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Award Number: DAMD17-01-1-0290

TITLE: Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk

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REPORT DATE: April 2008

TYPE OF REPORT: Final

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

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| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |             |                         |                            | Form Approved<br>OMB No. 0704-0188                       |                                           |
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| 1. REPORT DATE (DD-MM-YYYY)<br>01/04/08                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |             | 2. REPORT TYPE<br>Final |                            | 3. DATES COVERED (From - To)<br>1 Sep 2001 - 28 MAR 2008 |                                           |
| 4. TITLE AND SUBTITLE<br>Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |             |                         |                            | 5a. CONTRACT NUMBER                                      |                                           |
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| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br><br>Ernest Orlando Lawrence Berkeley National Laboratory<br>Berkeley, CA 94701                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |             |                         |                            | 8. PERFORMING ORGANIZATION REPORT NUMBER                 |                                           |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |             |                         |                            | 10. SPONSOR/MONITOR'S ACRONYM(S)                         |                                           |
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| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br>Approved for Public Release; Distribution Unlimited                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |             |                         |                            |                                                          |                                           |
| 13. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |             |                         |                            |                                                          |                                           |
| 14. ABSTRACT<br>This study investigated the hypothesis that widespread exposure to polycyclic aromatic hydrocarbons (PAHs) increases breast cancer risk. PAHs are products of incomplete burning of organic matter and are present in cigarette smoke, ambient air, drinking water, and diet. PAHs require metabolic transformation to bind to DNA, causing DNA adducts, which can lead to mutations and are thought to be an important pre-cancer marker. In breast tissue, PAHs appear to be metabolized to their cancer-causing form primarily by the cytochrome P450 enzyme CYP1B1. Because the genotoxic impact of PAH depends on their metabolism, we hypothesized that high CYP1B1 enzyme levels result in increased formation of PAH-DNA adducts in breast tissue, leading to increased development of breast cancer. We have investigated molecular mechanisms of the relationship between PAH exposure, CYP1B1 expression and breast cancer risk in a clinic-based case-control study. We collected histologically normal breast tissue from 56 women (43 cases and 13 controls) undergoing surgery and analyzed these specimens for CYP1B1 gene expression, CYP1B1 genotype and PAH-DNA adducts. CYP1B1 transcript levels were slightly lower in controls than cases, but the difference was statistically not significant. We did not detect any difference in aromatic DNA adduct levels of cases and controls, only between smokers and non-smokers. We did not find a correlation between the levels of CYP1B1 expression and DNA adducts. Due to the lack of statistical power these results should be treated with caution. |             |                         |                            |                                                          |                                           |
| 15. SUBJECT TERMS<br>xenobiotic metabolism, CYP1B1, gene expression, genetic polymorphism, DNA adducts                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |             |                         |                            |                                                          |                                           |
| 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                                   |
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**Table of Contents**

**Cover.....1**

**SF 298.....2-3**

**Introduction.....5**

**Body.....5-19**

**Key Research Accomplishments.....20**

**Reportable Outcomes.....20**

**Conclusions.....21**

**References.....21**

**List of Personnel.....23**

## INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment from incomplete combustion of fossil fuels and other industrial sources. The genotoxic impact of PAHs depends on their metabolism to more reactive intermediates. The major pathway by which ingested or inhaled PAHs are metabolized, is the stepwise oxidative activation by the cytochrome P450 isozymes, CYP1A1 and CYP1B1, followed by detoxification by phase II enzymes (Nebert and Gonzales, 1987). The highly reactive intermediate formed by CYP1A1 or CYP1B1 can bind to DNA, the resulting DNA adduct can cause a mutation that, if in a relevant gene, could initiate cancer. These adducts are considered biomarkers of potential cancer risk. Expression of both *CYP1A1* and *CYP1B1* is highly inducible by PAHs and other environmental toxins, such as dioxin (Safe, 1995). *CYP1B1* gene expression analysis captures the convergence of multiple genetic and environmental factors that influence metabolic enzyme levels (Gonzalez and Gelboin, 1994; Whitlock, 1999). PAH-DNA adducts provide the most direct link between exposure to PAHs and cancer and can be used as biomarkers of exposure and effect. CYP1B1 could be a key enzyme in the activation of carcinogens in the breast and therefore play a role in the development of breast cancer: the *CYP1B1* gene is highly expressed in human breast tissue (Larsen et al., 1998; Goth-Goldstein et al., 2003); besides activating lipophilic environmental carcinogens, such as PAHs and aromatic amines, the CYP1B1 enzyme hydroxylates 17  $\beta$ -estradiol at the C-4 position to the potentially carcinogenic 4-hydroxy estradiol (Shimida et al., 1996; Hayes et al., 1996).

Because the genotoxic impact of PAH depends on their metabolism, high CYP1B1 enzyme levels may result in increased formation of PAH-DNA adducts in breast tissue, subsequently leading to development of breast cancer (Lagueux et al., 1999). We have investigated the underlying molecular mechanisms of the relationship between PAH exposure and breast cancer risk. Because the influence of PAH exposure on disease risk would be easier to detect in a highly exposed population, we chose as study site Porto Alegre in the South of Brazil where we suspected that frequent barbecued meat consumption, smoking and limited air pollution controls might lead to higher PAH exposure of women than in the U.S.. Using a clinic-based case-control design, breast tissue was obtained from female patients undergoing either mastectomy or reduction mammoplasty surgery in Porto Alegre, Brazil. PAH exposure and potential confounding factor data were collected for all cases and controls via medical chart review and an interviewer-administered questionnaire. To characterize molecular level interindividual variation in PAH metabolism, *CYP1B1* gene expression and PAH-DNA adducts were measured in the surgically obtained histologically normal breast tissue from women undergoing mastectomy or reduction mammoplasty surgery.

## BODY

### **Task 1: Identify study participants undergoing reduction mammoplasties or mastectomies and collect data and tissue**

Two collaborating surgeons, one specializing in breast diseases, the other in breast reconstruction and reduction mammoplasty, identified candidate patients invited them to participate in the study. To assure that procedures were consistently followed at the recruitment hospitals in Porto Alegre,

Brazil, various forms and protocols (Recruitment Form listing eligibility criteria, Participant Tracking Form, a questionnaire, Medical abstraction form, protocols for collection and processing of breast tissue, blood and urine) were developed (Forms and Protocols submitted as part of the 2004 Annual Report). Using these various protocols two part-time Research Coordinators were trained to oversee day-to-day operations of the study, which included: (1) getting consent from study participants prior to surgery, (2) collecting, processing and storing biologic specimens immediately after surgery, (3) coordinating interviews, (4) reviewing and abstracting information from participants' medical records, and (5) managing data and study records. Informed consent to participate in the study was obtained before surgery. Urine samples were collected before surgery, breast tissue and blood samples were obtained during scheduled surgeries. The collected specimens were processed by the clinical research coordinator and stored in a stabilizing buffer (RNA Later, Ambion) at -20°C until shipment to LBNL.

**Recruitment of Study Participants:** Based on power calculation to test our hypothesis our goal had been to collect during the span of one year normal breast tissue from at least 37 cases undergoing mastectomy and 74 controls undergoing reduction mammoplasty surgery (or alternatively 74 cases and 34 controls) with the following inclusion criteria for all: no prior diagnosis of cancer, usual residence in the state of Rio Grande do Sul, Brazil; additionally for cases: no chemotherapy or radiation therapy prior to mastectomy; additionally for controls: no history of atypical hyperplasia, atypia, or other benign proliferative disease.

Due to reasons not anticipated in the study design and that were beyond our control, enrollment of participants was much slower than anticipated and even though we extended the recruitment time to three years we only recruited 54 cases and 18 controls. The reason for the slow recruitment of cases was a change in standard of care for women undergoing a full mastectomy surgery since we designed the study, in that chemotherapy is now administered before surgery. This conflicted with our eligibility criteria of no prior chemotherapy. Therefore, we only collected tissue from women undergoing partial mastectomy. Recruitment of controls turned out to be even more difficult. Our Brazilian Co-Investigators have given two reasons: (1) the economic situation in Brazil made the often elective surgery less affordable for many women; (2) a change in beauty perception had occurred in recent years making breast enlargement rather than reduction a more frequent choice.

In spite of training the Research Coordinators in the procedure for consenting we found some deficiencies when reviewing all study records: we had to exclude 3 control participants for whom the consent forms could not be located. Further, for one woman it was discovered after specimen collection that she had had prior chemotherapy, and only the CYP1B1 polymorphism results were included. For another 4 participants the date of consent was unclear. So, only the information collected by questionnaire or Medical Record Abstraction was used, but not data obtained from tissue analysis. The consent forms of 8 participants continue to reside with former co-investigator Dr. Christine Erdmann at the University of Michigan and we could not access and include these data. This left 46 cases and 15 controls for descriptive data collection and 43 cases and 13 controls for tissue analysis.

**Characteristics of Study Participants:** Through a questionnaire administered by trained interviewers and through medical record abstraction we collected information regarding

residential, occupational, dietary, environmental exposure, medical histories and known or suspected breast cancer risk factors.

**Table 1.** Characteristics of study population

|                                      | Cases<br>n = 46              | Controls<br>n = 15             |
|--------------------------------------|------------------------------|--------------------------------|
| Age, mean $\pm$ SD<br>(range)        | 53.26 $\pm$ 9.2<br>(37 – 71) | 47.27 $\pm$ 11.29<br>(27 – 70) |
| Past menopause                       | 29 (63%)                     | 7 (47%)                        |
| Education – highest degree           | Of 43                        | Of 13                          |
| High school completed                | 9 (21%)                      | 7 (54%)                        |
| University degree                    | 27 (63%)                     | 4 (31%)                        |
| Family history of breast cancer      | Of 46                        | Of 13                          |
| In 1 <sup>st</sup> degree relative   | 6 (13%)                      | 1 (8%)                         |
| In 2 <sup>nd</sup> degree relative   | 10 (22%)                     | 3 (23%)                        |
| Benign breast disease before surgery | 4 of 44 (9%)                 | 0 of 13                        |
| Age at menarche                      | 12.2                         | 12.2                           |
| Age at 1 <sup>st</sup> child         | 26.2 $\pm$ 5.6 (of 39)       | 24.8 $\pm$ 4.0 (of 12)         |
| # of birth                           | 2.2                          | 2                              |
| Total months of breastfeeding        | 10.0 (0 – 44)                | 8.5 (0 – 30)                   |
| Nulliparous                          | 7 (15 %)                     | 1 (7.7 %)                      |
| Use of HRT                           | 14 (48 %)                    | 4 (57 %)                       |
| Exercise (in hours per week)         | 2.2                          | 2.5                            |

**Table 2:** Potential Exposure to PAHs

|                                                                               | Cases<br>N = 43 | Controls<br>N = 13 |
|-------------------------------------------------------------------------------|-----------------|--------------------|
| Heating/Cooking with wood/open fire                                           | 9 (21 %)        | 4 (31 %)           |
| Smoking – Former smoker                                                       | 6 (14 %)        | 4 F (31 %)         |
| Current smoker                                                                | 9 (21 %)        | 2 C (15 %)         |
| Second Hand Smoke                                                             | 11 (25 % )      | 5 (38 %)           |
| Diet - Portions per week of                                                   |                 |                    |
| Grilled meat                                                                  | 3.3 (0 - 13)    | 2.2 (0 – 8)        |
| Smoked meat                                                                   | 1.7 (0 – 14)    | 0.1 (0 – 1)        |
| Toasted cheese and bread                                                      | 3.7 (0 - 26)    | 0.8 ( 0 – 3)       |
| Total                                                                         | 8.7             | 3.1                |
| Use of coal tar containing shampoo 3 times or more per week for last 10 years | 2               | 1                  |

**Characteristics of breast tumors:**

Tumor Histology: 32 (70%) invasive ductal carcinoma, 7 (15%) DCIS, 7 (15 %) other

Tumor Size: 0.1 – 5.1 cm

Axillary node involvement: for 8 (17 %)

ER status: positive 41, negative 5; PR status: positive 42, negative 4; ER<sup>+</sup> PR<sup>+</sup>: 40 (87 %)

p53 status: positive in 12 of 44 (27 %)

Her2 overexpression: 6 of 44 (14 %)

From the questionnaires we learned that all participants were white and were mostly well educated with about half of participants holding a university degree and frequently working as teachers. As can be seen in Table 1, the control population is somewhat younger (mean 47 versus 53). Alcohol consumption in participants was low with 4 women reporting that they drank 0-1 drink per day and two women who reported that they drank 2 or more drinks per day.

Most of the study participants when asked wanted to be informed about the results of the study and we are preparing a document that can be shared with them.

**Task 2: Characterize the breast tissue samples in respect to *CYP1B1* expression and *CYP1B1* polymorphism****a. Isolate DNA and RNA from breast epithelial cells**

DNA was isolated by the Proteinase K - phenol/chloroform methods from the 57 specimens received. We had originally proposed to isolate the DNA together with RNA in the TRI Reagent procedure, but found that this method yielded only small amounts of low-quality DNA. DNA was quantitated and its purity determined by its 260/280 nm absorption. Samples were aliquoted for later measurements of *CYP1B1* genotype and DNA adducts and stored at -80°C.

Total RNA of the 57 specimens was isolated using TRI Reagent following the manufacturer's protocol (Molecular Research Center, Inc., Cincinnati, OH) and RNA samples stored at -80°C. The quality and quantity of RNA was determined spectrophotometrically.

**b. Reverse transcribe RNA and****c. Measure *CYP1B1* gene expression**

Breast tissue specimens represent a complex mixture of varying cell types and this mixture differs between individuals. We had originally planned to evaluate *CYP1B1* expression levels relative to the  $\beta$ -actin housekeeping gene, but found that  $\beta$ -actin expression varied considerably between breast tissue fractions and therefore could not be used (see Progress Report 2004).

Instead of determining *CYP1B1* expression levels relative to a housekeeping gene, we quantitated the absolute amount of *CYP1B1* transcript found in each specimen using real-time RT-PCR (with the LightCycler instrument) and as quantitation standard (QS) a cloned *CYP1B1* PCR fragment. Each LightCycler run included a dilution series of this standard. The standard and the *CYP1B1* gene transcript were reverse transcribed and amplified together at equal efficiencies to control for each step of the assay. The *CYP1B1* expression level in an unknown sample was determined by

extrapolating from a curve produced from the dilution series of the quantitation standard. To control for variations in specimen quantities, an equal amount of total RNA isolated from each specimen was added to each RT-PCR reaction.

Two possible sources of variation in the quantitative PCR assay were characterized: (1) reproducibility of the QS dilution series and (2) variability in the RNA isolation. When analyzing a dilution series of the QS at five different concentration levels (from 10,000 fg to 1 fg), each in triplicate the variance was less than 1% at each concentration level (see Progress Report 2006). To measure the variability in the isolation procedure, the RNA from one specimen was isolated in triplicate and then analyzed by the RT-PCR assay for CYP1B1. The variance in transcript level was 6.6 %.

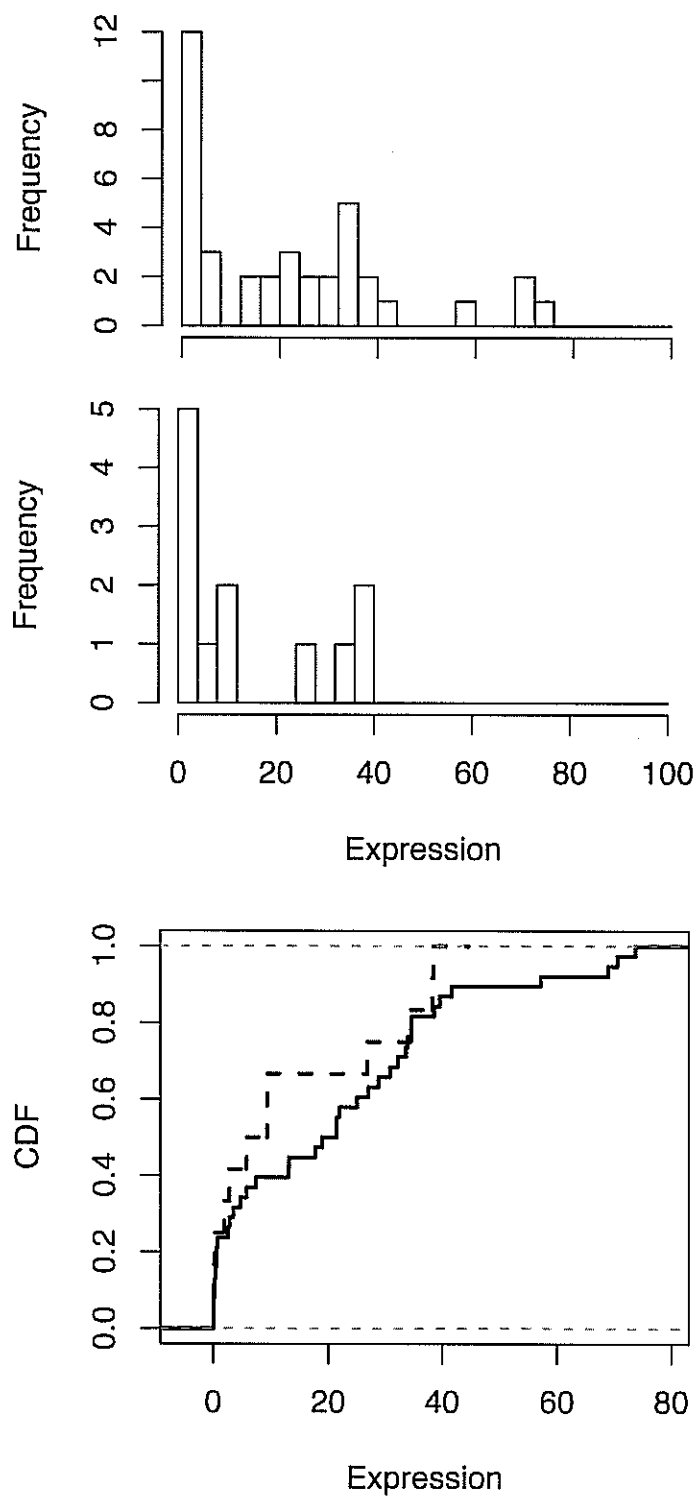
RNA $\textit{Later}$  turned out to be very effective in preserving the RNA in the specimens, so that even specimens from the first shipment, which arrived at room temperature, provided RNA and the quantitation of CYP1B1 transcript was comparable to repeat measurements with additional tissue sent in the second shipment.

Of the 56 breast tissue specimens analyzed for CYP1B1 transcript levels, 6 did not give any results or only unspecific PCR product. The values for the 50 specimens that gave results are summarized in Table 3 and the distribution of expression levels shown in Figure 1.

**Table 3.** *CYP1B1* expression in 50 breast tissue specimens analyzed. Transcript levels are expressed as fg/500ng total RNA used in the reverse transcription.

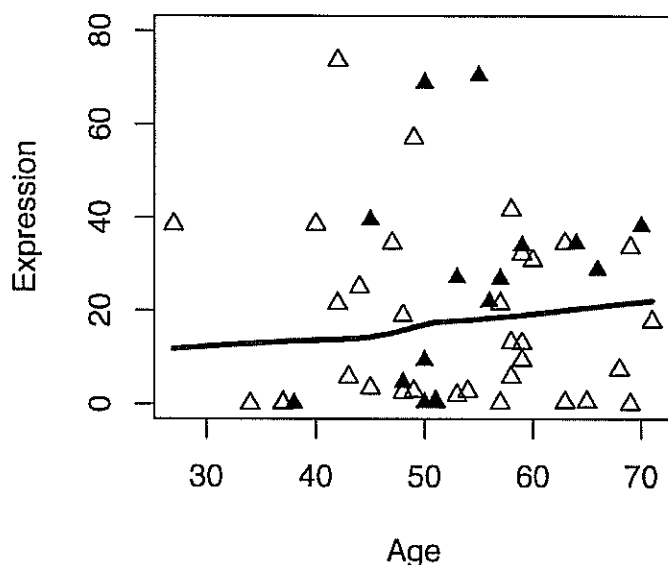
|                       | CYP1B1<br>Mean (SD) | CYP1B1<br>Median | range        |
|-----------------------|---------------------|------------------|--------------|
| Total (n = 50)        | 19.9 (20.1)         | 15.46            | 0.06 – 73.7  |
| Control (n = 12)      | 14.0 (15.7)         | 7.56             | 0.08 – 38.2  |
| Cases (n = 38)        | 21.8 (21.2)         | 20.22            | 0.06 – 73.7  |
| Inv. Duct. Carc. (26) | 16.5 (17.3)         |                  | 0.06 – 68.95 |
| DCIS (n = 6)          | 40.6 (24.5)         |                  | 0.54 – 73.7  |

As we had already observed previously (Goth-Goldstein et al., 2003), expression levels vary over a broad range. We observed a 1000 fold variation. The mean and median transcript level of controls was lower than that of cases, but the difference was statistically not significant (p-value = 0.1823). This might be due to lack of statistical power. Surprisingly cases with DCIS had a higher transcript level than cases with invasive ductal carcinoma (Table 3); but as we tested only six cases with DCIS these results should be treated with caution.



**Figure 1:** CYP1B1 transcript levels displayed as histogram (top panel – cases, middle panel – controls) and as cumulative distribution function (bottom panel; solid line - cases, dotted line - controls)

In contrast to our earlier findings (Goth-Goldstein et al., 2003) we did not observe lower CYP1B1 transcript levels in post menopausal women (Figure 2).



**Figure 2:** *CYP1B1* expression levels displayed as function of age. Open triangles – nonsmokers, solid triangles – current and former smokers

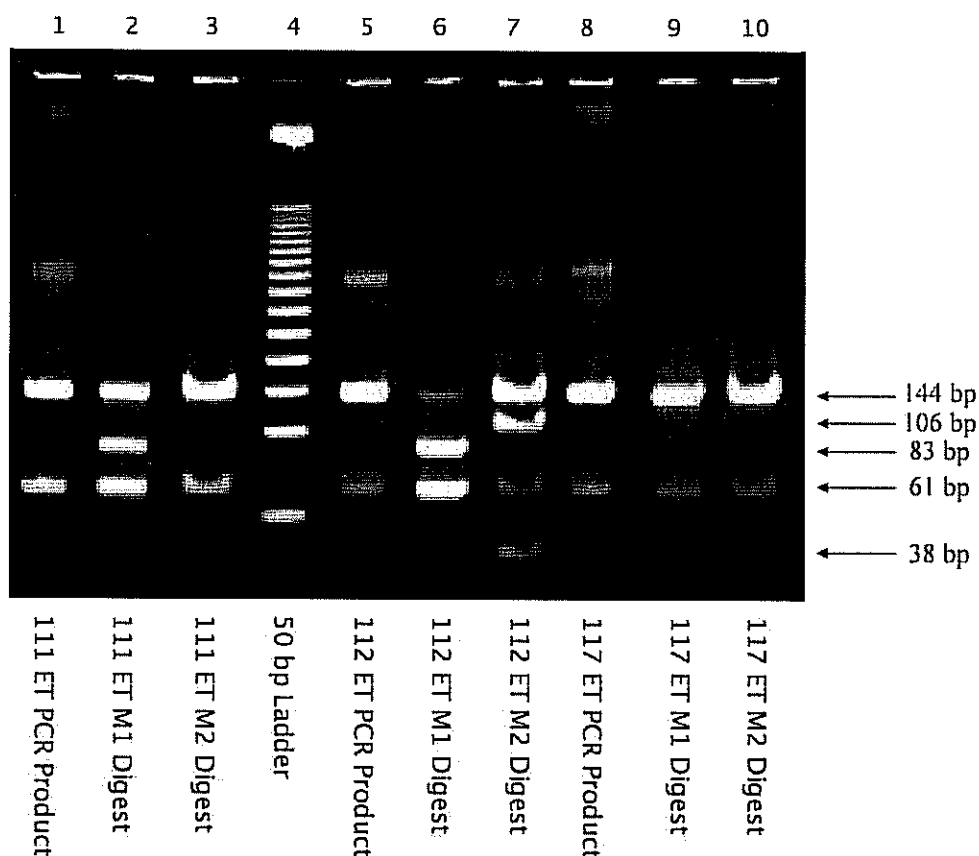
To test whether lymphocytes could be used as a predictor of *CYP1B1* expression levels in breast tissue, blood samples had been collected from participants just before surgery, lymphocytes were separated, stored in a stabilizing buffer and shipped to the US. However, we could isolate RNA only from very few lymphocyte samples and it appears that separation procedure was not properly performed. For the few samples which yielded sufficient RNA, *CYP1B1* expression levels in peripheral lymphocytes did not correlate with levels in breast tissue.

#### d. Perform *CYP1B1* genotype analysis

Beside the overall level of exposure the genetic background of the exposed individual may alter the health consequences of exposure. Genetic variation in the *CYP1B1* gene may play a role in interindividual differences in the metabolism of PAHs and estrogen.

Therefore the *CYP1B1* genotype at two polymorphic sites located in the catalytic side of the enzyme at codon 432 (m1) and at codon 453 (m2) was analyzed by PCR /RFLP. Using the primers described by Bailey et al. (1998, corrections, 1999) a 144 bp product is amplified. This product can be used to detect both the m1 and m2 polymorphisms. The m1 (Val to Leu) polymorphism is detected by digestion with the restriction enzyme Eco571, which produces 83bp and 61bp fragments in the variant. The m2 (Asp to Ser) polymorphism is detected by digestion with Cac8I,

which produces 106bp and 38bp fragments in the variant. The digestion products are separated on a 10% native polyacrylamide gel stained with SYBR Gold (Figure 3).



**Figure 3:** This gel shows the results for 3 specimens. Lane 1, 5, 8 contain the undigested PCR product (144 bp); lane 2, 6 and 9 contain the m1 digestion products (144bp, 83 bp and 61 bp); lane 3, 7, 10 contain the m2 digestion products (144bp, 106 bp and 38 bp). Extraneous bands are visible at approximately 65 bp and 400 bp.

The results from the genotype analysis are summarized in Table 4.

**Table 4.** *CYP1B1* genotype of 56 participants

| CYP1B1 genotype |         | Total | Controls | Cases |
|-----------------|---------|-------|----------|-------|
| m1              | Val/Val | 8     | 2        | 6     |
|                 | Val/Leu | 33    | 10       | 23    |
|                 | Leu/Leu | 15    | 1        | 14    |
| m2              | Asn/Asn | 41    | 13       | 30    |
|                 | Asn/Ser | 15    | 3 (2)    | 13    |
|                 | Ser/Ser | 0     | 0        | 0     |

We calculated the allele frequencies for the two polymorphic sites and compared them to reports of allele frequencies observed in different ethnic groups. The allele frequencies determined in our small sample set resemble those seen in populations of European descent.

**Table 5.** Comparison of *CYP1B1* allele frequency in participants and in different ethnic groups (given as means of several published reports)

| Codon   | Participants | African Descent <sup>1,3</sup> | Asian Descent <sup>2,3</sup> | Europ. Descent <sup>1,2,3</sup> |
|---------|--------------|--------------------------------|------------------------------|---------------------------------|
| 432 Val | 0.437        | 0.703                          | 0.154                        | 0.404                           |
| 432 Leu | 0.563        | 0.297                          | 0.846                        | 0.596                           |
| 453Asn  | 0.866        | 0.975                          | 0.997                        | 0.807                           |
| 453Ser  | 0.134        | 0.025                          | 0.003                        | 0.193                           |

<sup>1</sup> Bailey et al., 1998; <sup>2</sup> Inoue et al., 2000; <sup>3</sup> Mammen et al., 2003.

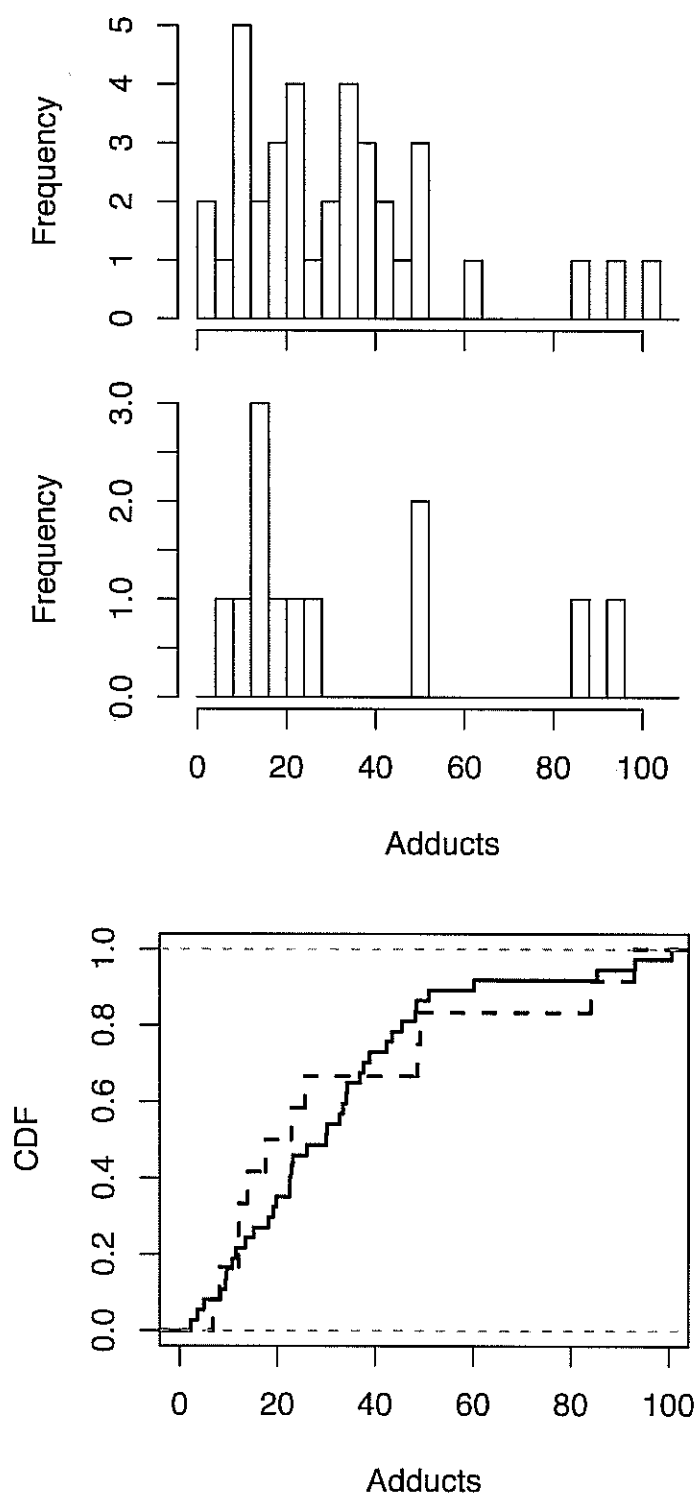
A number of studies have investigated the role of the *CYP1B1* Val432Leu polymorphism in breast cancer risk and results have been inconclusive. The small number of participants included in our study does not allow any conclusions on the relationship of these polymorphisms to breast cancer risk. A recent meta-analysis of all published case control studies found that results depended on ethnic background and that a possible association of the Val genotype with breast cancer was suggested only for Caucasians (Paracchini et al., 2007). This genotype was also identified as a modifier of breast cancer risk in Finnish Caucasian women who smoke and/or who carry the N-acetyl transferase slow acetylator genotype (Sillanpää et al. 2007).

### **Task 3 Measure aromatic DNA adducts by <sup>32</sup>P-postlabeling in breast tissue**

Aliquots of DNA isolated from breast epithelial tissue were shipped to Dr. Donghui Li, a Co-Investigator at the M.D. Anderson Cancer Center, for DNA adduct analysis. Aromatic DNA adduct levels were determined by the nuclease P1-enhanced version of the <sup>32</sup>P-postlabeling assay as described in Li *et al.* (1996), which involves stepwise DNA digestion to nucleosides, conversion to <sup>32</sup>P-labeled deoxyribonucleosides, purification and separation by multidirectional TLC. Adducts were detected and quantitated by image analysis. Adduct levels are expressed as a relative adduct level (RAL) value, which is a ratio of the counts per minute (cpm) of modified nucleotides over the cpm of total nucleotides in the reaction. The detection limit of adducts is 1 per 10<sup>9</sup>. For 6 specimens, not enough DNA was obtained for adduct analysis. But even for the remaining DNA samples problems were encountered. Preserving tissue in *RNA Later*, a supersaturated salt buffer, appears to be a problem for DNA adduct measurements. We presume that carry-over of salts from the *RNA Later* inhibits nuclease digestion of the isolated DNA. We modified the DNA isolation protocol to using a low-salt buffer and reprecipitating the DNA to remove these salts. In 1 specimen no adducts were detected.

**Table 6.** PAH-DNA adducts determined in breast tissue (expressed as RAL x 10<sup>9</sup>)

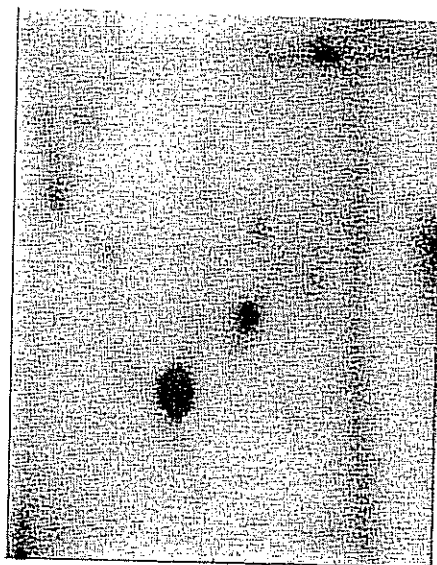
|                   | Mean (SD)   | Median | Range       |
|-------------------|-------------|--------|-------------|
| Total (n = 50)    | 32.3 (24.8) | 25.66  | 3.6 – 100.6 |
| Controls (n = 13) | 32.8 (29.6) | 20.24  | 0.5 – 92.9  |
| Cases (n = 37)    | 32.2 (23.5) | 30.07  | 3.6 – 100.6 |



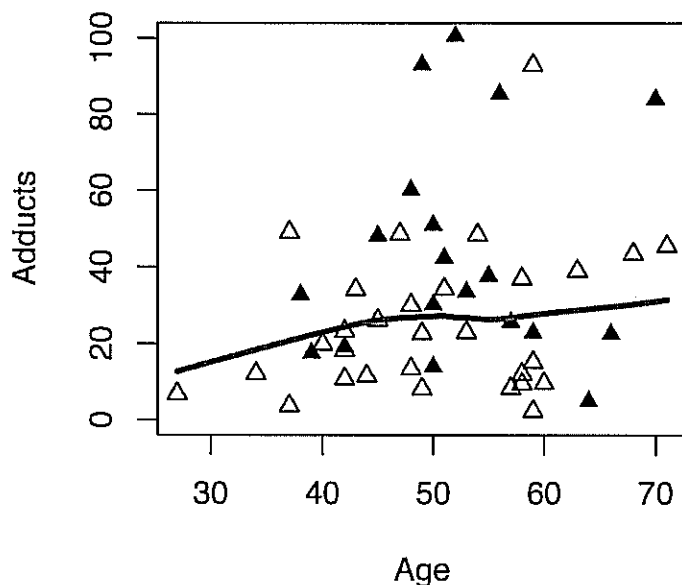
**Figure 4:** Aromatic DNA adduct levels displayed as histogram (top panel – cases, middle panel – controls) and as cumulative distribution function (bottom panel; solid line - cases, dotted line - controls).

We had chosen Porto Alegre, Brazil as study site because we expected high PAH exposures due to limited air pollution controls and the local diet which includes frequent barbecued meat consumption. However, even though DNA adduct measurements were done by the same method and in the same laboratory, the mean DNA adduct levels ( $32.3 \pm 24.8$  per  $10^9$  nucleotides, Table 6) in women from this area were only one third of those found by Li et al. (1996) in normal breast tissue of women with breast cancer (97.4 per  $10^9$  nucleotides, range 3.8 – 1737 per  $10^9$  nucleotides) recruited from around Houston. In contrast to the findings of Li et al. (1996) we did not find a difference in adduct levels of cases and controls (Table 6). In the Long Island breast cancer study where PAH DNA adducts in blood samples of over 2200 women were measured by ELISA only a modest support for an association between PAH DNA adducts and breast cancer development was found (Gammon et al., 2002).

A benzo[a]pyrene-like DNA adduct observed by Li et al. (1996) in normal breast tissue of about 40 % of cases, but not in any controls, was detected only in a single participant (Figure 5). The origin of this adduct is still unclear (Li et al., 2002). Interestingly, this woman, a nonsmoker and control, reported using coal tar-containing shampoo daily in the last 10 years. Weyand et al. (2000) found that coal tar-containing shampoo applied to the shaved backs of mice produced significant levels of DNA adducts in lung tissue indicating dermal absorption of the coal tar. These various adducts included a BaP-like adduct (Weyand, personal communication). This might indicate that coal tar containing shampoo leads to dermal uptake of coal tar and if frequently used can result in detectable DNA adducts at distant sites. We identified one other participant who reported using coal tar containing shampoo daily, but the DNA adduct panel was not as pronounced as in the first one. The amount of tissue obtained from the participant was not sufficient for further isolation and characterization of this adduct.



**Figure 5:**  $^{32}\text{P}$ -labeled DNA adduct profile in normal breast tissue of woman who uses daily coal tar containing shampoo.



**Figure 6:** DNA adduct levels as function of age. Open triangles – nonsmokers, solid triangles – current and former smokers

DNA adduct levels appear to increase with age. However, this might be due to the higher adduct levels in smokers.

#### **Task 4 Analyze data and write reports**

Because the genotoxic impact of PAH depends on their metabolism, we hypothesized that high CYP1B1 enzyme levels result in increased formation of PAH-DNA adducts in breast tissue, leading to increased development of breast cancer. We set out to test the following hypothesis:

1. Increased *CYP1B1* gene expression is associated with increased risk of invasive breast cancer.
2. Increased PAH-DNA adduct formation is associated with increased risk of invasive breast cancer.
3. Increased *CYP1B1* gene expression is associated with increased PAH-DNA adduct formation in breast cells.
4. The positive association between *CYP1B1* gene expression and increased risk of invasive breast cancer is not due to genotype variation.

Regarding hypothesis (1), we report that the mean and median CYP1B1 transcript levels in breast tissue of controls were lower than those of cases, but this difference was not statistically significant. The  $p$ -value of the mean is greater than 0.18 ( $p$ -value on  $\log(\text{means}) \simeq 0.4$ ,  $p$ -value on variance  $\simeq 0.3$ ). This might be due to low sample number and lack of statistical power.

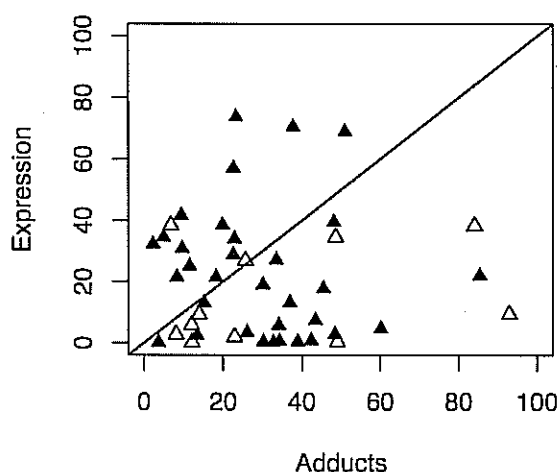
Significantly higher median levels of *CYP1B1* expression in breast tissue of breast cancer patients than in benign breast disease have been reported recently (Wen et al., 2007).

At the onset of our study we had assumed that *CYP1B1* gene expression was predominantly regulated by the Ah receptor pathway (Gonzalez and Gelboin, 1994; Whitlock, 1999). In the

meantime it has become apparent that the *CYP1B1* gene expression regulation is very complex and can include *CYP1B1* gene silencing through hypermethylation of promoter CpG islands (Widschwendter et al., 2004) and translational repression through microRNA (Tsuchiya et al., 2006).

Regarding hypothesis (2), there was no evidence for a difference in DNA adduct levels of cases and controls) in contrast to the findings of Li et al. (1996). Comparing the means using the two-tailed Student's t-test the  $p$ -value = 0.95 ( $p$ -value on log (means)  $\simeq$  0.89,  $p$ -value on variance  $\simeq$  0.29). This does not indicate that cases and controls are the same, but merely reflect the fact that with so few control samples, the statistics do not allow us to discriminate between them. In the Long Island breast cancer study where PAH DNA adducts in blood samples of over 2200 women were measured by ELISA only a modest support for an association between PAH DNA adducts and breast cancer development was found (Gammon et al., 2002).

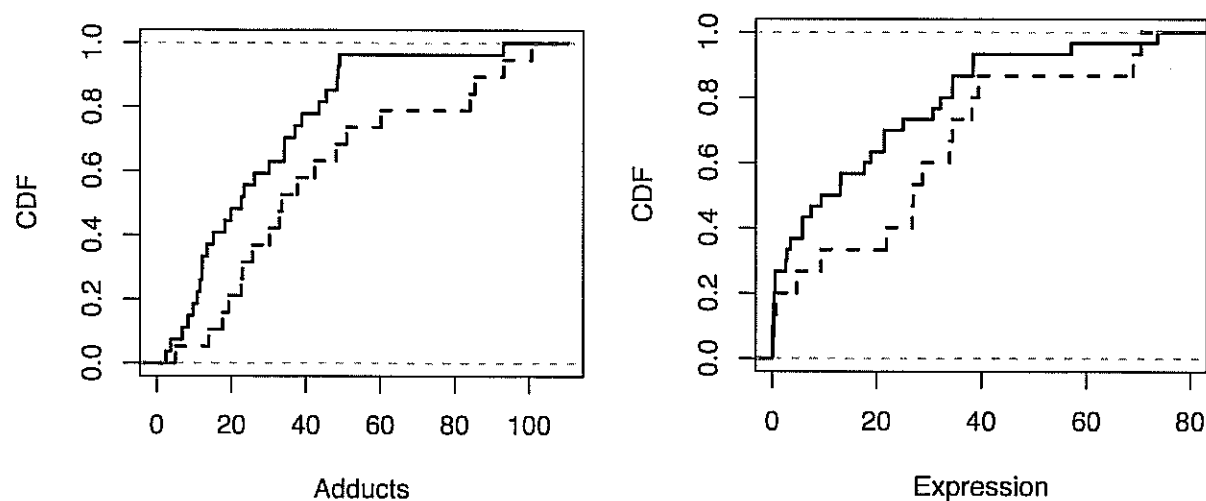
Regarding hypothesis (3), we did not find any indication of a linear relationship between *CYP1B1* expression level and PAH-DNA adducts (Figure 7). The Spearman coefficient is  $\rho = -0.05$ .



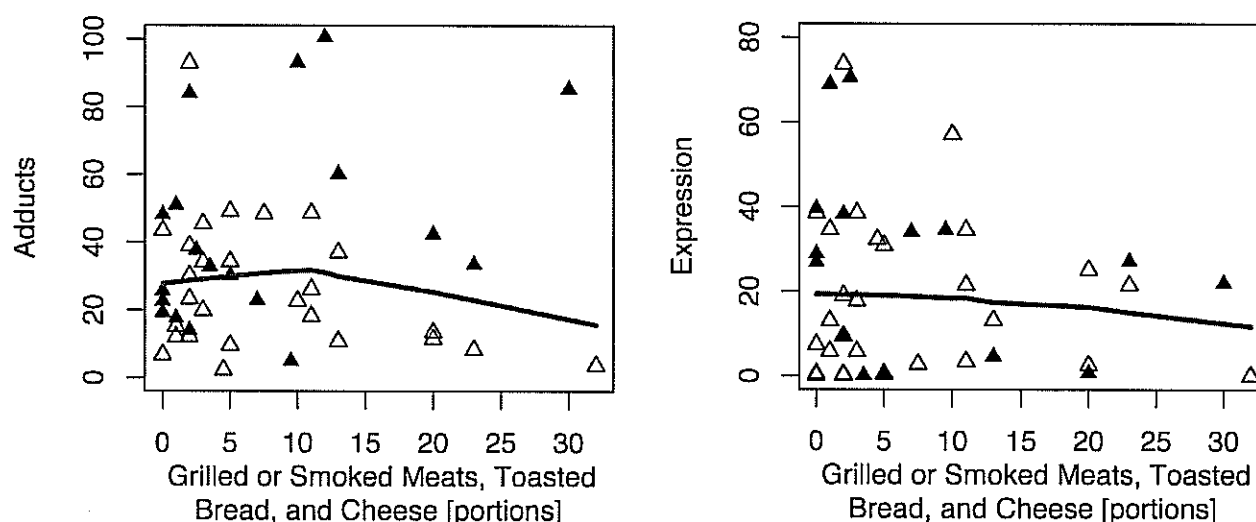
**Figure 7:** Level of *CYP1B1* expression (fg/500ng total RNA) versus aromatic DNA adducts (RAL  $\times 10^9$ ). Open triangles – controls; solid triangles – cases.

Hypothesis (4) could not be tested adequately because power was too limited to draw valid conclusions. There was no difference in DNA adducts or *CYP1B1* gene expression levels between the various *CYP1B1* polymorphic subgroup.

Clearly the major limitation of our study is the lack of statistical power as we did not succeed in recruiting as many controls as planned. Also, cases and controls were not quite matched by age (mean age 53 versus 47). While we had worried that the use of reduction mammoplasty patients as controls might lead to a selection bias, because only wealthier patients would consider this selective surgery, but it turns out that cases and controls were recruited from a similar socio-economic group.



**Figure 8:** The effect of smoking on DNA adducts levels (left panel) and CYP1B1 transcript level (right panel) shown as cumulative distribution. Solid lines represent non smokers and dotted lines current and former smokers



**Figure 9:** The effect of diet (combined portions per week of grilled or smoked meat and toasted cheese and bread) on DNA adducts levels (left panel) and CYP1B1 transcript level (right panel). Solid triangles represent current or former smokers and open triangles nonsmokers.

We considered the influence of various PAH exposure sources on *CYP1B1* gene expression and PAH-DNA adduct levels in an exploratory analysis. Estimates of exposure to environmental PAHs had been obtained through interviewer-administered questionnaires. As can be seen in Figure 8, participants who identified themselves as current or former smokers showed increased DNA adduct levels, and increased CYP1B1 transcript levels. In comparing the DNA adducts of smokers versus nonsmokers, we see a statistically significant difference in the means ( $p$ -value on means = 0.034,  $p$ -value on  $\log(\text{means}) \simeq 0.024$ ), but less so in the variance ( $p$ -value on variance  $\simeq 0.09$ ). If

we extrapolate from these data, this may suggest that smokers have more DNA adducts, but the inter-individual variation in DNA adduct levels are the same for smokers and nonsmokers due to genetic variation in metabolism of PAHs and DNA repair of adducts. We do not see a difference in the expression between smoker and nonsmokers ( $p$ -value on means = 0.21,  $p$ -value on  $\log(\text{means}) \simeq 0.43$ ,  $p$ -value on variance  $\simeq 0.45$ ). This might suggest that the effect of smoking on *CYP1B1* expression in breast tissue is not strong and would not be a powerful biomarker for smoking.

No association of aromatic DNA adduct levels or CYP1B1 transcript level with heating or cooking sources, second hand smoke exposure or intake of grilled, smoked or toasted food was observed (Figure 9). Even in much larger studies the relationship between environmental exposures and DNA adduct levels remain unclear (Steck et al., 2007).

Shantakumar et al.(2005) reported inconsistent associations between detectable PAH-DNA adducts, such as grilled and smoked foods or a summary measure of total dietary BaP intake during the year prior to the interview. They concluded that the PAH-DNA adducts detected in a population-based sample of adult women with ambient exposure levels reflect some key residential PAH exposure source, such as cigarette smoking. The authors suggested that season may be a better surrogate of dietary PAH intake and recent ambient PAH exposure than the measures used in the study. Because of this observation, we evaluated the association of DNA adduct levels and season at time of tissue collection (Table 7). Surprisingly, we observed the highest adduct level in winter and spring, even when all current and former smokers were eliminated and this seemed independent from the indoor heating and cooking source reported.

**Table 7:** Seasonable variation in mean DNA adducts

| Tissue collected<br>between            | Winter<br>6/22 – 9/21 | Spring<br>9/22 – 12/21 | Summer<br>12/22 – 3/21 | Fall<br>3/22 – 6/21 |
|----------------------------------------|-----------------------|------------------------|------------------------|---------------------|
|                                        | n                     | n                      | n                      | n                   |
| All values                             | 9 52.8 $\pm$ 34.9     | 11 33.5 $\pm$ 14.1     | 18 24.7 $\pm$ 19.6     | 11 26.8 $\pm$ 24.6  |
| Current /former<br>smokers<br>excluded | 5 42.7 $\pm$ 31.9     | 6 35.7 $\pm$ 16.7      | 11 17.6 $\pm$ 11.8     | 9 17.1 $\pm$ 10.4   |

## **KEY RESEARCH ACCOMPLISHMENTS**

- Enrolled 56 participants and brought collected specimens in five shipments to LBNL
- Collected information on possible PAH exposure by interviewer-administered questionnaire
- Determined *CYP1B1* expression in all specimens, detected a lower, but statistically not significant level in controls compared to cases
- Determined *CYP1B1* polymorphism at codon 432 and codon 453 in all specimens
- Determined DNA adducts, found no difference in adduct levels between cases and controls, but a statistically significant difference in adduct levels of smokers and non-smokers

## **REPORTABLE OUTCOMES**

Erdmann, C. A., Goth-Goldstein, R. (2002) Gene-environment interactions and breast cancer risk: The potential role of metabolic enzymes and PAH exposures. *Epidemiology* 13(4) Supplement: S113.

Erdmann CA, Goth-Goldstein R. (2002) Gene-environment interactions and breast cancer risk: The potential role of metabolic enzymes and PAH exposures. The 12th Conference of the International Society of Exposure Analysis/14th Conference of the International Society for Environmental Epidemiology, August, 2002. Vancouver, BC, Canada.

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## CONCLUSIONS

We have tested the hypothesis that individuals with higher CYP1B1 expression are at a higher risk for breast cancer because they produce higher amounts of ultimate carcinogen. We found a slight, but statistically not significant difference in *CYP1B1* expression levels of cases and controls. We did not detect any difference in aromatic DNA adduct levels of cases and controls, only between smokers and non-smokers. We did not find a correlation between the levels of *CYP1B1* expression and DNA adducts.

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