PERICONCEPTIONAL EXPOSURE TO NITRATES IN DRINKING WATER AND RISK FOR NEURAL TUBE DEFECTS

THESIS

Presented to the Graduate Council of
Southwest Texas State University
in Partial Fulfillment of
the Requirements

For the Degree

Master of SCIENCE in HEALTH PROFESSIONS

By

Janus McCallum Olive, B.S.

San Marcos, Texas
May, 2002

DEDICATION

I dedicate this paper to my husband, Kelly Olive, and my daughter, Jessica Olive, for their loving support and encouragement of my academic endeavors and completion of this research project.

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. Jean Brender, who chaired my thesis, for her patient mentoring, enthusiasm, and expertise in guidance of this research project and paper; to Dr. Charles Johnson, Chair of Health Services Research and thesis committee member, whose fervor for his profession has been an inspiration throughout my current academic pursuit; and, to Dr. Marilyn Felkner, thesis committee member, for her very helpful advice, ideas, and positive input the entirety of my thesis endeavor. Acknowledgements are extended to Dr. Kate Hendricks, principal investigator for the Texas Neural Tube Defect Project, and the Texas Department of Health for providing data used in this research project.

Sincere appreciation is extended to the SWT physical therapy faculty and co-workers who gave me continuous positive encouragement throughout my graduate program experience.

Finally, I wish to express heartfelt appreciation to my larger family and circle of friends for their support and for helping me to keep a healthy perspective on life in general.

TABLE OF CONTENTS

LIST OF TABLES

ABSTRACT

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by

JANUS McCALLUM OLIVE, B.S.
Southwest Texas State University
May 2002

SUPERVISING PROFESSOR: Jean D. Brender

Epidemiologic studies investigating associations between drinking water nitrate levels and neural tube defect (NTD)-affected pregnancies have been inconclusive. This case-control study investigated the association between drinking water nitrate levels and NTD-affected pregnancies in 43 Mexican-American case-women and 67 Mexican-American control-women who were living along the Texas-Mexico border and who had either NTD-affected pregnancies/births or normal births between June 1995 and May 2000. The study subjects were interviewed in person about periconceptional maternal exposures, and samples of usual drinking water during the periconceptional period were collected and measured for nitrate concentration levels. Women exposed to periconceptional drinking water nitrate concentration levels $\geq$3.52 mg/liter were more likely to have NTD-affected pregnancies, adjusted odds ratio (OR) = 1.76, 95% confidence interval (CI) = 0.76 - 4.07. This increased association between nitrate

exposure and risk for NTD was modified by body mass index (BMI). Women with BMI ≥ 30 kg/m^2 who were exposed to nitrate concentration levels $\geq$3.52 mg/liter had 9.4 times the risk for NTD-affected pregnancies (95% CI: 1.02 - 98.39) compared to women with BMI < 30 kg/m^2 who were exposed to the same nitrate levels. Further research is warranted to investigate the causal inferences suggested by these findings. The observed increased rates in this unique study population are important in terms of public health implications for the entire Texas-Mexico border region.

CHAPTER I

INTRODUCTION

Definitions and Classification of Neural Tube Defects

Neural tube defects (NTDs) are congenital anomalies that occur when the neural tube, which ultimately forms the brain and spinal cord, fails to properly close during the first few weeks of gestation. The embryologic process of neural tube development (neurulation) begins at approximately day 17 of gestation and is completed at approximately day 30. Failure of the fetal neural tube to properly close during this earliest phase of gestation results in major malformations of the developing brain and/or spinal cord. (Lemire, 1988; Elwood, Little, & Elwood, 1992; Finnell et al., 2000).

There are three general categories of NTDs: anencephaly, myelomeningocele (spina bifida), and encephalocele. Anencephaly occurs with failure of the neural tube to close in the cranial region, a defect that typically occurs 23 to 26 days of gestation. As the fetus develops, a portion of the skull fails to close over a malformed brain. A major portion of the brain, skull, and scalp are missing. Most of these infants are stillborn or die within a few days of birth. Spina bifida occurs when a portion of the neural tube fails to close along the vertebral column in the spinal region, typically 26 to 30 days of gestation. This results in an incomplete closure of the vertebra arches and exposure of the spinal

cord during fetal development. Most infants born with spina bifida will require some level of surgical repair and most will survive. However, they will live with varying amounts of disability, including lower body paralysis, problems with bladder and bowel control, skeletal deformities, and possible mental impairment. The third type of NTD, encephalocele, is characterized by protrusion of a sac containing meninges and other neural tissue through a skull defect. Most encephaloceles can be surgically repaired without the infant having any major residual disabilities. Of the three NTD types, anencephaly and spina bifida are the most common. (Lemire, 1988; Elwood et al., 1992).

Neural tube defects occur in approximately one in every 1,000 births in the U.S. and contribute substantially to infant mortality and childhood morbidity (Elwood et al., 1992). Most epidemiologic research suggests a multifactorial etiology, having both a genetic and an environmental component. Suspected teratogenic agents must be active during the critical first month of pregnancy for consideration as causative factors of NTDs (Finnell et al., 2000; Campbell, Dayton, & Sohal, 1986).

In 1991, a cluster of six anencephalic births over a six-week period was reported in Brownsville (Cameron County), Texas. An investigation conducted by the Texas Department of Health (TDH) reported a prevalence rate of 27.1 per 10,000 live births for women conceiving from 1990 - 1991. In 1992, in response to the high prevalence rate of NTDs and public concerns that the cluster was due to environmental contamination, the TDH implemented NTD surveillance, risk reduction activities, and a case-control study in a 14 county region that included Cameron County (Hendricks, Simpson, & Larsen, 1999). This study investigates one of the numerous environmental agents identified as potential risks for NTD-affected pregnancy and for which data were gathered.

Purpose of Study

Studies investigating drinking water contaminants as potential environmental exposures associated with risk for NTDs have been given substantial attention in the past several decades. Of the numerous contaminants, nitrates have been a foremost concern. However, epidemiologic studies have provided no conclusive evidence that women who are exposed to nitrates in drinking water are at an increased risk for NTD-affected pregnancies (Sever, 1995). The purpose of this study was to investigate the association between maternal periconceptional exposure (3 months before to 3 months after conception) to nitrates in drinking water and NTD-affected pregnancies in Mexican-American women in a 14 county Texas-Mexico border region. In order to identify an association, the following research question was asked: Are women who have NTD-affected pregnancies exposed to higher periconceptional drinking water nitrate levels than women who have normal pregnancies? This research question is based upon the contradictory findings in preceding studies. It is hoped that findings from this study will provide further clarification of the role that drinking water nitrate exposure may or may not play in association with NTD-affected pregnancies.

CHAPTER II

LITERATURE REVIEW

Trends in Neural Tube Defect Incidence and Prevalence

Worldwide NTD rates vary widely among geographical regions. Data from the International Clearinghouse for Birth Defects Monitoring Systems (1991) show that between 1974 and 1988 rates for combined anencephaly and spina bifida varied from exceptionally high in Mexico and Northern Ireland (29.0 and 34.3 per 10,000 births, respectively) to as low as 3.7 and 4.3 per 10,000 births in Finland and France, respectively. The estimated rates for the U.S. (1985 - 1994) ranged between 7.2 and 15.6 per 10,000, all gestational ages included (MMWR, 1995). In a 1992-1995 surveillance study of the Texas-Mexico border region, Hendricks et al. (1999) reported an overall NTD prevalence rate of 14.6 per 10,000 births, all gestational ages included.

There has been an overall decrease in worldwide NTD prevalence rates since the 1970's. A major reason for this decrease, especially for anencephaly, has been due to widespread antenatal screening, prenatal diagnosis and therapeutic abortion. In addition, primary prevention of NTDs by periconceptional multivitamin/folic acid supplementation has been credited for a decrease in both occurrence and recurrence (Elwood et al., 1992; Velie & Shaw, 1996). Despite the declining trend, NTDs continue to comprise one of

the most prevalent birth defects in the U.S. with an estimated annual occurrence of approximately 4,000 (MMWR, 1992).

In the United States, Hispanic Whites have a higher prevalence when compared to prevalence in non-Hispanic Whites and Blacks (Canfield, Annegers, Brender, Cooper, & Greenberg, 1996; Elwood et al., 1992; Shaw, Jensvold, Wasserman, & Lammer, 1994; Shaw, Velie, & Wasserman, 1997). In a recent study of NTD prevalence in California, the Hispanic prevalence was 11.2/10,000 births followed by non-Hispanic Whites, 9.6/10,000; Blacks, 7.5/10,000; and Asians, 7.5/10,000 (Feuchtbaum et al., 1999). Hendricks et al. (1999) reported NTD prevalence along the Texas-Mexico border for 1993 - 1995 to be 14.9/10,000 for Hispanics and 10.6/10,000 for Anglos.

Etiology of Neural Tube Defects

As with most human congenital malformations, neural tube defects are believed to be due to the interaction of both genetic and environmental influences. Observations supporting a prominent role for genetically inherited factors in the occurrence of NTDs include parental consanguinity, recurrence risk among women who have had previous NTD-affected pregnancies, recurrence risk among siblings of affected individuals, and race/ethnicity (Campbell et al., 1986). Environmental exposures are traditionally considered to be all nongenetic aspects of etiology, including demographic and health characteristics, ambient (community) exposures, and occupational exposures (Sever, 1995). Numerous environmental agents have been identified as potential causative factors (physical agents, pharmaceuticals, agricultural and industrial pollutants) capable of increasing the risk for NTDs in genetically susceptible fetuses. An understanding of the

interactions between environmental factors and target genes is critical in effectively identifying the etiology of NTDs (Finnell et al., 2000).

Risk Factors for Neural Tube Defects

A number of traditional demographic and socioeconomic risk factors are associated with the occurrence of NTDs. These include extremes of maternal age (less than 20 years or greater than 35 years old), low household income, low maternal education, and inadequate prenatal care (Blatter, Van der Star, & Roeleveld, 1994; Elwood et al., 1992; Strassburg, Greenland, Portigal, & Sever, 1983; Feldman, Stein, Klein, Schuyler, & Casey, 1982).

Race and ethnicity have been shown to be associated with risk for NTDs. Studies have shown that the risks of Hispanic women in the U.S. for delivering offspring with NTDs are 1.5 to 3 times higher than for non-Hispanic Whites, Blacks, and Asians (Shaw et al., 1994; Brender, Carmichael, Preece, Larimer, & Suarez, 1989; Feldman et al., 1982; Strassburg et al., 1983; Canfield, Annegers, Brender, Cooper, & Greenberg, [II], 1996; Feuchtbaum et al., 1999).

Investigations of associations of maternal illnesses and medications with increased risk of NTDs have produced significant results. Maternal epilepsy as well as the use of the anticonvulsant agents valproic acid and carbamazepine have been found to increase the incidence of NTDs (Lindout, Meinhardi, Meijer, & Nau, 1992; Rosa, 1991; Lammer, Sever, & Oakley, 1987; Robert & Guibaud, 1982). Maternal insulin-dependent diabetes has also been shown to be a risk (Becerra, Khoury, Cordero, & Erickson, 1990; Mills, Baker, & Goldman, 1979). Comparing non-diabetic mothers with insulin-

dependent diabetic mothers, Becerra et al. (1990) found a fifteen-fold increased risk for anencephaly and spina bifida combined, relative risk (RR) = 15.3, 95% confidence interval (CI): 2.3-102.1.

Studies have consistently reported an association between maternal obesity and risk for NTD-affected pregnancies (Richards, 1969; Naye, 1990; Waller, Mills, & Simpson, 1994; Werler, Louik, Shapiro, & Mitchell, 1996; Shaw, Velie, and Schaffer, 1996; Kallen, 1998). Waller et al. (1994) observed a nearly two-fold increased risk among women with prepregnancy body mass indexes (BMI) > 31 kg/m^2 compared with women who were of average prepregnancy weight, odds ratio (OR) = 1.8, 95% CI: 1.1-3.0. Risks were more elevated with spina bifida, OR = 2.6, 95% CI: 1.5 - 4.5. In a follow-up study, Shaw et al. (1996) also observed similar increased risk among periconceptionally obese women (OR = 1.9, 95% CI 1.3 - 2.9). The associated risk was greater for spina bifida than for anencephaly. Despite the fact that the associations found in the Waller et al. (1994) and Shaw et al. (1996) studies were examined in the presence of numerous potentially relevant covarites, including dietary factors and diabetes, they were unable to identify a particular covariable that might explain the increased risk among obese women.

Maternal heat exposure has been suggested as risk factor for NTD-affected pregnancies. In a large cohort study (Milunsky, et al., 1992), women reporting any heat exposure (fever, electric blanket, sauna, hot tub) in early pregnancy had a slightly elevated risk, crude RR = 1.6, 95% CI: 0.9 - 2.9. Hot tub exposure had the strongest effect of any single heat exposure, adjusted RR = 2.8, 95% CI: 1.2 - 6.5.

Studies investigating maternal and/or paternal smoking as risk factors for NTDs have produced conflicting results. A 1988 study by Ericson, Kallen, and Lofkvist investigating maternal smoking reported an increased risk for NTD-affected pregnancies, OR = 2.0, 95% CI: 0.9-4.6). In contrast, a more recent study (Kallen, 1998) found a surprising protective effect of maternal smoking on the incidence of NTDs, OR = 0.75, 95% CI: 0.61-0.91. Zhang, Savitz, Schwingl, and Cai (1992) observed an elevated two-fold risk for NTD-affected pregnancies associated with paternal smoking (anencephaly OR = 2.1, 95% CI: 0.90 - 4.50, spina bifida OR = 1.9, 95% CI: 0.70 - 4.70).

Numerous studies in the past decade have shown a significant protective effect among women who take folic acid periconceptionally and/or during pregnancy (Czeizel & Dudas, 1992; Werler, Shapiro, & Mitchell, 1993; Milunsky et al., 1989). Well-established evidence strongly suggests that a substantial portion of NTDs is due to folate deficiency and can be prevented (MRC Vitamin Study, 1991). Contrary to protective effect findings, recent studies have observed that folic acid supplementation does not protect against NTDs among populations of Mexican descent (Harris & Shaw, 1995; Shaw, Schaffer, Velie, Morland, & Harris, 1995). Suarez et al. (2000) replicated these findings in their recent study of Mexican-Americans living on the Texas-Mexico border. No protective benefit of folic acid supplements was observed with an OR = 1.12, 95% CI: 0.22-5.78); however, supplement use was rare resulting in a study with insufficient power to conclude that supplements were ineffectual.

Although it is generally accepted by the scientific community that folic acid supplementation has a preventative effect, little progress has been made in answering the question of how it actually affords protection against NTDs and why some populations

may experience a greater protective effect. Interest in defective folate absorption as a cause has led to current research investigating abnormal polymorphisms in folate-related enzymes and other folate pathway gene variants (Boddie et al., 2000).

Findings from studies investigating maternal and paternal occupations and occupational exposures associated with increased NTD risks factors have been inconsistent. Occupational exposures investigated include solvents, ionizing radiation, anesthetic gases, pesticides, and mercury. Evidence of statistically significant risks has been observed for maternal occupations such as nursing and dentists/dental assistants and for paternal occupations such as farmworker, painter, and food and beverage processing (Blatter et al, 1994; Sever, 1995). In a 1996 study by Blatter, Roeleveld, Zielhaus, and Gabreels, investigating maternal occupational exposures, agricultural workers had higher odds ratios for NTDs but the associations were unrelated to either pesticides or any other specific exposures. Women employed in cleaning also had elevated NTD rates but they were unrelated to disinfectant exposures. Brender and Suarez (1990) found a significantly increased risk for anencephaly related to paternal occupations associated with solvent exposure (OR = 2.53, 95% CI: 1.56-4.10) with painters showing the highest risk in that group (OR = 3.43, 95% CI: 1.83-6.43). In a recent study by Shaw et al. (1999), maternal occupational exposures and hobby chemical exposures were studied. Exposures to 74 different chemical agent groups were investigated. No associations were observed.

Ambient environmental pollutants suggested to be associated with NTDs include vinyl chloride, organic solvents, metals and chemicals from hazardous waste sites, agricultural chemicals, radiation from nuclear contamination, drinking water contaminants and by-products of water treatment procedures. Studies investigating

exposure to vinyl chloride and risk for NTDs have found slightly increased risks but they were not statistically significant (Blatter et al., 1994; Sever, 1995). Increased risks associated with proximity to hazardous waste sites were found in studies conducted in New York state and California (Geschwind et al., 1992; Croen, Shaw, Sanbonmatsu, Selvin, & Buffler, 1997).

Studies examining drinking water contaminants have focused on inorganic contaminants such as arsenic and nitrates or on by-products of water disinfection, primarily trihalomethanes. Shalat, Walker, and Finnell (1996) present a comprehensive review of studies investigating the role of arsenic as a risk for NTDs and conclude that findings are inconsistent and further research is needed. Sever (1995) discusses the results of several New Jersey studies performed in 1992 that investigated the relationships between NTD malformations and different water contaminants. These studies reported significant associations with total trihalomethanes and weak associations for trichloroethylene and nitrates. Research design and exposure assessment methods were questionable in these studies. In a more recent New Jersey study, Klotz and Pyrch (1999) conducted a population-based case control study of NTDs and drinking water chlorination disinfection by-products, correcting for methodology flaws in the earlier studies. They observed a two-fold risk for total trihalomethanes and NTDs, OR = 2.1, 95% CI: 1.1 - 4.0.

Nitrate Exposure as a Risk Factor for Neural Tube Defects

Nitrates, nitrites, and related *N*-nitroso compounds are well recognized as a major class of carcinogens. Ingestion of nitrates in drinking water can cause a potentially fatal

condition in infants known as methemoglobinemia (Canter, 1997).

Animal models have linked a variety of *N*-nitroso compounds to central nervous system abnormalities, including NTDs, during early fetal development. Experimental teratogenesis in genetically susceptible mice, rats and hamsters has resulted in gross abnormalities of the brain and spinal cord upon administration of particular doses of specific *N*-nitroso compounds during the early gestational period (Givelber & DiPaolo, 1969; Koyama, J. Handa, H. Handa , & Matsumoto, 1970; Diwan, 1974; Pfaffenroth, Das, & McAllister, 1974).

The toxicity of nitrate to humans is due to the body's reduction of nitrate to nitrite and nitrite to nitrosamines and other N-nitroso compounds by bacteria in the saliva, stomach, and intestine. Exposures include pharmaceuticals, tobacco smoke, food and drinking water (Choi, 1985; Canter, 1997).

Groundwater (underground reservoirs, aquifers and springs) is the source of drinking water to approximately 50% of the overall population in the U.S., and to over 90% of the rural population. Nitrates are one of the most problematic and widespread of the vast number of potential groundwater contaminants. Small amounts of nitrate are present in groundwater from the natural degradation of plant materials. Excessive levels of nitrates are a result of excessive applications of commercial fertilizers or animal manure, or seepage from septic tanks or municipal sewage facilities (Canter, 1997).

The U.S. maximum contaminant limit (MCL) for drinking water nitrates is 10 mg/liter for nitrate-nitrogen (NO_3-N), converted to 45 mg/liter for total nitrate (NO_3). This level was established by World Health Organization guidelines based upon the potentially fatal condition in infants, methemoglobinemia, caused from extremely low

oxygen levels in the blood that occurs following ingestion of groundwater containing concentrations greater than 10 mg/liter NO_3-N (Canter, 1997).

Findings from epidemiologic studies investigating the association of human exposure to drinking water nitrates with NTDs have been inconsistent. Scragg, Dorsch, McMichael, and Baghurst (1982) conducted a case-control study investigating congenital malformations in South Australia between 1968 - 1976 and found strong correlations between drinking water nitrate levels and risk for NTDs, RR = 3.5, 95% CI: 1.1-14.6. A dose-response relationship was observed using estimated nitrate concentrations. A subsequent study in the same region was carried out two years later (Dorsch, Scragg, McMichael, Baghurst, & Dyer, 1984) that investigated a larger group of subjects from years 1951-1979 and similar relative risk and dose-response results were observed. In a 1988 Swedish study, investigators did not find associations between the occurrence of NTDs and average water nitrate content (Ericson, Kallen, & Lofkvist, 1988). In a 1988 Canadian case-control study of drinking water nitrate with 130 cases and 264 controls, Arbuckle, Sherman, Corey, Walters, and Lo found an increased risk for central nervous system malformation associated with exposure to increased levels of nitrates in water from private wells but not public water source. However, the effect estimates were not statistically significant, (RR = 2.3, 95% CI 0.73-7.29). A New Jersey statewide case-control study of NTDs and drinking water contaminants was conducted for the years 1993 - 1994 (Klotz & Pyrch, 1998). Tap water samples were analyzed for 265 individuals (cases = 89, controls = 176) and odds ratios were calculated. Nitrates were not observed to be associated with NTDs. A recent population-based case-control study in California (Croen, Todoroff, & Shaw, 2001) investigated maternal exposure to nitrate from drinking

water and diet and risk for NTDs for 436 case-mothers and 432 control-mothers. Analyses were conducted for NTDs combined and for anencephaly and spina bifida separately. Exposure to nitrate in drinking water at concentrations above the 45 mg/liter MCL was associated with increased risk for anencephaly (OR = 4.0, 95% CI: 1.0-15.4) but not for spina bifida or NTDs combined. Statistically significant dose-response relationships for anencephaly were also observed at nitrate levels below the MCL among groundwater drinkers only (OR = 2.1 for 5-15 mg/liter; OR = 2.3 for 16-35 mg/liter; and OR = 6.9 for 36-67 mg/liter. Dietary nitrate exposure was not associated with increased risk.

Many of the aforementioned studies investigating the association of nitrate exposure and risk of NTDs have shown increased risks that were not statistically significant. Inability to produce statistically significant results may have been due to the fact that NTDs are a rare occurrence in the general population and also that nitrate exposure levels in these studies were lower than the established drinking water nitrate MCL. Therefore, increased risks may have been present but not demonstrable because of inadequate statistical power to detect a low risk and also because of the increased probability of exposure misclassification at low exposure levels (Sever, 1995; National Research Council, 1997).

CHAPTER III

METHODS

Data for this study came from the population-based case-control study of neural tube defects conducted by the TDH Texas Neural Tube Defect Project (TNTDP) between June 1995 and May 2000. The TNTDP consisted of multisource active surveillance, a folic acid intervention program, and a case-control study designed to examine various historical, biological, and environmental variables between NTD-affected pregnancies and normal births among Mexican-American women residing in any of the 14 counties along the Texas-Mexico border. The counties included Cameron, Hidalgo, Starr, Webb, Zapata, Maverick, Kinney, Val Verde, Terrell, Brewster, Presidio, Jeff Davis, Hudspeth, and El Paso.

The TNTDP was evaluated and approved by the TDH Institutional Review Board for the Protection of Human Subjects (Appendix A). The TNTDP principal investigator, Dr. Kate Hendricks, granted permission to access data for this research project (Appendix B). This study was reviewed and approved by the Southwest Texas State University Institutional Review Board (Appendix C). Per stipulation of the TDH, all personal identifiers were removed from the data prior to removal from the data repository for subsequent analyses.

Research Design

This study was a retrospective population-based case-control study investigating the relationship between maternal periconceptional exposures to drinking water nitrates and risk for NTD-affected pregnancies.

Study Population

Participants for the current study were drawn from the TNTDP population-based case-control study from June 1995 through May 2000. In the TNTDP, Mexican-American women comprised 94.3 % of the race/ethnicity group. Therefore, only the data for this group were analyzed because the small numbers of other racial and ethnic groups represented in the data would make adjustment for ethnicity and race difficult.

A case-woman was defined as a Mexican-American woman with a NTD-affected pregnancy or delivery (spontaneous or induced abortion or delivery of a live or stillborn infant with a NTD). A neural tube defect was defined as anencephaly, spina bifida, or encephalocele, classified according to *International Classification of Diseases*, Ninth Revision (ICD-9) code designations 740, 741, and 742. The case-woman must have been a resident in one of the 14 counties at the time of delivery or termination and throughout the study and must have signed a consent form before participating in any part of the study. Case-women were identified through an active surveillance system of hospitals, birthing centers, abortion facilities and delivering midwives located in the 14-county study area.

A control-woman was defined as a Mexican-American woman who had a normal live birth during the same time period. The control-woman must have given birth in a

hospital or birthing center in one of the 14 counties of the study region. The control-woman also must have resided in the 14 county study area at the time of delivery and throughout the study and must have signed a consent form before participating in any part of the study. A control-woman was chosen through a random number table process. Control-women were frequency matched to case-women by year of index birth or pregnancy termination and hospital or facility of delivery.

Potential participants were approached about study enrollment either prenatally or in the hospital at the time of delivery or pregnancy termination. For those participants who enrolled in the study, the TNTDP collected data through an extensive mother questionnaire, a food frequency questionnaire, laboratory blood tests, and, when available, drinking water samples.

Interviews

TNTDP face to face interviews were scheduled four to six weeks postpartum and were conducted at home in Spanish or English using a standardized instrument modeled after the Centers for Disease Control and Prevention's 1993 mother questionnaire for birth defects risk factor surveillance. The complete instrument assessed maternal health and reproductive history; demographic and socioeconomic information; family medical history; use of medications, nutritional supplements, herbal preparations, tobacco, alcohol, illegal drugs and inhalants; and parental environmental and occupational exposures. Participants were asked to report all addresses and dates of residence for places where they had lived during the periconceptional period.

Determination of Nitrate Exposure

The reported usual periconceptional drinking water source (bottled or tap water) was ascertained and a water sample was collected and measured for level of nitrates at the visit that occurred four to six weeks postpartum. If the reported water source was bottled water, the sample was collected from the current available source and measured for nitrate levels. If the reported water source was tap water, it had to be taken from the residence at the time of conception, not the current residence if there was a difference. If the periconceptional residence was not accessible or if consent could not be obtained for sampling, the water sample was not collected. Nitrate levels were tested with the Nitrate Kit (Products for Analysis, 1995) by mixing a water sample with a reagent followed by measurement of nitrates with a pocket colorimeter. Three measures were taken. Results were shown in mg/liter nitrate as nitrogen (NO_3-N). For the purposes of this study, these values were converted to mg/liter total nitrate (NO_3) by multiplying NO_3-N values by 4.4 and an average of the three values was calculated. Information regarding individual daily drinking water intake during the periconceptional period was not collected as a part of the TNTDP interview or water collection process.

Statistical Analysis

A nitrate exposure data file was created from water sample information abstracted from individual subject files. This file was merged with a TNTDP data file of variables considered potential effect modifiers and/or confounders in the association between drinking water nitrate levels and risk for NTD-affected pregnancies. These variables included maternal age, education, income, prepregnant BMI, preconception folic acid

use, preconception smoking, and preconception alcohol use. Each variable was stratified and recoded. Working data files were developed using Microsoft Excel 2000® (1999). Data were analyzed using SPSS® 10.0 (SPSS Inc., 1999) and Computer Programs for Epidemiologists - PEPI (Abramson & Gahlinger, 2000).

Descriptive analyses were performed to compare the characteristics of the water sample study participants and non-participants and to compare the characteristics of the study case- and control-women. Mean and median levels for drinking water nitrate levels were calculated. The percent case-women and control-women above and below the mean and median, based on both overall values and control-women distribution values, were calculated. A Mann-Whitney *U*-test was performed to determine if significant differences in nitrate levels existed between groups. The case- and control-women were also compared with respect to reported drinking water sources, either bottled or tap water.

Odds ratios with 95% CIs were calculated as a measure of strength of associations found between drinking water nitrate level and NTDs. Analyses were based on the 50th percentile (median) cutpoint for control-women distribution. The 50th percentile was used instead of quartile or quintile due to the small sample size, which would result in low numbers within multiple cutpoint categories. Stratified analyses were conducted to adjust the odds ratios for other risk factors and to identify any potential effect modification and/or confounding. Odds ratios with 95% CIs, using the 50th percentile cutpoint, were also calculated for nitrate levels among tap water and bottled water drinkers. Since many of the odds ratio calculations involved small numbers, confidence limits were calculated using Fisher's Exact method.

Logistic regression analysis was performed to model the effect of drinking water nitrate exposure on the risk of having an NTD-affected pregnancy while controlling for other potentially confounding risk factors and adjusted odds ratios with 95% CIs were calculated.

Due to the small sample size, all analyses were performed with NTDs combined; no comparisons were made between separate NTD categories. An alpha level of 0.05 was used for all statistical tests.

Abbreviated analyses were performed using nitrate exposure category cutpoints established in the recent study by Croen et al. (2001). Cutpoints were less than 5mg/liter, 5-15 mg/liter, and greater than15 mg/liter. Calculation of crude and adjusted odds with 95% CIs was performed, enabling direct comparison of results from this study to the Croen et al. (2001) findings.

CHAPTER IV

RESULTS

Descriptive Analyses

A total of 110 water samples were collected. Characteristics of the water sample study participants (N = 110) and non-participants (N = 299) were similar (Table 1). There were no differences greater than 6.4% for any of the comparisons.

Table 1. Comparison of characteristics of study participants and non-participants

	Water Sample Study Participants		Non-Participants	
	N	(%)	N	(%)
Maternal age (years)				
<20	28	(25.5)	73	(24.4)
20-24	35	(31.8)	104	(34.8)
25-29	23	(20.9)	76	(25.4)
≥ 30	24	(21.8)	46	(15.4)
Education (years)				
< 7	15	(13.6)	47	(15.7)
7-11	35	(31.8)	106	(35.5)
≥12	60	(54.5)	146	(48.8)
Household income[a]				
<$15,000/year	64	(58.2)	179	(61.3)
$15-25,000/year	21	(19.1)	60	(20.5)
>$25,000/year	25	(22.7)	53	(18.2)
Body mass index[b]				
< 30 kg/m^2	85	(77.3)	225	(76.5)
≥ 30 kg/m^2	25	(22.7)	69	(23.5)
Preconception folic acid				
Yes	4	(3.6)	17	(5.7)
No	106	(96.4)	282	(94.3)
Preconception smoking				
Yes	14	(12.7)	56	(18.7)
No	96	(87.3)	243	(81.3)
Preconception alcohol use				
Yes	30	(27.3)	88	(29.4)
No	80	(72.7)	211	(70.6)

[a] Seven non-participants missing on household income
[b] Five non-participants missing on body mass index

Of the study participants, 43 (39.1%) were case-women and 67 (60.9%) were control-women. Table 2 shows the comparison of demographic and health characteristics between the case-and control-women.

Table 2. Comparison of characteristics of case-women and control-women

	Case-women		Control-women	
	N	(%)	N	(%)
Maternal age (years)				
< 20	8	(18.6)	20	(29.9)
20-24	15	(34.9)	20	(29.9)
25-29	9	(20.9)	14	(20.9)
≥ 30	11	(25.6)	13	(19.4)
Education (years)				
< 7	8	(18.6)	7	(10.4)
7-11	9	(20.9)	26	(38.8)
≥ 12	26	(60.5)	34	(50.7)
Household Income				
< $15,000/year	24	(55.8)	40	(59.7)
$15-25,000/year	8	(18.6)	13	(19.4)
> $25,000/year	11	(25.6)	14	(20.9)
Body mass index				
< 30	30	(69.8)	55	(82.1)
≥ 30	13	(30.2)	12	(17.9)
Preconception folic acid				
No	42	(97.7)	64	(95.5)
Yes	1	(2.3)	3	(4.5)
Preconception smoking				
Yes	7	(16.3)	7	(10.4)
No	36	(83.7)	60	(89.6)
Preconception alcohol				
Yes	11	(25.6)	19	(28.4)
No	32	(74.4)	48	(71.6)

Case-women were slightly older than control-women. Case-women ranged in age from 15 to 44 years with a mean of 25.1 years. Control-women ranged in age from 14 to 36 years with a mean of 23.8 years. Over 50% of both case- and control-women were 20-29 years of age.

A higher proportion of case-women had 12 years or more of education compared to control-women (60.5% versus 50.7%) and a higher proportion of case-women had less than seven years education compared to control-women (18.6% versus 10.4%). The mean level of education for each group was 11 years.

Household incomes for case- and control-women were similar. A slightly higher proportion of case-women had household incomes greater than $25,000. For both groups, greater than 50% of participants had incomes less than $15,000 per year.

Case-women were heavier compared to control-women but there was no statistically significant difference between the groups. Prepregnant BMI (kg/m^2) ranged from 17.4 to 42.2 for case-women and from 14.3 to 41.6 for control-women. The mean BMI for case-women was 27.2 and for control-women was 25.4. Based on the Expert Panel on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults (1998) definition of obesity (BMI 30 kg/m^2 or greater), a higher proportion of case-women (30.2%) were obese compared to control-women (17.9%).

Fewer than 5% of case- and control-women reported using preconception folic acid supplements. Fewer than 17% of case- and control-women reported preconceptional smoking and fewer than 30% reported that they used alcohol preconceptionally.

No water sample collected exceeded the MCL of 45 mg/liter NO_3. Overall drinking water nitrate levels ranged from 0 to 28.16 mg/liter, median = 4.69 mg/liter and mean = 5.89 mg/liter. Drinking water nitrate levels of case-women ranged from 0 to 28.16 mg/liter and those of control-women ranged from 0 to 23.76 mg/liter. Medians were 5.43 and 3.52 mg/liter for case- and control-women, respectively. Means were 6.55 and 5.48 mg/liter for case- and control-women, respectively (Table 3).

Table 3. Distribution of drinking water nitrate levels (mg/liter)

	N	Median	Mean	(Std. Dev.)	Range
Overall	110	4.69	5.89	5.29	0-28.16
Case-women	43	5.43	6.55	5.55	0-28.16
Control-women	67	3.52	5.48	5.11	0-23.76

Based on overall median and mean values, 56% of case-women had nitrate values above the median compared to 43% of control-women; 45% of case-women had values above the overall mean compared to 43% of control-women. Based on control-women distribution, 65% of case-women had nitrate values above the median value of 3.52 mg/liter compared to 49% of control-women; 48.8% of case-women had nitrate level values above the mean compared to 38.8% of the control-women (Table 4).

Table 4. Nitrate level median and mean distribution based on control-women values[a]

	Below Median		Above Median		Below Mean		Above Mean	
	N	(%)	N	(%)	N	(%)	N	(%)
Case-women	15	(35)	28	(65)	22	(51.2)	21	(48.8)
Control-women	34	(51)	33	(49)	41	(61.2)	26	(38.8)

[a] Control-women nitrate level median = 3.52 mg/liter and mean = 5.48 mg/liter

The individual nitrate concentration levels, measured in mg/liter, were rank ordered and a Mann-Whitney *U*- test was used to compare the ranks for the case-women (N = 43) and the control-women (N = 67). For sample sizes larger than 20, the distribution of the *U*-statistic approximates a normal shape and the test is evaluated using a *z*-score statistic. The results indicated no significant difference between groups, $Z = 1.37, p > 0.05$. (Table 5).

Table 5. Mann-Whitney *U*-test of differences in nitrate levels

	Nitrate Levels
Mann-Whitney *U*-value	1217.0
Z	-1.369
Sig. (2-tailed)	.171

Frequency analyses of study subject drinking water sources revealed that 54% (N = 59) reported bottled water and 42% (N = 46) reported tap water. Five subjects did not report the source. For the case-women, 56% (N = 24) reported bottled water sources and 37% (N = 16) reported tap water sources. There were three case-women with unreported sources. For the control-women, 52% (N = 35) reported bottled water sources and 45% (N = 30) reported tap water sources. There were two control-women with unreported sources.

Bivariate Analyses

An increased risk for NTD-affected pregnancy was observed among women with periconceptional exposure to drinking water with nitrate levels greater than 3.52 mg/liter, OR = 1.92, 95% CI: 0.82 - 4.60 (Table 6).

Table 6. Association between drinking water nitrate levels[a] and NTDs

Nitrate (mg/liter)	Case-women N	Control -women N	Odds Ratio	95% CI[b]
<3.52	15	34	1.0	Referent
≥3.52	28	33	1.92	0.82 - 4.60

[a] Based on control group distribution (50th percentile)
[b] CI (Fisher's Exact Method)

Stratification analyses and examination of each stratum-specific odds ratio showed increasing risks for NTD-affected pregnancies associated with drinking water nitrate levels ≥ 3.52 mg/liter among women 25 years and older; with less than 7 or more than 12 years of education; with household incomes >$15,000; with BMI ≥ 30 kg/m^2; with no reported preconception folic acid use; with reported preconceptional smoking; and with reported preconceptional alcohol use. Significant chi-square for heterogeneity tests were observed for BMI and for preconception folic acid use, indicating effect modification by these two factors (Table 7).

Table 7. Stratified odds ratios[a] for association of nitrate level and NTDs by risk factor

Stratification Characteristic	Case-women (N)	Control-women (N)	Nitrate Level (mg/l) <3.52	Nitrate Level (mg/l) ≥ 3.52
			Referent	OR (95% CI)[b]
Overall	43	67	1.0	1.92 (0.82-4.60)
Maternal Age (years)				
< 20	8	20		0.90 (0.12-6.43)
20-24	15	20		1.64 (0.33-8.28)
25-29	9	14		2.63 (0.30-27.27)
> 29	11	13		3.11 (0.42-25.22)
Maternal Education (years)				
< 7	8	7		4.00 (0.30-68.93)
7-11	9	26		0.50 (0.08-3.04)
≥ 12	26	34		2.71 (0.80-9.46)
Household Income				
< $15,000	24	40		1.07 (0.34-3.34)
$15-25,000	8	13		1.94 (0.23-17.35)
>$25,000	11	14		13.33 (1.10-365.04)
Body Mass Index (kg/m^2)				
< 30	30	55		1.17 (0.44-3.16)
≥ 30	13	12		11.00 (1.24-134.23)[c]
Preconception Folic Acid				
No	42	64		2.27 (0.94-5.55)[c]
Yes	1	3		0.00 (0.00-7.36)
Preconception Smoking				
No	36	60		1.68 (0.67-4.25)
Yes	7	7		4.50 (0.23-166.72)
Preconception Alcohol Use				
No	32	48		1.73 (0.64-4.73)
Yes	11	19		3.27 (0.44-29.54)

[a] Mantel-Haenzel Method
[b] CI (Fisher's Exact Method)
[c] Significant chi-square test for heterogeneity ($p < 0.05$)

The association between drinking water nitrate and risk for NTDs was modified by BMI. For drinking water nitrate levels ≥3.52 mg/liter, obese women (BMI ≥ 30 kg/m^2) had a significantly increased risk, OR = 11.00, 95% CI: 1.24-34.23, compared to women with BMI < 30 kg/m^2 exposed to the same nitrate levels, OR = 1.17, 95% CI: 0.44-3.18. The heterogeneity test was significant, $\chi^2_{HET} = 4.57$, $p = 0.03$, (Table 8).

Table 8. Association between nitrate levels[a] and NTDs by body mass index

Body Mass Index (kg/m^2)	Nitrate Level (mg/liter)	Case-women N	Control-women N	Stratum Specific Odds Ratio (95% CI)[b]	Summary Odds Ratio[c] (95% CI)	χ^2_{HET}[d] (p)
< 30	< 3.52	13	26	1.0 (Referent)		
	≥ 3.52	17	29	1.17 (0.44-3.18)	1.83 (0.85-3.98)	4.57 (0.03)
≥ 30	< 3.52	2	8	1.0 (Referent)		
	≥ 3.52	11	4	11.00 (1.24-134.23)		

[a] Based on Based on 50th percentile control group distribution
[b] CI (Fisher's Exact Method)
[c] Mantel-Haenzel Method
[d] Test for Heterogeneity (Cornfield-Gart Procedure)

Effect modification was also observed with preconception folic acid use. Women who did not use folic acid preconceptionally had a more than two-fold risk of NTD-affected pregnancies, OR = 2.27, 95% CI: 0.94-5.52. The heterogeneity test was significant, $\chi^2_{HET} = 6.37$, $p = 0.01$ (Table 9). Due to the extremely low numbers of study subjects who reported use of preconception folic acid (less than 5%), it was deemed likely that these findings were imprecise as a result of sparse data and cell numbers less than one.

Table 9. Association between nitrate levels[a] and NTDs by preconception folic acid use

Folic Acid Use	Water Nitrate (mg/liter)	Case-women N	Control-women N	Stratum Specific Odds Ratio (95% CI)[b]	Summary Odds Ratio[c] (95% CI)	χ^2_{HET}[d] (p)
No	< 3.52	14	34	1.0 (Referent)		
	≥ 3.52	28	30	2.27 (0.94-5.55)		
					1.91 (0.87-4.15)	6.37 (0.01)
Yes	< 3.52	1	0	1.0 (Referent)		
	≥ 3.52	0	3	0.00 (0.00-13.00)		

[a] Based on Based on 50th percentile control group distribution
[b] CI (Fisher's Exact Method)
[c] Mantel-Haenzel Method
[d] Cornfield-Gart Procedure

When the overall effect estimate for nitrate was singly adjusted for age, education, income, BMI, preconception folic acid use, smoking and alcohol use, adjusted estimates were similar to the crude odds ratio (less than a 10% difference). Therefore, confounding by individual covariates did not explain the increased risk among women with nitrate levels ≥ 3.52 versus lower nitrate levels (Table 10).

Table 10. Adjusted odds ratios for individual risk factors for NTDs

Risk Factor	Nitrate < 3.52 mg/liter	Nitrate ≥ 3.52 mg/liter
	Referent	OR (95% CI)[a]
Crude Odds Ratio	1.0	1.92 (0.82-4.60)
Maternal Age		1.80 (0.81-4.03)
Maternal Education		1.76 (0.79-388)
Household Income		1.85 (0.85-4.04)
Body Mass Index		1.83 (0.85-3.98)[b]
Preconception Folic Acid		1.91 (0.87-4.15)[b]
Preconception Smoking		1.86 (0.84-4.11)
Preconception Alcohol Use		1.99 (0.89-4.43)

[a] CI (Fisher's Exact Method)
[b] Significant chi-square test for heterogeneity (< 0.05)

Calculation of risks among tap water drinkers and bottled water drinkers produced comparable overall odds ratios (Table 11). An increased risk was observed among women who reported bottled water source with nitrate levels $\geq$ 3.52 mg/liter, OR = 2.1, 95% CI: 0.65 - 6.91. A similar increased risk was observed for women at this nitrate level who reported tap water as their periconceptional drinking water source, OR = 2.3, 95% CI: 0.52 - 11.88. These elevated odds ratios were similar to the previously reported risk for all water sources combined (crude OR = 1.92).

Table 11. Association between nitrate levels and NTDs for bottled water and tap water sources

Source	Water Nitrate[a] (mg/liter)	Case-women (N)	Control-women (N)	Odds Ratio	95% CI[b]
Bottled Water	< 3.52	10	21	1.0	Referent
	$\geq$ 3.52	14	14	2.1	0.65 - 6.91
Tap water	< 3.52	4	13	1.0	Referent
	$\geq$ 3.52	12	17	2.3	0.52 - 11.88

[a] Based on control group distribution (50th percentile)
[b] CI (Fisher's Exact Method)

Analyses using the nitrate level cutpoints established in the Croen et al. (2001) study showed no significant association with risk for NTD-affected pregnancy. The small sample size resulted in sparse data and cell numbers less than one for the >15 mg/liter category. For nitrate exposure category >15 mg/liter, effect modification was found with household income and preconception folic acid use ($\chi^2_{HET} < 0.05$). However, results were deemed imprecise due to cell numbers less than one. There was no evidence of confounding; adjusted odds ratios were similar (less than 10% difference) to the crude odds ratios (Table 12).

Table 12. Adjusted odds ratios[a] for individual risk factors based on Croen et al. (2001) cutpoints[b]

Risk Factor	Nitrate Levels		
	< 5 mg/liter	5-15 mg/liter	>15 mg/liter
	Referent	OR (95% CI)[c]	OR (95% CI)[c]
Crude Odds Ratio	1.0	1.53 (0.63-3.17)	1.11 (0.16-6.39)
Maternal Age		1.46 (0.65-3.25)	1.02 (0.20-5.28)
Maternal Education		1.40 (0.61-3.19)	1.11 (0.23-5.30)
Household Income		1.53 (0.68-3.44)	1.10 (0.26-4.68)[d]
Body Mass Index		1.57 (0.70-3.54)	1.01 (0.21-4.91)
Preconception Folic Acid		1.54 (0.69-3.43)	1.14 (0.25-5.27)
Preconception Smoking		1.51 (0.67-3.39)	1.10 (0.24-5.07)
Preconception Alcohol Use		1.62 (0.71-3.66)	1.12 (0.26-4.68)[d]

[a] Mantel-Haenzel Method
[b] Exposure level cutpoints <5, 5-15, >15 mg/liter
[c] CI (Fisher's Exact Method)
[d] Significant chi-square test for heterogeneity (< 0.05)

Multivariate Analyses

A series of multivariate logistic regression analyses were performed to model the effect of drinking water nitrate exposure on the risk of having a NTD-affected pregnancy. Four models were produced. The first model examined the effect of nitrate levels on the risk for NTD. The second model examined the nitrate factor and all other potentially confounding covariates. Model 3 examined the nitrate and BMI main effect factors together with the nitrate * BMI interaction factor. The fourth and final model included the aforementioned factors (nitrate, BMI, and nitrate * BMI interaction) and all other covariates identified as potential confounders (Table 13).

Table 13. Logistic regression analysis of nitrate level[a] and BMI[b] for case-women and control-women

Model #	Predictor Variable	Beta	S.E.	Odds Ratio	95% CI	Wald Statistic	*p*
1	Nitrate level	0.65	0.40	1.92	0.87-4.23	2.64	0.104
2	Nitrate level	0.57	0.43	1.76	0.76-4.07	1.75	0.187
	Other potential risk factors[c]						
3	Nitrate level	0.16	0.46	1.17	0.48-2.87	0.12	0.728
	BMI level	-0.69	0.86	0.50	0.09-2.70	0.65	0.421
	Nitrate level * BMI level	2.24	1.08	9.38	1.12-78.49	4.27	0.039
4	Nitrate level	0.05	0.49	1.06	0.40-2.76	0.01	0.913
	BMI level	-0.87	0.94	0.42	0.07-2.65	0.86	0.354
	Nitrate level * BMI level	2.30	1.17	9.99	1.02-98.39	3.89	0.049
	Other potential risk factors[c]						

[a] Based on 50th percentile control group distribution, < 3.52 mg/liter and ≥ 3.52 mg/liter
[b] Based on BMI cutpoint, < 30 kg/m^2 and ≥ 30 kg/m^2
[c] Age, education, income, BMI, preconception folic acid, preconception smoking, preconception alcohol (Refer to Appendix D for complete summary of statistics)

The first model that examined the nitrate level factor only produced the crude odds ratio result, OR = 1.92, 95% CI: 0.87 - 4.23. The Wald statistic for this model was 2.64 (p >0,05). Nagelkerke R square was 0.03 (3%). The overall predicted percent correct was 61% (100% of control-women and 0% of case-women correctly predicted).

In model 2 (nitrate level factor and other covariates identified as potential risk factors for NTD), the resultant odds ratio for exposure to drinking water nitrates and risk for NTDs was slightly lower, adjusted OR = 1.76, 95% CI: 0.76 - 4.07. The Wald statistic was 1.75 (p > 0.05). The overall predicted percent correct for this model was improved to 66.4% (82% of control-women and 42% of case-women correctly predicted).

Model 3 (nitrate level, BMI level, and nitrate * BMI interaction factors) produced a significant odds ratio for the interaction, OR = 9.38, 95% CI: 1.12 - 78.49, Wald statistic = 4.27 (p < 0.05). The model chi-square of 9.56 was significant (p < 0.05) and Nagelkerke R-Square = .11 (11%). This model had an overall predicted percent correct of 67.3% (94% of control-women and 26% of case-women predicted correctly).

Model 4 (nitrate, BMI, nitrate * BMI, and other potentially confounding covariates) produced odds ratios for the main effect and interaction factors similar to model 3. The odds ratio for the nitrate * BMI interaction was increased slightly and was the only significant factor, OR = 9.99, 95% CI: 1.02 - 98.39. The Wald statistic was 3.89 ($p < 0.05$). The overall predicted percent correct was 67.3% (90% control-women and 33% case-women correctly predicted). Nagelkerke R square was increased to 0.16 (16%).

Model 3 was selected as the best model over model 4 because adjusting for the covariates (age, education, income, preconception folic acid use, smoking, and alcohol) changed the odds ratios for drinking water nitrate levels ≥3.52 mg/liter less than 10% for women with BMI <30 kg/m^2 and women with BMI ≥30 kg/m^2. It was therefore preferable to go with the simpler model 3, which indicated a 9.4 times risk for NTD-affected pregnancies for women with BMI ≥ 30 kg/m^2 who were exposed to drinking water nitrate levels ≥ 3.52 mg/liter during the periconceptional period. The low predicted percent correct for case-women (26%) in this model suggests that caution should be used with interpretation of these results. Appendix D contains a complete summary of statistics for the logistic regression analyses. The logistic function for this model was $logit\ P = \beta_0 + \beta_1$Nitrate level $+ \beta_2$ BMI $+ \beta_3$ Nitrate level * BMI.

The logistic function for Model 3 was used to relate the dependent variable (NTD occurring = 1 or NTD not occurring = 0) to the independent variables (Nitrate, BMI, Nitrate * BMI interaction) in order to estimate probability. The estimated probability that an obese Mexican-American woman exposed to periconceptional drinking water nitrate levels ≥ 3.52 mg/liter would have a NTD-affected pregnancy was 0.73 for the interaction term, indicating that although this predicted value was approaching the value of 1 (NTD

occurring), other factors beyond those included in the logistic function needed to be considered in order to produce a higher predictive value. The remaining independent variables had estimated probabilities less than 0.50, indicating uncertainty in the prediction of outcome (Appendix E).

Multivariate analysis was also performed using nitrate level cutpoints established in the Croen et al (2001) study, with the same identified potential risk factors for NTD (maternal age, education, income, BMI, folic acid use, smoking and alcohol use) being entered into the model. No significant model for prediction was produced.

CHAPTER V

DISCUSSION

The results of this population-based study demonstrated an association between exposure to nitrate contaminated drinking water and risk for NTDs. Overall, a nearly two-fold risk was associated with drinking water nitrate exposure during the periconceptional period and risk for NTD-affected pregnancies in Mexican-American women. The increased risk was modified by body mass index. Women who were obese at the time of conception and who were exposed to periconceptional drinking water nitrate levels $\geq$ 3.52 mg/liter had greater than nine times the risk for NTDs.

Study Limitations and Strengths

Limitations of this study included small sample size, the potential for recall bias, potential fluctuations in water samples, and looking at NTDs as one group.

Of the 110 study participants there were 43 (39%) case-women and 67 (61%) control-women. There were only 36% more control-women than case-women. The small sample size and less than two control-women for each NTD case limited the study's power and may have caused some analyses to fail to reach significance.

Ascertainment of exposures from the interview process was limited by the accuracy of what the mothers remembered about their periconceptional period and this could have resulted in recall bias. For this study, recall bias was of particular concern with respect to self-report of prepregnancy weight. Numerous studies investigating risk of NTD among obese women have addressed this issue and conclude self reported past body weight to be accurate (Waller et al., 1994; Shaw et al., 1996; Kallen, 1998).

This study was unable to control for seasonal fluctuations in tap water nitrate concentration levels. Water samples collected from tap water may not have been obtained during the same season as the periconceptional time period. Also, water samples collected from current bottled water sources could not account for any possible fluctuations in nitrate levels. The assumption that nitrate concentration levels are constant during all seasons and across all types of bottled water sources may have weakened the validity of exposure measurements.

Neural tube defects were analyzed as one group. Separate defect-specific (anencephaly, spina bifida) risks associated with nitrate exposure were not calculated. Sever (1995) and Blatter et al. (1997) discuss evidence that NTDs are etiologically heterogeneous. They recommend that separating anencephaly and spina bifida (and even splitting spina bifida) may improve the ability to detect a genetic or environmental exposure for a specific defect. Investigation of risk in terms of specific types of NTDs was not considered because of the small number of cases in the study.

Strengths of this study included the case-control study design, case ascertainment using active surveillance, availability of a variety of potential confounders, objective

analyses of drinking water nitrate levels and ascertainment of nitrate levels from tap water samples collected from periconceptional residences of study participants.

A case-control study design was utilized due to its strength in studying relatively rare outcomes such as NTDs. The active surveillance efforts of the TNTDP and inclusion of all gestational ages for NTD cases maximized study power and minimized misclassification bias. Although only 27% of the 409 enrolled TNTDP participants had water samples collected and measured, similarities in demographic and health characteristics of the study participants and non-participants minimized selection bias issues.

Data gathered from the detailed TNTDP questionnaire enabled the evaluation of and controlling for potential risk factors for NTD-affected pregnancies. The exposure of interest, nitrate levels in drinking water, consisted of objective, verifiable data and was not subject to recall bias on the part of the respondents.

Using nitrate exposure levels that were directly measured from the usual periconceptional drinking water sources instead of surrogate measures minimized exposure misclassification. Ascertainment of nitrate concentration levels from community monitoring systems instead of index residences has been a limitation of some of the previous studies (Scragg et al, 1982; Dorsch et al, 1984; Ericson et al., 1988; Croen et al, 2001). For this study, tap water samples were collected at addresses where study subjects lived at the time of conception instead of at the time of delivery. Some previous studies have measured nitrate exposures at the current (post-delivery) addresses, which could have differed from the periconceptional address (Scragg et al, 1982; Dorsch et al., 1984; Arbuckle et al., 1988). Given that closure of the neural tube occurs in weeks 3 to 4

of gestation, ascertainment of periconceptional drinking water nitrate levels is important for minimizing exposure misclassification.

Findings and Relation to Previous Studies

This population-based case-control study added to the weight of evidence that periconceptional drinking water nitrates may be a risk factor for neural tube defects. Comparison of data from this study with several previous studies (Dorsch et al., 1984; Arbuckle et al., 1988; Croen et al, 2001) suggests that an increased risk for NTDs exists at concentrations well below the standard MCL of 45 mg/liter, which was based on the risk for infant methemoglobinemia.

The suggestion of this study that periconceptional obesity interacts with nitrates to increase vulnerability to NTDs is preliminary but nonetheless interesting. This finding has not been reported in previous studies that have found associations between drinking water nitrates and increased risk of NTDs (Scragg et al, 1982; Dorsch et al., 1984; Arbuckle et al., 1988; Croen et al, 2001). Shaw et al. (1996) and Kallen (1998) point out that although obesity has been a consistently observed risk factor for NTD-affected pregnancies, there is no known evidence about whether the increased risk may be due to an innate metabolic abnormality (other than diabetes) associated with being obese. Could the increased risk for NTDs among women with BMI $\geq$ 30 kg/m^2 who are exposed to drinking water nitrate levels $\geq$ 3.52 mg/liter be the result of a metabolic peculiarity associated with obesity?

Another distinction of this study was an exclusively Mexican-American study population. Evidence indicates that this is an ethnic group identified at higher risk for

NTDs in the U.S. However, there has been a paucity of studies to date investigating the uniqueness of this group and the association of genetic-environmental risks (Suarez et al., 2000; Harris and Shaw 1995; Shaw et al., 1995). Could a nitrate-obesity interaction and risk of NTDs in a genetically susceptible population be due to a metabolic oddity spurred by a genetic bias? Is it possible that the Mexican-American population in this study has a genetic predisposition resulting in an unusual metabolism, rather than obesity per se, which results in an altered handling of nitrates and, consequently an increased risk of NTDs?

Conclusions

Although the findings of this study are preliminary and should be interpreted with caution, they suggest a possible biochemical interaction of obesity with exposure to nitrates in drinking water. These observations together with previous reports of an increased NTD risk for exposure to nitrate levels below the MCL and, separately, for obese women warrant further investigations before causality can be determined. Future studies with larger study samples should continue to investigate the Mexican-American population; should study NTDs as separate groups (e.g. anencephaly and spina bifida); should analyze the different types of drinking water sources; and, should examine the effects of dietary nitrate exposure levels.

The observed increased rates in this unique study population are important in terms of public health implications for the entire Texas-Mexico border region, and a major effort should be made to provide counseling about the importance of maintaining a healthy weight during the childbearing years.

APPENDICES

Appendix A

Texas Department of Health

William R Archer III, M D
Commissioner of Health

http://www.tdh.state.tx.us

1100 West 49th Street
Austin, Texas 78756-3199
512/ 458-7111

Patti J Patterson, M D., M P H.
Executive Deputy Commissioner

January 7, 2000

Kate Hendricks, M.D., Director
Infectious Disease Epidemiology & Surveillance Division
Texas Department of Health
1100 West 49th Street
Austin, Texas 78756

Dear Doctor Hendricks:

The Texas Department of Health's Institutional Review Board (IRB) for the Protection of Human Subjects has received your request for extension and subsequent changes required by the IRB

The IRB approves your survey "Texas Neural Tube Defect Project", IRB #940008-99 , for another year.

Should any adverse event related to this project occur, the TDH IRB needs to be notified immediately If your study should continue for longer than one year, you will need to submit a request for an extension of IRB approval at that time If you have any questions, you may contact me at 512/490-2515.

Sincerely,

Linda Bultman, Ph.D.
Vice-Chairperson
TDH Institutional Review Board

cc: IRB Members

Appendix B

August 23, 2001

Ms. Janus Olive, Graduate Student
Dr. Jean Brender, Associate Professor
Department of Health Services and Research
Southwest Texas State University
601 University Drive
San Marcos, Texas 78666-4616

Dear Ms. Olive and Dr. Brender:

Ms. Janus Olive, graduate student in the Health Services and Research Program at Southwest Texas State University, has requested permission to use data from the Texas Department of Health (TDH) Texas Neural Tube Defects Project (TNTDP) for her master's thesis. She has indicated that Dr. Brender, a collaborator in the TNTDP from 1982-1998, will serve as chair for her thesis committee. I have read and approve of her proposal to study nitrate exposure in drinking water as a risk factor for neural tube defects in Mexican-Americans.

As Principal Investigator of the TNTDP, I, Kate Hendricks, M.D., grant both of you permission to use our data from the case-control component for the purpose of Ms. Olive's thesis project. You are permitted to access data with personal identifiers but will sign confidentiality agreements with the TDH. Removal of data with identifiers from the TDH data repository will not be allowed.

Our Institutional Review Board for the Protection of Human Subjects has previously approved the TNTDP. If you need any additional information, please feel free to contact me at 512/458-7676.

Sincerely,

Kate Hendricks

Kate Hendricks, M.D.
Director, Infectious Disease & Surveillance Division
Principal Investigator, Texas Neural Tube Defect Project

Appendix C

Institutional Review Board

SWT

Certification of Review and Approval by the Southwest Texas State University Institutional Review Board

IRB Reference Number
02-007

The project titled:

Periconceptional Exposure to Nitrates in Drinking Water and Risk for Neural Tube Defects

by Janus Olive under the supervision of Jean Brender

has been APPROVED, effective 10/9/2001.

The Southwest Texas Institutional Review Board shall conduct continuing review of this research appropriate to the degree of risk and the length of the project period, but not less than once per year.

Charles Garofalo
Chair, Institutional Review Board

Billy C. Covington
Associate Vice President, Office of Sponsored Programs/
Director, Federal Relations

Southwest Texas State University
601 University Drive San Marcos, Texas 78666-4605
512-245-2414
SWT is a member of the Texas State University System

Appendix D

Logistic Regression Analysis

Logistic Regression – Nitrate Level

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	110	100 0
	Missing Cases	0	0
	Total	110	100 0
Unselected Cases		0	0
Total		110	100 0

a If weight is in effect, see classification table for the total number of cases

Dependent Variable Encoding

Original Value	Internal Value
control	0
case	1

Categorical Variables Codings

		Frequency	Parameter coding (1)
50%ile	lowest - 3 52	49	000
	3 53 - highest	61	1 000

Block 0: Beginning Block

Iteration History[a,b,c]

Iteration		-2 Log likelihood	Coefficients Constant
Step 0	1	147 215	- 436
	2	147 214	- 443

a Constant is included in the model

b Initial -2 Log Likelihood 147 214

c Estimation terminated at iteration number 2 because log-likelihood decreased by less than 010 percent

Classification Table[a,b]

Observed			Predicted		
			CASE_1		Percentage Correct
			control	case	
Step 0	CASE_1	control	67	0	100 0
		case	43	0	0
	Overall Percentage				60 9

a Constant is included in the model

b The cut value is 500

Variables in the Equation

		B	S E	Wald	df	Sig	Exp(B)
Step 0	Constant	- 443	195	5 151	1	023	642

Variables not in the Equation

			Score	df	Sig
Step 0	Variables	NITREV_2(1)	2 668	1	102
	Overall Statistics		2 668	1	102

Block 1: Method = Enter

Omnibus Tests of Model Coefficients

		Chi-square	df	Sig
Step 1	Step	2 696	1	101
	Block	2 696	1	101
	Model	2 696	1	101

Model Summary

Step	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	144 518	024	033

Classification Table[a]

Observed			Predicted		
			CASE_1		Percentage Correct
			control	case	
Step 1	CASE_1	control	67	0	100 0
		case	43	0	0
	Overall Percentage				60 9

a The cut value is 500

Variables in the Equation

		B	S E	Wald	df	Sig	Exp(B)	95 0% C I for EXP(B)	
								Lower	Upper
Step 1[a]	NITREV_2(1)	654	403	2 639	1	104	1 923	874	4 234
	Constant	- 818	310	6 970	1	008	441		

[a] Variable(s) entered on step 1 NITREV_2

Logistic Regression – Nitrate Level and Other Potentially Confounding Risk Factors

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	110	100 0
	Missing Cases	0	0
	Total	110	100 0
Unselected Cases		0	0
Total		110	100 0

[a] If weight is in effect, see classification table for the total number of cases

Dependent Variable Encoding

Original Value	Internal Value
control	0
case	1

Categorical Variables Codings

		Frequency	Parameter coding		
			(1)	(2)	(3)
AGECAT	<20	28	000	000	000
	20-24	35	1 000	000	000
	25-29	23	000	1 000	000
	eq_gt 30	24	000	000	1 000
EDUCAT	<7	15	000	000	
	7-11	35	1 000	000	
	eq_gt 12	60	000	1 000	
INCOMCAT	<15K	64	000	000	
	15K-25K	21	1 000	000	
	gt_25K	25	000	1 000	
BMICAT	<30	85	000		
	30-highest	25	1 000		
alcohol use preconception	no	80	1 000		
	yes	30	000		
any folic acid preconception	no	106	1 000		
	yes	4	000		
any smoking preconception	no	96	000		
	yes	14	1 000		
50%ile	lowest - 3 52	49	000		
	3 53 - highest	61	1 000		

Block 0: Beginning Block

Iteration History[a,b,c]

Iteration		-2 Log likelihood	Coefficients Constant
Step 0	1	147 215	- 436
	2	147 214	- 443

a Constant is included in the model

b Initial -2 Log Likelihood 147 214

c Estimation terminated at iteration number 2 because log-likelihood decreased by less than 010 percent

Classification Table[a,b]

			Predicted		
			CASE_1		Percentage Correct
	Observed		control	case	
Step 0	CASE_1	control	67	0	100 0
		case	43	0	0
	Overall Percentage				60 9

a Constant is included in the model

b The cut value is 500

Variables in the Equation

		B	S E	Wald	df	Sig	Exp(B)
Step 0	Constant	- 443	195	5 151	1	023	642

Variables not in the Equation

			Score	df	Sig
Step 0	Variables	NITREV_2(1)	2 668	1	102
		BMICAT(1)	2 264	1	132
		AGECAT	1 968	3	579
		AGECAT(1)	306	1	580
		AGECAT(2)	000	1	997
		AGECAT(3)	586	1	444
		EDUCAT	4 362	2	113
		EDUCAT(1)	3 858	1	050
		EDUCAT(2)	998	1	318
		INCOMCAT	330	2	848
		INCOMCAT(1)	011	1	917
		INCOMCAT(2)	327	1	567
		VIT_FA1(1)	346	1	556
		SMOKE1(1)	802	1	371
		ALCOHOL(1)	102	1	750
	Overall Statistics		9 212	12	685

Block 1: Method = Enter

Omnibus Tests of Model Coefficients

		Chi-square	df	Sig
Step 1	Step	9 484	12	661
	Block	9 484	12	661
	Model	9 484	12	661

Model Summary

Step	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	137 730	083	112

Hosmer and Lemeshow Test

Step	Chi-square	df	Sig
1	12 939	8	114

Classification Table[a]

			Predicted		
			CASE_1		Percentage
	Observed		control	case	Correct
Step 1	CASE_1	control	55	12	82 1
		case	25	18	41 9
	Overall Percentage				66 4

a The cut value is 500

Variables in the Equation

								95 0% C I for EXP(B)	
		B	S E	Wald	df	Sig	Exp(B)	Lower	Upper
Step 1[a]	NITREV_2(1)	565	428	1 745	1	186	1 759	761	4 066
	BMICAT(1)	553	516	1 147	1	284	1 738	632	4 778
	AGECAT			792	3	851			
	AGECAT(1)	443	595	555	1	456	1 558	485	5 003
	AGECAT(2)	015	655	001	1	982	1 015	281	3 663
	AGECAT(3)	266	662	161	1	688	1 304	357	4 772
	EDUCAT			2 614	2	271			
	EDUCAT(1)	-1 075	689	2 431	1	119	341	088	1 318
	EDUCAT(2)	- 448	654	468	1	494	639	177	2 305
	INCOMCAT			096	2	953			
	INCOMCAT(1)	175	569	095	1	758	1 191	391	3 633
	INCOMCAT(2)	035	604	003	1	954	1 035	317	3 384
	VIT_FA1(1)	.614	1 299	223	1	.636	1 848	145	23 559
	SMOKE1(1)	177	735	058	1	810	1 194	283	5 040
	ALCOHOL(1)	194	591	108	1	743	1 214	382	3 862
	Constant	-1 335	1 463	833	1	361	263		

a Variable(s) entered on step 1 NITREV_2, BMICAT, AGECAT, EDUCAT, INCOMCAT, VIT_FA1, SMOKE1, ALCOHOL

Logistic Regression – Nitrate level, BMI Level, Nitrate * BMI Interaction

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	110	100 0
	Missing Cases	0	0
	Total	110	100 0
Unselected Cases		0	0
Total		110	100 0

a If weight is in effect, see classification table for the total number of cases

Dependent Variable Encoding

Original Value	Internal Value
control	0
case	1

Categorical Variables Codings

		Frequency	Parameter coding (1)
BMICAT	<30	85	000
	30-highest	25	1 000
50%ile	lowest - 3 52	49	000
	3 53 - highest	61	1 000

Block 0: Beginning Block

Iteration History[a,b,c]

Iteration		-2 Log likelihood	Coefficients Constant
Step 0	1	147 215	- 436
	2	147 214	- 443

a Constant is included in the model

b Initial -2 Log Likelihood 147 214

c Estimation terminated at iteration number 2 because log-likelihood decreased by less than 010 percent

Classification Table[a,b]

			Predicted		
			CASE_1		Percentage Correct
	Observed		control	case	
Step 0	CASE_1	control	67	0	100 0
		case	43	0	0
	Overall Percentage				60 9

a Constant is included in the model

b The cut value is 500

Variables in the Equation

		B	S E	Wald	df	Sig	Exp(B)
Step 0	Constant	- 443	195	5 151	1	023	642

Variables not in the Equation

			Score	df	Sig
Step 0	Variables	NITREV_2(1)	2 668	1	102
		BMICAT(1)	2 264	1	132
		BMICAT(1) by NITREV_2(1)	8 553	1	003
	Overall Statistics		9 549	3	023

Block 1: Method = Enter

Omnibus Tests of Model Coefficients

		Chi-square	df	Sig
Step 1	Step	9 557	3	023
	Block	9 557	3	023
	Model	9 557	3	023

Model Summary

Step	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	137 656	083	113

Hosmer and Lemeshow Test

Step	Chi-square	df	Sig
1	000	2	1 000

Classification Table[a]

			Predicted		
			CASE_1		Percentage
	Observed		control	case	Correct
Step 1	CASE_1	control	63	4	94 0
		case	32	11	25 6
	Overall Percentage				67 3

a The cut value is 500

Variables in the Equation

								95 0% C I for EXP(B)	
		B	S E	Wald	df	Sig	Exp(B)	Lower	Upper
Step 1[a]	NITREV_2(1)	159	457	121	1	728	1 172	479	2 870
	BMICAT(1)	- 693	860	649	1	421	500	093	2 700
	BMICAT(1) by NITREV_2(1)	2 239	1 084	4 267	1	039	9 382	1 121	78 491
	Constant	- 693	340	4 164	1	041	500		

a Variable(s) entered on step 1 NITREV_2, BMICAT, BMICAT * NITREV_2

Logistic Regression – All factors and Nitrate* BMI interaction factor entered

Case Processing Summary

Unweighted Cases[a]		N	Percent
Selected Cases	Included in Analysis	110	100 0
	Missing Cases	0	0
	Total	110	100 0
Unselected Cases		0	0
Total		110	100 0

[a] If weight is in effect, see classification table for the total number of cases

Dependent Variable Encoding

Original Value	Internal Value
control	0
case	1

Categorical Variables Codings

			Parameter coding		
		Frequency	(1)	(2)	(3)
AGECAT	<20	28	000	000	000
	20-24	35	1 000	000	000
	25-29	23	000	1 000	000
	eq_gt 30	24	000	000	1 000
EDUCAT	<7	15	000	000	
	7-11	35	1 000	000	
	eq_gt 12	60	000	1 000	
INCOMCAT	<15K	64	000	000	
	15K-25K	21	1 000	000	
	gt_25K	25	000	1 000	
BMICAT	<30	85	000		
	30-highest	25	1 000		
alcohol use preconception	no	80	000		
	yes	30	1 000		
any folic acid preconception	no	106	1 000		
	yes	4	000		
any smoking preconception	no	96	000		
	yes	14	1 000		
50%ile	lowest - 3 52	49	000		
	3 53 - highest	61	1 000		

Block 0: Beginning Block

Iteration History[a,b,c]

Iteration		-2 Log likelihood	Coefficients Constant
Step 0	1	147 215	- 436
	2	147 214	- 443

a Constant is included in the model

b Initial -2 Log Likelihood 147 214

c Estimation terminated at iteration number 2 because log-likelihood decreased by less than 010 percent

Classification Table[a,b]

			Predicted		
			CASE_1		Percentage Correct
	Observed		control	case	
Step 0	CASE_1	control	67	0	100 0
		case	43	0	0
	Overall Percentage				60 9

a Constant is included in the model

b The cut value is 500

Variables in the Equation

		B	S E	Wald	df	Sig	Exp(B)
Step 0	Constant	- 443	195	5 151	1	023	642

Variables not in the Equation

			Score	df	Sig
Step 0	Variables	NITREV_2(1)	2 668	1	102
		BMICAT(1)	2 264	1	132
		BMICAT(1) by NITREV_2(1)	8 553	1	003
		AGECAT	1 968	3	579
		AGECAT(1)	306	1	580
		AGECAT(2)	000	1	997
		AGECAT(3)	586	1	444
		EDUCAT	4 362	2	113
		EDUCAT(1)	3 858	1	050
		EDUCAT(2)	998	1	318
		INCOMCAT	330	2	848
		INCOMCAT(1)	011	1	917
		INCOMCAT(2)	327	1	567
		VIT_FA1(1)	346	1	556
		SMOKE1(1)	802	1	371
		ALCOHOL(1)	102	1	750
	Overall Statistics		13 437	13	415

Block 1: Method = Enter

Omnibus Tests of Model Coefficients

		Chi-square	df	Sig
Step 1	Step	13 832	13	386
	Block	13 832	13	386
	Model	13 832	13	386

Model Summary

Step	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
1	133 382	118	160

Hosmer and Lemeshow Test

Step	Chi-square	df	Sig
1	8 945	8	347

Classification Table[a]

			Predicted		
			CASE_1		Percentage
	Observed		control	case	Correct
Step 1	CASE_1	control	60	7	89 6
		case	29	14	32 6
	Overall Percentage				67 3

a The cut value is 500

Variables in the Equation

								95 0% C I for EXP(B)	
		B	S E	Wald	df	Sig	Exp(B)	Lower	Upper
Step 1[a]	NITREV_2(1)	054	491	012	1	913	1 055	403	2 763
	BMICAT(1)	- 872	941	858	1	354	418	066	2 647
	BMICAT(1) by NITREV_2(1)	2 302	1 167	3 894	1	048	9 997	1 016	98 394
	AGECAT			465	3	926			
	AGECAT(1)	383	601	407	1	524	1 467	452	4 758
	AGECAT(2)	203	676	091	1	763	1 226	326	4 609
	AGECAT(3)	108	673	026	1	872	1 114	298	4 163
	EDUCAT			2 786	2	248			
	EDUCAT(1)	-1 101	696	2 504	1	114	333	085	1 300
	EDUCAT(2)	- 405	663	373	1	541	667	182	2 446
	INCOMCAT			154	2	926			
	INCOMCAT(1)	140	579	058	1	809	1 150	370	3 575
	INCOMCAT(2)	- 144	629	053	1	818	865	252	2 971
	VIT_FA1(1)	476	1 293	136	1	713	1 609	128	20 273
	SMOKE1(1)	275	768	129	1	720	1 317	293	5 927
	ALCOHOL(1)	- 120	608	039	1	843	887	269	2 919
	Constant	- 697	1 548	203	1	653	498		

a Variable(s) entered on step 1 NITREV_2, BMICAT, BMICAT * NITREV_2 , AGECAT, EDUCAT, INCOMCAT, VIT_FA1, SMOKE1, ALCOHOL

Appendix E

Estimated Probability for Neural Tube Defect

Logistic Regression Model 3:

Variables in the Equation

		B	S E	Wald	df	Sig	Exp(B)	95 0% C I for EXP(B)	
								Lower	Upper
Step 1[a]	NITREV_2(1)	159	457	121	1	728	1 172	479	2 870
	BMICAT(1)	- 693	860	649	1	421	500	093	2 700
	BMICAT(1) by NITREV_2(1)	2 239	1 084	4 267	1	039	9 382	1 121	78 491
	Constant	- 693	340	4 164	1	041	500		

[a] Variable(s) entered on step 1 NITREV_2, BMICAT, BMICAT * NITREV_2

NITREV_2 = Nitrate levels ≥ 3 52 mg/liter
BMICAT = BMI ≥ 30 kg/m^2
BMICAT * NITREV_2 = BMI ≥ 30 kg/m^2 · Nitrate levels ≥ 3 52 mg/liter Interaction

Logistic Function:

$$logit\ P = \beta_0 + \beta_1 \text{Nitrate level} + \beta_2 \text{ BMI} + \beta_3 \text{ Nitrate level * BMI}$$

Estimated Probability (*P*) for NTD

$$P = 1 / 1 + e^{-z} \text{ with } z = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2$$

$\beta_0 =$ -0.693 (constant)
$\beta_1 =$ 0.159
$\beta_2 =$ -0.693
$\beta_3 =$ 2.239

Nitrate Level (X_1) 1 = ≥ 3 52 0 = < 3 52	BMI (X_2) 1 = ≥ 30 0 = < 30	BMI * Nitrate Level (X_1X_2)	*P*
0	0	0	0.33
0	1	0	0 20
1	0	0	0 37
1	1	1	0 73

Probability of NTD Occurring = 1; Not Occurring = 0

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VITA

Janus McCallum Olive was born in Bryan, Texas, on May 21, 1948, the daughter of Evelyn and Raymond McCallum. She received a Bachelor of Science in Physical Therapy from the University of Texas Health Science Center - San Antonio, Texas, in 1982. During the following years and to the present time, she has been employed as a licensed physical therapist in the San Antonio, Seguin, Gonzales, and San Marcos areas, working in hospital-, rehabilitation-, outpatient-, and home healthcare-settings. In addition, she has worked as part-time faculty with the Department of Physical Therapy at Southwest Texas State University since 1994. In September 1999, she entered the Graduate School of Southwest Texas State University, San Marcos, Texas. In April 2002, as a graduate student of the Department of Health Services Research, she received the College of Health Professions awards for Outstanding Research Presentation as well as Academic Excellence and Outstanding Student in the Department of Health Services Research.

Permanent Address: 322 West Krezdorn Street
Seguin, Texas 78155

This thesis was typed by Janus McCallum Olive.