (yes, no).

To evaluate effect magnitude, statistically significant metabolites were examined in Cox regression models after pooled categorization into quintiles defined by fasting status (≥8 hours, <8 hours since last meal). Hazard ratios (HRs) and 95% confidence intervals (CIs) were also calculated per standard deviation (SD) change in log-transformed metabolite levels. We calculated median survival time for subjects in each quintile adjusted for covariates using direct adjusted survival estimation (27,28). This method uses proportional hazards models to estimate probabilities of survival at each follow-up timepoint for individual subjects and averages them to obtain an overall survival estimate. Partial Spearman correlation coefficients were calculated for metabolites, adjusted for cohort and fasting time.

We assessed heterogeneity of metabolite associations with pancreatic cancer survival across cohorts using Cochran's Q-statistic (29). We also conducted analyses stratified by time interval between blood collection and cancer diagnosis, sex, cancer stage, fasting status, and BMI. 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 examined tagging SNPs in *ACO1* and survival by including each three-level genotype as a continuous variable (additive model) in multivariable-adjusted Cox regression models. SNPs were considered statistically significant if *P* values were less than .002 (.05/33 genotyped variants). The same approach was followed for SNPs genotyped or imputed at *ACO1* and *ACO2* in the PanScan GWAS. Survival curves by genotypes were generated using the Kaplan-Meier method, and statistical significance measured using the log-rank test. We identified SNPs highly correlated with our most statistically significant tagged SNPs (r^2 > 0.7 in 1000G CEU data) and used HaploReg v2 (30) to explore noncoding functional annotation. We also assessed cis associations of these SNPs and expression of nearby genes in peripheral tissues from subjects of European descent (31–33).

The proportionality of hazards assumption was satisfied (*P* > .05) for Cox proportional hazards models by evaluating a time-dependent variable, which was the cross-product of time and metabolite or genotype. All analyses were performed with SAS 9.3 statistical package. All *P* values were two-sided.

Results

Characteristics of 484 pancreatic cancer case patients are shown in Table 1 and by cohort in Supplementary Table 2 (available online). The median time between blood collection and cancer diagnosis was 8.0 years. Among the case patients with known stage, 16.8% had localized disease, 28.7% had locally advanced

Table 1. Baseline characteristics of patients with pancreatic cancer

Characteristic*	Pancreatic cancer cases (n = 484)
Age at blood collection, Mean (SD), y	63.3 (8.4)
Age at cancer diagnosis, mean (SD), y	72.2 (8.0)
Female sex, No. (%)	322 (66.5)
Race/ethnicity, No. (%)	
White	430 (88.8)
Black	16 (3.3)
Other	14 (2.9)
Missing	24 (5.0)
Body mass index, mean (SD), kg/m^2	26.4 (4.8)
Physical activity, mean (SD), MET-h/wk	20.2 (32.9)
History of diabetes mellitus, No. (%)	28 (5.8)
Tobacco use, No. (%)	
Never	206 (42.6)
Past	216 (44.6)
Current	58 (12.0)
Missing	4 (0.8)
Alcohol (≥1 drink/day), No. (%)	120 (24.8)
Median time from blood draw to cancer diagnosis, y	8.0
Fasting (≥ 8 h) at blood collection, No. (%)	355 (73.3)
Diagnosis period, No. (%)	
1984–1995	45 (9.3)
1996–2005	370 (76.4)
2006–2010	69 (14.3)
Cancer stage, No. (%)	
Localized	65 (13.4)
Locally advanced	111 (22.9)
Metastatic	211 (43.6)
Unknown	97 (20.0)
Median survival time, mo	
All patients	6
By stage	
Localized	16
Locally advanced	10
Metastatic	3
Unknown	6

* Continuous variables reported as mean (SD) and categorical variables reported as No. (%) at time of blood collection, unless otherwise noted.

disease, and 54.5% had metastatic disease. The median survival by cancer stage was 16 months for those with localized disease, 10 months for those with locally advanced disease, and three months for those with metastatic disease. By the end of follow-up, 448 (93%) patients were deceased. In Cox regression models, two metabolites were statistically significant to *P* values of .0006 or less, the predefined statistical significance threshold after Bonferroni correction (Figure 1; Supplementary Table 3, available online).

The two statistically significant plasma metabolites after multiple-hypothesis correction were intermediates of the tricarboxylic acid (TCA) cycle: isocitrate and aconitate (Table 2). Compared to case patients in the bottom quintile, those in the top quintile had hazard ratios for death of 1.89 (95% CI = 1.06 to 3.35, P_{trend} < .001) for isocitrate and 2.54 (95% CI = 1.42 to 4.54, P_{trend} < .001) for aconitate, corresponding to a reduction in median survival of four to six months. Within the oxidative TCA cycle, isocitrate is generated from citrate with aconitate as an intermediate step, and plasma levels of isocitrate and aconitate were highly correlated in our patients (ρ = .82, *P* < .001) (Supplementary Table 4, available online). Thus, we examined the ratio of citrate to the sum of aconitate plus isocitrate and noted a hazard ratio for death of 0.46 (95% CI = 0.27 to 0.79, P_{trend} < .001) comparing extreme quintiles (Table 2). No statistically significant effect modification was identified in stratified analyses by cohort, cancer stage, fasting status at blood collection, or BMI (Supplementary Table 5, available online).

Because cancer diagnoses occurred at varying timepoints after blood collection, we performed secondary analyses adjusting for this time in our multivariable models and noted little change in the hazard ratios for death for isocitrate, aconitate, and their ratio with citrate (Table 2). We also examined survival among patients stratified by time between blood collection and cancer diagnosis. The strongest associations were seen among case patients with blood collected within 10 years of diagnosis (Table 3). Interestingly, it has been estimated that eight to 10 years elapse from the formation of the initial invasive founder cell within the pancreas to a patient's diagnosis (34). Thus, we also analyzed the association of all metabolites with survival among the 305 case patients with blood collected within 10 years of diagnosis (Supplementary Table 6, available online). Aconitate (P_{trend} < .001) and isocitrate (P_{trend} < .001) remained strongly associated with survival, with proline also reaching statistical significance (P_{trend} < .001) in this exploratory analysis. Further adjustment of multivariable models by BMI and diabetes history demonstrated similar results (Table 2).

Within the cytosol of cells, the enzyme aconitase 1, encoded by *ACO1*, catalyzes interconversion of citrate and isocitrate via aconitate. Thus, we examined genotypic variation in *ACO1* in relation to patient survival. Seven tagging SNPs in *ACO1* were

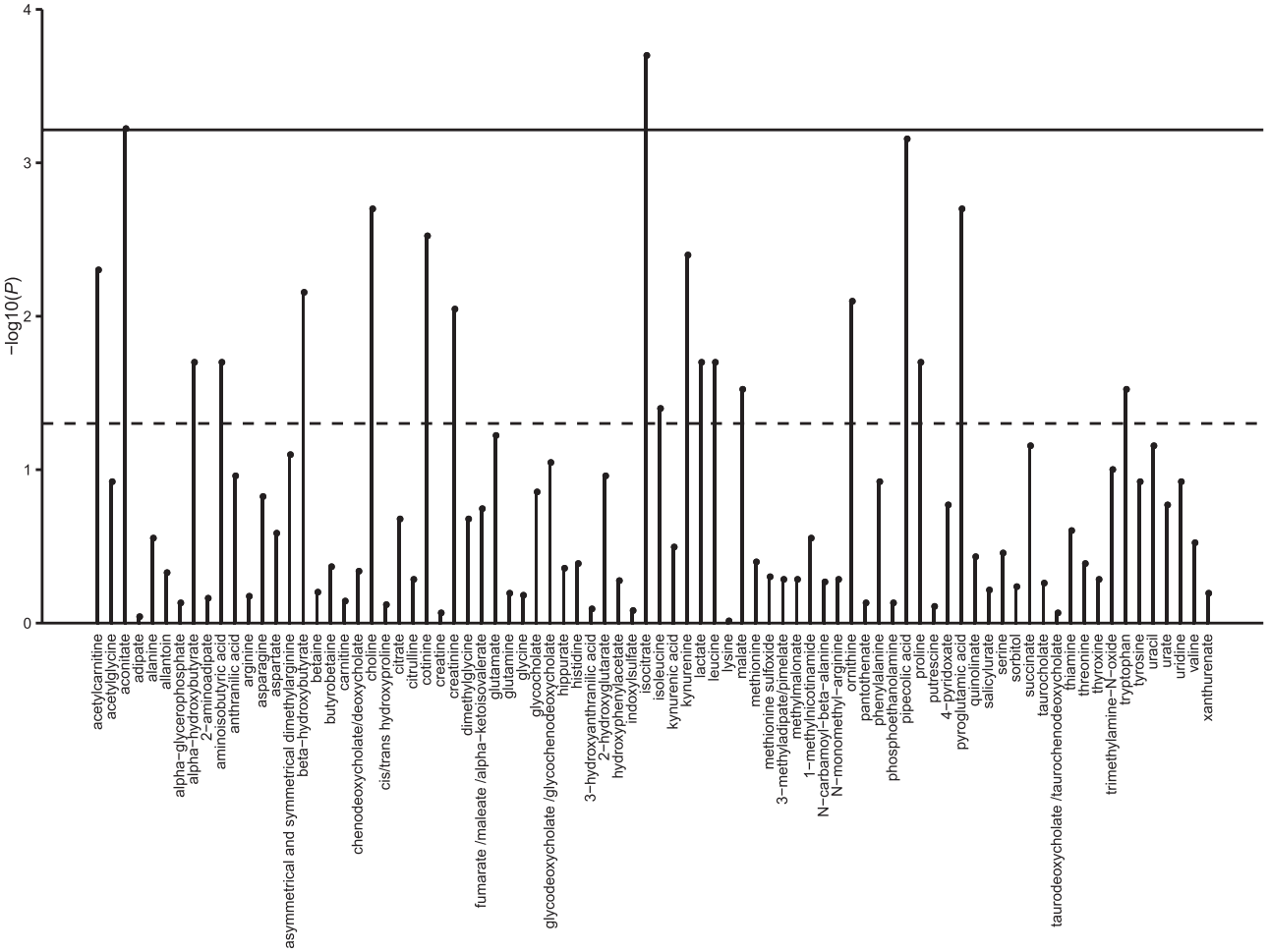

Figure 1. Association of prediagnostic levels of plasma metabolites with overall survival among pancreatic cancer case patients. Two-sided *P* value of the log-transformed metabolite as a continuous variable in Cox regression models adjusted for age at diagnosis (years, continuous), cohort (Health Professionals Follow-up Study, Nurses' Health Study, Physicians' Health Study, Women's Health Initiative; also adjusts for sex), race/ethnicity (white, black, other, missing), stage at diagnosis (localized, locally advanced, metastatic, unknown), fasting time (<4, 4–8, 8–12, ≥12 hours, missing), and year of diagnosis (1984–1995, 1996–2005, 2006–2010). **Solid line** indicates the statistically significant *P* value threshold after Bonferroni correction for multiple-hypothesis testing (*P* value ≤ .0006, .05/82). **Dashed line** indicates *P* value of .05.

Table 2. Hazard ratios for death among pancreatic cancer case patients by prediagnostic levels of plasma metabolites

Model	Quintile of plasma metabolite					P*
	1	2	3	4	5	
Isocitrate						
Person-mo	1078	1145	1341	1195	884	
Cases/deaths	96/92	97/89	97/89	97/84	97/94	
Median survival, mo	9	8	7	6	5	
HR (95% CI)†	1	1.16 (0.73 to 1.85)	1.27 (0.72 to 2.23)	1.61 (0.90 to 2.85)	1.89 (1.06 to 3.35)	<.001
HR (95% CI)‡	1	1.17 (0.73 to 1.86)	1.38 (0.78 to 2.42)	1.65 (0.93 to 2.94)	1.96 (1.10 to 3.48)	<.001
HR (95% CI)§	1	1.14 (0.71 to 1.82)	1.30 (0.73 to 2.29)	1.56 (0.87 to 2.80)	1.77 (0.98 to 3.19)	.002
Aconitate						
Person-mo	1113	1294	1195	1020	1021	
Cases/deaths	96/92	97/88	97/91	97/89	97/88	
Median survival, mo	11	8	6	5	5	
HR (95% CI)†	1	1.49 (0.93 to 2.37)	2.15 (1.21 to 3.80)	2.33 (1.31 to 4.14)	2.54 (1.42 to 4.54)	<.001
HR (95% CI)‡	1	1.53 (0.96 to 2.45)	2.26 (1.27 to 4.01)	2.39 (1.34 to 4.26)	2.70 (1.50 to 4.86)	<.001
HR (95% CI)§	1	1.47 (0.91 to 2.37)	2.10 (1.17 to 3.77)	2.25 (1.25 to 4.05)	2.44 (1.33 to 4.48)	.003
Citrate/(isocitrate+ aconitate)						
Person-mo	875	875	1460	1260	1173	
Cases/deaths	96/94	97/91	97/84	97/85	97/94	
Median survival, mo	5	5	6	9	9	
HR (95% CI)†	1	0.88 (0.65 to 1.19)	0.69 (0.50 to 0.94)	0.45 (0.32 to 0.64)	0.46 (0.27 to 0.79)	<.001
HR (95% CI)‡	1	0.90 (0.66 to 1.22)	0.73 (0.53 to 1.00)	0.45 (0.32 to 0.64)	0.47 (0.27 to 0.80)	<.001
HR (95% CI)§	1	0.90 (0.66 to 1.22)	0.74 (0.54 to 1.02)	0.48 (0.34 to 0.69)	0.50 (0.29 to 0.87)	<.001

* P_{trend} calculated by entering the log-transformed metabolite as a continuous variable in Cox regression models. All *P* values are two-sided. CI = confidence interval; HR = hazard ratio.
† Hazard ratios (95% confidence intervals) from Cox regression models adjusted for age at diagnosis (years, continuous), cohort (Health Professionals Follow-up Study, Nurses' Health Study, Physicians' Health Study, Women's Health Initiative; also adjusts for sex), race/ethnicity (white, black, other, missing), stage at diagnosis (localized, locally advanced, metastatic, unknown), fasting time (<4, 4–8, 8–12, ≥12 hours, missing), and year of diagnosis (1984–1995, 1996–2005, 2006–2010).
‡ Model further adjusted for time between blood collection and cancer diagnosis (0-<5, 5-<10, ≥10 years).
§ Model further adjusted for body mass index (World Health Organization categories) and history of diabetes (yes, no).

Table 3. Hazard ratios for death among pancreatic cancer case patients by prediagnostic levels of plasma metabolites stratified by time between blood collection and cancer diagnosis

Blood collection to cancer diagnosis	No. of cases	HR (95% CI)*					
		Isocitrate		Aconitate		Citrate/(isocitrate+aconitate)	
		Extreme quartiles	Per SD	Extreme quartiles	Per SD	Extreme quartiles	Per SD
0-<5 y	143	2.32 (1.29 to 4.18)	1.40 (1.11 to 1.78)	2.73 (1.49 to 5.00)	1.49 (1.15 to 1.93)	0.26 (0.14 to 0.51)	0.69 (0.55 to 0.88)
P†		.004		.002		.002	
5-<10 y	162	2.32 (1.06 to 5.09)	1.42 (1.04 to 1.94)	2.45 (1.10 to 5.49)	1.62 (1.18 to 2.24)	0.48 (0.21 to 1.08)	0.76 (0.57 to 1.02)
P†		.02		.003		.05	
≥10 y	179	1.58 (0.71 to 3.50)	1.14 (0.82 to 1.61)	1.11 (0.48 to 2.56)	0.98 (0.64 to 1.50)	1.01 (0.47 to 2.17)	0.87 (0.60 to 1.25)
P†		.28		.87		.38	

* Hazard ratios (95% confidence intervals) for the comparison of the fourth quartile to the first quartile (referent) or per standard deviation change of the metabolite from Cox regression models adjusted for age at diagnosis (years, continuous), cohort (Health Professionals Follow-up Study, Nurses' Health Study, Physicians' Health Study, Women's Health Initiative; also adjusts for sex), race/ethnicity (white, black, other, missing), stage at diagnosis (localized, locally advanced, metastatic, unknown), fasting time (<4, 4–8, 8–12, ≥12 hours, missing), and year of diagnosis (1984–1995, 1996–2005, 2006–2010). CI = confidence interval; HR = hazard ratio.
† P_{trend} calculated by entering the log-transformed metabolite as a continuous variable in Cox regression models. All *P* values are two-sided.

associated with survival to *P* values of less than .05 in an additive model of inheritance (Supplementary Table 7, available online). One SNP met the predefined statistical significance threshold after multiple-hypothesis correction, rs7874815, with a per-allele hazard ratio for death of 1.37 (95% CI = 1.16 to 1.61, *P* < .001) that was similar across cohorts ($P_{heterogeneity}$ = .26) (Supplementary Figure 2, available online). In a dominant model, the hazard ratio for death was 1.64 (95% CI = 1.32 to 2.04) comparing case patients with the AG/GG genotypes to those with the AA genotype with median overall survival times of four months and seven months, respectively (log-rank *P* < .001) (Figure 2). In linear regression models, we did not observe statistically significant associations between rs7874815 genotype and metabolite levels (Supplementary Table 8, available online). When considered together, patients with at least one minor allele at rs7874815 and higher levels of aconitate or isocitrate had an approximate three-fold hazard for death (Table 4), although *P* values for interaction were not statistically significant (*P* > .05). Using *ACO1* genotypes generated in the PanScan GWAS in the same patient population, rs7874815 and several highly correlated SNPs remained

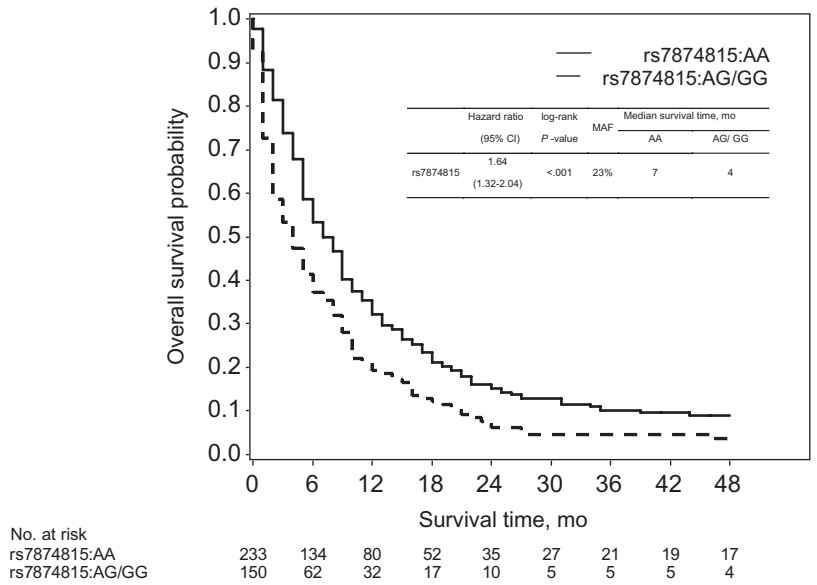

Figure 2. Overall survival among pancreatic cancer case patients by rs7874815 genotypes in *ACO1*. A table of the numbers of patients at risk in each group at various timepoints is given below the curves. Survival curves were generated by the Kaplan-Meier method, and the two-sided *P* value calculated using the log-rank test. CI = confidence interval; HR = hazard ratio; MAF = minor allele frequency.

most strongly associated with survival (Supplementary Figure 3, available online). Bioinformatic analyses for rs7874815 and highly correlated SNPs using HaploReg (30) demonstrated histone marks that tag enhancers in multiple tissue types, indicating possible regulatory elements in the region encompassing rs7874815 (Supplementary Table 9, available online). No statistically significant eQTLs were noted for these SNPs in MuTHER or blood eQTL databases (31–33). We also investigated genotypes at *ACO2*, which encodes mitochondrial aconitase, in the PanScan population (Supplementary Figure 4, available online). The SNP most statistically significantly associated with survival was rs112622778 (MAF = 0.05, per-allele HR = 1.98, 95% CI = 1.34 to 2.91, $P < .001$), intronic to *ACO2*.

Three other TCA cycle intermediates were measured in our patients, but these circulating metabolites were not associated with survival, including fumarate ($P_{trend} = .18$), malate ($P_{trend} = .03$), and succinate ($P_{trend} = .07$).

Table 4. Hazard ratios (95% CIs) for death among pancreatic cancer case patients by combined categories of plasma metabolite levels and rs7874815 genotypes in *ACO1**

		rs7874815	
Metabolite	Tertiles	AA HR (95% CI)	AG/GG HR (95% CI)
Isocitrate	1	1.0	1.47 (1.00 to 2.16)
	2	1.31 (0.84 to 2.05)	2.42 (1.50 to 3.91)
	3	1.64 (1.05 to 2.58)	2.72 (1.65 to 4.49)
Aconitate	1	1.0	1.67 (1.14 to 2.45)
	2	1.16 (0.73 to 1.83)	1.74 (1.08 to 2.80)
	3	1.44 (0.92 to 2.25)	2.67 (1.62 to 4.39)
Citrate/ (isocitrate + aconitate)	3	1.0	1.62 (1.10 to 2.40)
	2	1.64 (1.05 to 2.56)	2.33 (1.44 to 3.79)
	1	1.83 (1.14 to 2.92)	3.32 (2.02 to 5.47)

* Adjusted for age at diagnosis (years, continuous), cohort (Health Professionals Follow-up Study, Nurses' Health Study, Physicians' Health Study, Women's Health Initiative; also adjusts for sex), race/ethnicity (white, black, other, missing), stage at diagnosis (localized, locally advanced, metastatic, unknown), fasting time (<4, 4–8, 8–12, ≥12 hours, missing), and year of diagnosis (1984–1995, 1996–2005, 2006–2010). CI = confidence interval; HR = hazard ratio.

Discussion

Prior studies of altered metabolism and pancreatic cancer survival have demonstrated that obese patients (3,4) and those with diabetes (5–7) in the years before diagnosis have reduced survival. However, the association of circulating metabolites with survival has remained largely unexplored. In the current study, we identified associations of circulating intermediates of the TCA cycle and polymorphisms in *Aconitase 1* with survival of patients with pancreatic cancer. The TCA cycle is a fundamental pathway of cellular metabolism comprised of eight enzyme-catalyzed reactions that provide reducing agents to drive mitochondrial ATP production and intermediates that can fuel anabolic pathways to produce lipids, nucleic acids, and proteins. Mutations in TCA cycle enzymes are known to promote cancer development and growth, including mutations in succinate dehydrogenase, fumarate hydratase, and isocitrate dehydrogenase 1 and 2 (10,35). Furthermore, these mutations have been associated with distinct cancer subsets with different patient prognoses (36,37) and measureable changes in levels of TCA cycle metabolites (38–40). Although mutations in genes encoding TCA cycle enzymes have not been a prominent feature of pancreatic adenocarcinoma, the importance of reprogrammed cellular metabolism has become increasingly apparent for this disease, including alterations that involve the TCA cycle and mutant KRAS-induced tumor cell dependencies for glucose, glutamine, and extracellular protein (11–15).

Aconitase functions as a TCA cycle enzyme, but it also plays a critical role in intracellular iron homeostasis and is alternatively known as iron regulatory protein-1 (IRP1). Depending on intracellular iron levels, it may function primarily as an aconitase enzyme or bind to iron-responsive elements within the untranslated regions of specific mRNA molecules, altering

translation of proteins that regulate uptake, storage, and utilization of iron (41). Interestingly, these iron-regulatory functions of aconitase/IRP1 have also been linked to cancer development and progression (41,42). Additional studies will be required to determine whether the identified alterations in circulating TCA metabolites and *ACO1* single-nucleotide variants reflect perturbations in one or both of these aconitase/IRP1 activities.

Median survival times were shorter by four to six months in patients with high levels of circulating aconitate and isocitrate. For context, two multi-agent chemotherapy programs have recently been adopted for treatment of patients with metastatic pancreatic cancer (43,44). These two chemotherapy regimens improved median overall survival by 1.8 months (gemcitabine plus nab-paclitaxel) and 4.3 months (FOLFIRNOX) in comparison with single-agent gemcitabine in patients with excellent functional status. Understanding the metabolic dependencies of cancer is a promising approach to identifying novel prognostic markers and therapeutic programs (45). The current study demonstrates that circulating metabolites may provide prognostic information in patients with pancreatic cancer, while focusing attention on enzymatic steps within the TCA cycle as potentially influencing disease progression.

Patients in this study were drawn from four prospective US cohorts. An important strength of a prospective cohort design is the ability to fully capture the spectrum of patients with pancreatic cancer in terms of disease aggressiveness and stage of disease, as individuals are enrolled prior to their diagnosis and are not identified at select tertiary care centers. Notably, survival times and stage distribution were highly similar to the 121 713 patients included in the National Cancer Data Base, which is thought to capture 76% of pancreatic cancer case patients diagnosed in the United States each year (41). Another important feature of a prospective cohort design is the collection of blood samples prior to cancer diagnosis, which limits metabolic changes induced by advanced disease and cancer-induced complications, such as an altered diet, biliary obstruction, and pancreatic insufficiency. Interestingly, the strongest associations of aconitate and isocitrate with survival were seen in patients with blood drawn within 10 years of cancer diagnosis, when subclinical invasive cancer is likely present (34). Whether these associations would be seen using blood samples collected at the time of cancer diagnosis requires further study. We rigorously piloted LC-MS methods in samples from our study subjects (21,22), removing metabolites unduly influenced by blood processing conditions or with poor reproducibility, which reduced the likelihood of false-negative results.

Limitations of the current study also require consideration. We did not collect systemic treatment information across the studies. Nevertheless, chemotherapy options are limited (2) and it is unlikely that chemotherapy differed by prediagnostic circulating metabolite levels, such that confounding by systemic treatment is not likely to have materially affected our results. We utilized overall mortality in our analyses, as opposed to pancreatic cancer–specific mortality. However, less than 5% of patients with pancreatic cancer are cured of their disease, such that almost all patients die from their cancer rather than other causes. We cannot rule out that our findings may be influenced by residual confounding by unmeasured factors. Nonetheless, we included relevant covariates in multivariable models and also noted survival associations with inherited variants within *ACO1*. We used strict Bonferroni corrections to define statistical significance. This approach to multiple-hypothesis testing correction reduces the likelihood of false-positive results but is likely to under-report statistically significant associations, particularly given that metabolite levels are correlated with one another and therefore not entirely independent tests. Our study participants were predominantly of European descent and further studies in other populations are warranted.

In nearly 500 patients with pancreatic cancer from four large US cohort studies, we found that elevated prediagnostic circulating levels of aconitate and isocitrate, two TCA cycle intermediates, were associated with an approximate two-fold increased hazard for death. Furthermore, survival was associated with tagging SNPs within *ACO1*, which encodes the enzyme that interconverts citrate, aconitate, and isocitrate. Taken together, these data identify circulating aconitate and isocitrate as novel prognostic markers in patients with pancreatic cancer and implicate altered metabolism related to the TCA cycle in pancreatic cancer progression.

Funding

HPFS is supported by National Institutes of Health (NIH) grant UM1 CA167552. NHS is supported by NIH grants UM1 CA186107, P01 CA87969, and R01 CA49449. PHS is supported by NIH grants CA 97193, CA 34944, CA 40360, HL 26490, and HL 34595. The Women's Health Initiative (WHI) program is funded by the NIH through contracts N01WH22110, 24152, 32100–2, 32105–6, 32108–9, 32111–13, 32115, 32118–32119, 32122, 42107–26, 42129–32, and 44221.

Additional support came from a Nestle Research Center award to the Broad Institute; from the Robert T. and Judith B. Hale Fund for Pancreatic Cancer, Perry S. Levy Fund for Gastrointestinal Cancer Research, Pappas Family Research Fund for Pancreatic Cancer, NIH R01 CA124908, and NIH P50 CA127003 to CSF; and from Department of Defense CA130288, Howard Hughes Medical Institute, Lustgarten Foundation, and Promises for Purple to BMW.

Notes

The study sponsors had no role in the design of the study; the collection, analysis, or interpretation of the data; the writing of the manuscript; or the decision to submit the manuscript for publication.

The authors would like to thank the participants and staff of the HPFS, NHS, PHS, and WHI for their contributions, as well as the following state cancer registries for their help: AL, AZ, AR, CA, CO, CT, DE, FL, GA, ID, IL, IN, IA, KY, LA, ME, MD, MA, MI, NE, NH, NJ, NY, NC, ND, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA, WY. The authors assume full responsibility for analyses and interpretation of these data.

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Prediagnostic Plasma 25-Hydroxyvitamin D and Pancreatic Cancer Survival

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Running Head: Vitamin D and pancreatic cancer survival

Counts:
Abstract: 249 words (maximum= 250 words)
Text: 2,702 words (maximum= 3,000 words)
References: 35
Tables:5
Figures: 1
Supplementary Tables: 3

ABSTRACT

Purpose: Although vitamin D inhibits pancreatic cancer proliferation in laboratory models, the association of plasma 25-hydroxyvitamin D (25[OH]D) with patient survival is largely unexplored.

Patients and Methods: We analyzed survival among 493 patients from five prospective U.S. cohorts diagnosed with pancreatic cancer in 1984-2008. We estimated hazard ratios (HRs) for death by plasma level of 25(OH)D (insufficient, <20 ng/ml; relative insufficiency, 20-<30 ng/ml; sufficient ≥30 ng/ml) using Cox proportional hazards models adjusted for age, cohort, race/ethnicity, smoking, diagnosis year, stage, and blood collection month. We also evaluated 33 tagging single nucleotide polymorphisms (SNPs) in the vitamin D receptor gene (*VDR*), requiring $P<0.002$ (0.05/33) for statistical significance.

Results: The mean prediagnostic plasma level of 25(OH)D was 24.6 ng/ml, and 165 (33%) patients were vitamin D insufficient. Compared to patients with insufficient levels, multivariable-adjusted HRs for death were 0.79 (95% CI, 0.48-1.29) for patients with relative insufficiency and 0.66 (95% CI, 0.49-0.90) for patients with sufficient levels (P-trend=0.01). These results were unchanged after further adjustment for body-mass index and history of diabetes (P-trend=0.02). The association was strongest among patients with blood collected within 5 years of diagnosis, with a HR of 0.58 (95% CI, 0.35-0.98) comparing patients with sufficient to patients with insufficient 25(OH)D levels. No SNP at *VDR* met our corrected significance threshold of $P<0.002$; rs7299460 was mostly strongly associated with survival (HR per minor allele, 0.80; 95% CI, 0.68-0.95; $P=0.01$).

Conclusion: We observed longer overall survival in patients with pancreatic cancer who had sufficient prediagnostic plasma levels of 25(OH)D.

INTRODUCTION

Pancreatic cancer is the fourth-leading cause of cancer-related death in the United States, and most patients die within 12 months of diagnosis.[1] Aside from disease stage at presentation, few patient characteristics or circulating markers have been identified that predict survival time in patients with pancreatic ductal adenocarcinoma.[2]

Vitamin D is a fat-soluble vitamin that can be ingested in foods and is produced endogenously when sunlight triggers synthesis in the skin. Metabolites of vitamin D bind to the vitamin D receptor (VDR) within cells, regulating transcription of target genes by interacting with vitamin D response elements.[3] In addition to the essential roles of vitamin D in calcium homeostasis and bone health, emerging evidence indicates that vitamin D and its analogues may inhibit cancer development and growth. [3-5] Recent studies in laboratory models of pancreatic cancer have demonstrated the therapeutic potential of vitamin D analogues by engagement of VDR in tumor cells and supportive cells within the tumor stroma.[6-8] Although studies evaluating circulating vitamin D and pancreatic cancer incidence have been conflicting[9,10], few studies have been conducted to assess the association of vitamin D levels with survival in patients with pancreatic cancer.[11,12]

To assess the potential prognostic effect of vitamin D status, we evaluated the association between prediagnostic plasma 25-hydroxyvitamin D (25[OH]D) and overall survival among patients with pancreatic cancer drawn from five large U.S. prospective cohorts. Additionally, we examined survival in relation to common germline genetic variants in the *VDR* gene.

PATIENTS AND METHODS

Study Population

Our study population included pancreatic cancer cases from five U.S. prospective cohort studies. The Health Professionals Follow-Up Study (HPFS) was established in 1986 when 51,529 male health professionals aged 40 to 75 years responded to a mailed questionnaire. The Nurses' Health Study (NHS) was initiated in 1976 when 121,700 female registered nurses aged 30 to 55 years responded to a mailed questionnaire. The Physicians' Health Study I (PHS I) is a randomized clinical trial of aspirin and β-carotene that enrolled 22,071 male physicians aged 40 to 84 years in 1982. After completion of the randomized components, study participants were followed as an observational cohort. The Women's Health Initiative (WHI) Observational Study enrolled 93,676 postmenopausal women aged 50 to 79 years between 1994 and 1998. The Women's Health Study (WHS) is a randomized, clinical trial of low-dose aspirin and vitamin E and enrolled 39,876 female professionals 45 years of age or older between 1992 and 1995. The trial was completed in 2004, and participants are followed as an observational cohort. Details of these cohorts have been described previously (HPFS[13], NHS[14], PHS[15], WHI[16], and WHS[17]). The study was approved by the Human Research Committee at the Brigham and Women's Hospital, Boston, MA, and participants provided informed consent.

We included 493 incident pancreatic adenocarcinoma cases diagnosed through 2008 with available plasma and no prior history of cancer, except non-melanoma skin cancer. Incident cases of pancreatic cancer were identified by self-report or during follow-up of a participant's death. Deaths were ascertained from next-of-kin or the U.S. postal service and by searching the National Death Index; this method has been shown to capture>98% of deaths.[18]

Diagnoses were confirmed by review of medical records, death certificates and/or tumor registry data by study physicians blinded to exposure data. Patients with non-adenocarcinoma histology or unclear survival time were excluded.

Individual characteristics and habits were obtained from baseline questionnaires at study enrollment in PHS, WHI, and WHS and from questionnaires preceding the date of blood draw in HPFS and NHS. In all cohorts, data were available for age at blood draw, sex, race/ethnicity, weight, height, smoking status, and history of diabetes. Date of diagnosis and pancreatic cancer stage at diagnosis were obtained from medical record review. Cancer stage was classified as: local disease amenable to surgical resection; locally advanced disease with extrapancreatic extension rendering it unresectable, but without distant metastases; distant metastatic disease; or unknown.

Plasma Samples

Blood samples were collected from 18,225 men in HPFS from 1993 to 1995, 32,826 women in NHS from 1989 to 1990, 14,916 men in PHS from 1982 to 1984, 93,676 women in WHI-OS from 1994 to 1998, and 28,345 women in WHS from 1992 to 1995. All blood samples were continuously stored in well-monitored freezers. Details on blood draw procedures, transportation, and storage of plasma samples in these cohorts have been described previously.[19] As described previously, plasma levels of 25(OH)D were assayed in the laboratory of Dr. Nader Rifai (Children's Hospital, Boston, MA), using the 25-Hydroxyvitamin D Enzyme Immunoassay Kit from Immunodiagnostic Systems, as per manufacturer's instructions.[9] The mean intraassay coefficients of variance were ≤9% for blinded, replicate, quality control samples.[9]

SNP Selection and Genotyping

We selected 36 tagging SNPs in the *VDR* gene +/- 20 kb by using the tagger algorithm implemented in Haploview, with a cutoff of $r^2 = 0.8$ and a MAF ≥5% in Whites from the HapMap Project database, forcing in one previously reported SNP related to prostate cancer risk (rs11574143).[20] From 397 of the pancreatic cancer cases, DNA was extracted from archived buffy coat samples with QIAGEN QIAmp and whole genome amplified with GE Healthcare Genomiphi. All genotyping was carried out at the Partners HealthCare Center for Personalized Genetic Medicine using a custom-designed Illumina Golden Gate genotyping assay. Three tagging SNPs were not supported by the Golden Gate platform so could not be genotyped. Replicate samples included for quality control (N=44 sample groups) had mean genotype concordance of 97.7% across the 33 SNPs. No SNPs deviated from Hardy–Weinberg Equilibrium at $P<0.01$.

Statistical Analysis

The association of plasma 25(OH)D with overall survival was examined using Cox proportional hazards regression to calculate hazard ratios (HR) and 95% confidence intervals (CI). Overall survival time was calculated from the date of cancer diagnosis until the date of death or last follow-up if a participant was still alive. Although optimal levels of 25(OH)D have not been definitively determined, plasma 25(OH)D <20 ng/ml has been defined as "insufficiency," 20 to<30 ng/ml as "relative insufficiency," and ≥30 ng/ml as "sufficient".[21,22] Therefore, we investigated patient survival by these categories of plasma 25(OH)D. Two-sided tests for trend were calculated by entering log-transformed 25(OH)D as a continuous variable into Cox regression models, given that 25(OH)D was not normally distributed. In multivariable models, we adjusted for potential confounders, including age at diagnosis, cohort, race/ethnicity, smoking status, year of diagnosis, cancer stage, and time between

blood collection and cancer diagnosis. Subsequently, we adjusted for body-mass index (BMI) and history of diabetes, prognostic factors identified in prior studies of these populations.[23,24]

To compare more extreme levels of 25(OH)D in secondary analyses, we assessed the association between cohort-specific quintiles of 25(OH)D and survival. We also examined HRs for each cohort individually and computed a summary HR using the DerSimonian and Laird random effects model.[25] Heterogeneity was tested using Cochran's Q statistic.[26] The proportionality of hazards assumption was satisfied by evaluating a time-dependent variable, which was the cross-product of log-transformed 25(OH)D and time (P=0.58).

We estimated median survival time for subjects in each category adjusted for covariates using direct adjusted survival estimation.[27,28] This method uses Cox proportional hazards regression to estimate probabilities of survival at each follow-up time point for each individual and averages them to obtain an overall survival estimate. We assessed statistical interaction by BMI, tobacco use, cancer stage, and time between blood collection and cancer diagnosis by entering main effect terms and a cross-product term of log-transformed 25(OH)D and stratification variable into the model, evaluating likelihood ratio tests.

Pancreatic cancer and stromal cells express vitamin D receptors (*VDR*), which translocate to the nucleus and bind vitamin D response elements to regulate gene expression and mediate the activity of vitamin D. Thus, we examined the association of tagging SNPs in *VDR* with survival by including each three-level genotype as a continuous variable (additive model) in multivariate-adjusted Cox regression models. SNPs were considered statistically significant if P<0.002 (0.05/33 genotyped variants). We subsequently examined the joint association of

25(OH)D and *VDR* genotype in Cox regression models. All analyses were performed with SAS 9.3 statistical package. All *P*-values were two-sided.

RESULTS

Baseline characteristics of patients with pancreatic cancer by category of prediagnostic 25(OH)D and by cohort are listed in Table 1 and Supplementary Table 1, respectively. Median time between blood draw and pancreatic cancer diagnosis was 6.7 years. Mean prediagnostic 25(OH)D was 24.6 ng/ml, with 33% of patients classified as vitamin D insufficient. Among those with known disease stage, 16.0% had localized disease, 29.6% had locally advanced disease, and 54.3% had metastatic disease. Median survival by cancer stage was 17 months for those with localized disease, 10 months for those with locally advanced disease, and 3 months for those with metastatic disease. At the end of follow-up, 464 patients (94%) were deceased.

Higher plasma 25(OH)D levels were associated with greater survival (*P*-trend=0.01; Table 2). Compared to patients with insufficient levels of vitamin D, the multivariable-adjusted HRs for death were 0.79 (95% CI, 0.48-1.29) for patients with relative insufficiency and 0.66 (95% CI, 0.49-0.90) for patients with sufficient 25(OH)D levels. After adjustment for time between blood collection and diagnosis, BMI and history of diabetes, our results remained largely unchanged (Table 2). In analyses to compare more extreme values of 25(OH)D, a similar inverse relationship was noted between plasma 25(OH)D and patient survival (Table 3). Compared to those in the bottom quintile of 25(OH)D, patients in the top quintile had a multivariable-adjusted HR for death of 0.68 (95% CI, 0.48-0.95). We considered the possible influence of subclinical malignancy on plasma 25(OH)D. After excluding 19 patients diagnosed with pancreatic cancer within one year of blood collection, our results were not materially altered (Supplementary Table 2).

No statistically significant interactions were seen by BMI, tobacco use, cancer stage, or time between blood collection and diagnosis (Table 4). A stronger association of 25(OH)D and patient survival was identified in patients with blood collected within 5 years of diagnosis. Among patients with blood collected ≤5 years before cancer diagnosis, the HR for death was 0.58 (95% CI, 0.35-0.98), comparing those with sufficient levels to those with insufficient levels of 25(OH)D. We evaluated the association of 25(OH)D and patient survival across the cohort study populations (Figure 1), and noted no statistically significant heterogeneity (*P*-heterogeneity=0.78).

We next examined genotypic variation at *VDR* in relation to patient survival. Three tagging SNPs in *VDR* were associated with survival to $P<0.05$ in an additive model of inheritance, but no SNP met the predefined significance threshold after multiple-hypothesis testing correction (Table 5). The most significant SNP was rs7299460, which had a per-allele HR for death of 0.80 (95% CI, 0.68-0.95; $P=0.01$). In an exploratory analysis, patients homozygous recessive at rs7299460 and with sufficient 25(OH)D had the best overall survival, with a HR for death of 0.45 (95% CI, 0.22-0.93) (Supplementary Table 3).

DISCUSSION

In this prospective study of patients with pancreatic cancer from five large U.S. cohorts, patients with sufficient levels of prediagnostic 25(OH)D had a 35% lower hazard for death compared to those who were vitamin D deficient. The association of prediagnostic 25(OH)D with survival remained unchanged after considering multiple potential confounding factors, and appeared strongest when blood was collected within five years before pancreatic cancer diagnosis. Although several polymorphisms at the *VDR* locus were nominally associated with survival, no SNP met the threshold for statistical significance after multiple hypothesis testing correction. In aggregate, these results implicate prediagnostic plasma 25(OH)D as a prognostic factor in patients with pancreatic cancer.

In laboratory models, vitamin D and its analogues have been shown to induce differentiation, promote apoptosis, and inhibit proliferation of pancreatic cancers.[3,5,29-31] Furthermore, recent studies indicate direct effects of ligand binding to VDR in pancreatic cancer cells, but also indirect effects by VDR engagement in stromal cells.[6-8] In a study by Sherman and colleagues, VDR acted as a master transcriptional regulator of pancreatic stellate cells, which reside within the tumor microenvironment.[8] Treatment of pancreatic cancer in genetically-engineered mice with a vitamin D analogue led to remodeling of the stroma, increased concentration of intra-tumoral chemotherapy, and prolonged survival.

Few studies have investigated whether plasma levels of 25(OH)D predict patient survival. Circulating 25(OH)D is a preferred measure of vitamin D status, due to its 2-week half-life and reflection of both vitamin D ingested in the diet and synthesized in the skin.[32] In a retrospective study of 178 patients treated at a tertiary cancer center who underwent vitamin

D measurement as part of clinical care, serum 25(OH)D <20 ng/mL at the initial clinical visit was associated with poor prognosis (HR, 1.99; 95% CI, 1.16-3.43) in patients with advanced disease.[12] In contrast, baseline serum 25(OH)D levels were not associated with overall survival in 256 patients with advanced pancreatic cancer enrolled to a randomized clinical trial of gemcitabine with or without bevacizumab.[11] However, these studies were limited by relatively small sample size, different patient populations, and timing of 25(OH)D measurements after cancer diagnosis. Since inadequate nutrition and limited outdoor activity due to morbidity of recently diagnosed pancreatic cancer can reduce 25(OH)D levels, the measured levels likely do not reflect the long-term exposure to vitamin D prior to the acute illness.

Median survival times were longer by 3-4 months in patients with sufficient circulating 25(OH)D compared to patients with deficient levels. Two multi-agent chemotherapy programs have recently been adopted for treatment of patients with metastatic pancreatic cancer, improving median overall survival by 1.8 months (gemcitabine plus nab-paclitaxel)[20] and 4.3 months (FOLFIRINOX)[33] compared to single-agent gemcitabine. Whether the addition of vitamin D or its analogues to systemic chemotherapy can further improve patient outcomes is under active investigation (e.g., ClinicalTrials.gov identifier, NCT02030860)

Several strengths of this study are notable, including large sample size, inclusion of patients with all stages of disease and from all geographic regions of the United States, and a prospective cohort study design. An important aspect of the prospective cohort design is its ability to fully capture the spectrum of patients with pancreatic cancer in terms of disease aggressiveness and stage of disease, as individuals are enrolled prior to their diagnosis and are not identified at selected tertiary care centers. Notably, survival times and stage

distribution for patients in the five cohorts were highly similar to 121,713 patients included in the National Cancer Data Base, which is thought to capture 76% of pancreatic cancer patient cases diagnosed in the United States each year.[34] Furthermore, the prospective cohort design reduces bias due to reverse causation, since blood samples were collected prior to the nutritional deficiencies and limited performance status that commonly develop at the time of pancreatic cancer diagnosis. Circulating 25(OH)D was measured in a single laboratory as a single batch with low coefficients of variance for blinded, replicate quality control samples, and extensive covariate data from all five cohorts allowed for rigorous control of potential confounding factors and evaluation of effect modification.

Limitations of the current study also require consideration. Among pancreatic cancer cases, treatment programs likely varied, and we could not control for differences in treatment, because this information was not collected in our cohorts. Nevertheless, chemotherapy and radiation have had only a modest impact on patient survival,[2] and treatment programs were unlikely to have varied meaningfully by baseline 25(OH)D measured years before diagnosis. We used overall mortality data in our analyses, as opposed to pancreatic cancer-specific mortality. However, pancreatic cancer is a highly lethal malignancy with overall cure rates of <5%, such that overall mortality is a good surrogate for cancer-specific mortality. Although circulating 25(OH)D was measured at a single time-point, we have previously shown a high correlation of 0.70 for repeated measures of plasma 25(OH)D within individuals over time,[35] suggesting that a single measurement is a reasonable proxy for long-term levels of 25(OH)D. We cannot rule out that our findings may be influenced in part by residual confounding. Nonetheless, we included multiple possible confounding covariates in multivariate models without observing meaningful changes in risk estimates. Finally, our study participants were

predominantly individuals of European descent, and additional studies in other populations are warranted.

Higher prediagnostic plasma levels of 25(OH)D were associated with a statistically significant improvement in survival among patients with pancreatic cancer enrolled in five large prospective cohorts. When considering these findings together with previously reported preclinical data in pancreatic cancer models, agonists of the vitamin D receptor are a potentially attractive therapeutic approach for investigation in this highly lethal malignancy.

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ACKNOWLEDGMENTS

The authors would like to thank the participants and staff of the Health Professionals Follow-up Study, Nurses' Health Study, Physicians' Health Study, Women's Health Initiative, and Women's Health Study for their valuable contributions, as well as the following state cancer registries for their help: AL, AZ, AR, CA, CO, CT, DE, FL, GA, ID, IL, IN, IA, KY, LA, ME, MD, MA, MI, NE, NH, NJ, NY, NC, ND, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA, WY. The authors assume full responsibility for analyses and interpretation of these data.

Financial disclosures: None.

HPFS is supported by NIH grant UM1 CA167552. NHS is supported by NIH grants UM1 CA186107, P01 CA87969, and R01 CA49449. PHS is supported by NIH grants CA 34944, CA 40360, HL 26490, and HL 34595. The WHI program is funded by the NIH through contracts N01WH22110, 24152, 32100–2, 32105–6, 32108–9, 32111–13, 32115, 32118–32119, 32122, 42107–26, 42129–32, and 44221.

Additional support from NCI R35 CA197735 to SO; from the Robert T. and Judith B. Hale Fund for Pancreatic Cancer, Perry S. Levy Fund for Gastrointestinal Cancer Research, Pappas Family Research Fund for Pancreatic Cancer, NIH R01 CA124908, and NIH P50 CA127003 to CSF; and from Department of Defense CA130288, Lustgarten Foundation, the Noble Effort Fund, and Promises for Purple to BMW.

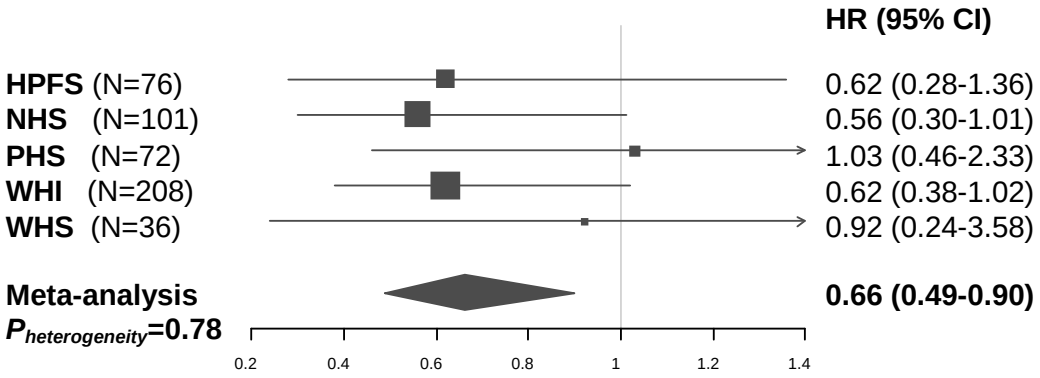

Figure 1. Forest plot and meta-analysis of hazard ratios for death among patients with pancreatic cancer comparing those with sufficient levels of 25(OH)D to those with insufficient levels in HPFS, NHS, PHS, WHI, and WHS

The solid squares and horizontal lines correspond to the cohort-specific multivariable-adjusted hazard ratios and 95% confidence intervals. The area of the solid square reflects the cohort-specific weight (inverse of the variance). The diamond represents the meta-analysis multivariable-adjusted hazard ratio and 95% confidence interval. The vertical line indicates an odds ratio of 1.0. Hazard ratios adjusted for age at diagnosis, race/ethnicity (White, black, other, unknown), smoking status (never, past, current, or missing), month of blood draw (2-month intervals), stage at diagnosis (localized, locally advanced, metastatic, unknown), and year of diagnosis (1984-2000, 2001-2008)

Abbreviations: CI, confidence interval; HPFS, Health Professionals Follow-up Study; HR, hazard ratio; NHS, Nurses' Health Study; PHS, Physicians' Health Study; WHI, Women's Health Initiative; WHS, Women's Health Study

Table 1. Baseline characteristics of pancreatic cancer cases by prediagnostic plasma 25-hydroxyvitamin D

Characteristic*	Plasma 25(OH)D		
	Insufficient	Relative insufficiency	Sufficient
25(OH)D levels			
ng/ml	<20	20 to <30	≥30
nmol/L	<50	50 to <75	≥75
No. of cases	165	212	116
Age at blood draw, years	63.9 (7.7)	64.0 (8.8)	61.6 (8.8)
Age at diagnosis, years	71.1 (8.1)	71.7 (8.1)	70.2 (8.7)
Female sex	126 (76.4)	152 (71.7)	67 (57.8)
Race/ethnicity			
White	142 (86.1)	188 (88.7)	106 (91.4)
Black	14 (8.5)	4 (1.9)	0 (0)
Other	5 (3.0)	8 (3.8)	2 (1.7)
Missing	4 (2.4)	12 (5.7)	8 (6.9)
Body mass index, kg/m^2	27.2 (5.0)	26.7 (5.5)	24.9 (3.5)
Physical activity, MET-hr/week	11.5 (14.7)	16.8 (20.6)	26.9 (29.4)
Diabetes at blood draw	10 (6.1)	12 (5.7)	7 (6.0)
Tobacco use			
Never	65 (39.4)	96 (45.3)	43 (37.1)
Past	69 (41.8)	90 (42.5)	62 (53.4)
Current	29 (17.6)	25 (11.8)	11 (9.5)
Missing	2 (1.2)	1 (0.5)	0 (0)
Multivitamin use	60 (36.4)	104 (49.1)	53 (45.7)
Alcohol (≥1 drink/day)	46 (27.9)	45 (21.2)	32 (27.6)
Diagnosis period			
1984-2000	94 (57.0)	111 (52.4)	65 (56.0)
2001-2008	71 (43.0)	101 (47.6)	51 (44.0)
Cancer stage			
Localized	21 (12.7)	25 (11.8)	19 (16.4)
Locally advanced	48 (29.1)	49 (23.1)	23 (19.8)
Metastatic	68 (41.2)	97 (45.8)	55 (47.4)
Unknown	28 (17.0)	41 (19.3)	19 (16.4)

* Continuous variables reported as mean (standard deviation) and categorical variables reported as number (percent), unless otherwise noted.

Abbreviations: 25(OH)D, 25-hydroxyvitamin D

Table 2. Hazard ratios for death among pancreatic cancer cases by plasma 25-hydroxyvitamin D

Model	Plasma 25(OH)D			P_{trend} [±]
	Insufficient	Relative insufficiency	Sufficient	
Range				
ng/ml	<20	20 to <30	≥30	
nmol/L	<50	50 to <75	≥75	
Median survival, months	5	7	8	
Person-months	1712	2843	1348	
Cases / Deaths	165/154	212/199	116/111	
HR (95% CI) [*]	1.0	0.79 (0.48, 1.29)	0.66 (0.49, 0.90)	0.01
HR (95% CI) [**]	1.0	0.83 (0.52, 1.32)	0.67 (0.49, 0.92)	0.02
HR (95% CI) [†]	1.0	0.77 (0.46, 1.29)	0.62 (0.44, 0.86)	0.02

[*] Hazard ratio (95% confidence interval) from Cox regression models adjusted for age at diagnosis, cohort (also adjusts for sex), race/ethnicity (White, black, other, unknown), smoking status (never, past, current, or missing), month of blood draw (2-month intervals), stage at diagnosis (localized, locally advanced, metastatic, unknown), and year of diagnosis (1984-2000, 2001-2008)

[**] Model further adjusted for time between blood collection and cancer diagnosis (0-<5, 5-<10, ≥10 years)

[†] Model further adjusted for body-mass index (continuous) and history of diabetes (yes, no)

[±] Test for trend calculated by entering log-transformed 25(OH)D as a continuous variable in Cox regression models

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HR, hazard ratio; CI, confidence interval

Table 3. Hazard ratios for death among pancreatic cancer cases by quintile of plasma 25-hydroxyvitamin D

Model	Quintiles of Plasma 25(OH)D[*]				
	1	2	3	4	5
Median 25(OH)D, nmol/L	35.7	48.2	58.3	68.8	88.1
Person-months	1024	1172	1371	1165	1171
Cases / Deaths	97/92	99/91	101/94	99/95	97/92
HR (95% CI) [*]	1.0	0.91 (0.64-1.30)	0.72 (0.51-1.01)	0.74 (0.50-1.09)	0.68 (0.48-0.95)
HR (95% CI) [**]	1.0	0.91 (0.64-1.31)	0.77 (0.54-1.09)	0.76 (0.53-1.09)	0.66 (0.47-0.94)
HR (95% CI) [†]	1.0	0.96 (0.67-1.39)	0.74 (0.52-1.06)	0.76 (0.51-1.13)	0.66 (0.46-0.95)

[*] Cohort-specific quintile ranges of plasma 25(OH)D: HPFS (<46.9, 46.9 to <62.0, 62.0 to <72.3, 72.3 to <83.9, ≥83.9), NHS (<44.3, 44.3 to <54.1, 54.1 to <64.7, 64.7 to <82.9, ≥82.9), PHS (<45.0, 45.0 to <58.3, 58.3 to <68.6, 68.6 to <86.9, ≥86.9), WHI (<38.2, 38.2 to <49.4, 49.4 to <59.7, 59.7 to <68.9, ≥68.9), and WHS (<44.9, 44.9 to <56.4, 56.4 to <61.8, 61.8 to <79.7, ≥79.7).

[*] Hazard ratio (95% confidence interval) from Cox regression models adjusted for age at diagnosis, cohort (also adjusts for sex), race/ethnicity (White, black, other, unknown), smoking status (never, past, current, or missing), month of blood draw (2-month intervals), stage at diagnosis (localized, locally advanced, metastatic, unknown), and year of diagnosis (1984-2000, 2001-2008)

[**] Model further adjusted for time between blood collection and cancer diagnosis (0-<5, 5-<10, ≥10 years)

[†] Model further adjusted for body-mass index (continuous) and history of diabetes (yes, no)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; HPFS, Health Professionals Follow-up Study; HR, hazard ratio; NHS, Nurses' Health Study; PHS, Physicians' Health Study; WHI, Women's Health Initiative; WHS, Women's Health Study

Table 4. Hazard ratios for death among pancreatic cancer cases by plasma 25-hydroxyvitamin D stratified by covariates

Stratification covariate	No. of cases	Hazard ratio (95% CI)* Plasma 25(OH)D			*P*-interaction
		Insufficient	Relative insufficiency	Sufficient	
BMI, kg/m2					0.44
< 25.0	216	1.0	0.92 (0.63-1.35)	0.86 (0.57-1.30)	
25.0-29.9	182	1.0	0.67 (0.46-0.97)	0.70 (0.44-1.12)	
≥ 30.0	95	1.0	0.64 (0.35-1.17)	0.37 (0.15-0.93)	
Tobacco use					0.60
Never	204	1.0	0.87 (0.60-1.25)	0.64 (0.41-1.01)	
Past	221	1.0	0.68 (0.48-0.96)	0.68 (0.46-1.00)	
Current	65	1.0	1.12 (0.47-2.72)	1.17 (0.41-3.32)	
Cancer stage					0.13
Localized	65	1.0	0.51 (0.21-1.24)	1.07 (0.39-2.96)	
Locally advanced	120	1.0	0.91 (0.53-1.56)	0.67 (0.35-1.27)	
Metastatic	220	1.0	0.91 (0.65-1.28)	0.63 (0.41-0.96)	
Time blood draw to diagnosis, years					0.20
≤ 5	165	1.0	0.64 (0.43-0.95)	0.58 (0.35-0.98)	
5- 10	183	1.0	1.03 (0.70-1.50)	0.75 (0.47-1.19)	
> 10	145	1.0	0.88 (0.54-1.44)	1.02 (0.60-1.75)	

* Adjusted for age at diagnosis, cohort (also adjusts for sex), race/ethnicity (White, black, other, unknown), smoking status (never, past, current, or missing), month of blood draw (2-month intervals), stage at diagnosis (localized, locally advanced, metastatic, unknown), and year of diagnosis (1984-2000, 2001-2008)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMI, body-mass index; CI, confidence interval

Table 5. Single-nucleotide polymorphisms in *VDR* ranked by association P-value with overall survival among pancreatic cancer cases

SNP*	No. Cases	Minor Allele Frequency (%)	Additive Model	
			HR (95% CI)	*P*-value
rs7299460	391	0.30	0.80 (0.68-0.95)	0.01
rs11568820	388	0.22	0.79 (0.65-0.96)	0.02
rs4334089	388	0.23	0.81 (0.68-0.98)	0.03
rs886441	392	0.18	0.82 (0.67-0.99)	0.04
rs2853564	396	0.38	1.16 (0.99-1.35)	0.07
rs2239179	392	0.44	1.14 (0.99-1.33)	0.08
rs2239182	392	0.47	0.89 (0.77-1.03)	0.12
rs7295021	395	0.20	0.86 (0.70-1.05)	0.13
rs11168293	392	0.34	1.13 (0.96-1.32)	0.15
rs2239186	395	0.20	0.89 (0.74-1.06)	0.20
rs7132324	391	0.35	1.10 (0.94-1.29)	0.24
rs7963776	388	0.46	0.91 (0.79-1.06)	0.24
rs2254210	396	0.39	1.09 (0.94-1.26)	0.28
rs2189480	390	0.34	0.92 (0.79-1.08)	0.30
rs2283342	392	0.15	0.90 (0.72-1.11)	0.31
rs4760648	391	0.43	0.92 (0.78-1.08)	0.32
rs10875693	389	0.33	1.08 (0.92-1.27)	0.35
rs2238136	393	0.27	0.92 (0.78-1.09)	0.35
rs11574077	391	0.05	1.18 (0.83-1.66)	0.36
rs1859281	395	0.07	1.13 (0.83-1.53)	0.44
rs4237855	390	0.48	1.05 (0.92-1.19)	0.48
rs1544410	359	0.37	1.05 (0.90-1.23)	0.53
rs7310552	393	0.40	1.04 (0.88-1.22)	0.64
rs1540339	333	0.39	0.97 (0.81-1.15)	0.70
rs10747524	329	0.49	0.97 (0.81-1.16)	0.71
rs2107301	394	0.28	0.97 (0.83-1.14)	0.71
rs11168275	392	0.24	0.97 (0.81-1.16)	0.73
rs4328262	388	0.42	0.98 (0.84-1.15)	0.82
rs2544037	394	0.42	1.02 (0.87-1.18)	0.84
rs11574032	297	0.09	1.02 (0.75-1.40)	0.89
rs12721364	391	0.14	1.01 (0.81-1.26)	0.93
rs11574143	391	0.11	1.01 (0.80-1.27)	0.96
rs3819545	393	0.39	1.00 (0.86-1.17)	0.96

* Effect of each minor allele of the single-nucleotide polymorphism on survival in Cox regression models adjusted for age at diagnosis (years, continuous), cohort (also adjusts for sex), race/ethnicity (White, black, other, unknown), smoking status (never, past, current, or missing), stage at diagnosis (localized, locally advanced, metastatic, unknown), and year of diagnosis (1984-2000, 2001-2008)

Abbreviations: CI, confidence interval; HR, hazard ratio; SNP, single nucleotide polymorphism; VDR, vitamin D receptor

Supplementary Table 1. Baseline characteristics of pancreatic cancer cases by cohort

Characteristics	HPFS	NHS	PHS	WHI	WHS	Total
No. of subjects	76	101	72	208	36	493
Age at blood draw, years	65.6 (7.8)	60.5 (6.7)	56.9 (8.6)	67.0 (7.3)	58.9 (8.9)	63.4 (8.5)
Age at diagnosis, years	72.7 (8.4)	70.7 (7.0)	72.0 (9.4)	71.9 (7.5)	63.1 (9.1)	71.2 (8.3)
Plasma 25(OH)D, nmol/L	68.5 (24.3)	65.4 (25.7)	65.8 (22.1)	55.2 (19.6)	60.4 (19.5)	61.3 (22.7)
Female sex	0 (0)	101 (100)	0 (0)	208 (100)	36 (100)	345 (70.0)
Race/ethnicity						
White	71 (93.4)	100 (99.0)	53 (73.6)	178 (85.6)	34 (94.4)	436 (88.4)
Black	1 (1.3)	1 (1.0)	1 (1.4)	14 (6.7)	1 (2.8)	18 (3.7)
Other	1 (1.3)	0 (0)	0 (0)	14 (6.7)	0 (0)	15 (3.0)
Missing	3 (3.9)	0 (0)	18 (25.0)	2 (1.0)	1 (2.8)	24 (4.9)
Body mass index, kg/m^2	25.7 (3.3)	25.6 (5.0)	25.7 (2.9)	27.4 (5.9)	26.4 (5.3)	26.4 (5.0)
Physical activity, MET-hr/week	36.9 (38.5)	15.4 (17.8)	12.0 (11.2)	13.0 (13.2)	17.8 (21.8)	17.4 (22.1)
History of diabetes	3 (3.9)	5 (5.0)	3 (4.2)	15 (7.2)	3 (8.3)	29 (5.9)
Tobacco use						
Never	26 (34.2)	40 (39.6)	29 (40.3)	96 (46.2)	13 (36.1)	204 (41.4)
Past	42 (55.3)	41 (40.6)	30 (41.7)	92 (44.2)	16 (44.4)	221 (44.8)
Current	8 (10.5)	20 (19.8)	13 (18.1)	17 (8.2)	7 (19.4)	65 (13.2)
Missing	0 (0)	0 (0)	0 (0)	3 (1.4)	0 (0)	3 (0.6)
Multivitamin use	41 (53.9)	46 (45.5)	21 (29.2)	99 (47.6)	10 (27.8)	217 (44.0)
Alcohol (≥1 drink/day)	32 (42.1)	24 (23.8)	24 (33.3)	36 (17.3)	7 (19.4)	123 (24.9)
Median time blood draw to diagnosis, years	6.5	10.6	16.6	5.2	4.1	6.7
Diagnosis period						
1984-2000	39 (51.3)	52 (51.5)	45 (62.5)	103 (49.5)	31 (86.1)	270 (54.8)
2001-2008	37 (48.7)	49 (48.5)	27 (37.5)	105 (50.5)	5 (13.9)	223 (45.2)
Cancer stage						
Localized	11 (14.5)	19 (18.8)	14 (19.4)	13 (6.3)	8 (22.2)	65 (13.2)
Locally advanced	11 (14.5)	12 (11.9)	14 (19.4)	77 (37.0)	6 (16.7)	120 (24.3)
Metastatic	33 (43.4)	44 (43.6)	32 (44.4)	91 (43.8)	20 (55.6)	220 (44.6)
Unknown	21 (27.6)	26 (25.7)	12 (16.7)	27 (13.0)	2 (5.6)	88 (17.8)
Median survival time, mo.						
All patients	5	5	6.5	8	5	6
By stage						
Localized	14	21	14.5	25.0	11.5	17
Locally advanced	10	9	8.5	12.0	10.5	10
Metastatic	4	3	3.5	4.0	4.5	3
Unknown	4	6	7.0	5.0	3.0	5

* Continuous variables reported as mean (standard deviation) and categorical variables reported as number (percent), unless otherwise noted.

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HPFS, Health Professionals Follow-up Study; NHS, Nurses' Health Study; PHS, Physicians' Health Study; WHI, Women's Health Initiative; WHS, Women's Health Study

Supplementary Table 2. Hazard ratios for death among pancreatic cancer cases by plasma 25-hydroxyvitamin D excluding patients diagnosed within 1 year of blood draw

Model	Plasma 25(OH)D			P_{trend} [±]
	Insufficient	Relative insufficiency	Sufficient	
Range				
ng/ml	<20	20 to <30	≥30	
nmol/L	<50	50 to <75	≥75	
Median survival, months	5	7	8	
Person-months	1662	2541	1325	
Cases / Deaths	159/148	201/189	113/108	
HR (95% CI) [*]	1.0	0.84 (0.53, 1.33)	0.62 (0.45, 0.85)	0.02
HR (95% CI) [**]	1.0	0.87 (0.55, 1.36)	0.63 (0.45, 0.87)	0.03
HR (95% CI) [†]	1.0	0.75 (0.41, 1.36)	0.59 (0.41, 0.84)	0.04

[*] Hazard ratio (95% confidence interval) from Cox regression models adjusted for age at diagnosis, cohort (also adjusts for sex), race/ethnicity (White, black, other, unknown), smoking status (never, past, current, or missing), month of blood draw (2-month intervals), stage at diagnosis (localized, locally advanced, metastatic, unknown), and year of diagnosis (1984-2000, 2001-2008)

[**] Model further adjusted for time between blood collection and cancer diagnosis (0-<5, 5-<10, ≥10 years)

[†] Model further adjusted for body-mass index (continuous) and history of diabetes (yes, no)

[±] Test for trend calculated by entering log-transformed 25(OH)D as a continuous variable in Cox regression models

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HR, hazard ratio; CI, confidence interval

Supplementary Table 3. Hazard ratios (95% CI) for death among pancreatic cancer cases by plasma 25-hydroxyvitamin D and stratified by rs7299460 genotypes in *VDR

SNP	Genotype	No. (%)	Plasma 25(OH)D		
			Insufficient	Relative insufficiency	Sufficient
rs7299460	GG	194	1.0	0.97 (0.69-1.38)	0.78 (0.51-1.18)
	AG	162	0.84 (0.57-1.25)	0.67 (0.46-0.98)	0.85 (0.56-1.30)
	AA	35	0.71 (0.31-1.59)	0.62 (0.33-1.16)	0.45 (0.22-0.93)

* Adjusted for age at diagnosis (years, continuous), cohort (also adjusts for sex), race/ethnicity (White, black, other, unknown), smoking status (never, past, current, or missing), stage at diagnosis (localized, locally advanced, metastatic, unknown), and year of diagnosis (1984-2000, 2001-2008).

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; SNP, single nucleotide polymorphism; VDR, vitamin D receptor

PHS 398/2590 OTHER SUPPORT

Brian M. Wolpin, MD, MPH

ACTIVE

W81XWH-14-1-0146 (Wolpin) 09/30/14 – 09/29/16 1.2 cal. mo.
U.S. Department of Defense $240,000
Comprehensive Evaluation of Altered Systemic Metabolism and Pancreatic Cancer Risk
This grant leverages a non-targeted metabolomics platform to comprehensively explore how thousands of small molecule metabolites may promote the initiation and early growth of pancreatic tumors.
Overlap: None

(Fuchs) 01/01/15-12/31/17 1.2 cal. mo.
Lustgarten Foundation $ 300,232
Obesity-driven PDAC: a comprehensive study to define mechanisms and new targets for prevention and therapy
We propose to explore putative mechanisms by which obesity/energy excess contribute to PDAC risk and outcome. Several hypotheses have been proposed to explain the influence of obesity/energy excess on PDAC risk and outcome, including elevations in insulin/IGF levels and associated signaling pathways, alterations in leptin/adipokines, and systemic inflammation and cytokine secretion, thereby promoting cell proliferation. Results from these efforts will identify new methods of chemoprevention and new targets for therapy in PDAC patients.
Overlap: None

5UM1CA186107-02 (Sub-BWH/Stampfer) 07/22/14-06/30/19 0.6 cal.mo.
NIH/NCI $ 30,754
Long Term Multidisciplinary Study of Cancer in Women: The Nurses Health Study
This UM1 application is to support the infrastructure of our Cancer Epidemiology Cohort, the Nurses' Health Study (NHS). The NHS began in 1976 with 121,700 women, aged 30-55 years, and has accumulated data from 19 biennial questionnaires over 37 years; the follow-up remains about 90%.
Overlap: None

Investigator Grant Program Award (Wolpin) 08/01/15 - 07/31/16 2.4 cal. mo.
Lustgarten Foundation $379,407
Clinical Science Coalition
We propose two overlapping pilot studies evaluating primary tumors and metastatic lesions for: (1) vitamin D receptor (VDR) activation and modulation of the tumor stroma, and (2) somatic mutations and copy number alterations, to include in genes governing DNA repair pathways (e.g. mutations in BRCA1, BRCA2, PALB2, and Fanconi anemia genes). These two pilot studies will enable rapid design of clinic trials based upon preclinical discoveries related to VDR stromal reprogramming and genomic alterations in pancreatic tumors.
Overlap: None

1U01CA187508-01A1 (Sub-BU/Palmer) 09/08/15-08/31/19 0.96 cal. mo.
NIH/NCI $168,300
A prospective investigation of the oral microbiome and pancreatic cancer
1.To perform a nested case-control study involving prediagnostic oral DNA samples using 125 incident pancreatic cancer cases and 450 matched controls from the SCCS and BWHS.
2.To evaluate racial differences in the oral microbiome using 165 AA and 165 EA controls (from the SCCS only), and to identify any racial differences that may explain the increased incidence of pancreatic cancer among AAs
3.To determine the association between recognized, modifiable risk factors for pancreatic cancer (cigarette smoking, obesity, red meat and processed meat consumption, alcohol consumption, type 2 diabetes) and oral microbiome attributes, and evaluate whether these exposures are associated with high-risk oral microbiome profiles.
Overlap: None

1UH2CA191284-01 (Sub- HSPH/Kraft) 09/01/15-03/31/17 0.96 cal. mo.
NIH/NCI $132,279
Leveraging GxE interaction to understand pancreatic cancer and altered metabolism
1. Conduct candidate and genome-wide association scans for SNPs associated with pancreatic cancer, leveraging potential interactions with known risk factors individually and in aggregate.
2. Conduct genome wide association scans for SNPs associated with 83 circulating metabolites, leveraging potential interactions with known risk factors individually and in aggregate.
3. Conduct analyses to assess whether the effects of genetic and environmental risk factors are mediated through alterations in metabolic profiles and whether genetic loci that alter metabolic profiles are associated with pancreatic cancer risk.
Overlap: None

PENDING

U01 07/01/16-06/30/21 3.0 cal. mo.
NIH/NCI $808,350
Circulating biomarker Consortium for Pancreatic Cancer Early Detection
1.) Generate extensively annotated human and murine sample banks to investigate PDAC early detection markers.
2.) Thoroughly investigate promising early detection markers for PDAC to define those ready for near-term clinical use.
3.) Evaluate multivariable models to define discriminatory capacity of currently available predisposition markers and added utility of new circulating markers.
Overlap: None