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We had previously determined that the NAT2 slow acetylator genotype and cigarette smoking is a risk factor for postmenopausal Caucasian women. This year, diet and meat consumption was not found to be a risk factor for breast cancer. Analysis for NAT1 did not reveal additional risks. Other genetic analyses are completed for *MEH3, MEH4, CYP2D6, SOD, GSTM1, GST-T and CYP1A1. CYP1A1* data have been studied in relation to PCB exposure, and a modest risk has been found in women with the highest PCB body burdens. Currently, 215 blocks have been received for p53 analysis, 93 have been immunohistochemical stained, and SSCP is in progress. To corroborate the epidemiological data, 38 breast cell strains have been established and metabolism is being studied in relation to genotypes. Methodological conditions are being addressed. Finally, to identify smoking-related risk, we have been studying smoking behavior and addiction, where, genetic polymorphisms in dopamine receptors have been associated with depression and smoking but tyrosine hydrolase, serotonin transporters, and dopamine D3 receptors are not. In 1997, 5 papers have been published, 10 have been accepted and 3 have been submitted.

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FOREWORD

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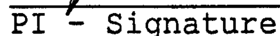

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INTRODUCTION

The first goal of this project is to determine the frequency of genetic polymorphisms for carcinogen metabolism and the p53 mutational spectra in a previously conducted breast cancer study. This study was designed to assess nutritional risk factors, seeking to identify risk factors related to inheritable susceptibilities and chemical etiologies. The workscope of this DOD grant was subsequently expanded to include the same goals, but for other epidemiological studies of breast cancer, and to perform studies of breast metabolism, p53 and smoking (including smoking cessation). The DOD grant allows us to examine a variety of risk factors (hormonal and non-hormonal; environment and diet; carcinogens and anticarcinogens) in relationship to p53 mutations and breast cancer with genetic polymorphisms as effect modifiers. The frequency of genetic polymorphisms themselves in relation to breast cancer and to p53 mutations are being determined.

A population-based case-control study of breast cancer was conducted between 1986 to 1991; blood and tissue have been stored. There were 371 postmenopausal and 301 premenopausal women with breast cancer and 438 and 316 age-matched controls, respectively. Genotyping for GSTM1, CYP1A1, CYP2D6, CYP2E1, APOE, aldehyde dehydrogenase, glutathione-*S*- transferase theta (GSTT), N-acetyltransferase 1 and 2 (NAT1 and 2), superoxide dismutase (SOD), microsomal epoxide hydrolase MEH)is being or has been determined for all subjects. The decision to study these genes was made in the context of a priori hypotheses relating to gene-environment interactions. They all have polymorphisms which are associated with changes in carcinogen metabolic activation, detoxification or carcinogen-DNA adduct formation.

We plan to determine the p53 mutational spectra to see if we can find associations with gene-environment interactions. The p53 mutational spectra is being determined for informative cases, who will be identified by single stranded conformational polymorphism analysis and immunohistochemical staining. Persons with mutations will be categorized by mutation and hypothesized chemical etiology will be compared to persons with other types of p53 mutations (four for each case) and also to controls without cancer (ten for each case). Odds ratios and logistic regression will address the association of genetic polymorphisms and exposures as a risk for p53 mutation and breast cancer, adjusting for other risk factors. We also will examine effect modification for other risk factors by genetic polymorphisms.

The current workscope was expanded to perform additional studies relating to findings in the first year of the award, specifically as they relate to smoking, smoking-related carcinogens and breast cancer. Thus, we are culturing human breast epithelial cells and examining the rate of adduct formation from cigarette-smoke carcinogens, as well as the p53 and apoptosis response. Interindividual variation will specifically be addressed. The purpose of these studies is to corroborate our epidemiological findings. We will also reproduce our findings in additional epidemiological studies. Finally, we will examine nicotine addiction and genetic risk factors for addictive behaviors, in the context of a smoking cessation project, in order to identify risks for smoking addiction and smoking cessation strategies.

BODY

1. Collection of Tissue Samples and Tissue Preparation

- Tumor blocks for 215 cases have been obtained and sectioned, and the DNA has been extracted. This represents an additional 122 blocks from last year. We now know that 32 blocks have been inadvertently destroyed at a local hospital, and one cancer center refuses to provide the blocks to us. We expect to obtain an additional 211 blocks this year (assuming that all the blocks will be found at the hospitals), and an approval for an additional 36 blocks is pending. In total then, we expect to analyze 426. This will represent the largest study to date of p53 mutational spectra and gene-environment interactions.

  The quality of the tumor blocks have been reviewed by Dr. Michael Slate, a local pathologist in buffalo, through a collaborative effort. Then, Dr. Andrew Borkowski at the University of Maryland will provide a second histological review of slides and he will circle areas of tumor for microdissection (150 subjects completed to date).

  We expect that this year, we will complete the immunohistochemistry staining and single stranded conformational polymorphism analysis, and about one half of the sequencing for persons with suspected mutations. We will therefore need to complete this aspect of the project after the DOD grant terminates, and this will occur using NIH intramural funds (which already supplements this project).

- A mechanism for receiving fresh breast tissues from autopsy cases and reduction mammoplasties continues to go well. To date, we have established 38 strains from a total of 75 breast tissues received (not all strains are established from tissues collected), and culturing is now routine from both autopsy and surgical donors. Additionally, we have previously collected 150 frozen breast tissues from autopsy and surgical donors, many of the former who have also donated liver. All surgical cases have completed an epidemiological questionnaire. The established cell strains come from a subset of these tissue donors, so that we are establishing a resource where we can look at in vitro cellular responses and then examine the parent tissue for carcinogen adducts and metabolism, and also have epidemiological questionnaire data.

- DNA has been extracted from approximately 600 smokers and non-smokers enrolled in a study of tobacco addiction in collaboration with Georgetown University. Outcome data at one year is now available for the ability to quit after smoking cessation counseling.

- Blocks are now being received for a multiracial study of breast cancer in collaboration with MD Anderson Cancer Center. The primary hypothesis is to examine gene-environment interactions for breast cancer risk and survival in a

case-series analysis Six hundred cases have been identified who were diagnosed from 1983 to 1993 and have had epidemiological questionnaires completed. These women include 400 Caucasians, 100 African Americans and 100 Hispanics. To date, 76 blocks have been received, but we are meeting resistance from MD Anderson pathologists to release their blocks, because of money and concerns that we are asking for too much tissue (about 250 um, which is only important for women with biopsy diagnosis only). In order to overcome this resistance, we have increased the inclusion criteria from diagnosis before 1988 (from 1986), so as to continue to have more than 10 years of follow-up, but increase the number of subjects outside MD Anderson (where all 76 blocks have come from so far). It will also increase the number of available and larger tissue samples. We also have arranged to supplement funding at MD Anderson ($16,000 from NIH intramural funds) to cover the costs of tissue collection and sectioning. Separately, chart reviews by our collaborators at MD Anderson is underway. We hope to have all blocks collected this year, have DNA extracted and NAT2 genotyping completed. Additional intramural funding is being sought to cover costs of additional analyses.

2. Genetic Polymorphism analysis

- Our initial focus was to study tobacco smoking as a risk factor for breast cancer. While smoking is generally considered not to be a risk factor for breast cancer, based on numerous epidemiological studies, it was our hypothesis that smoking would indeed be a risk factor in some women, but not others. When studied together as a homogenous population, the risk would not be observable. Thus, to test this hypothesis, we studied risk in the *N*-acetyltansferase gene (*NAT2*), because this gene functions as a detoxification pathway for aromatic amines, for which there is ample experimental evidence to suggest that aromatic amines would be a human breast carcinogen. The *NAT2* genetic polymorphism, which predicts rapid or slow acetylation, was tested in 304 breast cancer cases and 327 community controls. Neither smoking or the *NAT2* gene by themselves were risk factors, but when the women were stratified by smoking risk based on acetylation status, in postmenopausal women, smoking carried a risk of up to 4.4 (95% C.I.=1.4, 10.8) in slow acetylators, which was consistent with several different types of analyses for this data set. There was no similar findings for premenopausal women. A manuscript was published summarizing these findings in the Journal of the American Medical Association in 1996.

  We now also have examined the NAT2 genotypes in relation to consumption of meats, as a surrogate for heterocyclic amine consumption. While our questionnaire is appropriate for meat consumption, it is known that cooking practices is what determines the actual quantity of heterocyclic amines. Thus, our estimates of risk are approximate. In our study, we did not find a risk related to meat consumption modified by NAT2. A manuscript describing these results is in

press for the International Journal of Cancer. See Appendix A.

Because of a previous study which suggested that NAT2 might interact with smoking to increase the risk of spontaneous abortions, we examined our data but did not find a similar risk. See Appendix B.

NAT1 genotyping has been completed for postmenopausal women and premenopausal women (Appendix C). The genotypic frequency is similar to previous reports in the literature. Quality control analyses is were completed and the data did not increase a risk for the NAT1, either with or without smoking. See Appendix B. The current analysis examines allele numbers 3, 4, 10 and 11. But, while previous data has suggested the *10 allele was associated with increased activity and risk of colon and bladder cancer, subsequent data has indicated that *10 allele is actually not a functional polymorphism. Also since then, two additional low frequency alleles have been identified are functional, and we decided to examine these because they are likely more relevant. But, depending on the risk estimates, we might not have enough statistical power. These assays are now in progress. An abstract is submitted to the 1998 Annual meeting of the AACR summarizing the above results.

- A commonly accepted risk factor for breast cancer is alcohol consumption, and the findings are more frequently reported in premenopausal rather than postmenopausal women. It is currently unknown what might be the carcinogenic agents in alcoholic beverages. One candidate is ethanol, because ethanol is oxidized to acetaldehyde, which is mutagenic and carcinogenic in laboratory animals. The principle pathway for ethanol oxidation is through alcohol dehydrogenase. In order to study the risk of alcohol drinking in the context of ethanol metabolism, we studied the alcohol dehydrogenase 3 gene (*ADH3*). In this study, we found that women who would be predicted to have an increased capacity to form acetaldehyde ($ADH3^{1-1}$), had an odds ratio of 3.0 (95% C.I.=1.3, 6.6) in high drinkers compared to low or nondrinkers. Compared to women who would have a decreased capacity ($ADH3^{2-2}$), there was a 3.3-fold risk (95% C.I.=0.9, 12.9). This work has resulted in oral presentation at the 1997 American Association of Cancer Research Annual Meeting and the Society for Epidemiological Research. A manuscript has now been submitted to the Journal of the National Cancer Institute (See Appendix D for tables of data).

- Apolipoprotein E is involved in the production of VLDL and other parts of cholesterol metabolism. Several studies have related low cholesterol levels to breast cancer risk. The apoE gene is polymorphic, where some variants raise cholesterol levels and others lower them. We therefore measured apoE genotypes in both the pre- and postmenopausal women. The statistical analysis is continuing. Actually, no work was done on this data in 1997 (!), but a

postdoctoral fellow at the University of Buffalo (Dr. Kirsten Moysich) has now begun to continue this work.

- Our previous results indicated that a polymorphism in cytochrome P450IAI is related to breast cancer in postmenopausal women with low tobacco use. There also was a non-significant trend for GSTM1 in younger postmenopausal women. Both of the enzymes are involved in the activation and detoxification, respectively, of polycyclic aromatic hydrocarbons. The status of the genotyping for GSTM1, CYP1A1 and GST-T in premenopausal women is completed since last year, and the analysis is ongoing. Genotype frequencies are listed in Appendix E). We also have examined the postmenopausal data for *CYP1A1* in relation to polychlorinated biphenyl (PCBs) body burdens. It was previously published that PCBs might be related to breast cancer risk, although subsequent data, including from this study did not show this. But, we hypothesized that one way that PCBs might contribute to breast cancer risk was through the induction of cytochrome P450s and attendant increased metabolic activation. Our data indicated that the *CYP1A1* genetic polymorphism was a risk factor in breast cancer in women with PCB levels above the median (See Appendix F.) Thus, there may be an interactive effect. Analysis is ongoing.

- Another enzyme involved in this pathway is microsomal epoxide hydrolase. There are two polymorphic sites that result in a decrease of activity by 40%. The measurement of MEH in pre-and postmenopausal women is almost complete. In this past year, the genotyping analysis was completed, and statistical analysis is ongoing. See Appendix G for genotype frequencies.

- Cytochrome P450IID6 has been associated with lung cancer and breast cancer. Its metabolic substrate is unknown, but it may be a tobacco-specific nitrosamine. We are measuring the activity of this gene by PCR. The genotyping was completed in 1997, but there is still some additional quality control analyses to be completed. The current status is presented in Appendix H.

- A new genetic polymorphism analysis started in 1997 was for the manganese superoxide dismutase gene, which is a free radical scavenger. Genotyping is complete. The current frequencies are listed in Appendix I.

3. P53 Mutational Spectra Analysis

- Blocks from 215 individuals have been obtained and have been sectioned. P53 immunohistochemistry staining has been done for 93 and the rest are in progress. We have identified appropriate controls for sequencing to ensure quality control

and no contamination of wild-type DNA. We have identified these controls from lung cancer samples. There are 20 controls that contain mutations in each of the 4 exons of interest. We have also prepared blocks of cell lines with known p53 mutations, which also will be used as controls. The methods to perform the SSCP and sequencing are now being optimized and finalized for these samples. In 1998, we will complete the IHC and SSCP on all samples, and sequencing of exons for suspected mutations will begin (see above).

4. Ancillary Studies

- We have developed the technique in our laboratory, based upon previously published methods, to isolate breast epithelial cells and culture them in a sterile environment. Thus far we have established 38 cell strains. In these cells, we have determined that 4-aminobiphenyl is metabolically activated through cytotoxicity experiments, and have determined optimal timing and dose response relationships. Both metabolites of 4-ABP and parent 4-ABP are active in producing cell death, suggesting the presence of NAT1 and CYP1A2 in these cells. We are also now identifying the p53 induction in relation to the exposures, but have identified problems in reliable fixation without affecting P53 status. We are now trying alternative methods. We also have decided to delay analysis for apoptosis until the p53 studies are completed, which will avoid duplication of errors. The current data is presented in Appendix J.

  We have been attempting to measure DNA adducts using the postlabeling ADAM procedure. However, the procedure remains too variable to be reliable. However, we have made substantial progress in developing an alternative chemical postlabeling method. This method chemically acylates adducts using C^{14}-labeled acetic anhydride. The labeled adducts are resolved by high performance liquid chromatography and then detected using accelerator mass spectroscopy. Chemical standards have been synthesized to calibrate the assay and optimizing labeling conditions is in progress. The current data is presented in Appendix K.

- An understanding of why people smoke cigarettes can have an important impact upon smoking prevention and cessation. People smoke cigarettes to maintain nicotine levels in the body, and nicotine has been implicated in the stimulation of brain reward mechanisms via central neuronal dopaminergic pathways. We recruited smokers (n=283) and nonsmokers (n=192) through local media for a case-control study of smoking. Following informed consent and a behavioral questionnaire, smokers underwent a single minimal contact session of smoking cessation counseling, and then were followed for up to one year. Thus far, we have found that there is an interaction for polymorphisms with the dopamine transporter gene and the dopamine D2 receptor for smoking risk (P=0.001) and the combination of the two genotypes reduces the risk of smoking by more than half. This manuscript has been submitted to Health Psychology, and a manuscript

was submitted with last years report.

In this study, we also evaluated the association of smoking and smoking cessation with a dopamine D4 receptor 48 base pair variable nucleotide tandem repeat polymorphism, where the 7 repeat allele (D4.7) reduces dopamine affinity. The frequency of the dopamine D4 receptor genetic polymorphism using PCR was determined and individuals were classified by the number of repeat alleles (2-5 repeats as "S" and 6-8 repeats as "L"). Persons with those genotypes including only S alleles (homozygote S/S) were compared with those with at least one L allele (heterozygote S/L and homozygote L/L). The data showed that the S allele interacted with depression to increase the risk of smoking. A manuscript was published by Health Psychology (See Appendix L). We also found that the L allele increased smoking risk in African Americans. This paper has been accepted by Cancer, Epidemiology, Biomarkers and Prevention (See Appendix M). We also have studied genetic polymorphisms in the tyrosine hydrolase and serotonin transporter genes, which did not yield positive associations. These two manuscripts have been accepted for publication in Pharmacogenetics and Cancer, Epidemiology, Biomarkers and Prevention (See Appendices O and P). Finally, we have completed genetic analyses for the dopamine D3 receptor and dopamine hydroxylase, and have in progress another Dopamine D4 receptor.

CONCLUSIONS

The findings of an association of smoking and breast cancer in Caucasian women with the slow NAT2 acetylation genotype is very important because approximately 50% of women are slow acetylators. This results in a large attributable risk. The findings need to be reproduced and examined in other races. Such a study is underway in collaboration with the MD Anderson Cancer Center. Laboratory studies also need to corroborate this finding by examining the metabolic potential in rapid and slow acetylators. Recent studies showing that breast cells contain acetyltansferase activity and our studies described above are consistent with the epidemiological data, but adduct studies also are needed. The development of the acetic anhydride postlabeling procedure will provide data for intermediate endpoints. Thus, the application of this procedure for aromatic amine adducts in cell strains and parent tissues may provide important corroborative data for the epidemiological findings. Finally, the p53 mutational spectra will also provide data on intermediate endpoints and also possibly identify the effects of acetyltansferase on ultimate outcome. While it has been difficult to obtain blocks in the past, we now have in hand a significant number of samples, and more are expected.

As follow-up to smoking related risk, the ability to prevent smoking addiction and increase smoking cessation has the greatest potential impact from a public health and individual health perspective. The identification of polymorphisms in the dopamine receptor genes and dopamine transporter genes may be able to identify optimal prevention strategies.

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Shiva Krishnan

(Note that other persons are actively working on this project and are supported by NIH intramural funds, as described in the original proposal.)

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APPENDIX A

Breast Cancer Risk, Meat Consumption and N-Acetyltransferase (*NAT2*) Genetic Polymorphisms.

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Running title: Meat consumption and breast cancer risk

Keywords: breast neoplasms, meat products/adverse effects, fish, heterocyclic compounds/metabolism, arylamine acetyltransferases/genetics, polymorphism (genetics), epidemiology/molecular

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[1] This work was a collaborative effort by the Department of Social and Preventive Medicine, State University of New York at Buffalo, and The Laboratory of Human Carcinogenesis, National Cancer Institute. This work was supported, in part, by USAMRMC#DAMD17-94-J-4108, DAMD17-95-1-5022, CA-11535, and CA-62995. Dr. Freudenheim is the recipient of a PHS Research Career Development Award CA01633 from the National Cancer Institute. This publication is solely the responsibility of the authors and does not necessarily represent the views of the NCI.

The abbreviations used are: *NAT2*, *N*-acetyltransferase; HAs, heterocyclic aromatic amines; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5]pyridine; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline; OR, odds ratio; CI, confidence interval; BMI, body mass index; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; CYP1A2, cytochrome P4501A2.

SUMMARY

Although inconsistencies exist, some studies have shown that meat consumption is associated with breast cancer risk. Several heterocyclic amines (HAs), formed in the cooking of meats, are activated by polymorphic *N*-acetyltransferase (NAT2) and are mammary carcinogens in experimental animal models. We investigated whether ingestion of meat, chicken and fish, as well as particular concentrated sources of HAs, may increase breast cancer risk, and if associations may be modified by *NAT2* genotype. Caucasian women with incident breast cancer (n=810) and community controls (n=740) were interviewed and administered a food frequency questionnaire. A subset of these women (n=793) provided a blood sample. PCR-RFLP analyses were used to evaluate three polymorphic sites in *NAT2* that predict 90-95% of the slow acetylation phenotype among Caucasians. Consumption of red meats, as well as concentrated sources of HAs, was not associated with increased breast cancer risk. However, in postmenopausal women, fish consumption significantly decreased risk (odds ratio = 0.7; 95% confidence interval, 0.4-1.0); and among premenopausal women, there was the suggestion of inverse associations between risk and pork and chicken intake. Relationships between meat consumption and breast cancer risk were not modified by *NAT2* genetic polymorphisms. These results suggest that consumption of meats and other concentrated sources of HAs is not associated with increased breast cancer risk. However, due to the strong biologic plausibility for a role of some HAs in mammary carcinogenesis, and the likely measurement error in evaluation of sources of HAs in this study, further studies of these possible relationships are warranted.

INTRODUCTION

The incidence of breast cancer varies widely by geographic region (Willett, 1989), and there are indications that variability in diet, particularly intake of dietary fat and protein, may be related to this disparity in breast cancer rates (Freedman et al. 1990; Prentice et al. 1988). As summarized by Hunter and Willett (Hunter and Willett, 1993), the majority of epidemiologic studies have not supported an association between fat and breast cancer. Studies of the consumption of animal products, particularly meat, have also yielded inconsistent results. While some studies have shown that meat consumption increases breast cancer risk (Ronco et al. 1996; Gaard et al. 1995; Lee et al. 1991; Lubin et al. 1981; Vàtten et al. 1990; Richardson et al. 1991; Ewertz and Gill, 1990; Lubin et al. 1986; La Vecchia et al. 1987; D'Avanzo et al. 1991; Hislop et al. 1986; Hirayama, 1978), other researchers have found no association (Mills et al. 1988; Phillips et al. 1980; Kinlen, 1982; van den Brandt et al. 1993; Iscovich et al. 1989; Katsouyanni et al. 1986; Willett et al. 1990). A meta-analysis of 5 cohort and 12 case-control studies by Boyd and colleagues did reveal a summary relative risk of 1.18 (95% CI 1.06-1.32) associated with consumption of meat, fish and chicken combined, and a risk of 1.54 (95% CI 1.31-1.82) for red meat alone (Boyd et al. 1993).

The assessment of meat as a risk factor for breast cancer has focused primarily on its role as a source of dietary fat or animal protein. However, Toniolo and colleagues found that consumption of meat, but not total fat or protein, significantly increased breast cancer risk (Toniolo et al. 1994), and in a study in Uruguay, meat consumption was also associated with risk, even when controlling for protein and fat (Ronco et al. 1996). It is possible that if meat consumption does play a role in breast cancer etiology, the risk may not be related to meat as a source of fat or protein, but, rather as a source of mutagens

and/or carcinogens. When meat is cooked, particularly at high temperatures or for a long period of time, mutagenic heterocyclic amines (HAs) are formed (Sugimura, 1986). These compounds may be breast carcinogens in women, because experimental studies have shown that certain heterocyclic amines, such as 2-amino-1-methyl-6-phenylimidazo[4,5]pyridine (PhIP), 2-amino-3-methylimidazo[4,5-f] quinoline (IQ), and 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline (MeIQx), cause mammary cancer in rodents (Tanaka et al. 1985; Kato et al. 1989; Ito et al. 1991). In fact, a recent paper by De Stefani and colleagues, based on data from the case-control study in Uruguay, showed that risk was greatest for consumption of fried meats, a cooking method that results in high levels of heterocyclic amines (Destefani et al. 1997).

Metabolism of heterocyclic and aromatic amines varies among individuals, depending, in part, on polymorphisms in genes involved in xenobiotic metabolism. *N*-acetyltransferases NAT1 and NAT2 and cytochrome P4501A2 (CYP1A2) are involved in the metabolism of heterocyclic and aromatic amines (Lang et al. 1994). Several polymorphic sites have been identified at the *NAT2* locus, and result in decreased *N*-acetyltransferase activity (Blum et al. 1991; Bell et al. 1993). Slow NAT2 acetylation of aromatic amines is associated with increased risk for bladder cancer (Hanssen et al. 1985; Cartwright, 1984) and may increase postmenopausal breast cancer risk associated with cigarette smoking (Ambrosone et al. 1996). HAs appear to be poor substrates for *N*-acetylation at the liver, however, and rapid *O*-acetylation of the activated metabolites by NAT2 in the target tissue appears to be associated with increased risk of colon cancer, particularly among those with high consumption of red meat (Welfare et al. 1997; Roberts-Thomson et al. 1996; Wohlleb et al. 1990; Lang et al. 1994; Lang et al. 1986).

The purpose of these analyses was threefold; 1) we sought to evaluate relationships between breast cancer risk and consumption of meats, poultry and fish in pre- and postmenopausal women, 2) we were interested in determining if risk associated with meat consumption could be related to dietary HAs, as measured by consumption of products known to be concentrated sources of them, and 3) to determine if polymorphisms in *NAT2* might modify the association between breast cancer risk and consumption of sources of heterocyclic amines.

MATERIALS AND METHODS

Study population This study population and research methodology have been described in detail previously (Freudenheim et al. 1996; Ambrosone et al. 1996; Ambrosone et al. 1995; Graham et al. 1991). Briefly, cases were women diagnosed with incident, primary, histologically confirmed breast cancer, identified from all the major hospitals in Erie and Niagara counties; included were women ranging in age from 40 to 85. Women under age 50 were considered postmenopausal if they had ceased menstruation because of natural menopause, bilateral oopherectomy, or irradiation to the ovaries; all others were considered premenopausal. Women 50 years of age and over were considered postmenopausal if they were no longer menstruating. Cases were interviewed, on average, within two months of diagnosis. Controls under 65 years of age were randomly selected from the New York State Motor Vehicle Registry, and those 65 and over were identified from Health Care Finance Administration lists. Of premenopausal women contacted, 66% of eligible cases (n=301) and 62% of eligible controls (n=316) participated, and of postmenopausal women, 54% of cases (n=439) and 44% of controls (n=494) participated. Controls were frequency-matched to cases by age and

county of residence. The protocol for the study was reviewed by the Institutional Review Board of the State University of New York at Buffalo and each participating hospital, and informed consent was received from all participants. Cases and controls were interviewed in person by trained interviewers, with an in-depth food frequency questionnaire regarding usual diet two years prior to the interview, including frequency of consumption and usual portion size of over 300 specific foods. Reproductive, medical and family histories were obtained, as well as lifetime tobacco and alcohol histories. Of the women interviewed, approximately 45% of premenopausal and 63% of postmenopausal women agreed to have blood drawn for research purposes.

Analytic Methodology An extensive food frequency questionnaire was administered, assessing usual intake two years prior to the interview. Using food models, women were questioned about usual dietary intake two years prior to the interview, including quantity and frequency of intake, seasonal intake, and food preparation. Grams of meats per day were computed by multiplying frequency of consumption by portion size, estimated by food models. Participants were asked about portion size and frequency of consumption of steak, round steak, hamburger patties, ground beef, other beef, including roasts and stews, veal, lamb and beef liver. From this information, usual grams of consumption of each item were calculated and items were grouped to create a beef index. A pork index was based on queries regarding intake of pork roast, chops, and spareribs. A processed meats index, including ham, hot dogs, sausages, bacon, and cold cuts was also assessed. A poultry index included chicken and turkey. The fish index included fresh or frozen fish, canned fish, shrimp and other shellfish. In addition to frequency of consumption and usual portion size of various types of meat, women were also asked how frequently they used gravy made from pan drippings or fried foods in bacon grease. We also evaluated

associations between risk and grams consumed per month of bacon, breakfast sausages, and gravy made from pan drippings, all concentrated sources of heterocyclic amines, particularly PhIP (Skog et al. 1995; Murray et al. 1993). Data were not available on how well done the meat consumed was cooked, which is another indicator of exposure to heterocyclic amines.

Risks for pre- and postmenopausal women were examined separately, based on variability in some risk factors and the possibility that breast cancer may be different diseases in the two groups. Furthermore, mean levels of intake of certain meats varied significantly between the two groups. Quartiles of intake of types of meats were based on approximately uniform distribution in controls. Odds ratios (OR) with 95% confidence intervals (CI) were calculated by unconditional logistic regression for each category of the risk variables, with the lowest intake quartile as the referent category. P for trend was the level of significance of the beta coefficient for each independent variable as a continuous variable in the logistic regression model with the relevant adjusting variables.
Unadjusted ORs were calculated, as well as those adjusted for other breast cancer risk factors including age, education, body mass index (BMI), age at menarche, age at first pregnancy, family history of breast cancer, and age at menopause for postmenopausal women. BMI was computed as weight(kg)/height(m)^2, where weight was as reported for two years prior to the interview, and family history was defined as the presence of breast cancer in a mother and/or sister. Total calories consumed were not related to breast cancer risk in these data, and the addition of this variable to the model did not significantly alter estimates of risk. Models adjusted for cigarette smoking, found to increase risk among postmenopausal women with slow *NAT2* genotype in these data, also did not differ significantly from unadjusted. Because there may be a tendency for fish

and poultry eaters to also consume more fruits and vegetables, and because some components of fruits and vegetables, which were associated with reduced risk in this data (Freudenheim et al. 1996), may reduce mutagenic activity, an additional model was employed, adjusting for total fruit and vegetable consumption. To evaluate variable risk in relation to consumption of sources of HAs, cases and controls were stratified by acetylator status and the relationship between breast cancer risk and these foods was assessed within rapid and slow acetylator groups. Sample size for these latter determinations was restricted to those who provided a blood sample and for whom NAT2 data were available. This included 118 and 114 premenopausal cases and controls, and 185 and 213 postmenopausal cases and controls.

NAT2 Genotyping

Blood specimens were collected, serum was separated, and blood clots were stored at -70°C. Methods for DNA extraction from clots and determination of *NAT2* genotype have been described previously (Ambrosone et al. 1996). Briefly, DNA was extracted and amplified by polymerase chain reaction (PCR) in the presence of primers specific for *NAT2* (Bell et al. 1993). An aliquot (18μL) was then subjected to restriction fragment length polymorphism (RFLP) analysis for the $C^{481}T$ (*KpnI*; New England Biolabs, Beverly, MA), $G^{590}A$ (*TaqI*, New England Biolabs, Beverly, MA) and the $G^{857}A$ (*BamHI*, New England Biolabs, Beverly, MA) polymorphisms. Individuals were classified as genotypically determined rapid acetylator (carrying 0 or 1 slow acetylator mutation) or slow acetylator (individuals with two slow acetylator mutations) (Lin et al. 1993; De Stefani et al. 1994). Assays were performed in duplicate and were interpreted by two reviewers who were blinded to case-control status.

RESULTS

Table 1 shows reported mean values of consumption of various meats for all pre- and postmenopausal women. Premenopausal controls consumed significantly more pork and fish than cases. In interpretation of these reports, it is important to note that the diet assessment instrument used is a well-established tool for qualitative assessment of intake and that quantitative assessment may be less accurate. There were no significant differences in means for any of the variables tested among postmenopausal women. Associations between breast cancer risk and quartiles of consumption of various meats for pre- and postmenopausal women are shown in Tables 2 and 3. For premenopausal women, there was no increased risk associated with consumption of beef, processed meats, pork, chicken or fish (Table 2). In fact, there were inverse associations between breast cancer risk and consumption of pork, chicken and fish, although of borderline significance. However, the association between fish and chicken consumption and breast cancer risk was weaker after adjustment for fruit and vegetables.

Among postmenopausal women, there was no increase in breast cancer risk associated with higher consumption of beef, pork, or processed meats (Table 3). Both chicken and more notably, fish consumption were inversely associated with risk of postmenopausal breast cancer (4th quartile ORs and 95% CIs, respectively, 0.7, 0.5-1.0, and 0.6, 0.4-0.9). These relationships remained when adjustment was made for total fruit and vegetable consumption.

Tables 4 and 5 present analyses for the subset of women who provided blood specimens. When associations were assessed within categories of rapid and slow acetylators, there were no clear associations between risk and consumption of beef, pork, chicken, fish or processed meats among pre- or postmenopausal women by genotype

(data not shown). Evaluation of risk associated with consumption of foods that are concentrated sources of heterocyclic amines (bacon, gravy, breakfast sausages) also revealed no clear or significant associations, when groups were evaluated all together, or when stratified by NAT2 genotype. Associations with risk were also evaluated by frequency of consumption of various meats that were fried or grilled, but no effect was observed (data not shown).

DISCUSSION

In this case-control study of diet and breast cancer, we found that, in general, consumption of meats was not associated with increased breast cancer risk for pre- or postmenopausal women. Increased intake of fresh, frozen, or canned fish, as well as poultry, appeared to be associated with decreased risk among postmenopausal women. Among premenopausal women, there was a suggestion of a slight inverse association with pork consumption.

In studying associations between dietary sources of heterocyclic amines and breast cancer risk, we had extensive data regarding portion size and method of cooking for a number of meats. However, no data were available on how well-done the meat was cooked. Because a major determinant of HAs appears to be how well the meat is cooked (Sinha et al. 1995), it is possible that our measurement of sources of HAs by grams of meats consumed may be too crude to accurately assess dietary intake of HAs. However, bacon, breakfast sausages, and gravy made from pan drippings are documented sources of HAs, and these foods were also not associated with breast cancer risk.

We had hypothesized that consumption of all sources of HAs, including fish, chicken and pork, could be related to breast cancer risk. Reasons for the slight inverse associations between pork (premenopausal women) and chicken (postmenopausal

women) are unknown, although there is the possibility that they are due to chance, or to biased reports. However, the finding of reduced risk with fish consumption among postmenopausal women is supported by some human and animal data. Few epidemiologic studies have investigated the association of breast cancer risk with fish consumption. Some case-control studies did find that fish consumption, particularly poached fish, was associated with decreased risk (Hirose et al. 1995; Landa et al. 1994; Vatten et al. 1990; Hislop et al. 1986; Destefani et al. 1997), and ecologic studies show that populations with high fish consumption have lower breast cancer rates (Caygill et al. 1996; Kaizer et al. 1989; Lund and Bonaa, 1993). Additionally, laboratory studies in rodent models and with human mammary epithelial cells have shown that dietary omega-3 fatty acids, found in fish oil, suppress growth of carcinomas (Welsch et al. 1993; Rose and Connolly, 1993; Gonzalez et al. 1993). Fish that is pan-fried or broiled may be a source of HAs, however, which may counteract some of the anticarcinogenic effects that fish oil may have. Further investigations of breast cancer risk and fish consumption, particularly by method of cooking, may elucidate these issues.

The observation of an association between fish consumption and risk for postmenopausal, but not premenopausal breast cancer is consistent with other findings of differences in risk associated with some factors, such as body mass, among pre- and postmenopausal women. Recently, we found that among women with slow *NAT2* genotype, cigarette smoking was a risk factor for post-, but not premenopausal breast cancer. In light of the evidence that premenopausal and postmenopausal breast cancer may have different etiologies, (Hislop et al. 1986; Velentgas and Daling, 1994; Lubin et al. 1985; Janerich and Hoff, 1982; de Waard, 1979), this heterogeneity is plausible. The

disparity in results in these analyses by menopausal status may reflect different etiologic pathways associated with menopausal status.

This study may have been hampered by biases common to case-control studies, particularly those involving selection, dietary recall and measurement. Regarding selection bias, most case nonparticipation was due to physicians' refusals to allow contact with their patients (72%). Among postmenopausal women, non-participants were, on average, about three years older than participants. Thus, the most ill patients may not have been included, limiting generalizability. Among controls, a sample refusing interview (n=117) was compared with a sample of participants (n=372) in a telephone interview prior to data collection. No differences in reported meat, vegetable or fruit consumption were found. Thus, non-response among controls is unlikely to be related to dietary exposure.

For many cancers, illness may have caused changes in dietary habits, possibly influencing memory of past eating habits. Thus, recall bias may affect observed associations between dietary intake and cancer risk, although evidence for this bias is not consistent (Giovannucci et al. 1993; Freidenreich et al. 1991). With breast cancer, though, the growing tumor is often asymptomatic until diagnosis; it probably does not affect appetite. Questions in this study were focused on intake in the year two years before the interview. Regarding measurement error, clearly, the use of a food frequency questionnaire to assess macro- and micronutrients may result in misclassification of nutrient intake. Nonetheless, there is evidence that the instrument enables us to rank order subjects and identify at least strong relationships (Freudenheim et al. 1989). However, this questionnaire was not designed to estimate dietary intake of heterocyclic amines, and as such, allows only

use of surrogates for evaluation of associations between probable HA consumption and risk, which certainly include measurement error. It is also becoming clear that metabolic pathways are extremely complex, involving a number of Phase I and Phase II enzymes. It is possible that effects of NAT2 may only impact on risk if CYP1A2 phenotype is also rapid; that is, rapid activation at both junctures in the metabolic pathway. This phenomenon was observed by Lang and Kadlubar in a study of colon cancer, where risk was highest for those with rapid NAT2 and rapid CYP1A2 phenotypes (Lang et al. 1994). Lack of data on CYP1A2 may, therefore, be responsible for the lack of association between meats, NAT2, and breast cancer risk.

A final caution regarding these findings is related to the size of the study group. In the overall assessment of meat and fish consumption on risk, we have adequate power to detect an effect. However, these findings may be affected by numerous sources of bias. In the analyses stratified by acetylator status, where one would expect the bias to be nondifferential and thus, less of a problem, numbers are quite small. For some risk estimates, confidence intervals are wide and estimates of risk unstable. Thus, these findings must be viewed as tentative, and further studies of consumption of dietary heterocyclic amines, using a validated questionnaire for their assessment, are warranted, particularly in light of the laboratory data suggesting their association with mammary carcinogenesis.

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Table 1

Characteristics of cases and controls: Western New York Diet Study, 1986-1991[1]

	PREMENOPAUSAL		POSTMENOPAUSAL	
	Cases (n=301)	Controls (n=316)	Cases (n=439)	Controls (n=494)
Age (years)	46 (4)	46 (4)	64 (8)	63 (8)
Education (years)	14 (3)	14 (3)	12 (3)	12 (3)
Beef intake (gm/day)	60 (47)	60 (39)	52 (44)	51 (37)
Pork intake (gm/day)	12 (9) *	14 (11) *	11 (10)	11 (10)
Processed meat intake[2] (gm/day)	39 (41)	37 (31)	31 (30)	31 (31)
Poultry intake (gm/day)	31 (22)	34 (24)	23 (17)	25 (21)
Fish intake (gm/day)	27 (15) *	32 (28) *	25 (20)	28 (22)

Ratio of red meat/poultry and fish	1.5 (1.3)	1.5 (1.3)	2.8 (4.5)	2.4 (2.3)

[1]Values are expressed as mean (standard deviation)

* $p < 0.01$, Student's t-test for difference between means of cases and controls

[2] Includes bacon, breakfast sausages, ham, hot dogs, bologna and other cold cuts

Table 2

Meat consumption (grams per day) and premenopausal breast cancer risk; Western New York Diet Study, 1986-1991

Quartiles	Case (n)	Control (n)	OR (CI)[1]	OR (CI)[2]
Beef (gms/day)				
(< 33)	74	82	1.0	1.0
(33-51)	85	77	1.3 (0.8-2.0)	1.3 (0.8-2.1)
(51-78)	68	78	1.0 (0.6-1.5)	1.0 (0.6-1.6)
(> 78)	74	79	1.1 (0.7-1.7)	1.2 (0.8-1.9)
			p (trend) = 0.76	p (trend) = 0.3
Pork (gms/day)				
(< 6)	92	82	1.0	1.0
(6-10)	70	79	0.8 (0.5-1.2)	0.8 (0.5-1.2)
(10-20)	91	82	1.0 (0.6-1.5)	1.0 (0.6-1.5)
(> 20)	48	73	0.6 (0.4-1.0)	0.6 (0.4-1.0)
			p (trend) = 0.02	p (trend) = 0.05
Processed meats (gms/day)[3]				
(< 14)	65	80	1.0	1.0
(14-29)	94	79	1.6 (1.0-2.5)	1.5 (1.0-2.4)
(29-48)	60	78	1.0 (0.6-1.6)	1.0 (0.6-1.6)
(> 48)	82	79	1.4 (0.8-2.2)	1.4 (0.9-2.3)
			p (trend) = 0.3	p (trend) = 0.09

Table 2 (cont.)

Poultry (gms/day)				
(< 19)	95	79	1.0	1.0
(19-28)	66	79	0.7 (0.4-1.1)	0.7 (0.4-1.1)
(28-43)	84	79	0.8 (0.5-1.3)	0.9 (0.6-1.4)
(> 43)	56	79	0.6 (0.4-0.9)	0.7 (0.4-1.1)
			p (trend) = 0.2	p (trend) = 0.6
Fish (gms/day)				
(< 15)	83	80	1.0	1.0
(15-26)	85	79	1.1 (0.7-1.7)	1.1 (0.7-1.8)
(26-38)	71	75	0.9 (0.6-1.5)	1.0 (0.6-1.6)
(> 38)	62	82	0.8 (0.5-1.2)	0.9 (0.6-1.5)
			p (trend) = 0.03	p (trend) = 0.2
Ratio of red meat to chicken&fish				
(< .7)	71	80	1.0	1.0
(.7-1.2)	70	85	0.9 (0.6-1.4)	0.9 (0.5-1.4)
(1.2-1.8)	72	76	1.1 (0.7-1.7)	1.0 (0.6-1.7)
(> 1.8)	88	75	1.4 (0.9-2.2)	1.3 (0.8-2.0)
			p (trend) = 0.5	p (trend) = 0.8

[1] Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer

[2] Adjusted for the variables listed above, and total fruits and vegetables.

[3] Includes bacon, breakfast sausages, ham, hot dogs, bologna and other cold cuts

Table 3

Meat consumption (grams per day) and postmenopausal breast cancer risk; Western New York Diet Study, 1986-1991

Quartiles	Case (n)	Control (n)	OR (CI)[1]	OR (CI)[2]
Beef (gms/day)				
(< 28)	113	123	1.0	1.0
(28-45)	132	121	1.1 (0.8-1.7)	1.2 (0.8-1.7)
(45-62)	78	122	0.7 (0.5-1.0)	0.7 (0.5-1.0)
(> 62)	116	128	0.9 (0.6-1.3)	1.0 (0.7-1.4)
			p (trend) = 0.5	p (trend) = 0.3
Pork (gms/day)				
(< 4)	96	98	1.0	1.0
(4-8)	118	137	0.9 (0.6-1.3)	0.9 (0.6-1.3)
(8-15)	128	133	1.0 (0.7-1.4)	1.0 (0.7-1.4)
(> 15)	97	126	0.7 (0.5-1.1)	0.8 (0.5-1.2)
			p (trend) = 0.3	p (trend) = 0.5
Processed meats (gms/day)[3]				
(< 11)	101	122	1.0	1.0
(11-22)	117	126	1.1 (0.8-1.6)	1.1 (0.8-1.6)
(22-40)	112	124	1.1 (0.7-1.6)	1.1 (0.8-1.6)
(> 40)	109	122	1.0 (0.7-1.5)	1.1 (0.7-1.6)
			p (trend) = 0.9	p (trend) = 0.5

Table 3 (cont.)

Poultry (gms/day)				
(< 12)	126	120	1.0	1.0
(12-19)	119	125	0.8 (0.6-1.2)	0.8 (0.6-1.2)
(19-30)	80	122	0.5 (0.4-0.8)	0.5 (0.4-0.8)
(> 30)	114	127	0.7 (0.5-1.0)	0.8 (0.5-1.1)
			p (trend) = 0.01	p (trend) = 0.04
Fish (gms/day)				
(< 13)	129	124	1.0	1.0
(13-23)	117	131	0.9 (0.6-1.3)	0.9 (0.7-1.3)
(23-38)	112	120	0.8 (0.6-1.2)	0.9 (0.6-1.3)
(> 38)	81	119	0.6 (0.4-0.9)	0.7 (0.4-1.0)
			p (trend) = 0.03	p (trend) = 0.2
Ratio of red meat to chicken&fish				
(< 1.2)	107	130	1.0	1.0
(1.2-1.9)	94	125	0.9 (0.6-1.4)	0.9 (0.6-1.3)
(1.9-2.8)	94	109	1.0 (0.7-1.5)	1.0 (0.7-1.4)
(> 2.8)	144	130	1.4 (1.0-2.1)	1.3 (0.9-1.9)
			p (trend) = 0.1	p (trend) = 0.1

[1] Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer

[2] Adjusted for the variables listed above, and total fruits and vegetables.

[3] Includes bacon, breakfast sausages, ham, hot dogs, bologna and other cold cuts

Table 4

Consumption of concentrated sources of heterocyclic amines (bacon, breakfast sausage, gravy) and breast cancer risk

	Cases	Controls	OR (CI) [1]	Cases	Controls	OR (CI) [1]
Sources of heterocyclic amines(gms/month)	PREMENOPAUSAL			POSTMENOPAUSAL		
All women with genetic data						
< 58	25	28	1.0	45	53	1.0
58-149	26	31	0.8 (0.4-1.9)	39	55	0.8 (0.4-1.4)
149-464	45	27	2.0 (0.9-4.3)	43	59	0.7 (0.4-1.3)
> 464	22	28	0.9 (0.4-2.1)	58	46	1.4 (0.8-2.5)
NAT2 rapid						
< 58	11	9	1.0	25	31	1.0
58-149	9	13	0.8 (0.2-3.1)	20	31	0.8 (0.3-1.9)
149-464	21	11	2.7 (0.7-9.9)	29	29	0.3 (0.1-0.9)
> 464	10	16	0.9 (0.2-3.4)	31	22	1.0 (0.4-2.6)
NAT2 slow						
< 58	14	19	1.0	20	22	1.0
58-149	17	18	0.9 (0.3-2.8)	19	24	0.9 (0.4-2.0)
149-464	24	16	1.8 (0.6-5.4)	14	30	1.2 (0.5-2.6)
> 464	12	12	1.2 (0.3-3.9)	27	24	1.9 (0.9-4.3)

[1] Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer, and fruit and vegetable consumption.

Table 5

Frequency of consumption of gravy made from pan drippings and foods fried in bacon grease

	Cases	Controls	OR (CI) [1]	Cases	Controls	OR (CI) [1]
Frequency of gravy consumption	PREMENOPAUSAL			POSTMENOPAUSAL		
All women with genetic data						
never	10	10	1.0	23	34	1.0
< once per month	27	35	1.3 (0.4-3.6)	40	52	1.0 (0.5-2.1)
1-3 times per month	41	25	0.7 (0.4-1.6)	49	60	1.1 (0.5-2.1)
once per week-daily	40	44	1.9 (0.9-3.9)	73	67	1.6 (0.8-3.0)
Frequency of gravy consumption						
NAT2 rapid						
never	5	2	1.0	9	12	1.0
< once per month	11	14	3.8 (0.5-27.1)	21	28	0.9 (0.3-2.7)
1-3 times per month	19	9	0.7 (0.2-2.2)	19	26	0.8 (0.3-2.6)
once per week-daily	16	24	2.6 (0.8-8.7)	31	34	1.0 (0.3-3.0)
Frequency of gravy consumption						
NAT2 slow						
never	5	8	1.0	14	22	1.0
< once per month	16	21	0.6 (0.2-2.5)	19	24	1.0 (4-2.7)
1-3 times per month	22	16	0.7 (0.2-1.8)	30	34	1.3 (0.5-3.0)
once per week-daily	24	20	1.2 (0.4-3.1)	42	33	2.1 (0.9-5.0)
	Cases	Controls		Cases	Controls	

Table 5 (cont.)

Frequency of consumption of foods fried in bacon fat All women with genetic data	PREMENOPAUSAL			POSTMENOPAUSAL		
never	89	83	1.0	131	158	1.0
< once per month	16	17	0.8 (0.4-1.8)	22	28	0.9 (0.5-1.7)
1-3 times per month	6	7	0.7 (0.2-2.3)	20	16	1.6 (0.8-3.4)
once per week-daily	7	7	1.2 (0.4-3.9)	12	9	1.8 (0.7-4.5)
Frequency of consumption of foods fried in bacon fat						
NAT2 rapid						
never	38	31	1.0	58	75	1.0
< once per month	7	11	0.6 (0.2-1.8)	9	14	0.6 (0.2-1.6)
1-3 times per month	2	3	0.7 (0.1-4.9)	10	8	1.9 (0.6-5.5)
once per week-daily	4	4	1.1 (0.2-5.4)	3	2	1.7 (0.2-13.7)
Frequency of consumption of foods fried in bacon fat						
NAT2 slow						
never	51	52	1.0	73	83	1.0
< once per month	9	6	1.4 (0.4-4.8)	13	14	1.1 (0.5-2.6)
1-3 times per month	4	4	0.9 (0.2-4.0)	10	8	1.5 (0.5-4.3)
once per week-daily	3	3	1.3 (0.2-7.6)	9	7	1.6 (0.5-4.7)

[1] Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, body mass index, family history of breast cancer, and fruit and vegetable consumption.

APPENDIX B – Spontaneous abortions, *NAT2* and smoking

Data accepted by Epidemiology

Risk of Recurrent Spontaneous Abortion, Cigarette Smoking, and Genetic Polymorphisms in NAT2 and GSTM1

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Risk of Recurrent Spontaneous Abortion, Cigarette Smoking, and Genetic Polymorphisms in NAT2 and GSTM1

Abstract

Tobacco smoke exposure, a source of mutagenic aromatic amines (AAs), increases risk of spontaneous abortion. AAs are detoxified by polymorphic *N*-acetyltransferase (NAT2) and glutathione S-transferase (GSTM1). Genotypes and smoking were studied in women with recurrent spontaneous abortions (n=37) and those with at least two live births (n=211). Smoking increased risk (OR=1.62; CI, 0.80-3.31), but NAT2 or GSTM1 did not. Among smokers, however, risk was increased for those with slow NAT2 (OR=2.11; CI, 0.93-5.63), as well as those with null GSTM1 (OR= 1.79; CI=0.60-5.40), but not for women with wild-type alleles. Metabolizing enzymes may affect spontaneous abortion risk related to smoking.

keywords: N-acetyltransferase 2, glutathione S-transferase M1, spontaneous abortion, genetic polymorphism, gene-environment interaction

Introduction

Spontaneous abortion is a common reproductive event, occurring in approximately 12-15 percent of clinically recognized pregnancies. Studies of recurrent spontaneous abortion have suggested a role for genetics {1696,1697}and possibly for immunologic factors {1698}. Putative environmental factors implicated in spontaneous abortion include cigarette smoking and coffee consumption {1696}. Tobacco smoke contains numerous compounds, including mutagenic and carcinogenic aromatic amines {916,1016}. *N*-Acetyltransferase (NAT2) and glutathione S-transferase (GSTM1) are both involved in the detoxification of aromatic amines. The NAT2 slow acetylator phenotype is explained primarily by three point mutations, resulting in a protein that is either catalytically inefficient, has impaired stability, or is poorly expressed {1414}. Individuals with the null allele for GSTM1 are deficient for activity of that isozyme {75}. The association between polymorphic NAT2 and GSTM1 and risk of recurrent spontaneous abortion was previously investigated {1030}; the authors concluded that there was little evidence for an association. In this investigation, we sought to corroborate those findings in our study population. We were also interested in examining effects of a potential interaction between cigarette smoking and variability in metabolism of tobacco smoke carcinogens on risk for recurrent spontaneous abortion.

Study Subjects and Methods

This secondary analysis is based on data collected as part of a case-control study on the epidemiology of breast cancer. Caucasian postmenopausal women between the ages of 41 and 85 were enrolled between 1986 and 1991 in Western New York (439 women with breast cancer and

494 community controls). A more detailed description of the study population has been published elsewhere {166,1027}. Approximately 63% agreed to provide a blood sample (n=587) and DNA was extracted from specimens from 498 women (85%). Methods for DNA extraction from stored clots and genotyping for polymorphisms have been previously described {1027,47}. GSTM1 was evaluated for presence or absence (null) of alleles. For NAT2, 90-95% of the slow acetylation phenotype is predicted by mutations at $C^{481}T$, $G^{590}A$, and $G^{857}A$.

Reproductive histories and smoking data were obtained in a two-hour in-person interview. Women were asked about the outcome of each pregnancy they reported as part of a comprehensive reproductive history. Of those with genetic data available, the following were excluded from these analyses: nulliparous women (n=134), women who reported a history of only one spontaneous abortion (n=67), one livebirth (n=32), any stillbirth (n=11), or ectopic pregnancy (n=6). Cases were defined as women who had two or more spontaneous abortions, regardless of other pregnancy outcomes, and had genetic data available for NAT2 (n=37) and GSTM1 (n=32). The control groups were comprised of women with at least two livebirths and no spontaneous abortions who had NAT2 (n=211) and GSTM1 (n=166) data. All women with GSTM1 data also had NAT2 status available.

Participants gave a detailed smoking history including age started, times quit and amount smoked two, 10 and 20 years prior to the interview. Unfortunately, detailed smoking information during the reproductive years was usually unavailable since most women completed childbearing more than 20 years prior to the interview. For this analysis, women were crudely classified as ever or never smokers.

Statistical analyses included Student t-tests and chi-square tests to detect differences between means and proportions between cases and controls. Unconditional logistic regression analysis was utilized to obtain odds ratios (OR) with 95% confidence intervals (CI), computed from the standard error of the regression coefficient. Adjusted models did not change the point estimates appreciably; only unadjusted results are presented.

Results

Descriptive characteristics of the samples are shown in Table 1. The distributions for both genetic polymorphisms were within the expected range for Caucasian women. The reproductive factors are shown to illustrate the impact of the case and control definitions, such that women who are cases had more pregnancies than control women and a higher ratio of pregnancies to live births.

While smoking appeared to elevate risk (Table 2), no strong association was seen for either the NAT2 slow or GSTM1 null genotypes. However, genotype appeared related to risk in the presence of exposure to cigarette smoke (Table 3). Even with a crude measure of smoking (ever/never), risk was higher among smokers with the 'at risk' genotypes (NAT2 slow, GSTM1 null).

Discussion

In these data, we observed some evidence of effect modification by genetic polymorphisms in NAT2 and GSTM1 on the association of a crude measure of smoking and risk of recurrent spontaneous abortion.

Complete reproductive histories were available from postmenopausal women. Recall of pregnancy events including spontaneous abortions is reliable over time {1699}, although it is

somewhat limited for losses occurring early in gestation and by the length of time since the event {1700}. Misclassification of recurrent spontaneous abortion is unlikely and there is no evidence to suggest that reporting would be affected by genetic status (NAT2 or GSTM1) or "ever" smoking status. DNA analyses were conducted on a large proportion of women from the original cancer case-control study. All genotyping was done at the same laboratory with appropriate quality control protocols in place.

An extensive smoking history was taken in the interview including age started smoking, amount smoked two, 10 and 20 years prior to the interview and ages and time periods of quitting. However, the most relevant time frame for the present analysis, smoking at the time of pregnancies, was not available. We attempted to construct an index to reflect the likelihood of smoking during the reproductive years, but were not confident that this improved the smoking measure and the results were very similar to the data presented. Most women who reported smoking in these data appear to have smoked at some point during their reproductive years.

Additionally, the timing of the dietary exposure measurements did not allow us to analyze caffeine consumption, which was available for two years prior to the interview only. We did not feel confident extrapolating caffeine use back in time 20 to 60 years. Caffeine exposure may exhibit effect modification with NAT2 and GSTM1 similar to smoking with regard to spontaneous abortion risk {1701}.

Similar to a previous investigation, we did not observe a strong relation between polymorphisms in NAT2 and GSTM1 and risk of recurrent spontaneous abortion {1030}. It appears from our data that the interaction of exposure and genetic susceptibility is the important factor, rather than genetics alone. Both smoking and caffeine use have been associated with increased risk for spontaneous abortion. NAT2 and GSTM1 are important detoxification genes

with activity related to the metabolism of cigarette smoke and caffeine as well as other compounds.

Even with an extremely crude measure of lifetime smoking, we found some evidence of effect modification. Future studies of the potential influence of metabolism genes such as NAT2 and GSTM1 on spontaneous abortion risk should include appropriate measures of exposure and focus on gene-environment interactions.

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TABLE 1. Characteristics of the study samples, Western New York.

NAT2 Analysis		
	Cases (n=37)	Controls (n=211)
NAT2 Genotype	n (%)	n (%)
Rapid	16 (43)	100 (47)
Slow	21 (57)	111(53)
Smoking		
Ever	22 (59)	100 (47)
Never	15 (41)	111 (53)
Reproductive Factors	mean (sd)	mean (sd)
Number of pregnancies	7.2 (2.2)	3.4 (1.4)
Number of live births	4.4 (2.1)	3.4 (1.4)
Age at first pregnancy	22.4 (4.7)	23.3 (4.0)
***GSTM1* Analysis**		
	Cases (n=32)	Controls (n=166)
GSTM1 genotype	n (%)	n (%)
Wild-type	16 (50)	85 (51)
Null	16 (50)	81 (49)
Smoking		
Ever	19 (59)	80 (48)
Never	13 (41)	86 (52)
Reproductive Factors	mean (sd)	mean (sd)
Number of pregnancies	7.0 (2.1)	3.5 (1.6)
Number of live births	4.3 (1.9)	3.5 (1.6)
Age at first pregnancy	22.8 (4.6)	23.3 (4.2)

Note: Cases had two or more spontaneous abortions regardless of other pregnancy outcomes. Controls had two or more livebirths and no spontaneous abortions.

Table 2. Unadjusted Risk of Recurrent Spontaneous Abortion in Association with Smoking Status, and Polymorphic Detoxification Genes, Western New York.

	Odds Ratio (95% Confidence Interval)
Never Smoker	1.0 (Reference)
Ever Smoker	1.6 (0.8-3.31)
NAT2 Rapid	1.0 (Reference)
NAT2 Slow	1.2 (0.5-2.4)
GSTM1 Wild-type	1.0 (Reference)
GSTM1 Null	1.0 (0.5-2.2)

Table 3. Unadjusted Risk of Recurrent Spontaneous Abortion Associated with Smoking Stratified by Polymorphic Detoxification Gene Status, Western New York.

	NAT2 Rapid OR (CI)[1]	**NAT2 Slow** OR (CI)
Never Smoker	1.0 (reference)	1.0 (Reference)
Ever Smoker	1.2 (0.4-3.4)	2.1 (0.9-5.3)
	GSTM1 wild-type	**GSTM1 null**
Never Smoker	1.0 (reference)	1.0 (Reference)
Ever Smoker	1.3 (0.5-3.9)	1.8 (0.6-5.4)

[1] Odds ratios and 95% confidence intervals calculated using unconditional logistic regression
Interaction NAT2*smoking ever/never: Beta=-0.2182; p=0.16
Interaction: GST*smoking ever/never: Beta=-0.232; p=0.92

APPENDIX C - *NAT1* Unpublished results

Table 1
NAT1 genetic polymorphisms in premenopausal and postmenopausal cases and controls: Western New York Breast Cancer Study.

Genotype combinations	Cases (n=127) n	(%)	Controls (n=134) n	(%)	Cases (n=181) n	(%)	Controls (n=224) n	(%)
	PREMENOPAUSAL				POSTMENOPAUSAL			
4/3	4	(3)	2	(2)	7	(4)	7	(3)
4/4	78	(61)	79	(64)	116	(64)	130	(58)
10/3	1	(1)	1	(1)	1	(1)	0	(0)
4/10	33	(26)	29	(23)	40	(22)	59	(26)
4/11	3	(2)	8	(6)	9	(5)	10	(5)
10/10	8	(6)	4	(6)	5	(3)	14	(6)
11/10	0	(0)	1	(1)	1	(1)	4	(2)
11/11	0	(0)	0	(0)	2	(1)	0	(0)

Table 2
NAT1 Genotypes, Cigarette Smoking, and Risk of Breast Cancer: Western New York Breast Cancer Study.

NAT1 Genotype[1]	Cases (%)	Controls (%)	OR (CI) [2]	Cases (%)	Controls (%)	OR (CI) [2]
	PREMENOPAUSAL			POSTMENOPAUSAL		
All women with genetic data						
Slow	85 (67)	89 (72)	1.0	134 (74)	147 (66)	1.0
Rapid	42 (33)	35 (28)	1.3 (0.7-2.2)	47 (26)	77 (34)	0.7 (0.5-1.1)
Non-smokers						
Slow	57 (69)	64 (74)	1.0	62 (69)	73 (64)	1.0
Rapid	26 (31)	22 (26)	1.4 (0.7-2.8)	28 (31)	41 (36)	0.8 (0.5-1.6)
Smokers [3]						
Slow	28 (64)	25 (66)	1.0	72 (79)	74 (67)	1.0
Rapid	16 (36)	13 (34)	1.1 (0.4-3.0)	19 (21)	36 (33)	0.6 (0.3-1.1)

[1]Rapid genotype are women with any *NAT1*10* alleles, slow genotype are those with others (*3, *4, *11).
[2] Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, age at menopause, body mass index, family history of breast cancer.
[3] Women were classified as smokers if they had smoked more than 1 cigarette per day for at least one year.

Table 3
NAT1 and *NAT2* Genotypes, Cigarette Smoking, and Risk of Breast Cancer: Western New York Breast Cancer Study.

NAT1 Genotype[1]	Cases (%)	Controls (%)	OR (CI) [2]	Cases (%)	Controls (%)	OR (CI) [2]
	NAT1			*NAT2*		
Non-smokers						
Rapid	62 (69)	73 (64)	1.0	41 (49)	59 (54)	1.0
Slow	28 (31)	41 (36)	1.2 (0.6-2.2)	43 (51)	50 (46)	0.9 (0.5-1.7)
Smokers [3]						
Rapid	19 (21)	36 (33)	1.0	37 (37)	50 (48)	1.0
Slow	72 (79)	74 (67)	1.8 (0.9-3.5)	64 (63)	54 (52)	1.7 (0.9-3.0)

[1]Rapid genotype are women with any *NAT1**10* alleles, slow genotype are those with others (*3, *4, *11).
[2] Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, age at menopause, body mass index, family history of breast cancer.
[3] Women were classified as smokers if they had smoked more than 1 cigarette per day for at least one year.

Table 4
Risk of breast cancer related to combinations of *NAT1* and *NAT2* genotypes among postmenopausal women: Western New York Breast Cancer Study.

NAT1, NAT2	Cases n (%)	Controls n (%)	OR (CI) [1]
NON-SMOKERS			
rapid, rapid [2]	16 (23)	19 (21)	1.0
rapid, slow	7 (10)	12 (13)	0.6 (0.2-2.2)
slow, rapid	18 (26)	24 (26)	0.7 (0.3-1.9)
slow, slow	29 (41)	37 (40)	0.9 (0.4-2.2)
SMOKERS			
rapid, rapid [2]	6 (8)	18 (23)	1.0
rapid, slow	9 (12)	12 (15)	1.7 (0.5-6.3)
slow, rapid	21 (27)	19 (24)	2.6 (0.8-8.3)
slow, slow	41 (53)	31 (39)	4.0 (1.3-11.7)

[1] Odds ratios and 95% confidence intervals calculated by logistic regression, adjusted for age, education, age at menarche, age at first pregnancy, age at menopause, body mass index, family history of breast cancer.
[2] Reference category are women with rapid *NAT1* and rapid *NAT2* genotypes
[3] Women were classified as smokers if they had smoked more than 1 cigarette per day for at least one year.

APPENDIX D - Breast cancer, alcohol consumption and ADH genotypes

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Alcohol Dehydrogenase 3 Genotype and Risk of Breast Cancer Associated with Alcohol Consumption

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Running head: ADH_3 and breast cancer risk

Abbreviations used: Alcohol dehydrogenase (ADH), body mass index (BMI), confidence interval (CI), estrogen replacement therapy (ERT), reduced nicotinamide adenine dinucleotide (NADH), odds ratio (OR), polymerase chair reaction (PCR)

Key words: breast neoplasms, alcohol dehydrogenase, alcohol, epidemiology

Abstract

Background: Accumulating, although inconsistent, epidemiologic evidence implicates alcohol consumption in breast cancer risk. Alcohol dehydrogenase 3 (ADH_3), important in oxidation of alcohols, is polymorphic in Caucasian populations. The ADH_3^{1-1} genotype is associated with more rapid metabolism of ethanol to acetaldehyde, a possible human carcinogen. We conducted a case-control study of alcohol and breast cancer risk examining modifying effects of the Exon VIII ADH_3 polymorphism.

Methods: Caucasian women in western New York, aged 40-85 were included. Cases (134 premenopausal, 181 postmenopausal) had incident, primary, pathologically-confirmed breast cancer. Controls (126 premenopausal, 230 postmenopausal) were frequency-matched to cases on age and county. Lifetime alcohol intake was ascertained by interview. DNA, extracted from blood clots, was genotyped by PCR. Odds ratios (OR) and 95% confidence intervals (CI) were computed by unconditional logistic regression.

Results: For all analyses, women who were lighter drinkers with ADH_3^{2-2} or ADH_3^{1-2} genotypes were the referent. The ADH_3^{1-1} genotype was weakly and non-significantly associated with risk among premenopausal but not postmenopausal women. Among premenopausal women, increased risk associated with higher alcohol consumption was limited to women with ADH_3^{1-1} (OR 2.68, 95% CI 1.05-6.85); the OR was close to unity for all other groups. There was no association between risk and alcohol consumption for postmenopausal women.

Conclusions: Among premenopausal women, alcohol consumption is associated with breast cancer risk for those with the ADH_3^{1-1} genotype. If this relationship is seen in other studies, these findings would support the hypothesis of alcohol in breast cancer risk and provide indications of a role of acetaldehyde in breast carcinogenesis.

Introduction

While there is evidence that alcohol consumption may increase the risk of breast cancer (1-3), results have not been consistent and the mechanism of action is not well understood. It is possible that genetic differences in the metabolism of alcohol may alter the relation of alcohol exposure to breast cancer risk. Evaluation of heterogeneous groups may mask susceptible subgroups and impair estimation of risks. In this study, we evaluated the effect of a polymorphism in alcohol dehydrogenase, a key enzyme in alcohol metabolism, in terms of its effect on the relation between alcohol intake and breast cancer risk.

Alcohol dehydrogenase (ADH) plays a rate-limiting role in the metabolic pathway for most human ethanol oxidation and catalyzes the oxidation of ethanol to acetaldehyde (4). Dimeric class I ADH enzymes are comprised of subunits encoded by genes designated as ADH_1, ADH_2, and ADH_3. Genetic variants with altered kinetic properties have been identified at the ADH_2 and ADH_3 loci. In one study of the ADH_3 gene, approximately 58%, 91% and 88% of European whites, Asians and Africans, respectively had the ADH_3^1 allele (5). *In vitro,* there is more than two-fold difference in V_{max} between the ADH_3 genotypes (4). The ADH_3^1 allele codes for the more rapid form of the enzyme. There are also differences by ADH_3 genotype in release of NADH by the enzyme. The aldehyde dehydrogenase family of enzymes (ALDH) is also involved in alcohol metabolism. Variant alleles with altered kinetic activities have been identified in the $ALDH_2$ gene. Because polymorphisms in ADH_2 and $ALDH_2$ are rare in Caucasian populations, for our study of Caucasians, we examined modification only by the ADH_3 polymorphism.

While there are, to our knowledge, no reports on the effect of the genetically-determined differences in alcohol metabolism in relation to breast cancer risk, there

have been reports of an association of the ADH_3^{1-1} genotype with increased risk of cancer of the oral cavity and pharynx (6,7) and of hepatic cirrhosis and chronic pancreatitis (8). We report here on the results of a case-control study of breast cancer risk with an examination of differential effects of alcohol consumption among women with different ADH_3 genotypes.

Materials and Methods

We conducted a case-control study of breast cancer in pre- and postmenopausal women in western New York State. All participants provided written informed consent; procedures for protection of human subjects in this study were approved by the Human Subjects Review Board of the State University of New York at Buffalo School of Medicine and Biomedical Sciences and of each of the participating hospitals. The women in the study were between the ages of 40 and 85, residents of Erie and Niagara counties, alert, able to speak English and in sufficiently good health to be interviewed; all were Caucasian. Women were considered to be premenopausal if they were currently menstruating or, if they were not menstruating because of a hysterectomy or other medical intervention, if they had at least one of their ovaries and were less than age 50. All other women were considered to be postmenopausal.

Women with incident, primary, histologically-confirmed breast cancer were identified from pathology records of all the major hospitals in the two counties; case ascertainment was conducted in the period beginning November, 1986 and ending October, 1989 for postmenopausal cases, and ending April, 1991 for premenopausal cases. The physician of each woman identified with breast cancer was contacted to obtain consent to allow us to invite the woman for an interview. Of eligible cases, 66% of

premenopausal and 54% of postmenopausal cases were interviewed. Physician refusal to allow us to contact their patients accounted for most of the lack of participation, 74% and 71% of nonparticipation for pre- and postmenopausal women, respectively. Interviews were conducted, on average, two months after diagnosis.

Controls were frequency-matched to cases on age and county. The listing of licensed New York State drivers was used for random selection of women under age 65; women age 65 and over were randomly selected from the listing of the Health Care Finance Administration. Interviewed were 62% and 44%, respectively, of the eligible pre- and postmenopausal controls. Because controls under age 65 were licensed drivers, we asked the cases under 65 if they had driver's licenses. Nine did not hold a driver's license. Compared to cases with licenses, women without licenses were slightly less educated and slightly, though not significantly older. All are included in these analyses. For a subset of participating controls and those refusing to participate, we conducted a very brief phone interview querying usual frequency of consumption of several foods. These participants and non-participants did not differ in reported intake of vegetables, fruits, meat or coffee. Non-participants were somewhat more likely to smoke. Information was not collected on alcohol intake in this comparison of participants and non-participants (9,10).

Interviews Interviews were conducted in the participants' homes by trained interviewers. The interview lasted, on average, two hours. Details of the interview have been described elsewhere (9-11). Included in the interview were questions regarding usual diet in the year two years before interview, reproductive history, medical history, family history of cancer, smoking history (pack-years) and other breast cancer risk factors. Body mass index (BMI) was calculated from reported height and weight, weight (kg)/height^2(m^2). Family history of breast cancer was defined as having at least one first-degree relative (mother, sister, daughter) with breast cancer.

Questions regarding alcohol intake included queries of the usual frequency of intake and number of drinks per occasion for wine, beer and hard liquor during the year two years ago, 10 years ago, 20 years ago and at age 16. Total alcohol intake was calculated as the sum of the reported number of drinks of beer, wine and hard liquor under the assumption that the alcohol content for one glass of beer or wine or one shot of hard liquor were approximately the same. An index of usual alcohol consumption in the last 20 years was estimated by summing the intake reported for two years ago multiplied by six with the intake reported for 10 years ago multiplied by seven and with the intake reported for 20 years ago multiplied by seven.

At the end of the interview, participants were asked to provide a blood sample following an additional informed consent. About 45% of premenopausal and 63% of postmenopausal participants gave a blood sample.

Molecular Genetic Analyses All analyses were conducted at the Laboratory for Human Carcinogenesis at the National Cancer Institute. DNA was extracted from blood clots (11). As previously described (6), a 145 bp fragment including the Exon VIII polymorphism was amplified by the polymerase chain reaction (PCR) using a modification of the method of Groppi et al. (12). The highly homologous ADH_1 and ADH_2 genes were digested with the NlaIII restriction enzyme prior to the PCR. An aliquot of this digestion mixture was then subject to PCR and subsequent SspI enzymatic digestion to reveal the ADH_3 genotype (i.e., ADH_3^{1-1}, ADH_3^{1-2} , or ADH_3^{2-2}). Every 14 samples contained a positive and negative control. The results were scored separately by two authors independently who were blinded to all identifying data including subjects' case-control status. Twenty percent of samples were repeated for quality control. In the adjusted analyses, NAT2 genotype was included as an adjusting variable; methodology for the NAT2 analyses have been described previously (11).

The final sample for this report included 134 premenopausal cases and 126 premenopausal controls, 181 postmenopausal cases and 230 postmenopausal controls, those women whom we interviewed and whose ADH_3 genotype could be determined. Because we did not get blood samples from all participants who completed the interview nor were we able to successfully determine the ADH_3 polymorphism on all blood samples, we compared the characteristics of those included in this report with the entire group included in the case-control study; comparisons of means were made using the student's t-test. Those with and without ADH_3 data were largely similar with a few exceptions. Differences ($p<0.05$) among premenopausal women were that those with data tended to be older, have higher parity and to drink less beer than those without. Among postmenopausal women, the only characteristic that was significantly different was age; those with ADH_3 data were older.

Statistical Analysis Because there are indications that there are differences in the risk factors for pre- and postmenopausal breast cancer (13) and in particular because there may be differences in the relation of alcohol intake to risk by menopausal status (1), analyses were stratified on menopausal status. For potential confounding factors, means and standard deviations for groups defined by ADH_3 genotype and by case-control status were compared by one-way analysis of variance, with a two-tailed test of significance (14). Odds ratios (OR) and 95% confidence intervals (CI) were calculated using unconditional logistic regression (15). For analyses of categorical data, risk was calculated relative to the indicated referent category. Cutoffs for categories of alcohol intake were at the median level of intake for controls. Adjusted analyses included control for age, education, family history of breast cancer, reported history of benign breast disease, BMI, parity, age at first birth, age at menarche, fruit and vegetable intake, smoking history, NAT2 status, smoking by NAT2 interaction and, for postmenopausal women, age at menopause. Risk associated

with the genotype was calculated and then risks associated with alcohol intake both without and with stratification on ADH_3 genotype were calculated. Because of issues regarding differential recall for cases and controls in case-control studies, we also examined a case-case analysis for the effect of alcohol dehydrogenase status; alcohol intake was regressed on ADH genotype among the cases with the ADH_3^{2-2} and ADH_3^{1-2} groups combined as the referent with comparison to ADH_3^{1-1} (16,17).

Results

The associations between reported alcohol consumption in the last 20 years and risk of breast cancer in this sample of individuals with available genetic data are shown in Table 1. For all analyses, the cutoff between the low and high drinkers was 6.5 and 4.4 drinks per month, on average over the last 20 years, for the pre- and postmenopausal women, respectively. For both pre- and postmenopausal women, there was no strong evidence of an association between alcohol intake and breast cancer risk although for the premenopausal women there was a suggestion of increased risk among heavier drinkers with confidence intervals including the null. Similar results were obtained when all the data, including that from participants who did not provide a blood sample, were analyzed.

In Table 2, breast cancer risk factors are shown for groups defined by the three genotypes. In general, characteristics among the three groups were similar. In one-way analysis of variance, the reported alcohol intakes of premenopausal cases with the ADH_3^{2-2} genotype were significantly higher than those with the ADH_3^{1-2} ($p<0.05$), but not the ADH_3^{1-1} genotype. There were also some differences for smoking between the homozygotes and the heterozygotes among the premenopausal women. Among

postmenopausal women, alcohol consumption and smoking did not differ for the different groups; there was a difference by genotype for education among the cases.

In Table 3, the associations of the ADH_3 genotypes with risk are shown. There was a weak increase in risk for the premenopausal women associated with the ADH_3^{1-1} genotype; the confidence interval included the null value (adjusted OR 1.54; 95% CI 0.61-3.92). There was no evidence of an association of genotype with risk for the postmenopausal women. Odds ratios estimated without adjustment for alcohol intake were similar to those shown here.

We also examined risk of breast cancer associated with the ADH_3^{1-1} genotype, when the referent was the ADH_3^{2-2} and ADH_3^{1-2} genotype groups combined. For premenopausal women, the odds ratio was 1.86 (95% CI, 0.96-3.60); for postmenopausal women the odds ratio was 1.22 (95% CI, 0.72-2.08) (data not shown).

In Table 4, risk associated with alcohol intake by ADH_3 genotype is shown. The referent was women with lower intake of alcohol and either the ADH_3^{2-2} or ADH_3^{1-2} genotype. (We also examined these analyses with ADH_3^{2-2} as the referent. The results were similar to those shown here. However, the findings were less stable because the sample size in the reference group was small and confidence intervals were wider.) Among the premenopausal women, odds ratios were generally close to the null and confidence intervals included the null for all categories with one exception. Among women who drank more than the median intake and who had the ADH_3^{1-1} genotype, the odds ratio was 2.68 with 95% confidence interval 1.05-6.85. We also examined risk associated with alcohol within the group of women with the ADH_3^{1-1} genotype. With lighter drinkers as the referent, the adjusted odds ratio for drinking more than the median of alcohol was 3.51, 95% confidence interval 1.30-9.56 (data not shown). Among postmenopausal women, there was no evidence of an association of alcohol

intake and risk when modification by ADH_3 was taken into account. Because of reports that an increased risk associated with alcohol consumption among postmenopausal women may be restricted to those who have used estrogen replacement therapy (ERT) (18,19), we also looked at the risk among women who had ever used ERT. In that group, there was weak evidence of an increased risk among the heavier drinkers with the ADH_3^{1-1} genotype compared to lighter drinkers with the other ADH_3 genotypes (adjusted OR 1.29, 95% CI 0.88-1.69). Sample size was quite small for the cells in this analysis; there were only 10 cases and nine controls with the ADH_3^{1-1} genotype who had ever used ERT. Heavier alcohol intake and the ADH_3^{1-1} genotype was unrelated to risk among those who had never used ERT (adjusted OR 1.0; 95% CI 0.95-1.60). All of these analyses were based on reports of alcohol consumption in the last 20 years. We had also queried regarding alcohol intake at age 16. The number of drinkers at that age was too small to estimate whether there was a modifying effect of ADH_3 genotype.

In a case-case analysis, we examined risk associated with the ADH_3^{1-1} genotype again with the combined ADH_3^{1-2} and ADH_3^{2-2} groups as the comparison. As for the case-control analyses, there was evidence of some increase in risk associated with the ADH_3^{1-1} genotype for pre- but not postmenopausal women. For premenopausal women, there was almost a doubling of risk for women drinking more than the median compared to lighter drinkers but the confidence interval included the null (adjusted OR 1.88, 95% CI 0.86-4.18). For the postmenopausal women the adjusted OR was 0.95 and the 95% CI was 0.45-1.86 (data not shown).

Discussion

In this case-control study of breast cancer, we found evidence that, for premenopausal women, those with the ADH_3^{1-1} genotype and with heavier alcohol

intake, risk of breast cancer may be more than two-fold greater than risk for women with this genotype who drink less or who have the other ADH_3 genotypes. To our knowledge, this is the first study of the relation of the ADH_3 polymorphism with alcohol and breast cancer risk. As noted above, there is some indication of an increase in risk of other alcohol-related diseases among individuals with the ADH_3^{1-1} genotype, including reports of a 2.5 to 6-fold increase in risk of oral and pharyngeal cancer (6,7). In this study, we saw effects of alcohol at modest levels of intake. In the study of oral cancer, the effect of ADH_3 genotype was limited to individuals with very high intakes of alcohol. One possible reason for that difference may relate to the ethanol effect on oral tissues being a local effect while in breast cancer, it is a systemic exposure. There are a considerable number of studies that indicate that alcohol is related to increased risk of breast cancer (1-3). Some (20-24), but not all (1) studies find risk associated with alcohol intake particularly among premenopausal women. This association of alcohol consumption, ADH_3 genotype and risk of breast cancer may provide some indication as to the mechanism of effect of alcohol exposure.

There are reasons to believe that ADH_3 genotype, alcohol consumption and breast cancer etiology are related. Alcohol metabolism in humans is regulated primarily by the ADH system of enzymes. There is considerable evidence that acetaldehyde, the product of alcohol dehydrogenase oxidation of alcohol, has carcinogenic properties (25). Acetaldehyde is mutagenic and carcinogenic in experimental animals. In short term cell culture assays, including assays of human cells, acetaldehyde but not ethanol is mutagenic (26,27). Acetaldehyde effects *in vitro* include DNA adducts (28,29), DNA crosslinks and DNA-protein crosslinks (30,31) and inhibition of DNA repair (30). The International Agency for Research on Cancer (IARC) has indicated that the evidence regarding acetaldehyde is sufficient for it to be designated as a carcinogen in

experimental animals (32). *In vitro,* the V_{max} for ADH_3^{1-1} is more than two-fold greater than for ADH_3^{2-2} (4) and may therefore contribute to increased exposure to acetaldehyde. It should be noted, however, that in one study in Caucasians, a difference in ethanol levels was not found for different ADH_3 genotypes (33). There is evidence of measurable levels of circulating acetaldehyde in premenopausal women after consumption of moderate amounts of alcohol during the high estrogen phases of the menstrual cycle (34,35). There is also evidence of acetaldehyde excretion in human milk (36); however, the determinations in milk were not made in conjunction with alcohol consumption. ADH_3 expression is greatest in the liver; however there is evidence of ADH_3 activity in other organs (37-42) with an indication of expression particularly in epithelial cells (41).

Another possible mechanism involving ADH and alcohol is with regard to steroid hormone metabolism. There is strong evidence that estrogen exposure is an important contributor to breast cancer risk (43). Alcohol consumption appears to affect estrogen levels; there is evidence that both acute (44-46) and chronic (47-49) alcohol consumption lead to increased estrogen levels in premenopausal women and in postmenopausal women who take exogenous estrogen. ADH_3 also is involved in steroid hormone metabolism and is inhibited by testosterone (50,51). If the effect of ADH_3 on risk is the result of an interaction with steroid hormones, that mechanism might explain why we saw an effect only among premenopausal women with some indication of an effect among postmenopausal women who had ever used estrogen replacement therapy.

Given the toxic effects of acetaldehyde, the apparent likelihood of exposure to breast tissue of acetaldehyde and the interactions of alcohol, ADH_3 and estrogens, these mechanisms together may explain, at least in part, an effect of alcohol

consumption on breast cancer etiology. There are other possible mechanisms that may also explain an effect of alcohol on breast cancer and also need to be considered. These include effects on cell membrane integrity, immune function, DNA repair and effects of other components of alcoholic beverages (44).

In interpretation of these findings regarding breast cancer risk, several potential sources of bias need to be considered. In this study, all measures of alcohol intake were by self-report and contain error. However, there is some evidence that reliability of recall of intake of alcohol in the past five to ten years is relatively good (52,53), although current drinking practices may bias recall of intake (52). In data such as ours, there is also the concern of recall bias, that women with recently diagnosed breast cancer may report their previous alcohol intake differently than the healthy controls do. However, in one study, this potential source of bias accounted for only a small reduction in the risk estimate with bias toward the null (54). As for the measure of ADH_3 status, there may also be some misclassification of a clinically significant ethanol oxidation phenotype. Methodologically, however, laboratory personnel were blinded to case-control status; error with regard to ADH_3 status would therefore be non-differential and would contribute to an attenuation of the measure of risk (55). In terms of the selection of the sample, while every effort was made to include a population-based sample in this study, there were several sources of non-participation. For the cases, the largest source of non-participation was the refusal of physicians to allow us to contact the women. It may be that this lack of inclusion reflects physician rather than patient characteristics, but we could not verify whether or not this was true. Among the controls, we do have some evidence that at least for dietary intake, there were no differences among participants and those who did not participate (9,10). There may have been differences in alcohol

intake of those refusing to participate; in particular, it is possible that the heaviest drinkers in the population were underrepresented.

For both cases and controls, there is no reason to believe that participation would be related to ADH_3 polymorphism; the frequency of the ADH_3^1 and ADH_3^2 alleles measured in this population (59% and 41%, respectively among the controls) were similar to those reported by others (4,5,56). ADH_3 would be unlikely to affect alcohol consumption; studies of ADH_3 in Caucasians have not shown there to be differences in risk of alcoholism associated with the ADH_3 genotype (8,56). We did not find any difference in alcohol intake by ADH_3 polymorphism among the controls. Among premenopausal cases, reported alcohol intake was lower for the $ADH_3^{1\text{-}2}$ genotype than for the $ADH_3^{2\text{-}2}$. There were no differences in intake for the other comparisons within in the cases, for the controls or for the postmenopausal cases or controls.

Finally, there is also the possibility that these findings were the result of chance. Given the small samples in some of the cells of analysis and given the issues of potential bias, these results necessarily need to be considered as preliminary and await confirmation by other epidemiologic studies. Because of the restriction by sample size, we were only able to categorize participants into two levels of drinking. The group of heavier drinkers necessarily included women whose alcohol consumption was in fact rather low. Additionally, the group of lighter drinkers included both non-drinkers and those who drink less frequently.

From these data, it does appear genetic differences in alcohol metabolism by ADH_3 need to be considered as possible modifiers of the effect of alcohol intake on breast cancer. As in other studies of alcohol and breast cancer that have not included stratification by ADH_3, the effect was found particularly among premenopausal women (with a weak indication of possible increase in risk among postmenopausal women who

had ever taken exogenous estrogen). In other studies, consideration of genetic variation in ADH_2 and ALDH, which we were not able to study, is warranted. Our findings of a modification of effect by ADH_3 genotype, if confirmed in other studies, may shed some light on the possible mechanism of an alcohol consumption effect on breast cancer risk.

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Table 1

Alcohol Consumption in the Last 20 Years and Risk of Breast Cancer, Western New York, 1987-1991 (Subgroup of Women with Alcohol Dehydrogenase 3 (ADH_3) Genotype Measured)

Alcohol	Cases	Controls	Crude OR	Adjusted OR*	95% CI
Premenopausal					
Low	54	63	1.00	1.00	
High	80	63	1.48	1.42	(0.84-2.41)
Total	134	126			
Postmenopausal					
Low	93	113	1.0	1.00	
Moderate	88	117	0.93	0.96	(0.57-1.61)
Total	181	230			

*OR = odds ratio; CI = confidence interval. Adjusted for age, education, family history of breast cancer, history of benign breast disease, BMI, parity, age at first birth, age at menarche, fruit and vegetable intake, smoking history, NAT2 status, smoking x NAT2, and age at menopause (postmenopausal women only).

Table 2
Characteristics of Study Sample by Case and Control Status and Alcohol Dehydrogenase 3 (ADH_3) Genotype

Premenopausal Women

Characteristic*	Cases			Controls		
	ADH_3^{1-1}	ADH_3^{1-2}	ADH_3^{2-2}	ADH_3^{1-1}	ADH_3^{1-2}	ADH_3^{2-2}
Age (yrs)	46.2 (4.6)	46.8 (4.0)	45.0 (3.2)	46.2 (3.2)	46.9 (3.7)	47.5 (4.5)
Education (yrs)	13.6 (2.5)	14.0 (3.2)	14.1 (2.5)	14.1 (2.4)	13.6 (2.8)	14.2 (2.6)
Age at menarche (yrs)	12.6 (1.8)	12.4 (1.6)	12.5 (1.2)	13.1 (1.9)	12.9 (1.7)	13.3 (1.7)
Body Mass Index**	24.2 (5.2)	25.2 (6.0)	24.7 (4.8)	25.2 (4.6)	25.6 (4.1)	26.6 (6.0)
History of benign breast disease (% of cases or controls)	22	20	7	15	13	9
Family history of breast cancer (% of cases or controls)	7	7	2	0.01	0.03	0.01
Total Alcohol** (drinks/month)	16.8 (20.2)	9.7^{a} (11.3)	20.2^{a} (24.4)	14.2 (28.0)	13.4 (18.1)	12.5 (12.8)
NAT2 (% rapid of cases or controls)	24	13	5	13	18	10
Parity	2.5 (1.6)	2.1 (1.5)	1.9 (1.3)	2.4 (1.6)	2.8 (1.8)	3.0 (1.7)
Age at first birth (yrs)	24.0 (4.4)	24.0 (4.8)	23.8 (5.2)	22.8 (4.0)	21.9 (4.1)	21.8 (4.0)
Vegetable intake** (gm/day)	459(220)	395 (180)	419 (175)	462 (190)	473 (201)	450 (155)
Fruit intake** (gm/day)	239 (133)	210 (141)	170 (125)	272(170)	245 (149)	216 (112)
Smoking (pack-yrs)	11.7^{b} (16.4)	5.8^{b} (10.1)	12.6 (14.4)	5.7^{c} (11.4)	$11.7^{c,d}$ (16.6)	4.8^{d} (8.9)

Table 2 continued

Postmenopausal Women

	Cases			Controls		
Characteristic*	**$ADH_3^{1\text{-}1}$**	**$ADH_3^{1\text{-}2}$**	**$ADH_3^{2\text{-}2}$**	**$ADH_3^{1\text{-}1}$**	**$ADH_3^{1\text{-}2}$**	**$ADH_3^{2\text{-}2}$**
Age (yrs)	64.9 (6.4)	63.6 (7.8)	61.9 (7.5)	63.4 (7.7)	63.1 (7.2)	61.6 (6.7)
Education (yrs)	12.2[e] (2.6)	12.3 (2.9)	13.4[e] (3.2)	12.3 (2.6)	12.0 (2.3)	12.7 (2.5)
Age at menarche (yrs)	13.0 (1.8)	13.0 (1.6)	12.6 (1.4)	12.7 (1.7)	13.1 (1.6)	12.6 (1.3)
Age at menopause (yrs)	47.8 (5.3)	47.6 (6.1)	46.8 (5.5)	46.2 (6.0)	47.6 (5.3)	47.0 (6.0)
Body Mass Index**	25.7 (5.3)	26.0 (5.0)	25.6 (3.6)	25.2 (4.2)	25.7 (5.4)	25.4 (4.7)
History of benign breast disease (% of cases or controls)	6	12	2	8	8	3
Family history of breast cancer (%)	5	6	5	2	6	1
Total Alcohol** (drinks/month)	11.8 (21.9)	17.1 (31.4)	17.7 (29.8)	10.6 (16.6)	15.9 (25.2)	12.6 (15.2)
NAT2 (% rapid of cases or controls)	14	17	11	18	20	9
ERT (% ever used of cases or controls)	9	11	6	10	15	9
Parity	3.1 (2.0)	2.7 (2.0)	3.2 (2.8)	2.8 (2.2)	3.1 (2.0)	2.9 (1.8)
Age at first birth (yrs)	24.8 (5.0)	24.1 (4.9)	23.4 (5.2)	23.3 (4.6)	23.5 (4.6)	23.3 (3.8)
Vegetable intake** (gm/day)	451 (201)	406 (175)	417 (207)	458 (237)	456 (227)	484 (334)
Fruit intake** (gm/day)	298 (175)	254 (175)	287 (177)	306 (186)	282 (172)	308 (218)
Smoking (pack-yrs)	14.6 (21.0)	17.4 (21.3)	16.9 (29.1)	12.9 (16.5)	13.8 (19.3)	13.0 (23.0)

*Values shown are mean (SD) except for history of benign breast disease and family history of breast cancer which are percent with positive history and NAT2 which are percent with rapid genotype. Two-sided comparisons of means among the ADH_3 groups within cases or controls were computed by ANOVA; those with the same letter are significantly different, $p<0.05$.

**Body mass index (kg/m^2) calculated from reported height and weight two years before the interview. Alcohol values are average drinks per month during the last 20 years, calculated from the weighted average of reported consumption two, 10 and 20 years ago. Vegetable and fruit intake is reported intake in the year two years before the interview.

Table 3

Alcohol Dehydrogenase 3 Polymorphisms and Risk of Breast Cancer, Western New York, 1987-1991

ADH_3	Cases	Controls	Crude OR	Adjusted OR*	95% CI*
Premenopausal					
2-2	21	24	1.00	1.00	
1-2	50	60	0.95	0.77	(0.31-1.94)
1-1	63	42	1.71	1.54	(0.61-3.92)
Total	134	126			
Postmenopausal					
2-2	28	35	1.00	1.00	
1-2	89	114	0.98	0.98	(0.45-2.15)
1-1	64	81	0.99	1.16	(0.52-2.58)
Total	181	230			

*Adjusted for age, education, alcohol intake, family history of breast cancer, history of benign breast disease, BMI, parity, age at first birth, age at menarche, fruit and vegetable intake, smoking history, NAT2 status, smoking x NAT2, and age at menopause (postmenopausal women only).

Table 4

Lifetime Alcohol Consumption by ADH_3 Genotype and Risk of Breast Cancer, Western New York, 1987-1991

Alcohol	Cases	Controls	Crude OR	Adjusted OR*	95% CI*
Premenopausal					
$ADH_3^{2-2} + ADH_3^{1-2}$					
Low	33	38	1.00	1.00	
High	38	46	0.95	0.72	(0.31-1.66)
ADH_3^{1-1}					
Low	21	25	0.97	0.82	(0.31-2.18)
High	42	17	2.84	2.68	(1.05-6.85)
Postmenopausal					
$ADH_3^{2-2} + ADH_3^{1-2}$					
Low	60	69	1.00	1.00	
High	57	80	0.82	1.08	(0.56-2.07)
ADH_3^{1-1}					
Low	34	46	0.85	1.28	(0.63-2.57)
High	30	35	0.99	1.18	(0.53-2.64)

*Adjusted for age, education, family history of breast cancer, history of benign breast disease, BMI, parity, age at first birth, age at menarche, fruit and vegetable intake, smoking history, NAT2, NAT2xsmoking, and age at menopause (postmenopausal women only).

APPENDIX E – Unpublished *GSTT, GSTM1* and *CYP1A1* genetic polymorphism gene frequencies

Genotype	Premenopausal	Postmenopausal
CYP1A1		
WW	179 (0.87)	na
WM	24 (0.11)	na
MM	3 (0.1)	na
GSTM1		
PRESENT	105 (0.50)	na
NULL	107 (0.50)	na
GSTT		
PRESENT	145 (0.68)	250 (0.69)
NULL	67 (0.32)	113 (0.31)

APPENDIX F – Unpublished Interactions for PCB exposure and *CYP1A1* genetic polymorphisms and breast cancer risk.

CYP1A1	Case	Control	Crude OR	95% CI	Adj OR	95% CI
All Cases						
W/W	127 (83)	169 (88)	1.0		1.0	
WM + M/M	27 (17)	23 (12)	1.56	0.86-2.85	1.79	0.91-3.55
Low PCB						
W/W	63 (89)	85 (89)	1.0		1.0	
W/M + MM	8 (11)	11 (11)	0.98	0.37-2.58	0.81	0.25-2.61
High PCB						
W/W	65 (77)	83 (87)	1.0		1.0	
W/M + M/M	19 (23)	12 (13)	2.02	0.92 - 4.46	3.24	1.24 - 8.24

APPENDIX G – Unpublished microsomal epoxide hydrolase genetic polymorphism frequencies.

Genotype	Premenopausal	Postmenopausal
MEH3		
HA	88 (0.38)	101(0.35)
HH	119 (0.51)	125 (0.43)
AA	25 (0.11)	66 (0.23)
MEH4		
HA	63 (0.29)	109 (0.31)
HH	7 (0.03)	6 (0.02)
AA	147 (0.68)	242 (0.68)

APPENDIX H – Unpublished *CYP2D6* genotype frequencies

CYP2D6A				
	WW	WM	MM	Fraction of Samples Genotyped
Premenopausal	0.953	0.034	0.013	0.84 (233/278)
Postmenopausal	0.958	0.040	0.003	0.69 (379/553)

CYP2D6B				
	WW	WM	MM	Fraction of Samples Genotyped
Premenopausal	0.636	0.302	0.062	0.87 (242/278)
Postmenopausal	0.579	0.370	0.050	0.72 (397/553)

CYP2D6T				
	WW	WM	MM	Fraction of Samples Genotyped
Premenopausal	0.986	0.014	0	0.79 (219/278)
Postmenopausal	0.979	0.021	0	0.60 (332/553)

APPENDIX I – Unpublished manganese superoxide dismutase frequencies

Genotype	Premenopausal	Postmenopausal
AA	68	85
AV	115	191
VV	41	61

APPENDIX J – Results of breast cell culture studies

Metabolism of 4-ABP in primary human mammary epithelial cells
Elise Bowman, Peter Shields

Background:
Breast cancer has been found to be associated with cigarette smoking an NAT polymorphic status. Our goal is to study the actual metabolism of 4-aminobiphenyl (4-ABP; cigarette smoke component) by primary breast cell strains that have different genotypes (poor metabolizer vs extensive metabolizer). We will also look at p53 status in these cells after carcinogen damage and finally look at apoptotic status after said damage.

Progress:

Strain development: To date (from 1/24/96), we have received from UMD 75 fresh breast tissue samples. Of these, we have 38 successful cell strains.

Genotyping: 23 DNAs have been extracted from breast tissue and are being genotyped for GST-T, GST-M, CYP1A1, NAT1, NAT2 and MnSOD. Results are attached.

Cytotoxicity of 4-ABP: Cytotoxicity assays have been performed on 9cell strains in duplicate Results: Some metabolism of 4-ABP is going on. There are no *striking* differences in rates or doses.

Metabolite studies: Comparison of 4-ABP to OH-ABP for metabolism studies: A time course was performed using media at 4 different doses (300, 30, 3, or 0.3 uM) of either 4-ABP or HO-ABP . Cells were treated for 15 min, 1 hr, 6 hr or 24 hr before changing to fresh media and incubating for a total of 24 hrs. A cell proliferation assay was then performed on each. Results: H0-ABP appears to be more cytotoxic than 4-ABP at the same concentration. This may indicate that some CYP1A2 is present in these cells as well.

p53 staining: 1. Cells were plated on three plates and then fixed at 24, 48 and 120 hrs. Staining for p53 was not different at these different times. Five random fields on each treatment/slide were counted for p53 positive/total cell count for each time period. These epithelial cells do not demonstrate induction of p53 just from the trauma of exposure to trypsin and plating.

2. A time course was performed in which media at 4 different doses (300, 30, 3, or 0.3 uM) of 4-ABP were used to treat cells for 15 min, 1 hr, 6 hr or 24 hr before changing to fresh media. The slides were then fixed in methanol and stained with CM-1/FITC. Five random fields on each treatment/slide were counted for p53 positive/total cell count for each dose.
Results: More cells are positive for p53 after 6 hrs of treatment- questionable if this is significant. We need to score staining intensity as well as distribution and have Andrew Borkowsi (pathologist, UMD) will look at the slides.

4C11 Staining
A small amount of antibody has been received from Regina Santella (Columbia University, NY) for staining treated cells for 4-ABP adducts. Mouse hybridoma cells have also been received for

antibody overproduction in mouse acsites. Immunohistochemical staining conditions are being worked out for this adduct detection system.

Apoptosis
We are looking into different techniques for identifying apoptosis (tunel assay, annexin V).

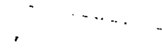
Immortalization: We have apparently successfully immortalized two cell mammary epithelial breast strains (LHC 8700 and LHC 7890) with E6 and SV40 Tag respectively. Both of these strains have superceded 15 passages (normal life span is approximately 9 maximum passages). PCR for the incorporation of E6 DNA was performed on strain 8700(see attached) as well as PCR for the SV40 Tag (data not shown). Cells were frozen at each passage and the population doublings were determined (plating efficiency is only 24%).

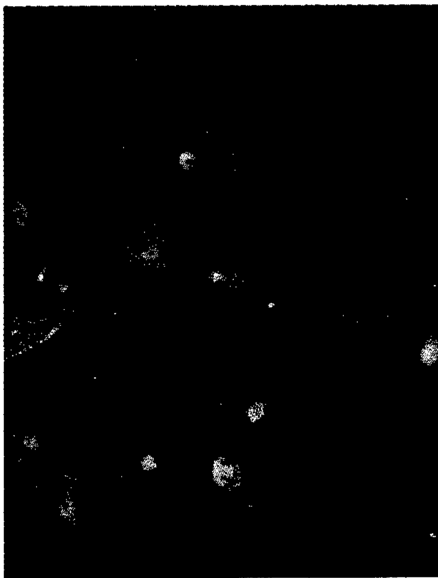
time 0

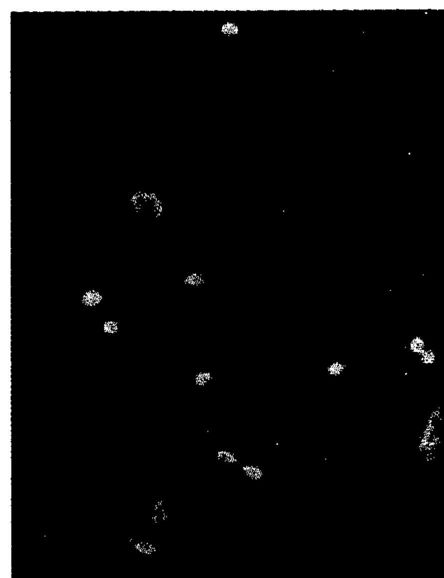
time 24 hr

treat ½ hr
30 μM 4-ABP
Strain 9007

p53







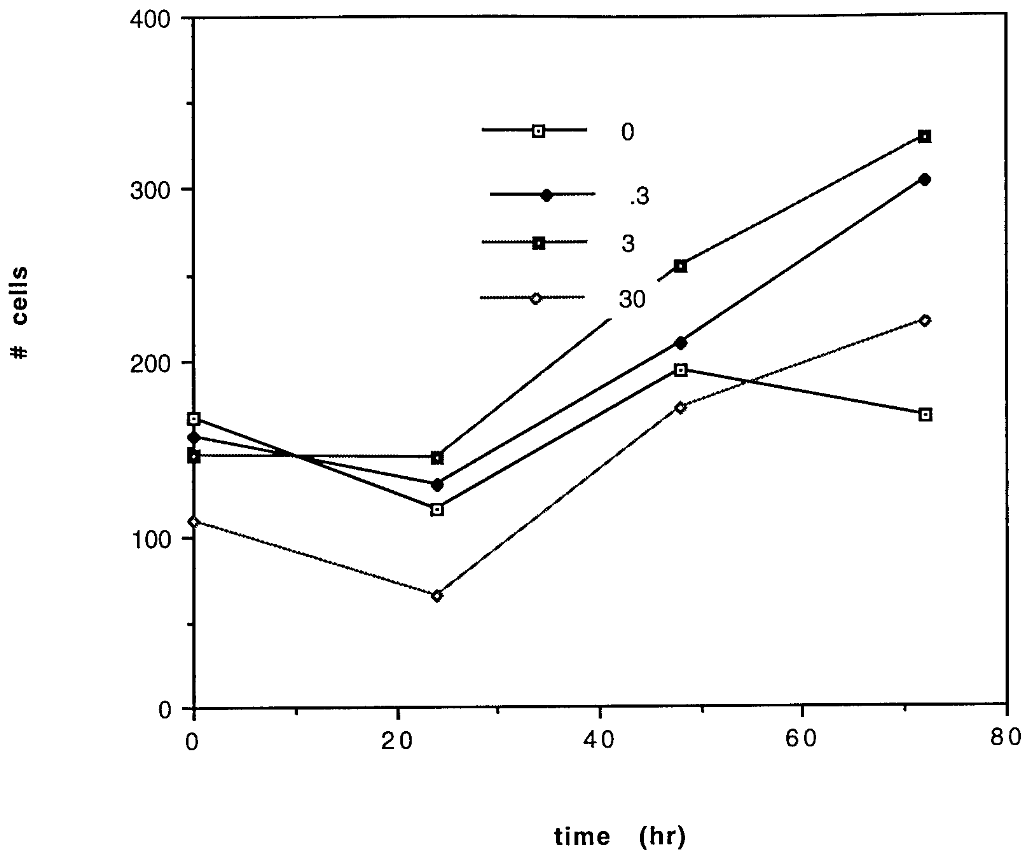
Annexin-V

Dapi

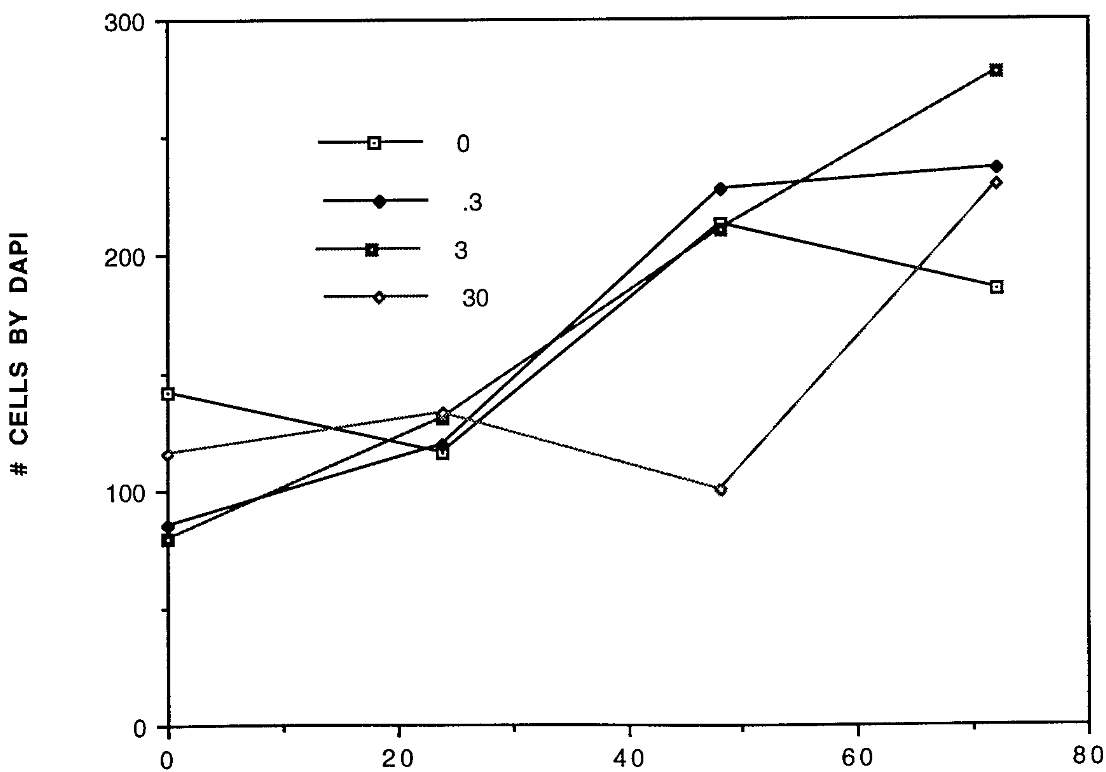
cells per field

FITC

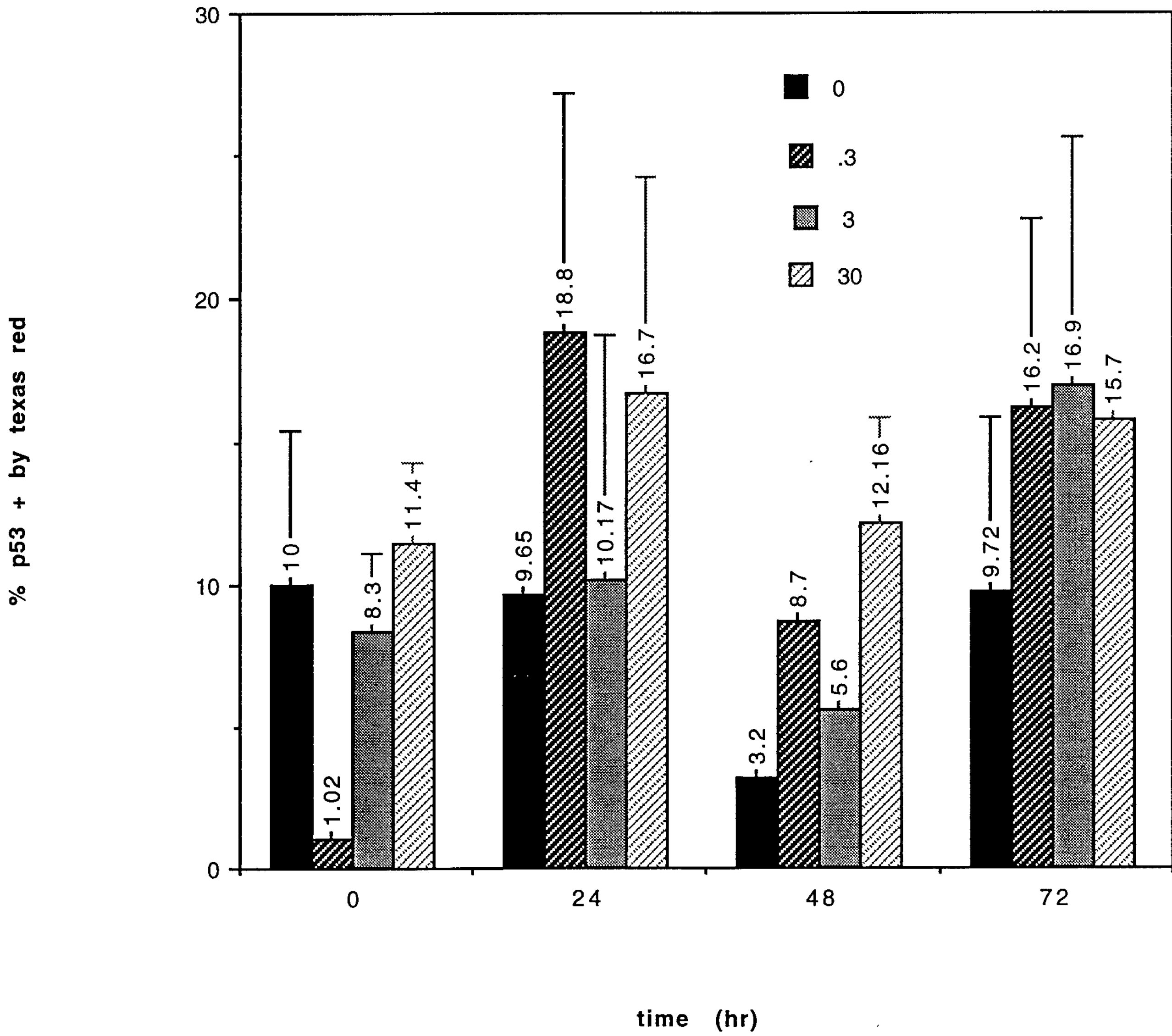
Strain 9007



CELLS IN TEXAS RED EXPERIMENT

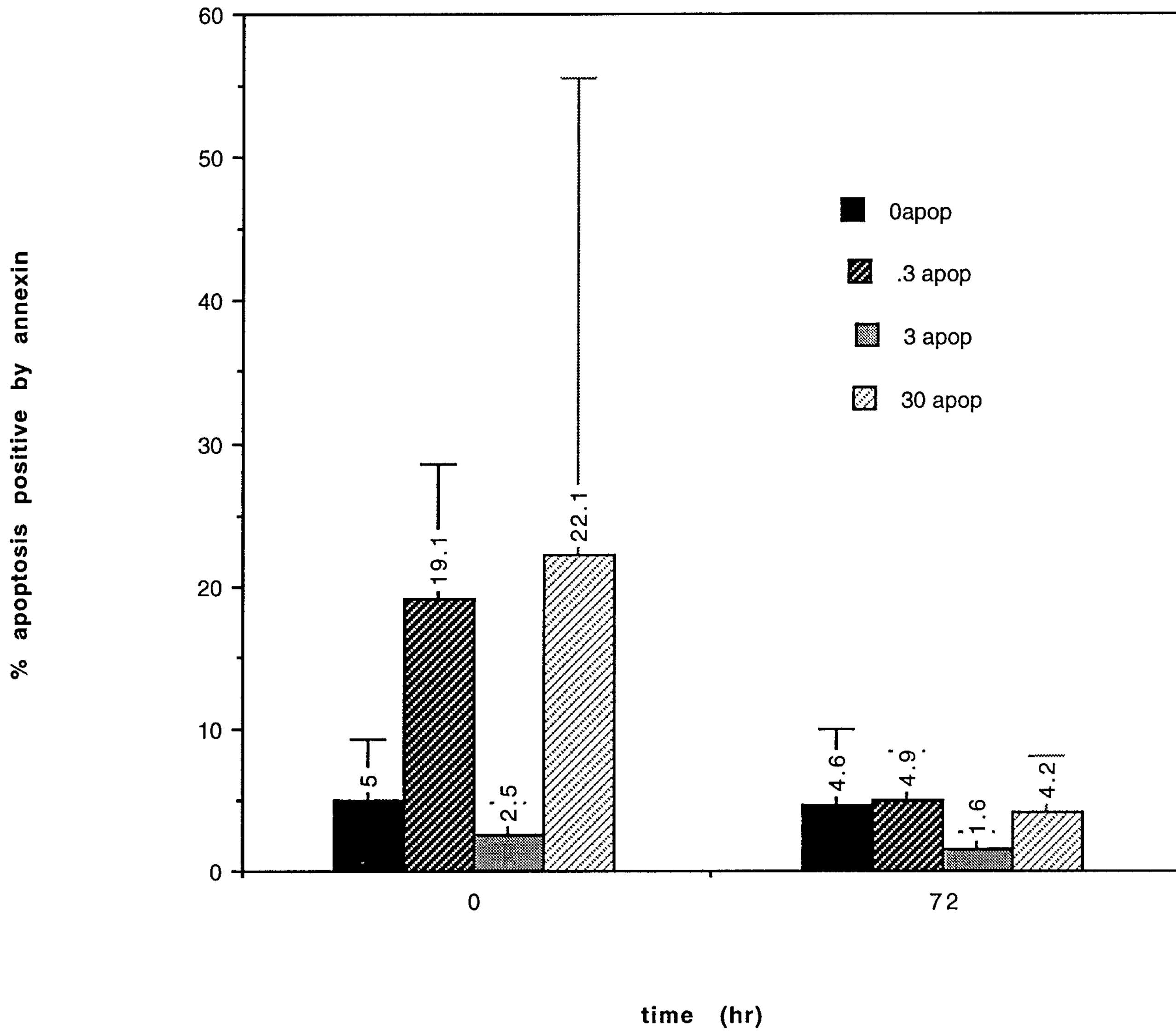


Data from "texas red" Strain 9007



Strain 9007

% cells positive by annexin staining for apoptosis



PE
Buffer
8700
8700/EG
EG+
—
Buffer B
8700
8700/EG
EG+
—

LHCNUM	ACCNUM	R/A	AGE	RACE	GENDE	GST-T	GST-M	CYP1A1	NAT2-1	NAT2-2	NAT2-3	NAT2-4	NAT-TOT	2 phen	1 phen	NAT1	MnSO	CYTOT	CYTOTOX-2	SLIDES	NO FROZ
7595	11422	RED	41	B	F	P	A	WW	WM	WW	WW	WW	WM	r	r	4,10	VV				2
7599	11436	AUT	34	W	F	A	P	WW	WM	WM	WW	WW	MM	s	s	4,4	AV				5
7809	11582	AUT	34	B	F	A	P	WW	WM	WW	WW	WM	MM	s	r	10,10	A V				10
7890	11591	RED	46	B	F	P	P	WW	MM	WW	WW	WW	MM	s	r	4,10	AV				11
7900	11599	AUT	48	W	F	P	A	WW	WW	WW	WW	WW	WW	r	s	4,4					7
7902	11601	RED	21	W	F	P	P	WM	WW	WM	WW	WW	WM	r	s	4,3					6
7932	11629	RED	20	B	F	P	P	WW	WW	WW	WW	WW	WW	r	r	4,10					9
7946	11648	RED	44	B	F	P	A	WW	MM	WW	WW	WW	MM	s	r	4,10					8
8068	11696	RED	35	B	F	P	P	WW	WW	WW	WW	WM	WM	r	r	4,10	VV				12
8078	11701	RED	22	B	F	P	P	WW	WM	WW	WW	WW	WM	r	s	4,4		Y			8
8079	11703	RED	37	B	F	P	A	WW	WW	WW	WW	WW	WW	r	r	10,10	VV	Y	Y		8
8080	11705	AUT	19	B	F	P	P	WW	WM	WW	WW	WM	MM	s	r	4,10	VV				10
8192	11707	RED	39	B	F	P	P	WW	WW	WM	WW	WW	WM	r	r	4,10	VV	Y	Y		8
8464	11775	RED	17	B	F	A	A	WW	WM	WM	WW	WW	MM	s	r	4,10	VV	Y	Y		9
8700	11811	RED	39	B	F	P	P	WW	WM	WW	WW	WW	WM	r	s	4,4	AV	Y	Y		7
8837	11817	RED	26	B	F	P	P	WW	WM	WM	WW	WW	MM	s	s	4,4	AV	Y	Y	Y	7
8872	11834	AUT	33	W	F	P	P	WM	WM	WW	WW	WM	MM	s	r	4,10	AV	Y		Y	10
8881	11844	RED	50	B	F	P	A	WW	WW	WM	WW	WW	WM	r	r	4,10	AV	Y	Y		9
8903	11866	AUT	24	B	F	A	P	WW	WM	WW	WW	WW	WM	r	r	4,10	VV	Y	Y		9
8904	11865	RED	49	W	F	P	P	WW	WM	WM	WW	WW	MM	s	r	4,10	AV	Y	Y	Y	9
8910	11869	RED	18	W	F	A	P	WW	WM	WM	WW	WW	MM	s	s	4,4	AV	Y	Y		9
8999	11895	RED	32	B	F															Y	
9001	11903	RED	24	W	F															Y	
9007	11930	RED	36	B	F															Y	
9058	11977	RED	32	B	F												AV				
9059	11985	RED	49	W	F																
9060	11990	RED	34	B	F															Y	
9065	12021	RED	31	B	F																
9222	12071	RED	42	B	F																
9185	12044	RED	53	W	F																
9231	12094	RED	27	B	F																
9232	12106	RED	29	B	F																
9482	12130	AUT	47	B	F																
9497	12184	RED	23	B	F																
9493	12166	RED	32	W	F																
9485	12123	RED	45	W	F																
9498	12193	RED	33	W	F																
9500	12200	RED	21	B	F																

APPENDIX K – Current results of acetic anhydride postlabeling studies

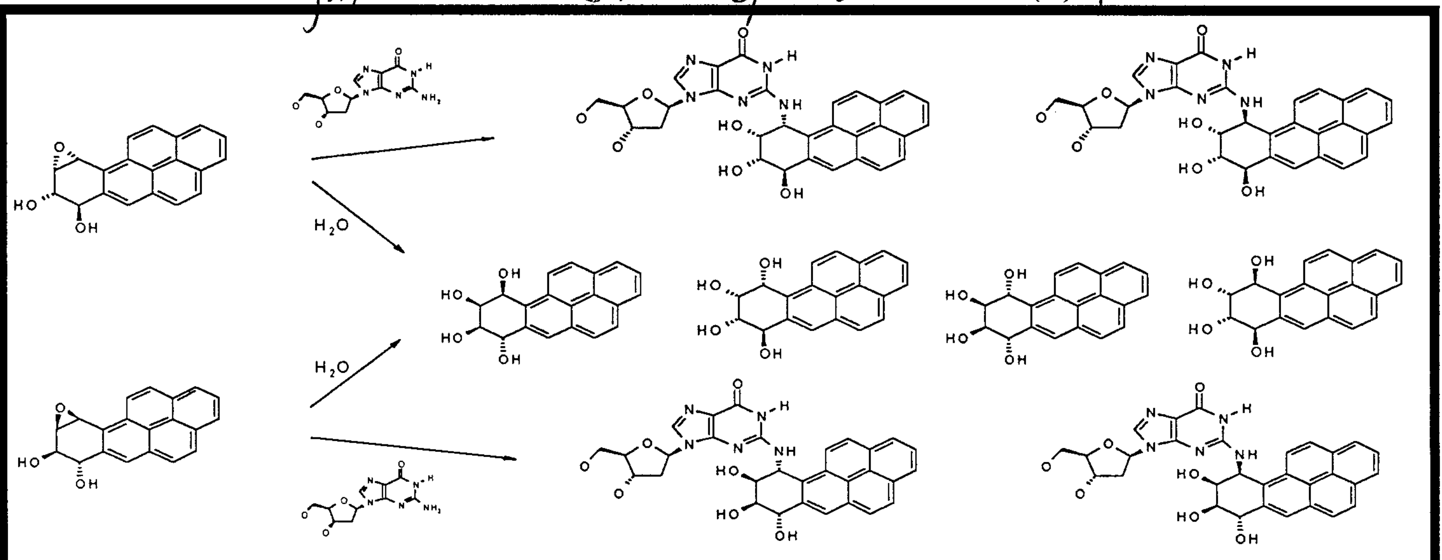
Molecular Epi Group Meeting 02.25.98

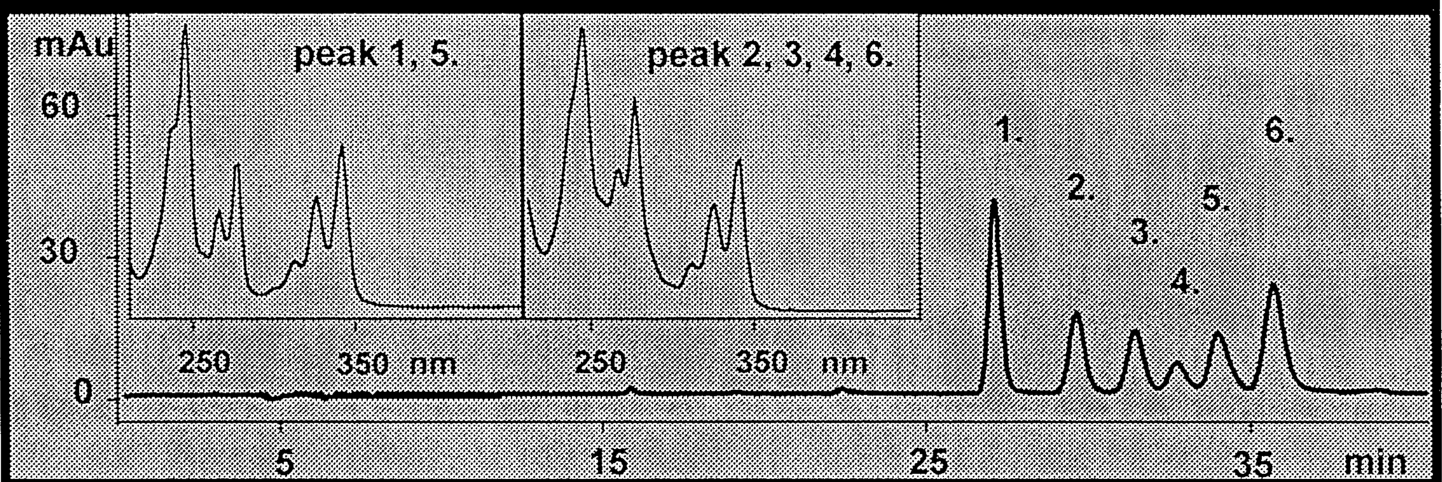
Rado Goldman

<u>Postlabeling of adducts with ^{14}C-acetic anhydride</u>

1. Revised analyses of BPdG from (+/-)BPDE+dG in $DMSO/ZnCl_2$
2. Optimization of the acetylation of BPdG with acetic anhydride
3. Acetylation of BPdG and dG8ABP in THF/MeIm
4. ^{14}C-Acetylation of deglycosylated BPdG : results

Synthesis of BP - dGp adduct Standard





Tetrols and deglycosylated adducts

Data File C:\HPCHEM\2\DATA\RADO\BPDG80.D Sample Name: (+)BPDE/DNA+BPdG

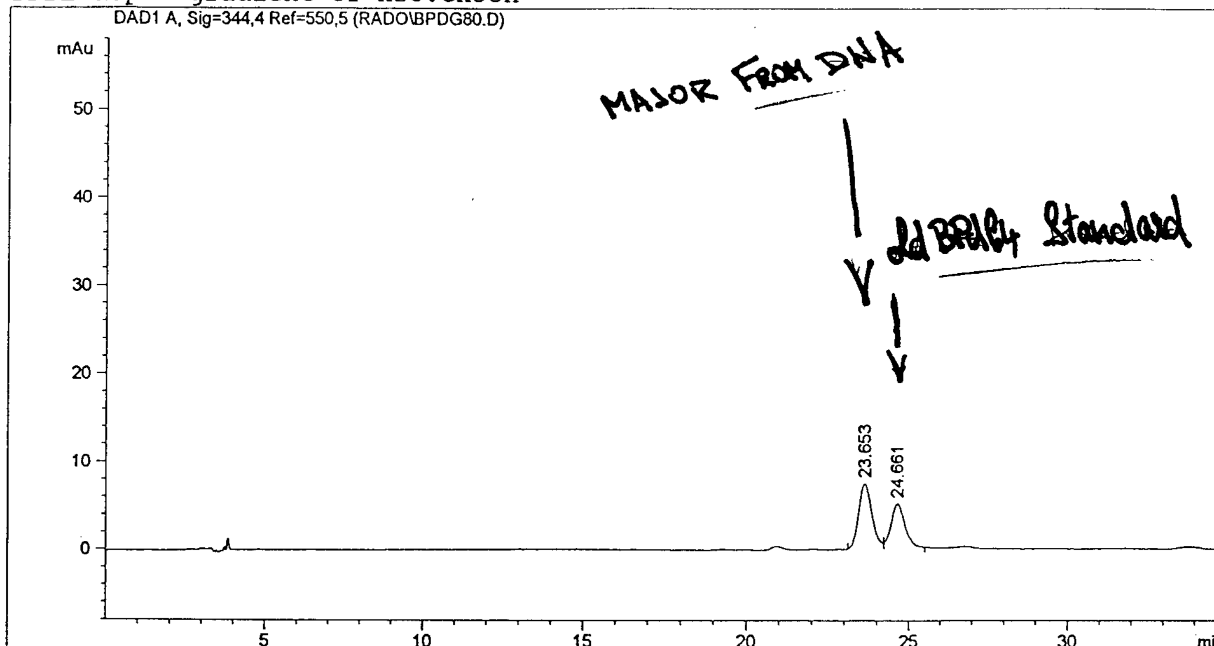
overlay of adducts from (+)BPDE/DNA syntheses (20ul) wi
th BPdG4 Standard (5ul)

HPLC of Synthesized Standard

===

Injection Date : 1/19/98 12:52:04 PM
Sample Name : (+)BPDE/DNA+BPdG Vial : 1
Acq. Operator : Rado
Method : C:\HPCHEM\2\METHODS\RADO\GOOD\BPDGSYN2.M
Last changed : 1/19/98 12:46:20 PM by Rado
(modified after loading)

BPDE-dGp - gradient of H2O:CH3OH



===

Area Percent Report

===

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=344,4 Ref=550,5
Results obtained with enhanced integrator!

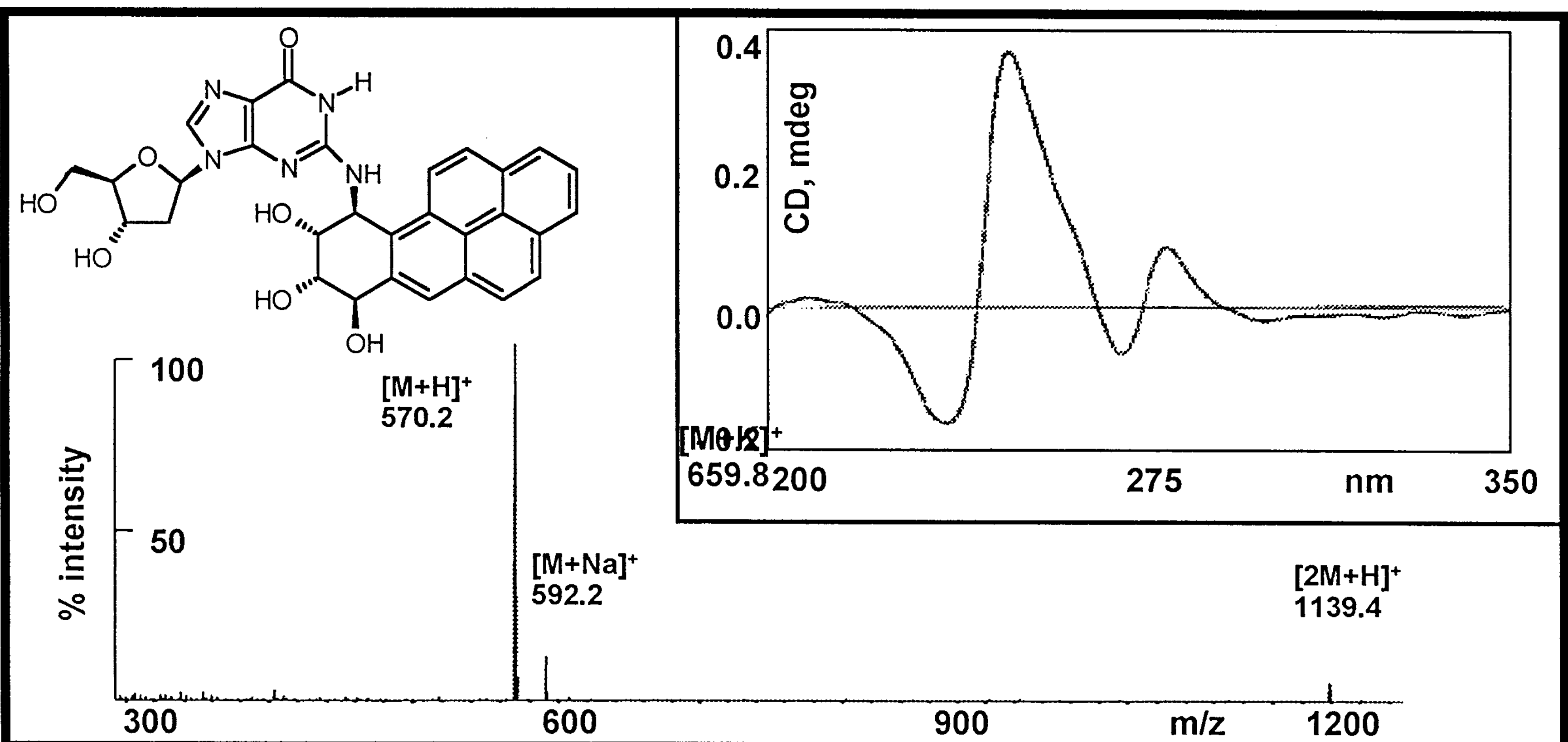
Peak #	RetTime [min]	Type	Width [min]	Area [mAu*s]	Height [mAu]	Area %
1	23.653	BV	0.3622	213.70901	7.47884	57.4927
2	24.661	VB	0.3694	158.00613	5.12305	42.5073
Totals :				371.71515	12.60189	

===

*** End of Report ***

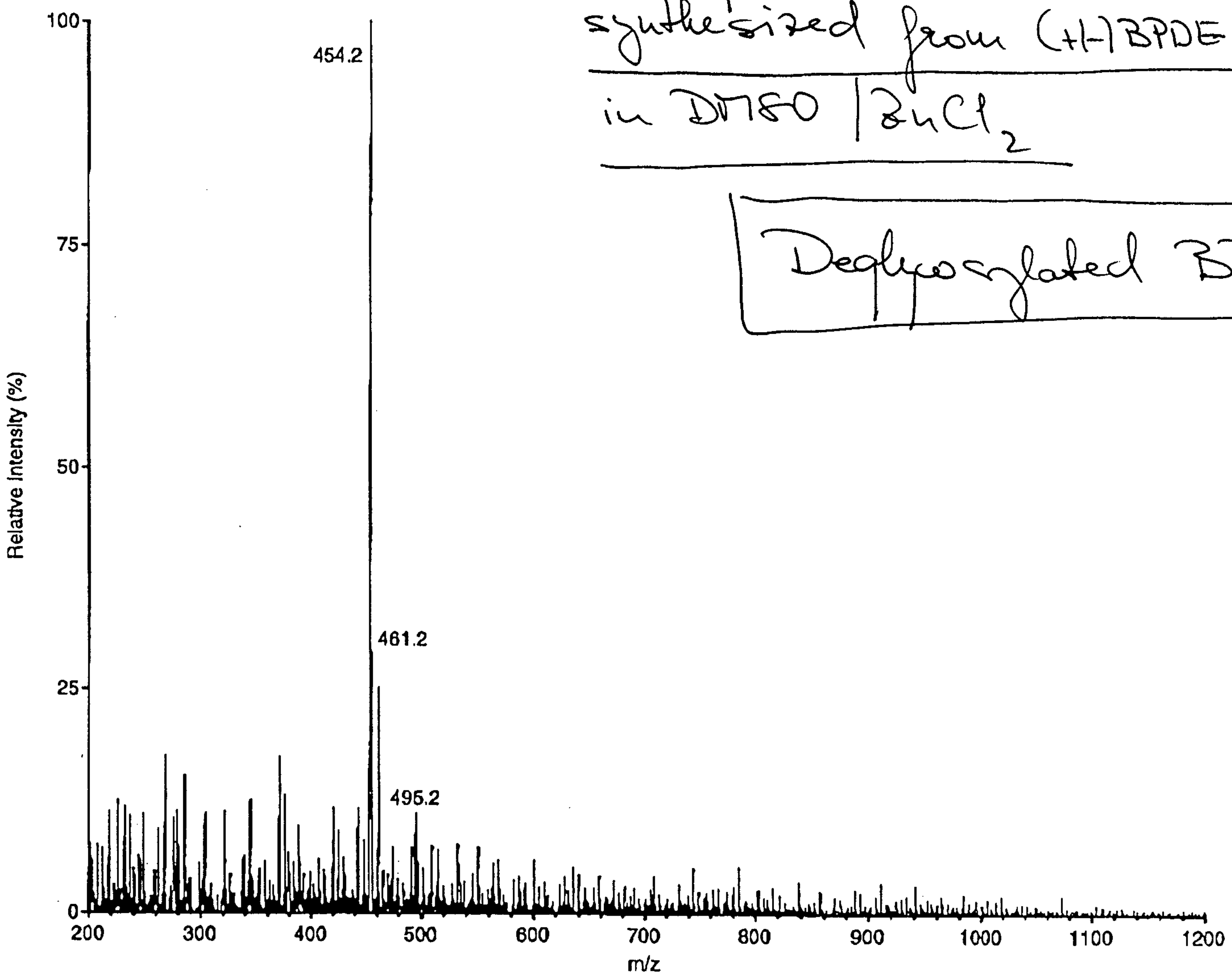
BPdG (rttr) : CD and LC-MS

Confirmation of Structure

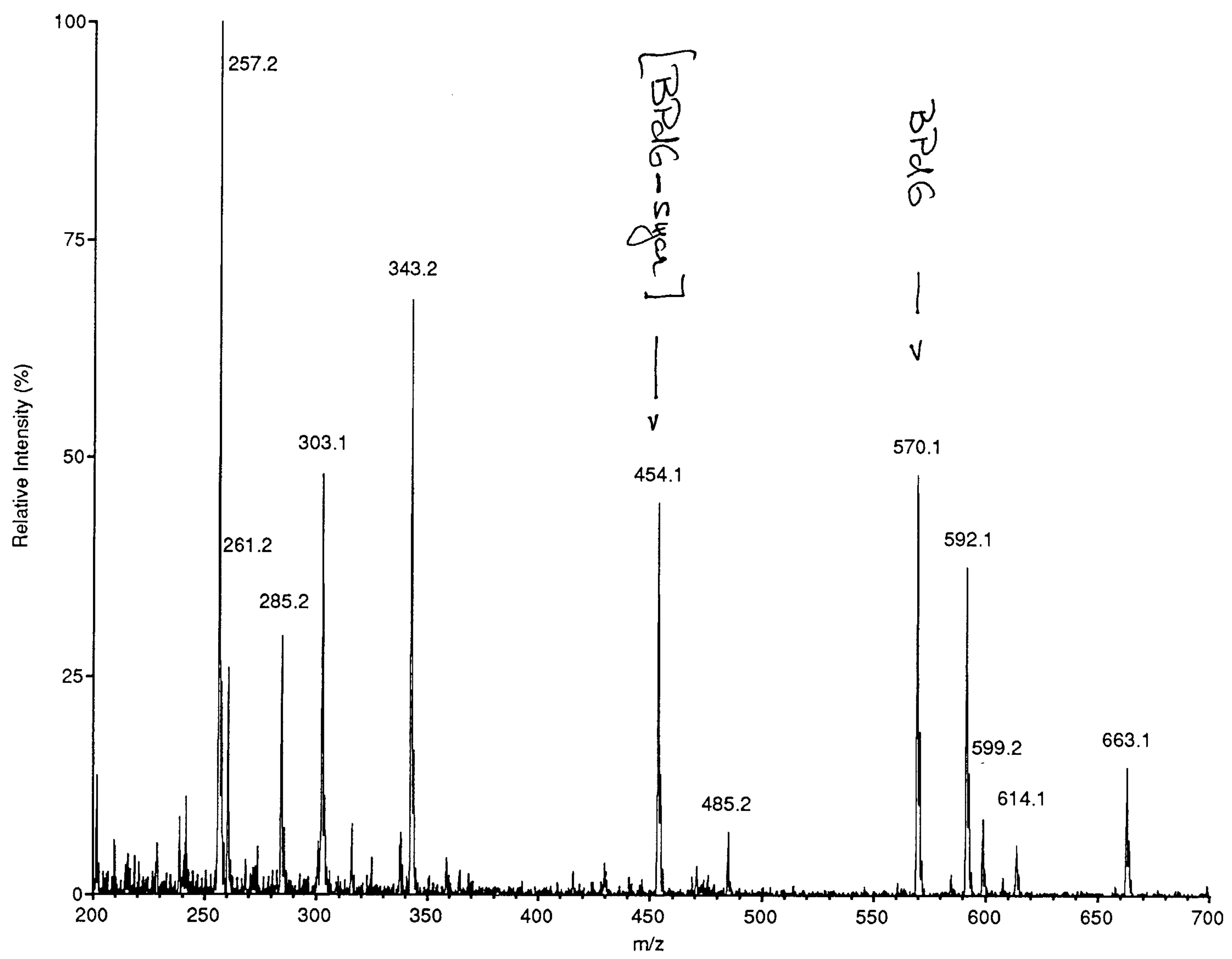


RG-005

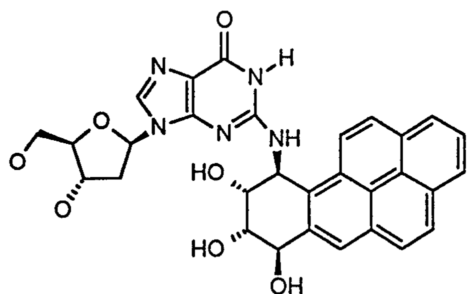
DI	50
ISV	5000
IN	[illegible]
OR	40
R0	30
M1	1000
RE1	117.5
DM1	0.100
R1	27.5
L9	-105
FP	-10
MU	-3900
CC	10
DI µA	10.4
IS v	5109.9
UV	92.8



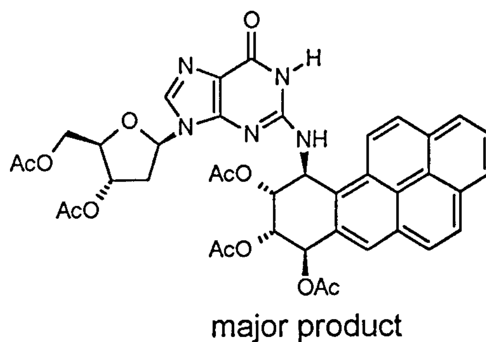
DI	50
ISV	5000
IN	650
OR	70
R0	30
M1	1000
RE1	118.4
DM1	0.130
R1	27
L9	-100
FP	-10
MU	-4000
CC	10
DI μA	4.03
IS v	5109.9
UV	83.05



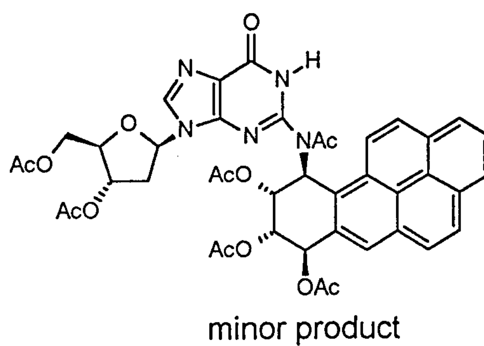
Acetic Anhydride postlabeling method.



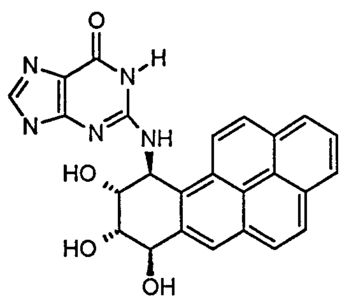
acetic anhydride
pyridine
→



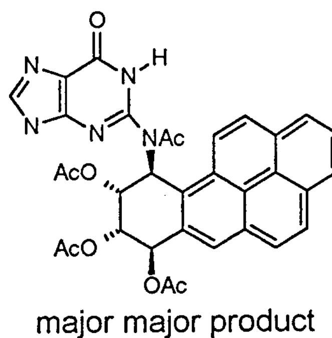
major product



minor product



acetic anhydride
pyridine
→



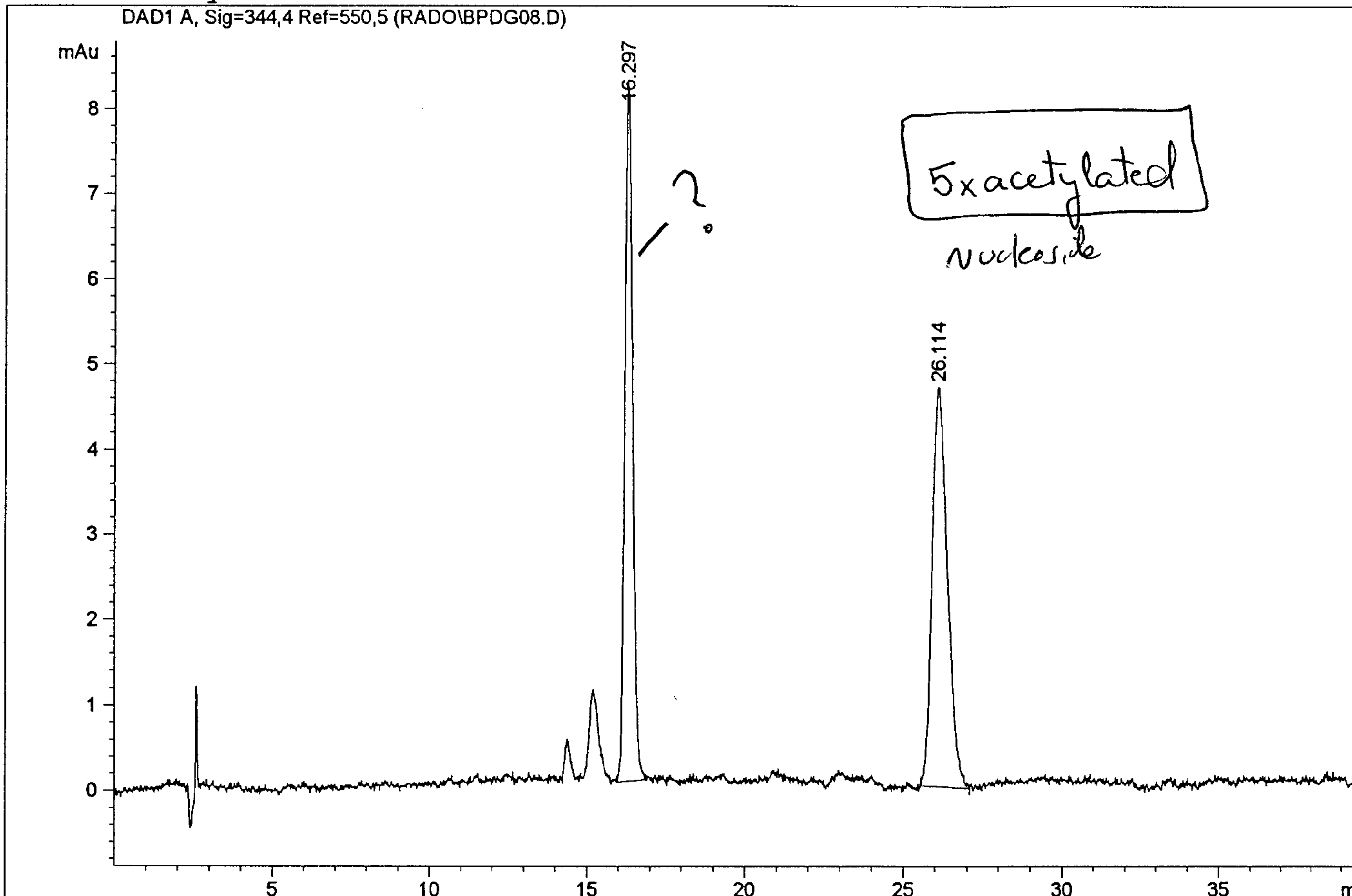
major major product

new standard from DNA, acetylation of 20mAu with 3ul co
ld acanh in 12ul py, test for hot
2 HOURS at 30°C

===

Injection Date : 2/19/98 9:12:07 AM
Sample Name : AcBPdG Vial : 1
Acq. Operator : Rado
Method : C:\HPCHEM\2\METHODS\RADO\GOOD\ACBPDG2.M
Last changed : 2/19/98 9:04:31 AM by Rado
(modified after loading)

BPdG - acetylated



===

Area Percent Report

===

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=344,4 Ref=550,5
Results obtained with enhanced integrator!

Peak #	RetTime [min]	Type	Width [min]	Area [mAu*s]	Height [mAu]	Area %
1	16.297	PB	0.2663	142.72063	8.23254	47.7926
2	26.114	BP	0.4305	155.90436	4.67716	52.2074

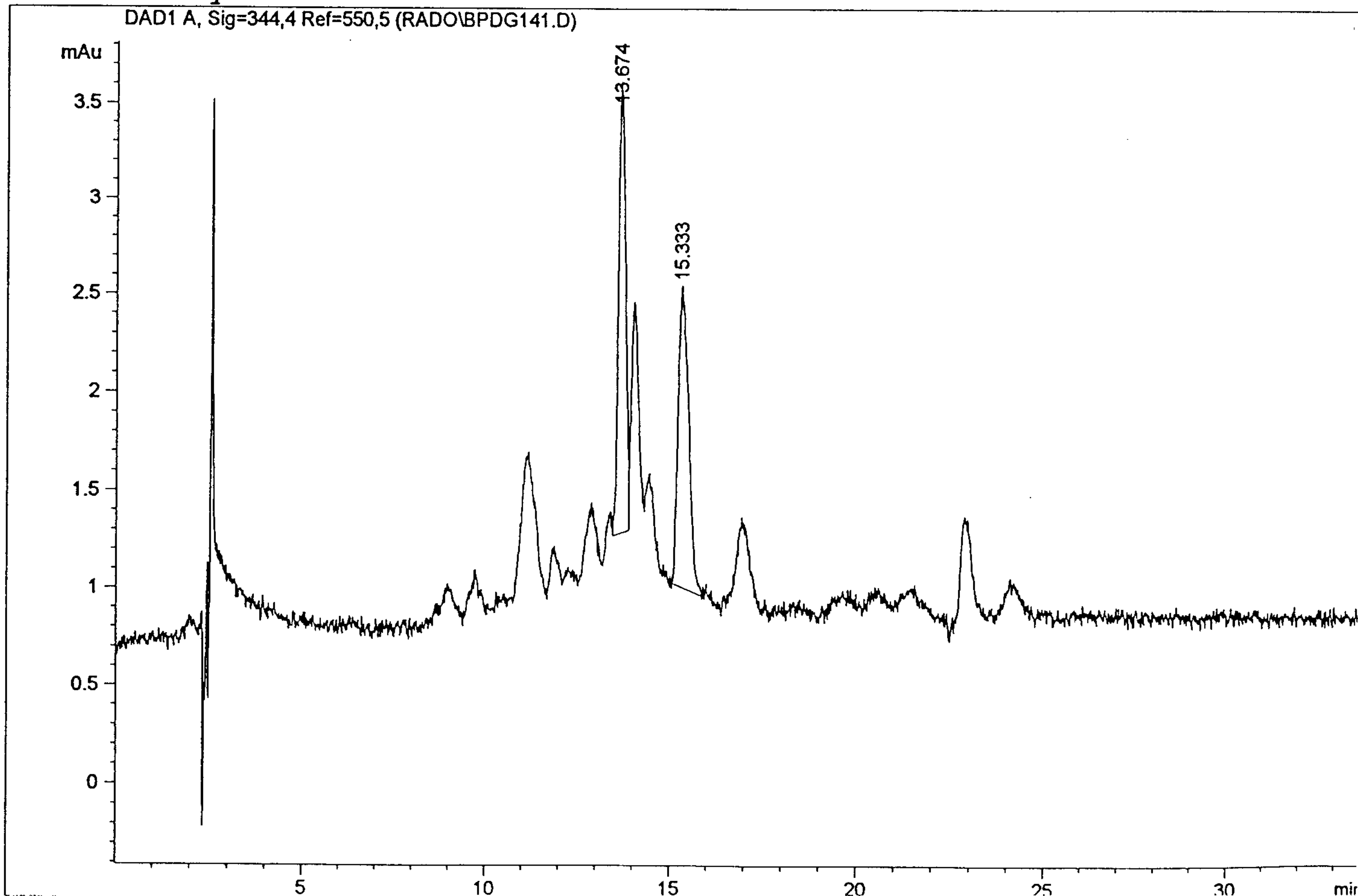
Optimization of the acetylation of BPdG : reaction yields in various solvents

BPdG, 500 pmol (400mAu*s), with 3ml of acetic anhydride in 17ml of solvent x

x = acetonitrile, ethyl acetate, dichloromethane, tetrahydrofuran, DMSO, DMF, and pyridine (all anhydrous)
Only pyridine gives reasonable profile.

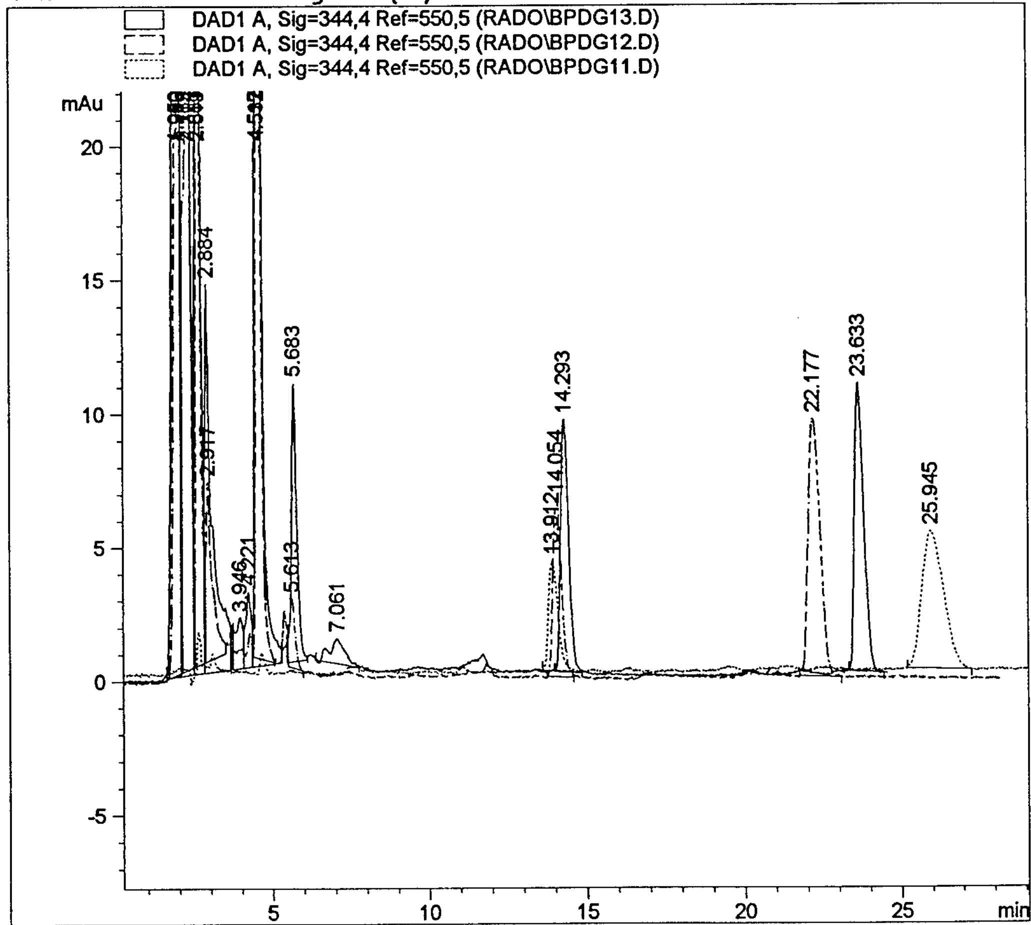
But tetrahydrofuran/methylimidazole (Applied Biosystems) gives good yield of one major product, even better than pyridine (with lots of unidentified sideproducts with short RT which absorb at 344 nm).

```
)ata File C:\HPCHEM\2\DATA\RADO\BPDG141.D                    Sample Name: AcBPdG

    BPdG-DNA after acanh, 3ul, in DMF, 17ul, at 35oC for 2
    hours 30min, quantitation of yield in different solvent
    s

    =====================================================================
    Injection Date  : 2/11/98 6:01:53 PM
    Sample Name     : AcBPdG                              Vial :   1
    Acq. Operator   : Rado
    Method          : C:\HPCHEM\2\METHODS\RADO\GOOD\ACBPDG2.M
    Last changed    : 2/11/98 5:15:53 PM by Rado
                      (modified after loading)
    BPdG - acetylated
```



Acetylation of BPdG in THF/MeIm (12 ul) with Acetic Anhydride (0.3-3 ul)

acetic anhydride ul	5xacetylated BPdG, RT 25min area, mAu*s	6xacetylated BPdG, RT 14 min area, mAu*s	ratio 5x/6x
3	224	168	1.3
1	260	125	2.1
0.3	252	84	3

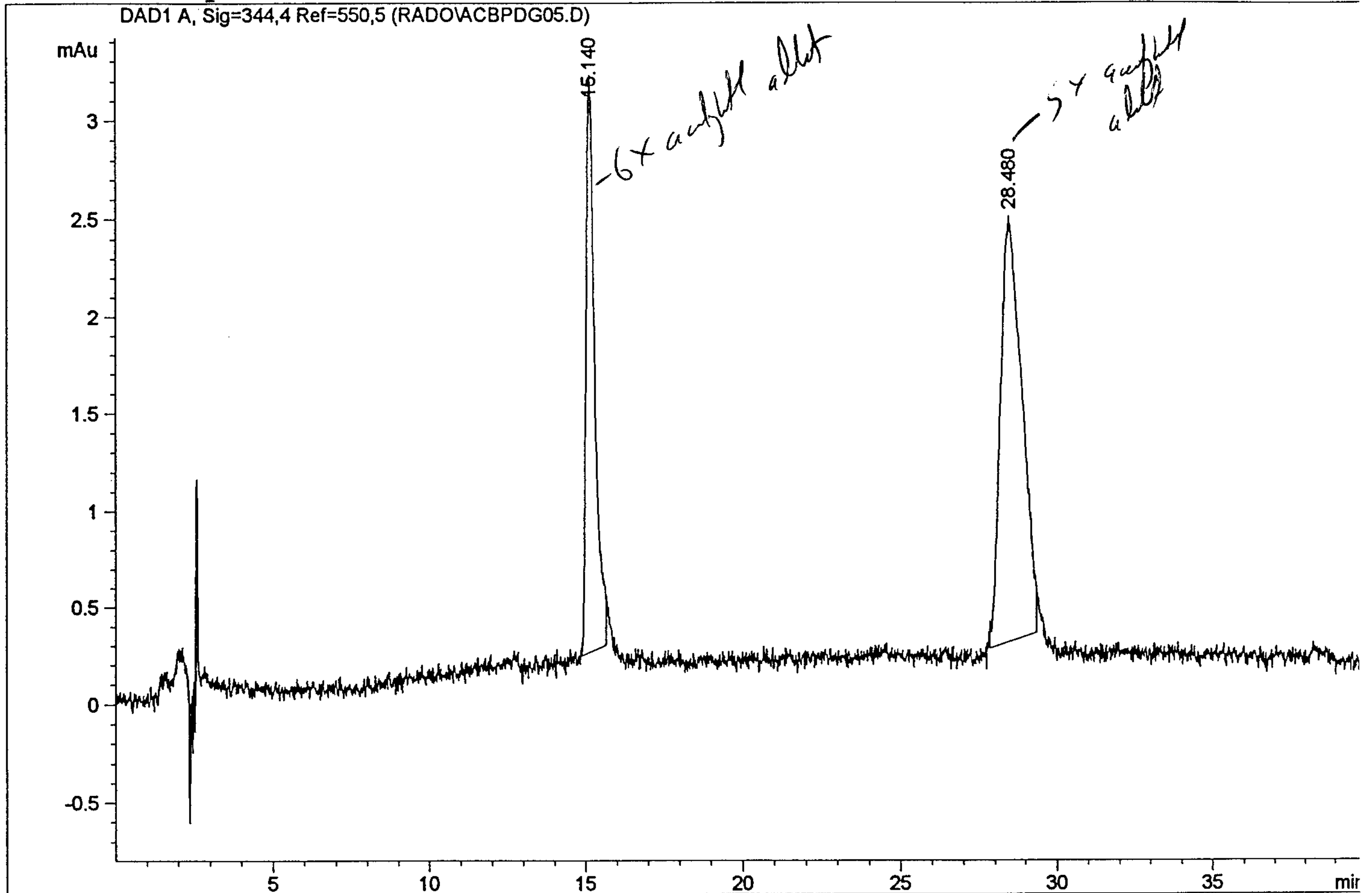


Data File C:\HPCHEM\2\DATA\RADO\ACBPDG05.D Sample Name: AcBPdG

acetylation in THF/mIm, pooled and reinjected, 2 peaks expected

===

Injection Date : 2/23/98 12:59:20 PM
Sample Name : AcBPdG Vial : 1
Acq. Operator : Rado
Method : C:\HPCHEM\2\METHODS\RADO\GOOD\ACBPDG2.M
Last changed : 2/22/98 10:15:28 AM by Rado
BPdG - acetylated



===

Area Percent Report

===

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

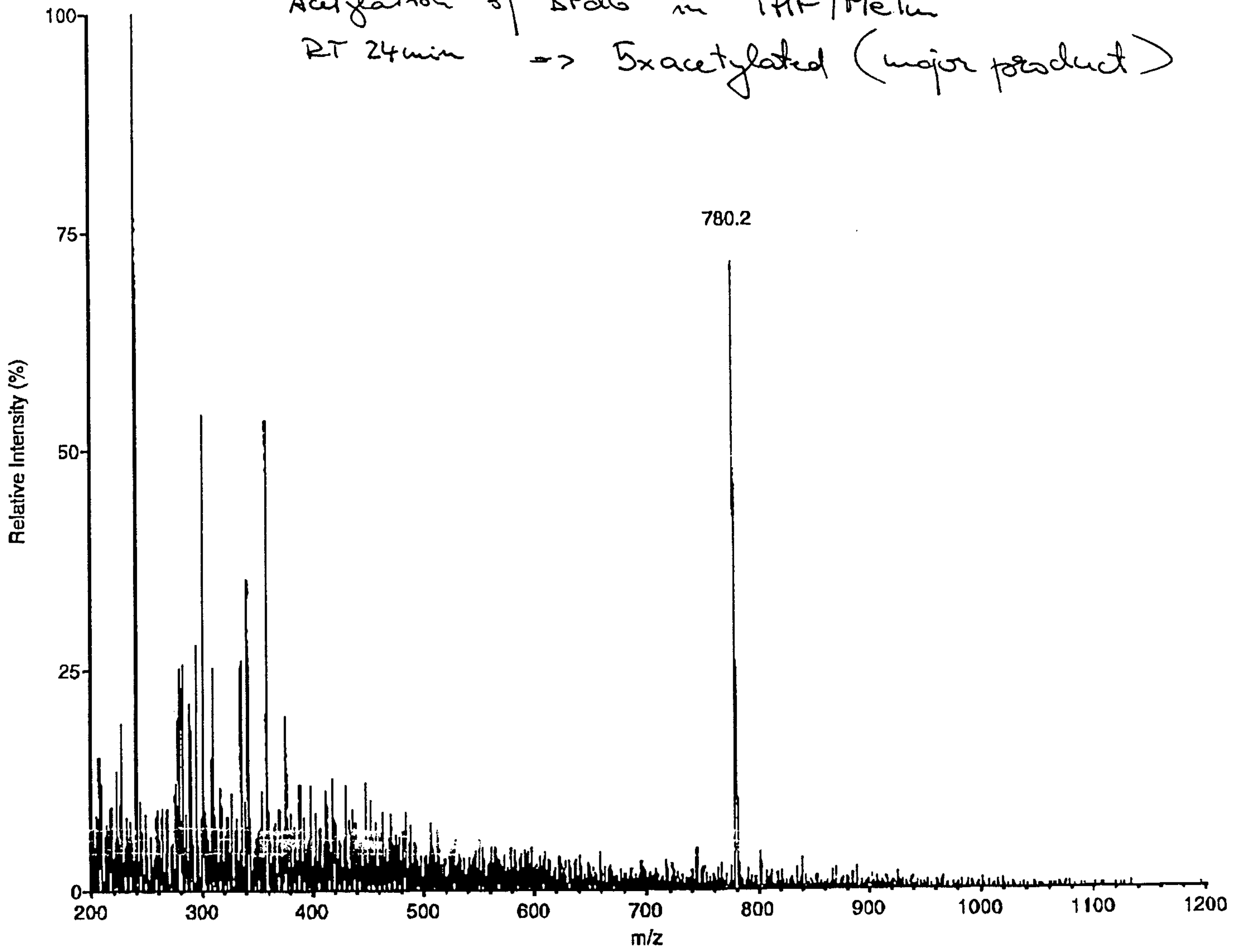
Signal 1: DAD1 A, Sig=344,4 Ref=550,5
Results obtained with enhanced integrator!

Peak #	RetTime [min]	Type	Width [min]	Area [mAu*s]	Height [mAu]	Area %
1	15.140	PB	0.2775	57.54465	2.96716	35.4284
2	28.480	BB	0.5633	104.88062	2.19710	64.5716
Totals :				162.42527	5.16426	

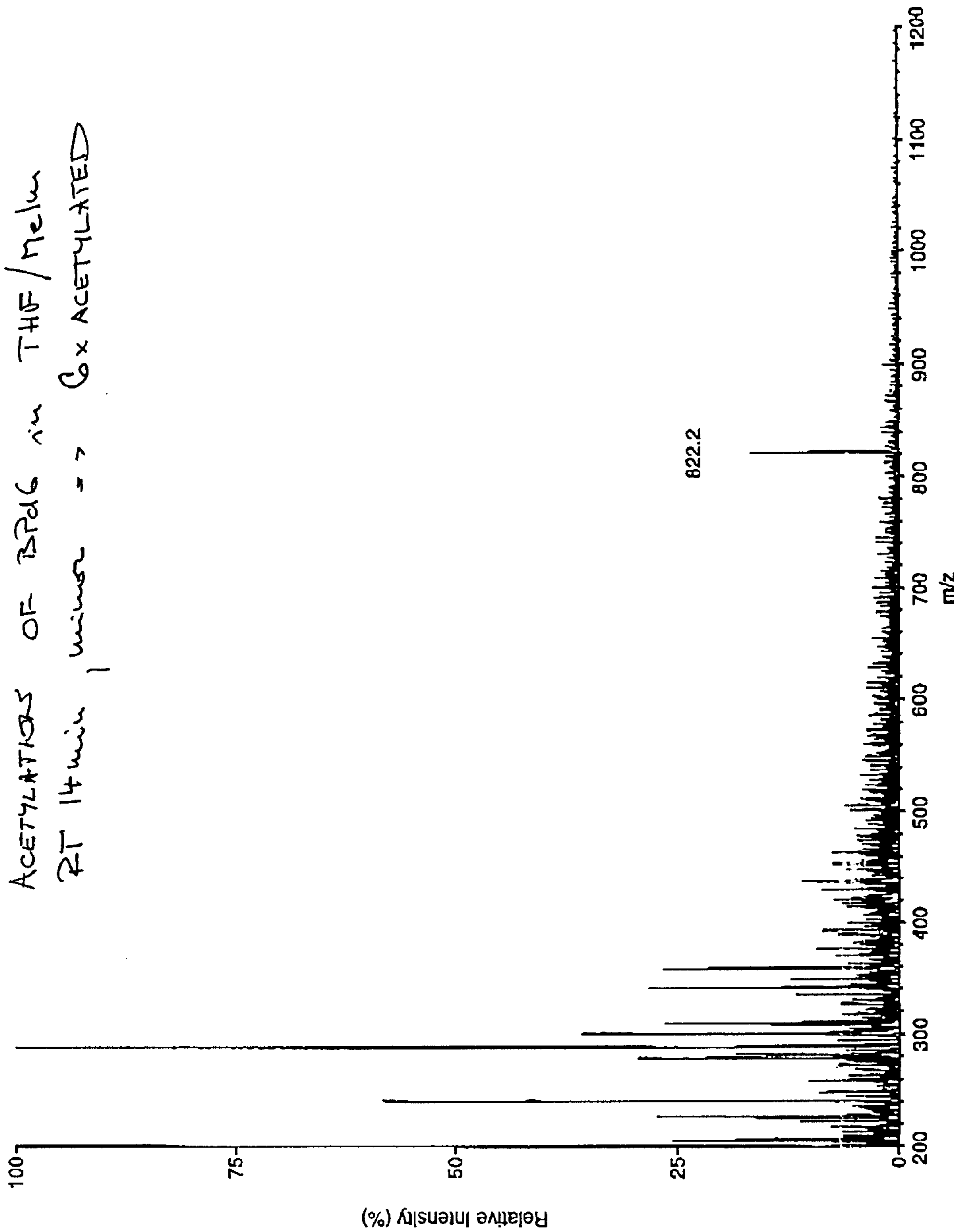
DI	50
ISV	5000
IN	650
OR	40
R0	30
M1	1000
RE1	119.0
DM1	0.100
R1	27.5
L9	-105
FP	-10
MU	-3800
CC	10
DI µA	4.6
IS v	5109.9
UV	97.7

rg22298_2/str/Scans 18-28

+Profile Q1SCAN
Scans 18-28 minus 7-9 & 36-38 Time=4.19 min
rg22298_2 - 2/22/98 - 12:31 PM
No Title
1 peak



+Profile Q1SCAN
Scans 18-29 minus 9-11 & 40-40 Time=4.15 min
rg22298_1 - 2/22/98 - 12:22 PM
No Title
1 peak



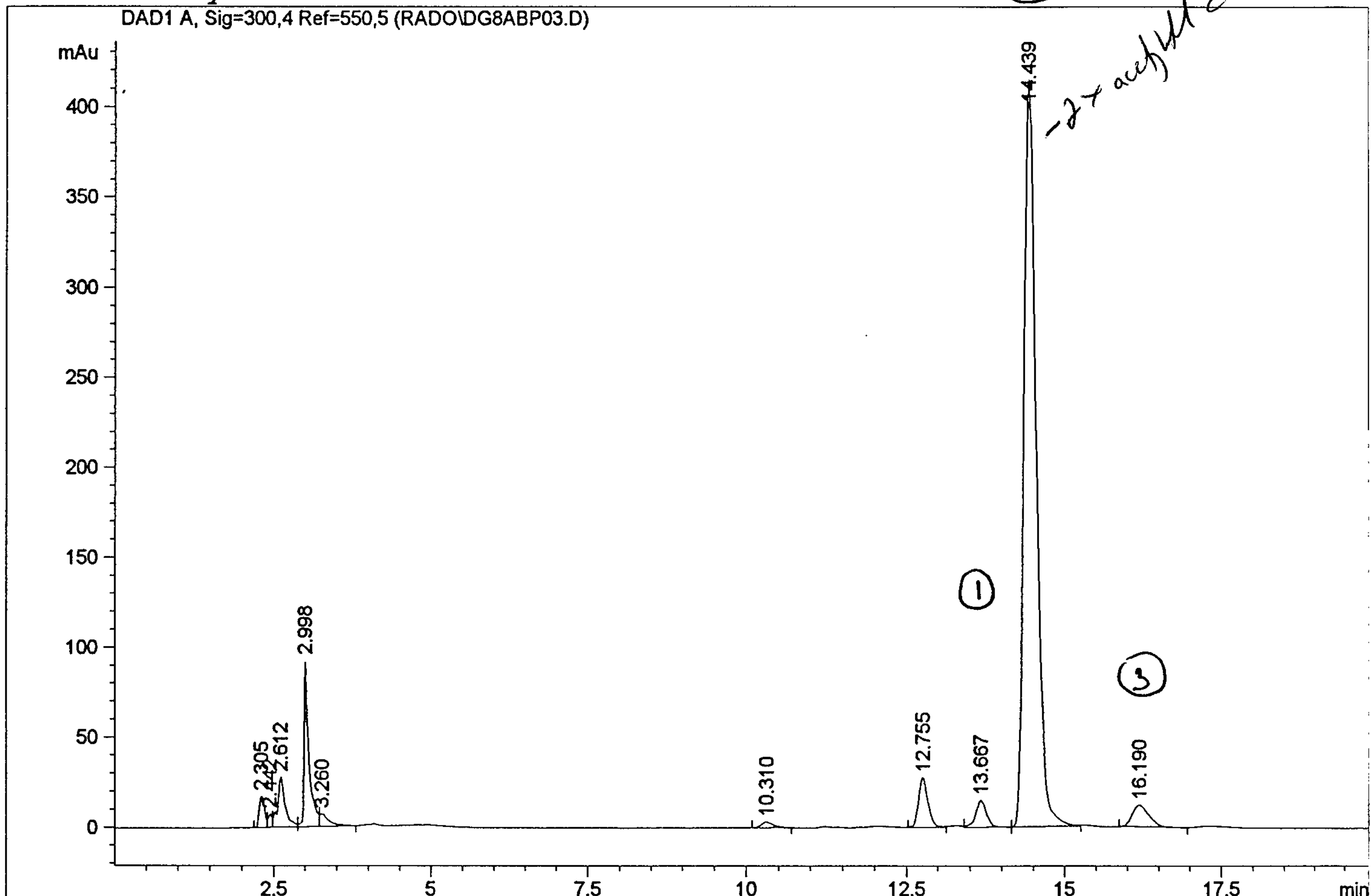
DI	50
ISV	5000
IN	650
OR	40
R0	30
M1	1000
RE1	119.0
DM1	0.100
R1	27.5
L9	-105
FP	-10
MU	-3800
CC	10
DI µA	4.5
IS v	5080.6
UV	92.8

acetylation of 100mAu with 0.3ul cold acanh in 12ul THF
/mIm

===

Injection Date : 2/19/98 4:08:42 PM
Sample Name : AcdG8ABP Vial : 1
Acq. Operator : Rado
Method : C:\HPCHEM\2\METHODS\RADO\GOOD\ACBPDG2.M
Last changed : 2/19/98 4:07:18 PM by Rado
(modified after loading)

BPdG - acetylated



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Area Percent Report

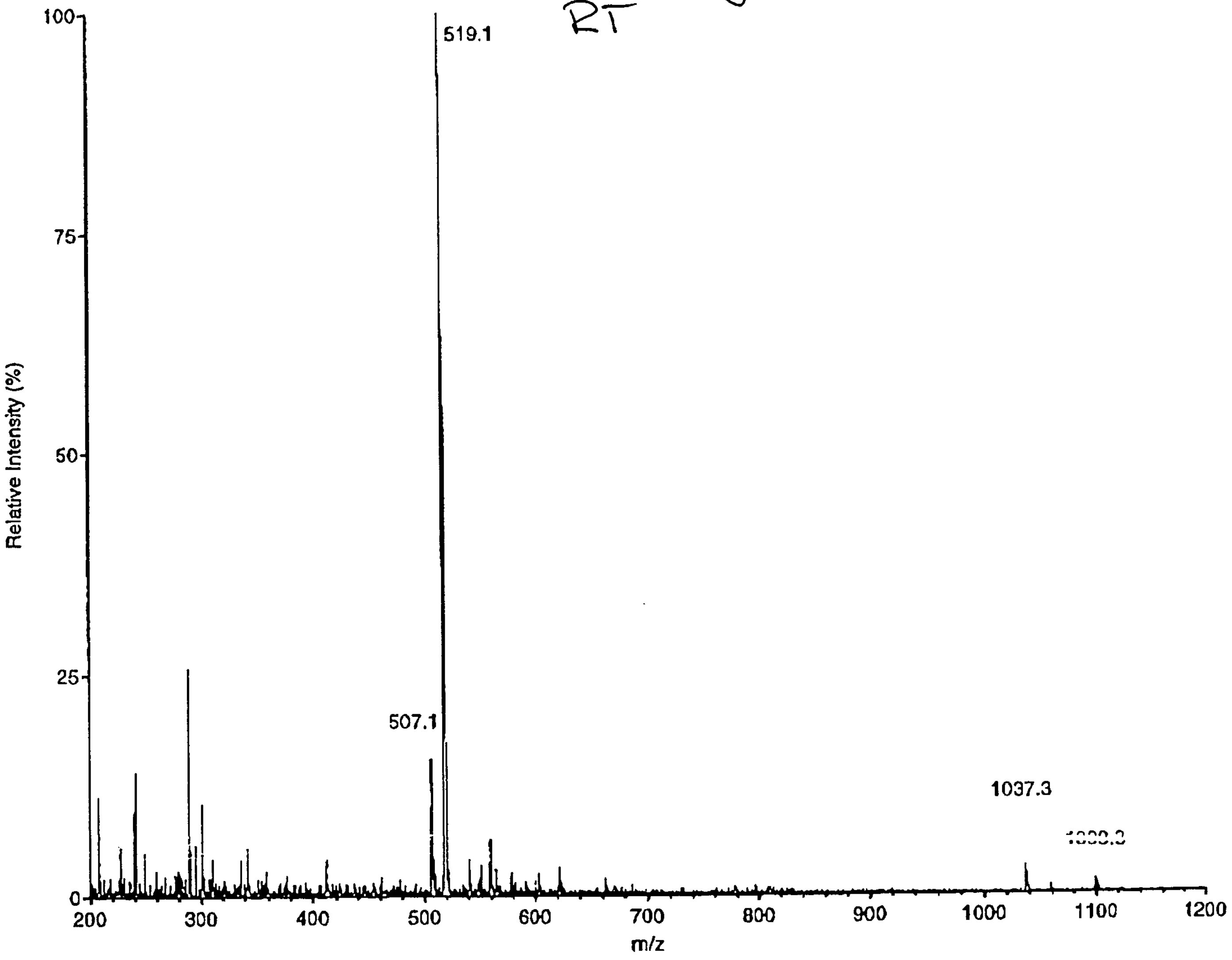
===

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: DAD1 A, Sig=300,4 Ref=550,5
Results obtained with enhanced integrator!

Peak #	RetTime [min]	Type	Width [min]	Area [mAu*s]	Height [mAu]	Area %
1	2.305	PV	0.0936	101.18351	17.35507	1.3848
2	2.442	VV	0.0582	31.96680	7.67860	0.4375
3	2.612	VV	0.1207	233.54565	27.71351	3.1963

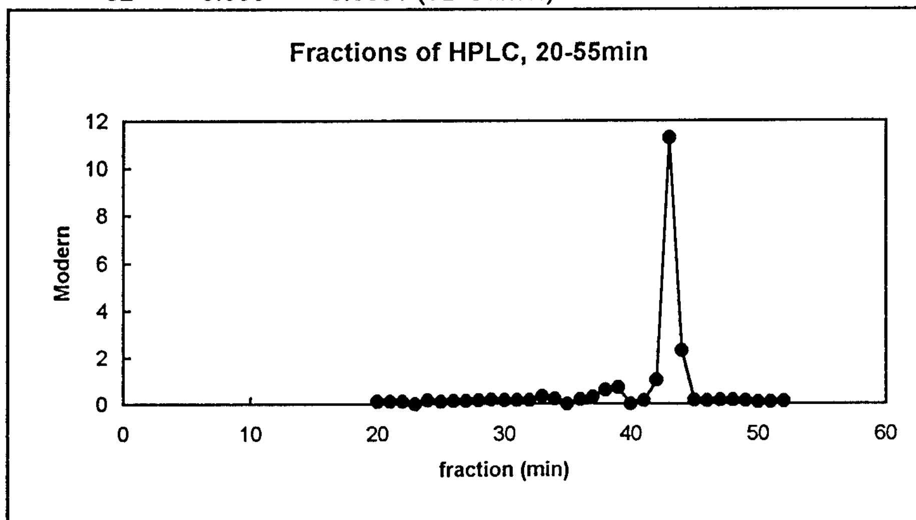
DI	50
ISV	5000
IN	650
OR	40
R0	30
M1	1000
RE1	119.0
DM1	0.100
R1	27.5
L9	-105
FP	-10
MU	-3800
CC	10
DI µA	3.5
IS v	5124.6
UV	97.7



HPLC FRACTIONS OF BPdG: ^{14}C-Acetylated deglycosylated BPdG
purified in our lab
injected in Ken's lab and analyzed by AMS

AMS of standard : HPLC fractions each minute after 20min

20	0.1032	0.0063
21	0.1034	0.0063
22	0.1072	0.0062
23	0.0063	
24	0.1653	0.0085
25	0.1096	0.0063
26	0.1233	0.0064
27	0.1328	0.0063
28	0.1489	0.0063
29	0.1876	0.0066
30	0.1658	0.0069
31	0.152	0.0063
32	0.1566	0.0065
33	0.3154	0.0068
34	0.2119	0.0065
35	0.0067	
36	0.1752	0.0064
37	0.2955	0.0068
38	0.5908	0.0087
39	0.6882	0.0088
40	0.0065	
41	0.1461	0.0063
42	1.0166	0.0106
43	11.2721	0.1153
44	2.2816	0.0248
45	0.1511	0.0063
46	0.1295	0.0063
47	0.1532	0.0067
48	0.1533	0.0062
49	0.1344	0.0062
50	0.0914	0.0062 (TB Carrier)
51	0.0937	0.0063 (TB Carrier)
52	0.098	0.0061 (TB Carrier)



Future Plans

1. Cleanup of ^{14}C background for AMS : washes with cold acetic acid
2. Finish labeling of BPdG in pyridine, determine sensitivity of measurement
3. Prepare new IAF columns for BPdG
4. Analyze a few DNA samples (make ^{3}H-BPdG derivatized DNA and some samples previously analyzed by ^{32}P)
5. Optimize acetylation with THF/MeIm for epi studies

APPENDIX L – Dopamine D4 receptors and the risk of cigarette smoking in African Americans and Caucasians

IN PRESS
Health Psychology
1997

Depression and Self-Medication with Nicotine:

The Modifying Influence of the Dopamine D4 Receptor Gene

Caryn Lerman

Georgetown University Medical Center

Lombardi Cancer Center

Neil Caporaso

National Cancer Institute

Laboratory of Human Carcinogenesis

David Main and Janet Audrain

Georgetown University Medical Center

Lombardi Cancer Center

Neal R. Boyd

Fox Chase Cancer Center

Elise D. Bowman and Peter G. Shields

National Cancer Institute

Laboratory of Human Carcinogenesis

4-8-97 c:\cl\paper\modify

ABSTRACT

A positive association between depression and self-medication with nicotine (i.e., smoking for stimulation or negative affect reduction) has been established previously. In this study, we evaluated whether there are genetic subgroups of depressed individuals who are more or less predisposed to engage in self-medication smoking practices. Two hundred and thirty-one smokers who volunteered for a smoking cessation treatment program completed self-report questionnaires of depression and smoking practices, and were genotyped for the dopamine D4 receptor *(DRD4)* gene. We found a significant interaction (*DRD4* genotype x depression) for stimulation smoking and negative affect reduction smoking. Specifically, these smoking practices were significantly heightened in depressed smokers with *DRD4* S/S genotypes, but not in those with S/L or L/L genotypes. These preliminary results suggest that the rewarding effects of smoking and the beneficial effects of nicotine replacement therapy for depressed smokers may depend, in part, on genetic factors involved in dopamine transmission.

Key words: smoking, genetics, depression

Depression and Self-Medication with Nicotine:

The Modifying Influence of the Dopamine D4 Receptor Gene

A positive association between depression and cigarette smoking has been well-established. Individuals who have a history of major depressive disorder are significantly more likely to be smokers and to have a DSM-III-R diagnosis of nicotine dependence (Glassman et al., 1990; Breslau, Andreski, & Kilbey, 1991; Breslau, Kilbey, & Andreski, 1993). Smokers are more likely than nonsmokers to report depressive symptoms (Pérez-Stable, Marín, Marín, & Katz, 1990), and the presence of such symptoms predicts relapse following smoking cessation attempts (Hall, Munoz, Reus, & Sees, 1993; Glassman et al., 1990). In fact, the likelihood of quitting smoking is about 40% lower among depressed smokers, compared to nondepressed smokers (Anda et al., 1990).

A "self-medication" model of smoking has been advanced to explain the heightened nicotine dependence among depressed individuals (Hughes, 1988; Carmody, 1989; Pomerleau & Pomerleau, 1984). According to this model, the mood-altering properties of nicotine are especially reinforcing for depressed individuals who are prone to experience negative affect. The self-medication model was supported by recent studies linking depression to self-reports of smoking to increase arousal and reduce negative affect (Lerman et al., 1996; Kinnunen, Doherty, Militello, & Garvey, 1996). Further support for the self-medication model was provided by studies showing greater responsiveness of depressed smokers to nicotine replacement therapy (Russell, 1994; Kinnunen et al., 1996), and to treatment with antidepressants (Berlin et al., 1995; Edwards, Simmons, Rosenthal, Hoon, & Downs, 1988). However, there is considerable individual variation in smoking practices and quitting rates among depressed smokers (Lerman et

al., 1996; Kinnunen et al., 1996).

Biological differences in the reinforcing properties of nicotine may underlie individual differences in propensity toward self-medication smoking in depressed persons (Pomerleau, Collins, Shiffman, & Pomerleau, 1993). These biological differences are likely to be mediated, in part, by genetic factors (Gilbert & Gilbert, 1995; Kendler et al., 1993). Twin studies suggest that 16% to 30% of the variance in self-reported depressive symptoms and as much as 50% of the variance in the initiation and maintenance of cigarette smoking is attributable to genetic influences (Gatz, Pedersen, Plomin, Nesselroade, & McClearn, 1992; Jardine, Martin, & Henderson, 1984; Carmelli, Swan, Robinette, & Fabsitz, 1992; Heath & Martin, 1993). Smokers' reports of self-medication smoking also have been shown to have a significant heritable component (Gynther, Hewitt, Heath, & Eaves, 1993).

Genes involved in the brain's reward mechanisms may be one source for genetic factors that influence self-medication smoking. Specifically, several converging lines of evidence point to genes in the dopaminergic system as being potentially important. As with other psychostimulants, the rewarding properties of nicotine are largely mediated by its effects on dopamine transmission (Di Chiara & Imperato, 1988; Corrigall, Franklin, Coen, & Clark, 1992). Of the five known types of dopamine receptors, the D4 receptor gene *(DRD4)* may be especially relevant to self-medication smoking because it is highly expressed in areas of the brain involved in emotion and reward-seeking behaviors (Van Tol et al., 1991; Seeman, 1995; Wise & Rompre, 1989). The *DRD4* gene has a variable number of tandem repeats (i.e., repeats in nucleotides in a particular DNA coding sequence) (Van Tol et al., 1991, 1992). The long version of the gene (referred to as "L allele") contains 6-8 repeats, while the short (S) alleles contain less than 6

repeats. The L alleles (specifically the 7-repeat [D4.7] allele) have been shown to alter receptor function and to blunt intracellular response to dopamine (Asghari et al., 1995; Van Tol et al., 1991, 1992). Novelty-seeking behavior (Benjamin et al., 1996; Ebstein et al., 1996) and attention-deficit disorder (LaHoste et al., 1996), two traits linked to reduced dopamine transmission, are more common in individuals who carry the *DRD4* L alleles. However, the S alleles have been related to lower scores for positive emotions (Benjamin et al., 1996).

These findings led us to consider two competing hypotheses for the role of *DRD4* in self-medication smoking: (1) self-medication smoking and nicotine dependence would be greater in depressed smokers who have *DRD4* L alleles, because they have a greater need to use nicotine to increase synaptic dopamine and pharmacologically overcome altered dopamine receptor function; or (2) self-medication smoking and nicotine dependence would be greater in depressed smokers who had *DRD4* S alleles, because normal dopamine receptor function is necessary for nicotine to be maximally rewarding. To test these hypotheses, we genotyped smokers who participated in a previous study of depression, self-medication smoking and nicotine dependence (Lerman et al., 1996). Specifically, we investigated whether self-medication smoking and nicotine dependence were heightened in depressed smokers who have the different *DRD4* alleles. The ultimate objective of this research was to better understand the genetic basis of self-medication smoking patterns in order to develop improved pharmacologic treatment strategies for depressed smokers, and to target these approaches to those most likely to benefit.

METHODS

Study Participants

Participants were 231 smokers who responded to media advertisements for a free

smoking cessation program. Eligible smokers were those smoking at least 5 cigarettes per day for at least one year. The exclusion criteria for the study included: being under age 18, pregnancy, having a personal history of cancer, undergoing current treatment for drug or alcohol addiction, or current use of psychotropic medications. A subset of this study population was examined in previous reports of depression and nicotine dependence (Lerman et al., 1996) and smoking cessation (Lerman et al., 1997). These participants also were included in a case-control study of *DRD4* and smoking risk (Shields et al., unpublished data).

Design and Procedure

Smokers responding to advertisements received a short telephone eligibility screening interview and a brief description of the study and participation requirements. During a visit to the clinic, participants completed an informed consent form and a set of self-report questionnaires described below. All participants donated blood for genetic analysis. DNA was extracted from whole blood using standard phenol extraction methods. PCR for the dopamine *D4* 48bp VNTR in exon 3 was performed based on the previously reported method (George, Cheng, Nguyen, Israel, & O'Dowd, 1993). Briefly, genomic DNA (25 ng) was amplified using 20 pmol of primers (5'-CTG CGG GTC TGC GGT GGA GTC TGG-3' and 5'-GCT CAT GCT GCT GCT CTA CTG GGC-3') in DMSO (5%), glycerol (10%), buffer (tris-HCL [10 mM, pH 8.3], KCl [50 mM], $MgCl_2$ [1.0 mM]), Amplitaq DNA polymerase (0.63 u, Perkin Elmer, Norwalk, CT) and 2-deoxynucleotides-5'-triphosphates (1.87 mM, Pharmacia, Piscataway, NJ) in a 25 μl volume. The primers were synthesized using a Beckman Oligo1000 DNA Synthesizer (Fullerton, CA). The PCR reaction had an initial melting temperature of 95°C (4 min), followed by 30 cycles of melting (95°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 1 min). An

extension period at 72°C (4 min) followed. The PCR reactions were performed using a Stratagene Robocycler Gradient 96 (La Jolla, CA). Fragments ranging from 270 to 570 base pairs (two to eight repeats) were resolved by agarose gel electrophoresis (Nusieve GTG [Gibco BRL, Gaithersburg, MD] and Agarose, 2:1 w/v, 3% total) and observed with ethidium bromide staining. The assay was validated by confirming polymorphic Mendelian inheritance patterns in seven human family cell lines (n = 134 family members), encompassing three generations (data not shown; samples were obtained from NIGMS Human Genetic Mutant Cell Repository, Coriell Institute, Camden, NJ). The genotype results were read by two independent investigators and 20% of the samples were repeated for quality control.

Measures

Center for Epidemiologic Studies Depression (CES-D) Scale. The CES-D is a 20-item Likert-style scale used to assess depressive symptomatology. This scale has high internal consistency (r = .85-.95), test-retest reliability (r = .57 for 2-8 weeks), and has been shown to correlate with clinical ratings of the severity of depression (Radloff, 1977).

Self-medication Smoking. Participants completed a modified version of the Horn-Waingrow Reasons for Smoking (RFS) Scale (Horn & Waingrow, 1966). As in our previous study (Lerman et al., 1996), we selected two specific factors that corresponded to self-medication smoking: smoking for stimulation (e.g., "I get a definite lift and feel more alert when smoking"; 4 items, range = 0-12), and smoking for negative affect regulation (e.g., "When I feel blue or want to take my mind off cares and worries, I smoke cigarettes"; 3 items, range = 0-9). Participants were asked to rate the statements on a Likert scale. "How much is each of the following characteristic of you" (0 = not at all to 3 = very much so). Both of these subscale

factors have been shown to correlate significantly with self-monitored smoking data (Joffe, Lowe, & Fisher, 1981; Shiffman & Prange, 1988; Tate & Stanton, 1990) and have stable factor structures and satisfactory test-retest reliability (Costa, McCrae, & Bosse, 1980).

Fagerstrom test for nicotine dependence (FTND). The FTND is a 6-item, self-report measure of nicotine dependence derived from the Fagerstrom Tolerance Questionnaire (FTQ) (Heatherton, Kozlowski, Frecker, & Fagerstrom, 1991). Sample items include the number of cigarettes smoked in the past seven days and the average length of time from waking to smoking. The FTND scale has satisfactory internal consistency (Cronbach's alpha = .64) and high test-retest reliability (r = .88) (Pomerleau, Carton, Lutzke, Flessland, & Pomerleau, 1994).

Statistical Analysis

The dopamine *D4* receptor data were coded as previously reported based upon the number of 48 bp repeats in exon 3, and also by genotypes representing the overall length (long (L) = ≥6 repeats and short (S) = <6 repeats) (Benjamin et al., 1996). Thus, participants were classified as having S genotypes (i.e., homozygous for the short alleles; S/S) or L genotypes (i.e., homozygous or heterozygous for the long alleles; S/L or L/L). The main effects of genotype on depression, self-medication smoking, and nicotine dependence were evaluated using Student T-Tests. Next, smokers were classified as depressed or nondepressed, using the standard cut-off on the CES-D (<16 vs. ≥16) (Radloff, 1977). To test our primary hypothesis, that *DRD4* genotype modifies the relationship of depression to self-medication smoking and nicotine dependence, we performed Student T-tests that were stratified by genotype (S/S vs. S/L or L/L). The main effects and interactions between *DRD4* and the dichotomized depression variable were examined in linear regression analyses controlling for race, gender, age and smoking rate (smoking rate was

not included in models of nicotine dependence since this variable is included in the FTND scale). Controlling variables having $p \leq 0.20$ associations with smoking outcome variables were retained in the final models. All *p* values reported are 2-tailed.

RESULTS

Descriptive Data on Study Sample

Fifty-three percent of participants were female and 47% were male. Eighty-three percent were Caucasian and 17% were African American. Twelve percent of participants were ages 18-29, 62% were ages 30-49, and 25% were ages 50 and older. Fifty-seven percent were college graduates.

The average score on the CES-D was 13.6 ± 0.6 (range = 0-54) (as compared to population reference value of 9.0; Radloff, 1977). One hundred forty-five (63%) smokers were classified as nondepressed and 86 (37%) were classified as depressed. These figures are consistent with rates of current depression found in other studies of smokers seeking treatment (Kinnunen et al., 1996). In the total sample, the average score for Stimulation Smoking was 6.0 ± 0.2 (range = 0-12) and for Negative Affect Reduction Smoking was 6.4 ± 0.1 (range = 0-9). These two scales were correlated ($r = 0.44$, $p = .0001$). The average number of cigarettes smoked per day in this sample was 21 ± 10, and the average score on the FTND scale was 5.1 ± 0.2. Nicotine dependence was correlated with stimulation smoking ($r = 0.35$, $p = .0001$) and negative affect reduction smoking ($r = 0.29$, $p = .001$).

In terms of *DRD4* genotype, 176 (76%) of participants had S/S genotypes, 44 (19%) of participants had S/L genotypes, and 11 (5%) of participants had L/L genotypes. As found previously (Ebstein et al., 1996), the L alleles consisted almost entirely of the D4.7 (95%). As

previously reported, we classified participants as having short genotypes (S/S, 76%) or long genotypes (S/L or L/L, 24%) (Benjamin et al., 1996). It was not possible to examine smokers with L/L genotypes separately due to the small sample size. Statistically significant race differences were found for the prevalence of the *DRD4* genotypes (78% of Caucasians had S/S genotypes, compared to 60% of African Americans) (χ^2 (1, N = 231) = 6.7, p = .03). Thus, race was controlled in all regression models.

DRD4 genotype (S/S vs. S/L or L/L) was not associated with depression (T(230) = 0.56, p = .56), stimulation smoking (T(230) = 0.44, p = .65), negative affect reduction smoking (T(230) = 0.42, p = .68), or nicotine dependence (T(230) = 0.56, p = .57).

Comparison of Depressed and Nondepressed Smokers on Smoking Variables, Stratified by *DRD4* Genotype

As shown in Table 1, among smokers with *DRD4* S/S genotypes, those who were depressed (compared to nondepressed) reported significantly greater stimulation smoking (p = .005) and greater negative affect reduction smoking (p = .0001). Among smokers with *DRD4* S/L or L/L genotypes, depression was not associated with stimulation smoking (p = 0.24) or negative affect reduction smoking (p = .51). The difference between depressed and nondepressed smokers in nicotine dependence was not statistically significant in smokers with S/S genotypes; however, the trend was consistent with other findings (p = .11). When nicotine dependence was dichotomized as very high versus very low to high (Heatherton et al., 1991), a significant association between depression and nicotine dependence was found. Specifically, among smokers with S/S genotypes, 14% of nondepressed smokers reported very high levels of dependence, compared with 27% of depressed smokers (χ^2 (1, N = 176) = 4.7, p = .03). There

was no association of depression with the continuous nicotine dependence variable in smokers with S/L or L/L genotypes ($p = .81$) or with the dichotomized nicotine dependence variable (χ^2 (1, N = 55) = 0.3, $p = .59$).

Insert Table 1 about here

Multiple Regression Models of Smoking Variables

The main and interacting effects of *DRD4* genotype and depression were tested in multiple linear regression models. Controlling for potential confounders (race, age, gender and smoking rate), we found evidence for statistically significant interactions between depression and *DRD4* genotype for stimulation smoking ($p = .04$) and negative affect reduction smoking ($p = .01$) (Table 2). As was found in the stratified univariate analyses, the associations of depression with the self-medication variables were modified by *DRD4* genotype; depression was positively related to self-medication smoking in smokers with S/S genotypes, but not those with S/L or L/L genotypes. After accounting for the effects of the confounders and depression, the changes in R^2 (variance accounted for) for the *DRD4* genotype effects were .02 for stimulation smoking and .03 for negative affect reduction smoking. The R^2 for the complete models of stimulation smoking and negative affect reduction smoking were 0.19 and 0.15, respectively. Significant main or interacting effects of *DRD4* on nicotine dependence were not found ($p = .22$ and .14, respectively).

Insert Table 2 about here

DISCUSSION

In this study, we sought to identify genetic subgroups of depressed individuals who are more or less predisposed to engage in self-medication smoking practices. We found that self-reported stimulation smoking and negative affect-reduction smoking were heightened significantly in depressed smokers with *DRD4* S/S genotypes, but not in those with S/L or L/L genotypes. Although the proportion of variance in smoking practices accounted for by genetic variables was relatively small, these effects are on the order of those observed in other studies of associations of single genes with complex behaviors (Lesch et al., 1996; Benjamin et al., 1996). The likelihood of being highly nicotine dependent was also significantly greater in depressed smokers with S/S genotypes; however, this effect did not remain significant in the multi-variable model.

A tentative biological explanation for these findings is that, in depressed individuals, normal dopamine receptor function (S/S genotype) is necessary for the effects of nicotine to be reinforcing. This hypothesis is consistent with both animal and human studies showing that the rewarding properties of nicotine are partly attributable to its effects on the neurotransmission of dopamine (DiChiara & Imperato, 1988; Henningfield, Schuh, & Jarvik, 1995) and that the S/L and L/L genotypes relate to a blunted intracellular response to dopamine (Ashgari et al., 1995; Van Tol et al., 1991, 1992).

While these results provide preliminary support for the *DRD4* genotype as a moderator of

the relationship of depression and smoking practices, significant main effects of genotype on depression or smoking practices were not detected. However, an association between *DRD4* genotype and smoking prevalence was found in a recent case-control analysis comparing smokers from this study to race-matched nonsmokers (Shields et al., unpublished data). In this study, African American smokers were significantly more likely to have *DRD4* S/L and L/L genotypes than African American nonsmokers. This finding, which suggests that *DRD4* long genotypes may predispose to initiation of smoking, appears at first to contradict the current findings. However, in non-depressed individuals, which account for the majority of participants in both studies, the findings are consistent. Inspection of Table 1 shows that, among non-depressed smokers, the levels of self-medication smoking variables were higher for smokers with S/L and L/L genotypes than those with S/S genotypes. The reverse was true for depressed smokers, however. Differences between depressed and nondepressed smokers in the effects of *DRD4* genotype on smoking may be attributable to an interaction of *DRD4* with other genes that influence depression (Gatz et al., 1992).

Given the prevalence of depressive symptoms in the general population (Radloff, 1977), and among smokers in particular (Kinnunen et al., 1996; Lerman et al., 1996), further research into the links between depression and nicotine dependence is warranted. Depressed smokers appear to be predisposed to initiate smoking and to become highly dependent (Perez-Stable et al., 1990; Glassman et al., 1990; Hall et al., 1993). Smokers who report depression may, therefore, derive significant benefit from nicotine replacement therapies and psychotropic medications (Edwards et al., 1988; Kinnunen et al., 1996). The present study is the first to suggest that the beneficial effects of such pharmacologic therapies may depend, in part, on genetic factors

involved in dopamine transmission. Current research on depression and smoking should be extended to account for individual differences in genes relevant to dopamine transmission, as well as other neurotransmitter such as serotonin. With additional research in this area, genotyping may become a useful strategy to design and target pharmacologic therapies to those smokers most likely to benefit.

Ultimately, an understanding of the genetic basis of smoking and smoking practices will require more complex analyses of the effects of multiple genes acting in conjunction with other personality factors (e.g., anxiety, extraversion) and environmental influences (e.g., tobacco advertising, peer pressure) (Gilbert & Gilbert, 1995). Therefore, the results of analyses involving a single gene should be viewed as preliminary. It should also be noted that our recruitment of study participants responding to newspaper advertisements for smoking cessation might generate a sample that is not representative of the general population. In addition, we utilized a measure of self-reported depressive symptoms, but did not assess major depression. However, given the high prevalence of depressive symptoms in the general population (Radloff, 1977), attention to these individuals is warranted. Despite these limitations, the current study provides some initial insights into genetic factors that influence smoking practices in depressed smokers and suggests a model for future research on the interacting effects of genetic and environmental factors in smoking.

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Table 1

Means and Standard Errors for Smoking Variables by Depression and Genotype

Dependent Variables	All Participants ($n = 231$)				S/S Genotypes ($n = 176$)				S/L or L/L Genotypes ($n = 55$)			
	Depressed ($n = 86$)	Nondepressed ($n = 145$)			Depressed ($n = 62$)	Nondepressed ($n = 114$)			Depressed ($n = 24$)	Nondepressed ($n = 31$)		
	$X \pm SE$	$X \pm SE$	$T_{(230)}$	p	$X \pm SE$	$X \pm SE$	$T_{(174)}$	p	$X \pm SE$	$X \pm SE$	$T_{(53)}$	p
Stimulation smoking	6.5 ± 0.3	5.8 ± 0.2	1.9	0.07	6.9 ± 0.4	5.6 ± 0.3	2.8	0.005	5.4 ± 0.5	6.2 ± 0.5	1.2	0.24
Negative affect-reduction smoking	7.1 ± 0.2	6.1 ± 0.2	3.7	0.0003	7.4 ± 0.2	5.9 ± 0.2	4.8	0.0001	6.3 ± 0.4	6.7 ± 0.3	0.7	0.51
Nicotine dependence	5.3 ± 0.3	4.9 ± 0.2	1.2	0.23	5.5 ± 0.3	4.9 ± 0.2	1.6	0.11	4.8 ± 0.5	5.0 ± 0.5	0.2	0.81

AUTHOR NOTES

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Table 2

Multiple Regression Models of Smoking Variables

Outcome variable	Predictor variable	Final β	p value
Stimulation smoking	Race	-0.5	.59
	Gender	1.8	.07
	# cigs./day	4.7	.0001
	Depression	2.7	.007
	DRD4	1.4	.17
	DRD4 x Depression	-2.1	.04
Negative affect smoking	Race	0.2	.83
	Age	-2.3	.02
	Gender	4.1	.0001
	# cigs./day	2.9	.004
	Depression	3.7	.0003
	DRD4	2.3	.02
	DRD4 x Depression	-2.4	.01
Nicotine dependence	Race	-0.8	.41
	Age	1.5	.13
	Gender	1.6	.11
	Depression	1.7	.10
	DRD4	0.4	.65
	DRD4 x Depression	-0.8	.44

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APPENDIX M – Dopamine D4 Receptors and the Risk of Cigarette Smoking in African Americans and Caucasians

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Dopamine D4 Receptors and the Risk of Cigarette Smoking
in African Americans and Caucasians

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ABSTRACT

Introduction: An understanding of why people smoke cigarettes can have an important impact upon smoking prevention and cessation. People smoke cigarettes to maintain nicotine levels in the body, and nicotine has been implicated in the stimulation of brain reward mechanisms via central neuronal dopaminergic pathways. In this study, we evaluated the association of smoking and smoking cessation with a dopamine D4 receptor 48 base pair variable nucleotide tandem repeat polymorphism, where the 7 repeat allele (D4.7) reduces dopamine affinity. *Methods:* We recruited smokers (n=283) and nonsmokers (n=192) through local media for a case-control study of smoking. Following informed consent and a behavioral questionnaire, smokers underwent a single minimal contact session of smoking cessation counseling, and then were followed for up to one year. The frequency of the dopamine D4 receptor genetic polymorphism using PCR was determined and individuals were classified by the number of repeat alleles (2-5 repeats as "S" and 6-8 repeats as "L"). Persons with those genotypes including only S alleles (homozygote S/S) were compared with those with at least one L allele (heterozygote S/L and homozygote L/L). Chi Square Tests of Association, Fisher's Exact Test, student T-tests and unconditional logistic regression were used. P values were two-tailed. *Results:* The data show that African Americans (n=72) who had at least one L allele had a higher risk of smoking (OR=7.7, 95% C.I.=1.5, 39.9; P=0.006), shorter time to the first cigarette in the morning (P=0.03) and earlier age at smoking initiation (P=0.09), compared with homozygote S/S genotypes. Following smoking cessation counseling, none of the African American smokers with an L allele were abstinent at two months, compared with 35% of the smokers who were homozygote S/S (P=0.02). The analysis of Caucasians (n=403) did not suggest a similar smoking risk for the D4

genotypes (O.R. = 1.0; 95% C.I. = 0.6, 1.6; P=0.90), or smoking cessation (P=0.75).

Implications: While the number of African Americans is small, this study is consistent with the hypothesis that the L alleles increase the risk of smoking because these individuals are prone to use nicotine to stimulate synaptic dopamine transmission. A single minimal contact session of cessation counseling is ineffective in African Americans smokers who have at least one L allele. These persons may need additional therapy, such as pharmacological intervention. Conversely, persons who are S/S may benefit from such minimal counseling. The response to such counseling based on genetic predispositions may be different by race.

INTRODUCTION

Tobacco smoking is a major cause of morbidity and mortality in the United States and other industrialized countries. To reduce the medical consequences of smoking, better means to prevent tobacco smoking initiation and addiction, and fostering smoking cessation are needed. While factors such as family history, peer pressure, advertising, and cost of cigarettes may contribute to smoking, the most significant determinant is nicotine dependence [1]. Currently, there is interest by governmental agencies to regulate nicotine in cigarettes to reduce cigarette smoking, and the attendant health effects. Host susceptibilities may play a role in nicotine dependence, such as through interindividual variation in nicotine metabolism or neurobehavioral factors that relate to the reinforcing value of nicotine. The former might dictate the initial pharmacological reactions to nicotine and how much smoking is needed to maintain nicotine levels [1,2], while the latter may affect why people need to maintain nicotine levels.

Nicotine has a "rewarding" property that serves to reinforce drug seeking behavior [1-3]. Nicotine stimulates central nicotinic acetylcholine receptors, which are upregulated and desensitized simultaneously by chronic exposure. These receptors stimulate the secretion of dopamine into the neuronal synapse, which then stimulates post-synaptic dopamine receptors, thereby satisfying craving. The relationship of nicotine to the dopaminergic system is well established, as is the effect of nicotine on psychiatric illness [4]. The stimulation of the dopaminergic system is not solely dependent upon nicotine, however; for example, cocaine, amphetamine, and food also affect dopamine pathways [5,6].

We have hypothesized that interindividual variation for dopamine pathways and the reward mechanism might alter smoking risk. To examine this hypothesis, we have studied

polymorphisms in genes that govern synaptic dopamine levels through active reuptake by the dopamine transporter and in dopamine receptors. These results have provided evidence that the risk of smoking is related to a genetic polymorphism in the dopamine reuptake transporter gene, and that there is an interaction with a dopamine D2 receptor polymorphism (unpublished data).

Post-synaptic dopamine receptors [7,8] can be classified as "D1-like"or "D2-like." The dopamine D4 receptor [9] is an example of the latter group. There are differences between the dopamine D2 and D4 receptors, however, such as dopamine affinity (greater for the D4 receptor in the low affinity receptor state) and levels of protein expression [7]. There also is a difference for the binding of the dopamine agonist clozapine, which is an order of magnitude higher for the D4 receptor compared with the D2 receptor [9-12]. Clozapine is used for the treatment of schizophrenia, a disease that has a hypothesized underlying defect of the dopamine reward system. The D4 receptor affects G-protein-mediated functional coupling [13], and has been reported to be increased in schizophrenic patients [10,13], although not consistently [14].

There is an imperfect 48 bp variable nucleotide tandem repeat polymorphism in the third exon of the dopamine D4 receptor, involving 18 amino acids, which codes for a proline rich protein domain in the third cytoplasmic loop [15,16]. The 7 repeat allele (D4.7) has been associated with increased competition for [^{3}H]spiperone binding in initial studies [15], accounting for increased clozapine binding. A subsequent *in vitro* study using transiently expressed COS-7 cells found that the cyclic AMP effect of dopamine for D4.7 was reduced about 2-fold, although the overall effects of the polymorphism were considered small [17]. Nonetheless, the data suggest that D4.7 is associated with a blunted response to dopamine. However, a clinical effect of the dopamine D4 receptor polymorphism on the response to clozapine therapy in schizophrenia

could not be shown [18].

If D4.7 has a blunted response to dopamine *in vivo*, given that the effect of nicotine is to increase synaptic dopamine, it is plausible that people with the D4.7 allele might have more nicotine dependence resulting in a greater risk of smoking and a lesser ability to quit smoking after cessation counseling. To test this hypothesis, we studied the dopamine D4 receptor polymorphism in smokers and nonsmokers, and then examined the ability of the smokers to abstain from smoking following minimal contact behavioral cessation treatment.

METHODS

Study Subjects: Smokers (n=283), ages 18 and over, seeking a free smoking cessation program were recruited through media advertising (newspapers and flyers) in Washington, D.C. and Philadelphia areas. Smokers were defined as smoking at least five cigarettes per day for at least one year. Controls (n=192) also were recruited through the newspaper advertisements and flyers, and were defined as having smoked less than 100 cigarettes in their lifetime. The exclusion criteria for the study were age less than 18, a personal history of cancer, undergoing current treatment for drug or alcohol addiction, or presence of a psychiatric disorder that precluded informed consent.

Procedures: During a visit to the clinic, subjects completed an informed consent form and a questionnaire assessing demographics and smoking history. Subjects then received a single minimal contact (1 hour) of behavioral smoking cessation counseling and self-help materials [19]. They were then followed for one year following the counseling to assess self reports of quitting.

The outcome measure was a 7-day point prevalence of smoking at two months and twelve months after smoking cessation treatment. All subjects donated blood for genetic analysis.

Dopamine D4 Receptor Genotyping: DNA was extracted from whole blood using standard phenol extraction methods. PCR for the dopamine D4 48bp VNTR in exon 3 was performed based on the method of George, et al [20]. Briefly, genomic DNA (25 ng) was amplified using 20 pmol of primers (5'-CTG CGG GTC TGC GGT GGA GTC TGG-3' and 5'-GCT CAT GCT GCT GCT CTA CTG GGC-3') in DMSO (5%), glycerol (10%), buffer (tris-HCl [10 mM, pH 8.3], KCl [50 mM], $MgCl_2$ [1.0 mM]), Amplitaq DNA polymerase (0.63 u, Perkin Elmer, Norwalk, CT) and 2'-deoxynucleosides-5'-triphosphates (1.87 mM, Pharmacia, Piscataway, NJ) in a 25 μl volume. The primers were synthesized using a Beckman Oligo1000 DNA Synthesizer (Fulleron, CA). The PCR reaction had an initial melting temperature of 95°C (4 min), followed by 30 cycles of melting (95°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 1 min). An extention period at 72°C (4 min) followed. The PCR reactions were performed using a Stratagene Robocycler Gradient 96 apparatus (La Jolla, CA). Fragments ranging from 270 to 570 base pairs (two to eight repeats) were resolved by agarose gel electrophoresis (Nusieve GTG [Gibco BRL, Gaithersburg, MD] and Agarose, 2:1 w/v, 3% total) and detected with ethidium bromide staining. The assay was validated by confirming polymorphic Mendelian inheritance patterns in seven human family cell lines (n=134 family members), encompassing three generations (data not shown; samples were obtained from NIGMS Human Genetic Mutant Cell Repository, Coriell Institute, Camden, NJ). Genotyping results were read by two independent investigators and genotyping for 20% of the subjects were repeated for quality control. The

investigators were blinded to each other's interpretations and to smoking status.

Statistical Analysis: The dopamine D4 receptor was classified as previously reported [21] based upon the number of 48 bp repeats in exon 3, and by genotypes representing the overall length ("S" consisting of 2, 3, 4 or 5 repeats and "L" alleles consisting of 6, 7 or 8 repeats). Most (89%) of the L alleles were the 7 repeats (D4.7) (Table 1). Associations of alleles and genotypes with current smoking and categorical smoking variables (age at smoking initiation [greater or less than 16 years old], and time to the first cigarette in the morning [greater or less than 30 minutes]), and 7-day point prevalence (persons who self-reported smoking within seven previous days) for two and twelve months of follow-up were examined using Chi Square Tests of Association, except where the Fisher's Exact Test was used. When examining continuos variables (e.g., smoking rate), student T-tests or ANOVAs were used. Odds ratios (ORs) and their 95% confidence intervals were calculated by unconditional logistic regression using SAS (Statistical Analysis System, Cary, NC). All p values were 2-tailed.

RESULTS

There were 283 smokers and 192 controls who were genotyped for the D4 genetic polymorphism (Figure 1). The allelic frequencies for smokers and nonsmokers by race are shown in Table 1, where D4.7 is more common in African Americans. There was a statistically significant difference for the prevalence of L and S alleles between Caucasians and African Americans for smokers (P=0.004), but not for nonsmokers (P=0.18). The frequency of

genotypes met Hardy Weinberg equilibrium for smokers and nonsmokers when examined separately by race. Given the differences in allelic frequency for Caucasians and African Americans, subsequent analyses are presented separately for each racial group.

For African Americans, there was a significant association of the homozygote S/S versus heterozygote S/L versus homozygote L/L genotypes with smoking (Table 2), although the number of study subjects was small (n=71; P=0.02 Fishers Exact test). A difference for the African Americans with the S/S compared with the S/L or L/L genotypes was highly significant (χ^2=7.56, P=0.006). The odds ratio for the risk of smoking in African Americans with at least one L allele was 7.7 (95% C.I.=1.5, 39.9). In addition, African American smokers who had the S/L or L/L genotypes were more likely to smoke within 30 minutes of waking (95% versus 69%, χ^2=4.62, P=0.03) and to have started smoking before the age of 16 years (47% versus 24%, χ^2=2.84, P=0.09). There was no association with the number of cigarettes smoked per day. Table 3 shows the data for smoking abstinence following minimal contact behavioral smoking cessation counseling. Overall, 23% and 17% of African Americans reported quitting at two months and twelve months, respectively. When analyzed by genotype, none of the African Americans with the S/L or L/L genotypes reported having quit smoking at two months follow-up, while 35% of the persons with the S/S reporting quitting (P=0.02). At twelve months, the data were identical, but because of the smaller number of subjects, the result was not statistically significant (P=0.29). The odds of resuming smoking for the S/L or L/L genotypes could not be estimated because the proportion of abstainers was 0%.

For Caucasians, the presence of the homozygote L/L versus heterozygote S/L versus homozygote S/S genotypes was not associated with smoking (Table 2). The odds ratio for the

risk of smoking in Caucasians with at least one L allele was 1.0 (95% C.I.=0.6, 1.6). There was no association for the S/L or L/L genotypes with number of cigarettes smoked per day (Students T=0.78, P=0.43), age when smoking began ($\chi 2$=0.00, P=0.94) or time to the first cigarette in the morning ($\chi 2$=1.96, P=0.16). There was no difference for the S/S versus S/L or L/L for the likelihood of having quit smoking at the 2-month (P=0.75) or 12-month (P=0.08) follow-up (Table 3). Overall, only 15% and 17% could quit smoking at two months and twelve months, respectively. For persons with the S/S genotype, 15% could abstain following cessation therapy at the 2- and 12-month follow-ups, while 16% and 27% of the S/L and L/L individuals continued to abstain at two and twelve months, respectively. The odds for continuing to smoke and having the S/L or L/L genotypes was 0.85 (95% C.I.=0.35, 2.11) at two months and 0.45 (95% C.I.=0.19, 1.09) at twelve months.

DISCUSSION

This study indicates that the 48 bp VNTR polymorphism in the third exon of the dopamine D4 receptor gene, specifically persons with D4.6, D4.7 or D4.8, may be a risk factor for smoking in African Americans, but not Caucasians. The data are internally consistent for African Americans in that those persons with the S/L or L/L genotypes had a higher risk of smoking at entry into the study, shorter time to the first cigarette in the morning, age at smoking initiation and an inability to quit after counseling. However, the number of African Americans in this study is small. Given that the D4.7 repeat VNTR for the dopamine D4 receptor alters the structure of the receptor, affects dopamine agonistic binding for clozapine, and reduces the effects of the receptor 2-fold [15,17,22], the relationships of this genetic polymorphism to the risk of

smoking might be due to a need for nicotine to increase synaptic dopamine.

The relationship of the dopamine D4 receptor exon 3 VNTR polymorphism to smoking has received little attention. One study [20] examined the polymorphism in relation to smoking in alcoholics and did not find an association, but alcoholism is an addictive disease so that the results from this study may not be applicable to the findings contained herein. However, this polymorphism, and specifically D4.7, has been related to neurological illness and personality, specifically in two of three studies investigating novelty seeking behavior [21,23,24], a personality pattern associated with smoking. Also, the D4.7 also has been seen with attention deficit disorder[25] and Tourette syndrome families [26]. While there was no evidence for an association with bipolar affective disorder [27], there also was a reported trend for one of two studies in schizophrenia [28,29].

The associations of the D4 S/L and L/L genotypes with smoking practice and cessation outcomes in African Americans, and the fact that these genotypes are significantly more common in African Americans than Caucasians, are consistent with a growing body of literature on race differences in smoking. The population prevalence of smoking is significantly higher in African American men than in Caucasion men (34% versus 28%) [30], and African Americans have higher rates of smoking-related morbidity and mortality [31,32]. The higher smoking-related health hazards seem inconsistent with the findings that African Americans tend to initiate smoking at a later age and smoke less than Caucasians [33,34]. However, African Americans tend to smoke cigarettes with a higher tar and nicotine content [35-37], and report higher levels of nicotine dependence than Caucasians [38]. Our results suggest that African Americans with the D4 S/L or L/L genotypes may be especially predisposed to smoke and to become nicotine dependent.

Although African Americans attempt to quit smoking as often as Caucasians [37,39,40], they are less likely to succeed [40,41]. Quit rates in the study contained herein for African Americans and Caucasians were 23% and 15% at two months, and 17% and 17% at twelve months, respectively. This is consistent with previous studies of minimal contact smoking cessation treatments [42,43]. Our preliminary results suggest that African Americans who have the D4 S/L or L/L genotypes may have an especially difficult time abstaining from smoking at two months, as none of these African Americans could quit. Because of the higher level of nicotine dependence in African Americans [38] and a possible genetic predisposition to become dependent on nicotine, African American smokers may be especially good candidates for treatments that use nicotine replacement [44,45]. Further, the dopamine D4 receptor polymorphism in African Americans may be useful in discerning who are better candidates for minimal contact behavioral therapy (S/S genotypes) and who should have other therapies, such as nicotine replacement or psychotropic medications (S/L or L/L genotypes).

There are several limitations in this study. First, smokers recruited through the media may not represent smokers in the general population, and especially when the enrollees are recruited for a smoking cessation program. Another limitation is the small number of subjects with the L alleles, due to the low gene frequency. Thus, there is limited statistical power to detect positive associations. Separately, there is a possibility that there may be an association with smoking due to specific haplotypes that we did not study. While we have examined the associations by genotypes characterized by L or S alleles, in actuality there are more than 25 different haplotypes that code for 18 different predicted amino acid sequences [46]. At this time, the functional effects of these haplotypes on clozapine binding or receptor structure are not

known, and the risk of these haplotypes can only be investigated in much larger studies in order to have sufficient statistical power.

Despite the limitations noted above, this study is the first to identify a genetic polymorphism associated with smoking practices and ability to quit in African Americans. It suggests that there may be differences in the effects of genetic susceptibilities by race. Additional studies are needed to corroborate the findings contained herein. Nonetheless, a better understanding of the genetic determinants of smoking could enhance current efforts to prevent and treat nicotine dependence in this population. Specifically, the dopamine D4 48 bp VNTR polymorphism might be used to triage individuals seeking smoking cessation therapy. In African Americans who have the S/S genotype, a single minimal contact behavioral smoking cessation therapy session might be successful. However, for African Americans with the S/L or L/L genotypes, additional interventions such as pharmacological treatment would be indicated.

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Figure 1: Representative genotypes for the dopamine D4 48 bp VNTR polymorphism. The following genotypes are shown: 2/2 (A), 2/4 (B), 3/4 (C), 4/4 (D), 4/7 (E), 7/7 (F).

Table 1

Allele Frequency for Smokers and Non-Smokers by Race

	Non-Smokers (%)		**Smokers (%)**	
Allele	**Caucasians**	**African Americans**	**Caucasians**	**African Americans**
D4.2	44 (13)	11 (23)	49 (10)	6 (6)
D4.3	15 (5)	1 (2)	23 (5)	2 (2)
D4.4	236 (70)	31 (65)	331 (70)	61 (64)
D4.5	2 (1)	3 (6)	4 (0.8)	2 (2)
D4.6	0 (0)	0 (0)	1 (0.2)	0 (0)
D4.7	37 (11)	2 (4)	59 (13)	24 (25)
D4.8	2 (1)	0 (0)	3 (0.6)	1 (1)
Total	336	48	470	96

Table 2

Association of Dopamine D4 Receptor Genotypes and Smoking

Genotype[1]	Non-Smoker (%)	Smoker (%)	P Value
Caucasians			
S/S	132 (79)	183 (78)	0.28[2,3]
S/L	33 (19)	41 (18)	0.90[4]
L/L	3 (2)	11 (4)	
African Americans			
S/S	22 (92)	29 (60)	0.02[2,3]
S/L	2 (8)	13 (27)	0.006[4]
L/L	0 (0)	6 (13)	

[1]S=D4.2, D4.3, D4.4, or D4.5; L=D4.6, D4.7, or D4.8

[2]Fisher's Exact test

[3]P value for S/S versus S/L versus L/L

[4]P value for S/S versus S/L or L/L

Table 3

Association of Dopamine D4 Receptor Genotypes and

Smoking Cessation at 2-Months and 12-Months

	2-Month Follow-Up							12-Month Follow-up						
Genotype [1]	Abstain			Smoking			P	Abstain			Smoking			P
	N	Row %	Column %	N	Row %	Column %		N	Row %	Column %	N	Row %	Column %	
Caucasians														
S/S	23	15	77	134	85	79	0.78[2,3]	18	15	64	106	85	80	0.12[2,3]
S/L	5	15	17	28	85	16	0.75[4]	7	24	25	22	76	17	0.08[4]
L/L	2	22	7	7	78	4		3	38	11	5	62	4	
Total	30	15		169	85			28	17		133	83		
African Americans														
S/S	9	35	100	17	65	55	0.06[2,3]	5	24	100	16	76	0.6	0.43[2,3]
S/L	0	0	0	8	100	26	0.02[4]	0	0	0	4	100	16	0.29[4]
L/L	0	0	0	6	100	19		0	0	0	5	100	20	
Total	9	23		31	77			5	17		25	83		

[1]S=D4.2, D4.3, D4.4, or D4.5; L=D4.6, D4.7, or D4.8

[2]Fisher's Exact test

[3]P value for S/S versus S/L versus L/L

[4]P value for S/S versus S/L or L/L

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APPENDIX N – The Role of Serotonin Transporter Gene in Cigarette Smoking

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The Role of the Serotonin Transporter Gene in Cigarette Smoking

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Abstract

Data from twin studies have suggested that cigarette smoking has a significant heritable component. The serotonin transporter gene (*5-HTT*) is a plausible candidate gene for smoking predisposition because of its association with psychological traits relevant to smoking behavior. The present investigation evaluated the associations of smoking practices and smoking cessation with a polymorphism in *5-HTT* which is manifested as either an inserted (long) variant or deleted (short) variant. A case-control study design (268 smokers, 230 controls) was used to evaluate the associations of *5-HTT* genotype with smoking status. Case series analysis of smokers was employed to evaluate the role of *5-HTT* in age at smoking initiation, previous quitting history, current smoking rate, and 12-month quit rate following a minimal contact smoking cessation program. There were no significant differences in the distribution of *5HTT* genotypes in smokers as compared with nonsmokers in either Caucasians or African Americans, nor was *5-HTT* genotype associated with the smoking outcome variables. However, the results did reveal significant racial differences in the distribution of *5-HTT* genotypes; Caucasians were significantly more likely to carry the short variant of the *5-HTT* gene than were African Americans (p=.005). These findings suggest that the *5HTT* gene may not play a significant role in cigarette smoking practices.

Introduction

Cigarette smoking is the greatest preventable cause of cancer mortality (1), yet approximately 26% of adults in the U.S. continue to smoke (2). Evidence from twin studies (3) indicates that smoking has a significant heritable component. Previously, we reported on the results of a smoking case control study which examined associations of smoking practices with polymorphic genes important in dopamine transmission. These studies were based upon evidence supporting the role of dopamine in the brain's reward mechanism (4) and nicotine stimulation of dopamine transmission (5). We found preliminary support for the interacting effects of the dopamine transporter (SLC6A3) and dopamine D2 receptor (DRD2) genes on the likelihood of smoking, age at smoking initiation, and previous quitting history (6). However, the tyrosine hydroxylase (TH) gene was not associated with any smoking outcomes (7).

In this study, we examined associations of a serotonin transporter *(5-HTT)* gene with smoking practices. The serotonin transporter gene is located on chromosome 17q11.2 (8), and gene transcription has been reported to be modulated by a polymorphism in its regulatory region (9). The polymorphism is manisfested as a 44 bp deletion or insertion, where the inserted variant (L=long) versus the deleted variant (S) occurs in 57% and 43% of Caucasians, respectively. The L variant has been found to have a 2-fold greater basal activity and 1.7-fold increase in mRNA levels (9).

The *5-HTT* gene is a plausible candidate gene for smoking predisposition because of its role in psychological traits relevant to smoking behavior. The *5-HTT* polymorphism has been linked with anxiety-related personality traits (10) and with depression (11); however, the former finding was not replicated in a recent analysis (12). Both anxiety and depression have been

linked with nicotine dependence (13,14). Further, preliminary clinical data suggest that serotonin reuptake inhibitors, such as fluoxetine hydrochloride, may promote smoking cessation (15,16). Of interest, smokers who are more nicotine dependent responded better to fluoxetine treatment than less dependent smokers (6).

In the analysis reported here, we used a case-control study design to evaluate the association of smoking practices with the *5-HTT* polymorphism. A case-series analysis of smokers was performed to examine associations of *5-HTT* with age at smoking initiation, previous quitting history, current smoking rate, and 12-month cessation rates following a minimal contact smoking cessation treatment program.

Materials and Methods

Subjects: Smokers (n = 268) who reported smoking at least 5 cigarettes/day for at least one year were recruited through varied newspaper advertisements and flyers in the metropolitan Washington and Philadelphia areas for a free smoking cessation program. Non-smoking controls (n = 230) who reported having smoked less than 100 cigarettes in their lifetimes were recruited through similar mechanisms. Exclusion criteria were: under age 18; a personal history of cancer; undergoing treatment for drug or alcohol addiction, or presence of a psychiatric disorder which precluded informed consent.

Procedures: During a visit to the clinic, subjects completed an informed consent form and a questionnaire assessing demographics and smoking history variables (age at smoking initiation, longest prior quitting period, current smoking rate). Subjects then received a single minimal contact (1 hour) of behavioral smoking cessation counseling and self-help materials (17). They were then followed for one year following the counseling to assess self reports of

quitting. The quitting outcome measure was a 30-day point prevalance of smoking twelve months after smoking cessation treatment. All subjects donated blood for genetic analysis.

Oligonucleotide primers flanking the 5-HTTLPR (5'ggcgttgccgctctgaattgc and 5'-gagggactgagctggacaacccac) (8) from the *5-HTT* gene 5'-flanking regulatory region generating 484-bp or 528-bp was amplified by PCR using 50 ng of genomic DNA, 2.5 mM deoxyribonucleotides, 0.1 ug of primers, 10 mM tris-HCL (pH 8.3), 50 mM Kcl, 1.5 mM MgC12, and 1 U Taq DNA polymerase. Cycling conditions included melting at 95°C (5 minutes), 35 cycles of 95°C for 30s, annealing at 62°C for 45 seconds, and an extension at 72°C for 1 minute. A final extension at 72°C for 4 minutes completed the PCR. The amplified product was resolved by agarose gel electrophoresis (1.5%). The assay was validated by confirming polymorphic Mendelian inheritance patterns in seven human family cell lines (n = 134), encompassing three generations each (data not shown; NIGMS Human Genetic Mutant Cell Repository, Coriell Institute, Camden, NJ) and 20% of the total number of samples were repeated for quality control.

Results

The study sample included 280 (56%) females and 218 (44%) males. Of the 268 smokers, 221 (84%) were Caucasian and 47 (16%) were African American. Of the 230 nonsmoking controls, 203 (88%) were Caucasian and 27 (12%) were African American. The average age of study participants was 43.8 ± 11.5 years; 89% of participants had education beyond high school. Among the smokers, the average smoking rate was 21.8 cigarettes per day.

As shown in Figure 1, significant racial differences in the distribution of *5-HTT* genotypes were found (Chi Sq. = 10.6, p = .005). Caucasians were significantly more likely to

carry the short variant of the *5-HTT* gene than were African Americans. Therefore, analyses of the associations of genotype with smoking practices were stratified by race.

The prevalence of *5-HTT* genotypes by smoking groups is presented in Table 1. There were no significant differences in the distribution of genotypes in smokers vs. nonsmokers in either Caucasians or African Americans. Among smokers, we used Chi Square Tests of associations of age at smoking initiation (<16 vs. ≥ 16 years) and the 12-month post-treatment quit rates with *5-HTT* genotypes; no associations were found in Caucasians or African Americans. As shown in Table 2, *5-HTT* genotype was not associated significantly with the longest prior quitting period (in days) or with current smoking rate (number of cigarettes/day) in either Caucasians or African Americans.

Discussion

The present case-control study was the first to evaluate whether the likelihood of smoking was associated with a polymorphism in the serotonin transporter *(5-HTT)* gene in Caucasians and African Americans. Previous evidence linking this polymorphism with anxiety (10) and supporting the potential benefits of serotonin reuptake inhibitors in smoking cessation (16) suggested that this gene may be a plausible candidate for predisposition to nicotine dependence. However, in this study, we found no evidence for associations of *5-HTT* with current smoking, smoking history, or with cessation rates in either racial group. These results are in contrast with previous studies supporting associations of polymorphic dopaminergic genes, particularly the dopamine transporter gene, with smoking practices and smoking cessation (6). Taken together, these findings indicate that the serotonin transporter gene, or the polymorphism studied here, may play a relatively less important role in cigarette smoking practices. Further investigation of

other polymorphic serotonergic genes, such as those regulating post-synaptic receptor function, are needed to evaluate fully the role of serotonin transmission in smoking behavior.

As in our previous studies of dopaminergic genes, we found evidence for significant racial variation in genotype frequencies. In the present study, Caucasians were significantly more likely than African Americans to carry the short variant of *5-HTT* which has been associated with anxiety-related traits (10). Previously, we found racial differences in the frequencies of the dopamine D2 receptor *(DRD2)*, and dopamine transporter *(SLC6A3)* genes (6). In both cases, African Americans were significantly more likely to have genotypes associated with reduced dopamine transmission. Evaluation of racial differences in the frequency of genes governing neurotransmitter function may enhance our understanding of genetic contributions to race differences in smoking practices (18).

To elucidate fully the influence of genetic factors in cigarette smoking, it will be necessary to examine the interplay of the genes involved in synthesis, release, and receptor function for a variety of neurotransmitters. Examination of genetic factors in nicotine metabolism may also be fruitful. A better understanding of these pharmacogenetic mechanisms can lead to the development of improved prevention and treatment strategies tailored to the needs of individual smokers.

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Table 1. Prevalence of *5-HTT* Genotypes within Smoking Groups by Race

Group	Caucasian			African Americans		
	S/S	S/L	L/L	S/S	S/L	L/L
Smokers, n (%)	43 (19.4)	108 (48.9)	70 (31.7)	5 (10.6)	18 (38.3)	24 (51.1)
Controls, n (%)	28 (13.8)	115 (56.6)	60 (29.6)	4 (14.8)	10 (37.0)	13 (48.2)
χ^2	3.40			0.28		
p	0.18			0.87		
Smokers, starting age < 16 yrs	17 (22.7)	38 (50.7)	20 (26.6)	1 (7.7)	5 (38.5)	7 (53.8)
Smokers, starting age ≥ 16 yrs	26 (17.9)	69 (47.6)	50 (34.5)	4 (11.8)	13 (38.2)	17 (50.0)
χ^2	1.61			0.17		
p	0.45			0.92		
Smokers, quit at 1-yr follow-up	6 (22.2)	12 (44.5)	9 (33.3)	0 (0)	2 (50.0)	2 (50.0)
Smokers, smoking at 1-yr follow-up	27 (18.6)	73 (50.3)	45 (31.0)	1 (4.4)	9 (39.1)	13 (56.5)
p*	0.85			1.00		

* Fisher Exact Test

Table 2. Smoking History by *5-HTT* Genotype and Race

Variable		Caucasians			African Americans		
		S/S	S/L	L/L	S/S	S/L	L/L
Longest quit period in days		421 ± 880	347 ± 753	334 ± 1109	189 ± 405	349 ± 670	183 ± 532
(X ± S.D.)	F	0.14			0.43		
	p	0.89			0.65		
# cigarettes/day (X ± S.D.)		24.0 ± 10.9	23.2 ± 10.1	22.2 ± 11.1	13.2 ± 4.7	15.3 ± 8.3	16.6 ± 7.6
	F	0.41			0.44		
	p	0.66			0.64		

Figure 1. Prevalence of *5-HTT* Genotypes by Race