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TITLE: Linking Cholesterol to Cancer: Circulating 27-Hydroxycholesterol and Breast Cancer Risk by Tumor Subtype and Expression of CYP27A1 and CYP7B1

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14. ABSTRACT

Following intriguing data from experimental models, this study investigates cholesterol metabolite 27-hydroxycholesterol and breast cancer risk in a case-control study nested in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Heidelberg cohort. This study capitalizes on the availability of pre-diagnostic serum samples, as well as tumor tissue samples on a subset of the cases. Grant year 1 was dedicated to study start-up, and included finalization of ethical approvals, selection and characterization of eligible breast cancer cases and matched controls from the EPIC-Heidelberg cohort, and identification and characterization of eligible cases for tissue microarray (TMA) analyses. A total of 530 cases and 1036 controls were selected into the overall investigation. A total of 360 of the cases are included on TMAs for immunohistochemistry analyses. Cases and controls were median age 53 at blood collection, and cases were median age 61 at diagnosis. Serum and TMA laboratory work will be completed in grant year 2.

15. SUBJECT TERMS

Breast cancer, cholesterol metabolism, oxysterols, risk

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1. INTRODUCTION:

Cholesterol metabolite 27-hydroxycholesterol (27HC) is linked to breast cancer in experimental models, however there are no prospective, human data characterizing the association between 27HC and breast cancer risk. The innovative proposed research project will provide the first epidemiologic data on pre-diagnostic circulating 27HC and (1) breast cancer risk, overall and by hormone receptor subtype, including the less well-characterized estrogen receptor (ER)-ß; (2) tumor expression of CYP27A1 and CYP7B1, key enzymes in 27HC metabolism and catabolism; and (3) tumor expression of the liver X receptor (LXR), linked to breast cancer metastases. This project addresses the following "Breast Cancer Research Program Overarching Challenges": (1) Determine why some, but not all, women get breast cancer; (2) Prevent breast cancer (primary prevention); (3) Identify what drives breast cancer growth, determine how to stop it; (4) Identify why some breast cancers become life-threatening metastasis.

2. KEYWORDS:

breast cancer, oxysterols, cholesterol metabolism, 27-hydroxycholesterol, CYP27A1, CYP7B1, liver X receptor, estrogen receptor-alpha, estrogen receptor-beta, nested case-control

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The project has three goals, with defined "major tasks" under each aim.
Specific aims of this proposal are:

1. Evaluate circulating 27HC and breast cancer risk overall, and
   - 1.1 Characterize risk by clinically measured hormone receptor subtype (ERα/PR).
   - 1.2 Investigate 27HC and risk by tumor expression of ERα and ERß .
   - 1.3 Investigate heterogeneity in risk by circulating endogenous sex steroids and use of exogenous hormones (i.e. postmenopausal hormones).
   - 1.4 Evaluate heterogeneity in risk by circulating cholesterol levels (i.e., total, HDL, LDL, triglycerides).
2. Investigate mechanisms linking 27HC breast cancer risk by evaluating circulating 27HC and tumor expression of CYP27A1 and CYP27B1.
3. Exploratory Aim: Investigate 27HC and tumor LXR expression.

What was accomplished under these goals?

Grant year 1 was dedicated to study start up, including study population selection, preparation for biospecimen assays, and preparation for tissue microarray (TMA) analyses. We outline the accomplishments under the major tasks defined in the project statement of work (SOW) to achieve the study aims.

Major Task 1: Study Start-Up, Case and Control Selection and Preparation for Biospecimen Assays
Status: Completed
Completion: August 2015

We received approval to proceed with research activities without further review from the Human Research Protection Office (HRPO) on May 29, 2015 (HRPO Log No. A-18626); Local ethical approval was obtained prior to the grant period (November 13, 2014, University of Heidelberg Medical Faculty Ethical Commission).

Subsequent to receiving ethical clearance, we identified eligible breast cancer cases (n=530) from the European Investigation into Cancer and Nutrition (EPIC) – Heidelberg cohort, verified their eligibility, and selected up to two matched controls for each case. For 506 cases, two matched controls were selected; for the remaining 24 cases, one matched control was identified (n=1036 controls; Complete August 2016). Baseline characteristics of the selected cases and controls are presented in **Table 1**; case characteristics are presented in **Table 2.**

Table 1. Baseline characteristics of 1,566 EPIC cohort participants

Study variables*	**Cases n=530**	**Controls n=1036**
Age at blood collection	53 (35 - 65)	53 (36 - 65)
Age at menarche	13 (9 - 18)	13 (8 - 20)
Age at menopause[1]	50 (45 - 59)	49 (45 - 60)
Age at last full-term pregnancy[2]	34 (25 - 41)	32 (23 - 45)
BMI	24 (18 - 48)	24 (17 - 43)
Fasting Status		
<3 hours	417 (79%)	817(79%)
3+ hours	109 (21%)	212 (20%)
Menopausal status		
Premenopausal	173 (33%)	340 (33%)
Peril/unknown	88 (17%)	172 (17%)
Postmenopausal/Surgical postmenopausal	269 (51%)	524 (51%)
Parity		
Nulliparous	102 (19%)	184 (18%)
1 child	124 (23%)	240 (20%)
2 children	216 (41%)	428 (41%)
3+ children	85 (16%)	181 (17%)
Breastfeeding[2]	337 (79%)	708 (84%)
Oral contraceptive (OC) use		
Never OC user	107 (20%)	199 (19%)
Past OC user	394 (78%)	778 (80%)
Current OC user	19 (12%)	39 (12%)

*median (range) or N (%); 1. Among menopausal women only; 2. Among parous women only,

Table 2. Case characteristics

Age at diagnosis*	61 (40 - 81)
Years between blood donation and diagnosis*	8 (0 - 18)
Hormone receptor status*	
ER+	328
ER-	77
ER+PR+	287
ER-PR-	69
HER2+	63

*median (range) or N; ER/PR status numbers based on data available February 2016

Major Task 2: Biospecimen Assays
Status: Ongoing; 25% complete
Estimated Completion: August 2016

We conducted a pilot study using the Biocrates LifeScience oxysterol platform in October 2015 to test laboratory precision. This pilot was very successful, with coefficients of variation (CVs) of 4% for replicate samples. We additionally tested the within-person stability of 27HC, observing good within-person stability over one year (intraclass correlation coefficient [ICC]: 0.75). To our knowledge, this is the first data on stability of 27HC.

Dr. Johnson identified and prepared serum samples for cases and matched controls for shipment to assay laboratories, with assay completion expected based on the proposed time line (expected completion August 2016: month 18 of grant period).

Major Task 3: Tumor Tissue Microarray (TMA) Analyses
Status: Ongoing; 50% complete
Estimated Completion: August 2016

Tumor tissue samples have been identified and included on TMA slides for evaluation by immunohistochemistry (IHC).

IHC antibodies for CYP27A1 and CYP7B1 have been optimized (complete February 2016). Antibody optimization for LXR and ERβ in progress. The initial antibody selected for ERβ was not able to be optimized with our samples (i.e., was not providing nuclear signal as expected), therefore we are optimizing an alternative antibody that has been used successfully by colleagues at a different institute, using tissue preserved with the same preservation method (formalin-fixed paraffin embedded [FFPE]). We expect TMA analyses to complete by August 31, 2016 (month 18 of grant period), as outlined in the SOW.

Major Tasks 4-6: Data Management, Analysis, and Manuscript Preparation
Status: To be initiated; 0% complete
Estimated Completion: Task 4, August 2017; Task 5, January 2018; Task 6, February 2018

As outlined in the SOW, we will begin work on Major Task 4, Data Management, Analysis, and Manuscript Preparation in grant year 2. Major tasks 5 and 6 will be accomplished in grant year 3.

What opportunities for training and professional development has the project provided?
Mr. Dalin Lu is a doctoral student in Dr. Fortner's group, and will use a component of the data generated from this project for his doctoral thesis. Dr. Lu has been involved in the data analysis in grant year 1 as part of his doctoral training.

How were the results disseminated to communities of interest?
Nothing to report. Dissemination activities will begin in Grant Year 2.

What do you plan to do during the next reporting period to accomplish the goals?
As proposed, all biochemical assays will be completed in the next reporting period (grant year 2). This includes the main analyte of interest, 27HC, as well as sex steroid hormones, and circulating blood lipids on serum samples of 530 cases and 1036 controls (n=1566). Further, all tumor tissue IHC

analyses will be completed in grant year 2 (n=360 cases). The completion of these assays and analyses will mark the successful completion of Major Tasks 2 and 3.

In grant year 2, we will integrate the biochemical and tumor tissue data generated in Major Tasks 2 and 3 into the master database. Further, we will evaluate 27HC and breast cancer risk as outlined in Specific Aim 1, and in accordance with the outlined SOW.

4. IMPACT:

As grant year 1 was dedicated to project start-up, we do not have progress to report under "Impact".

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report.

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

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

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

5. 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
As stated previously, we experienced minor delays in antibody optimization, particularly for the ER-beta antibody. We have obtained an alternative antibody and are currently optimizing this for use in our samples. We expect that this minor delay in antibody optimization will not impact the timeline for completion of the TMA work.

Changes that had a significant impact on expenditures
None to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
None to report.

Significant changes in use or care of human subjects
None to report. HRPO approval was confirmed on May 29, 2015 (HRPO Log No. A-18626)).

Significant changes in use or care of vertebrate animals.
Not applicable/None to report.

Significant changes in use of biohazards and/or select agents
Not applicable/None to report.

6. **PRODUCTS:**

Publications, conference papers, and presentations
None to report.

Journal publications.
None to report.

Books or other non-periodical, one-time publications.
None to report.

Other publications, conference papers, and presentations.
None to report.

Website(s) or other Internet site(s)
None to report.

Technologies or techniques
None to report.

Inventions, patent applications, and/or licenses
None to report.

Other Products
None to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?
The effort contributed by Drs. Fortner, Kaaks, and Johnson remains as proposed (Fortner, 3 calendar months; Kaaks, 0.6 calendar months; Johnson, 1.2 calendar months).

Cases and controls for this study were selected in concert with case and control selection for another study within the EPIC-Heidelberg cohort. Therefore, the data management effort for case and control selection and batching for the analytic laboratories was not charged to the grant in grant year 1. Based on recent experience with complex TMA and laboratory data, we plan to use this small personnel savings from grant year 1 (1.8 person-months) to extend data management support in grant years 2 and 3.

Dalin Lu contributed effort as part of his doctoral program, at no cost to the project.

Name:	*Dalin Lu*
Project Role:	*Graduate Student*
Nearest person month worked:	*3*
Contribution to Project:	*Mr. Lu assisted with preliminary analyses of the pilot study data, and basic characterization of the nested case-control study population as part of his doctoral training.*
Funding Support:	*Faculty and Staff Study/Work Abroad Plan of Wuhan University of Science and Technology (China)*

Name:	*Dr. Helena Schock*
Project Role:	*Postdoctoral researcher*
Nearest person month worked:	1
Contribution to Project:	*Dr. Schock oversaw the selection of the study population and batching of subjects for analytic labs.*
Funding Support:	*DKFZ Division of Cancer Epidemiology department funds*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Dr. Fortner and Dr. Kaaks had changes in other support since the last reported support.

Dr. Fortner and Dr. Kaaks each contribute 5% effort to the following project funded by the World Cancer Research Fund:
Continuous Update Project Mechanisms Validation Study; RFA2015/1436 (PI: Kaaks, Fortner Co-I)
Dates: 11/1/2015 – 5/30/2016
WCRF United Kingdom/International
Total: 50,000 £
The goals of this project are to identify biological mechanisms underpinning the link between body fatness and postmenopausal breast cancer, and systematically review and asses the strength of the evidence underlying one specific mechanism.

Dr. Kaaks additionally contributes 0.20 cal. months effort to the following project:
Deutsches Zentrum für Lungenforschung (DZL)“ to combat widespread lung diseases”,
Sub-project: Translational Lung Research Center Heidelberg (TLRC-H); 82DZL004A4 (PI: Mall, Kaaks (Co-PI)
Dates: 01/2016 – 12/2020
German Federal Ministry of Research and Education (“BMBF”) and Land Baden Württemberg
Total: 464,924 € (DKFZ department Kaaks)
The main objective of this project is a cooperation between German medical universities and partner institutions for coordination of translational research in the field of lung diseases.

Dr. Kaaks additionally contributes 0.20 cal. months effort to the following project funded by NIH –NCI:
Prospective Study of Serum MIS and Gynecologic Risk; 1R01CA163018-01A1
Kaaks (Co-PI)
Dates: 07/2012 – 06/2016
NIH-NCI
Total: 135,172 USD
The aim of the study is to combine the resources from nine existing cohorts to perform a prospective nested case-control study to evaluate the association of serum MIS (a novel biomarker) levels measured before age 46 with the subsequent development of endometrial and epithelial ovarian cancer. It is expected that high pre-diagnostic MIS concentrations will be inversely associated with risk of ovarian and endometrial cancers. Results will improve our understanding of the mechanism underlying the etiologies of endometrial and ovarian cancers.
Overlap: None

Dr. Kaaks additionally contributes 0.60 cal. months effort to the following project funded by NIH –NCI:
Screening and Risk Biomarkers for Ovarian Cancer in EPIC Specimens 1R01CA158119
Kaaks (Co-PI)
Dates: 04/2012 – 03/2017
NIH-NCI
Total: 526,346 USD (DKFZ)
The main objective is to combine standard risk factor data with blood-based (immune-related) biomarkers to develop maximally discriminant models for the prediction of ovarian cancer risk, and to test selected markers for the possible early diagnosis.
Overlap: None

Dr. Johnson: No changes in other support.

What other organizations were involved as partners?
Organization Name: National Center for Tumor Diseases (NCT)
Location of Organization: Germany
Partner's contribution to the project: Collaboration. The NCT manages the tumor tissue resources of the EPIC-Heidelberg cohort and is conducting the tumor tissue IHC for this project.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable.
QUAD CHARTS: Not applicable.

9. APPENDICES: Not applicable