UT-HOUSTON 25th ANNIVERSARY

School of Public Health

April 3, 1998

Priscilla Barry
Secretary to Admiral Zumwalt
1000 Wilson Boulevard, Suite 3105
Arlington, VA 22209-3901

Dear Ms. Barry:

Thank you for letting us know that the letter from Drs. Sweeney and del Junco and the research proposal, "Serum dioxin, cytochrome P-450 genes and reproductive health in Vietnam veterans" were received by Admiral Zumwalt's office. We were just in the process of contacting you to follow up on it when we received your letter. We look forward to hearing from Admiral Zumwalt when he has had the opportunity to review the research proposal. In the mean time, if more information is needed or there are any questions, please feel free to call. Thanks again for your reply.

Sincerely,

Suzanne Moore

Suzanne Moore
Research Associate
University of Texas – Houston
School of Public Health

Phone (713) 500-9248
Fax (713) 500-9249
Email smoore@utsph.sph.uth.tmc.edu

E. R. ZUMWALT, JR.
ADMIRAL, U.S. NAVY (RET.)

March 31, 1998

Ms. Betty Mekdeci
Executive Director
Association of Birth Defect Children, Inc.
827 Irma Street
Orlando, FL 32803

Dear Betty:

Thank you for having the researchers at the University of Texas, Houston School of Public Health, send me a copy of their research proposal on the reproductive health of Vietnam veterans. I had been alerted to the fact that a grant had been made, but didn't have the details.

I enclose a copy of their letter to me. Please give me a draft of what you would like to have me cosign pursuant to their request.

Sincerely,

E. R. Zumwalt, Jr.
Admiral, USN (Ret.)
Chairman, Agent Orange Coordinating Council

1000 Wilson Boulevard, Suite 3105
Arlington, VA 22209-3901

Tel: (703) 527-5380
Fax: (703) 528-5795

Enclosure

ADMIRAL ZUMWALT & CONSULTANTS, INC.

1000 WILSON BOULEVARD, SUITE 3105, ARLINGTON, VIRGINIA 22209-3901 (703) 527-5380 FAX: (703) 528-5795

E. R. ZUMWALT, JR.
PRESIDENT

March 26, 1998

Anne M. Sweeney, Ph.D.
Deborah J. del Junco, Ph.D.
Assistant Professors of Epidemiology
University of Texas - Houston
School of Public Health
P. O. Box 20186
Houston, TX 77225

Dear Professors Sweeney and del Junco:

This letter will acknowledge receipt of your recent letter enclosed a copy of your research proposal entitled, "Serum dioxin, cytochrome P450 genes and reproductive Health in Vietnam veterans."

Admiral Zumwalt has been out of town for a few weeks, but is due back in the office next week. Once he reviews the study, he will respond to your requests.

Sincerely,

Priscilla Barry

Priscilla A. Barry
Secretary to Admiral Zumwalt

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Arlington, VA 22209-3901

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E. R. ZUMWALT, JR.
PRESIDENT

MEMORANDUM

TO: Dick Christian
David Carter

FROM: Priscilla Barry

DATE: March 10, 1998

SUBJ: Agent Orange Study by the University of Texas

I am forwarding to you both a copy of the research proposal recently received.

David, this is being forwarded to you since you inquired about it last week.

Dick, I am forwarding the correspondence to you for your review in view of Admiral Zumwalt's absence from the office for the next few weeks.

Please let me know if any action needs to be taken. Should it be an agenda item at next AOCC?

FYI: Next AOCC -- Thursday, April 23, 2 PM, Admiral Zumwalt's office

ADMIRAL ZUMWALT & CONSULTANTS, INC.
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E. R. ZUMWALT, JR.
PRESIDENT

March 26, 1998

Roberta Shall Carlin, M.A., J.D.
Associate Executive Director
Spina Bifida Association of America
4590 MacArthur Boulevard, NW
Suite 250
Washington, DC 20007-4226

Dear Ms. Carlin:

Please find enclosed a letter from UT-House School of Public Health attaching a research project funded by EPA for investigation of herbicide exposure in Vietnam veterans.

Admiral Zumwalt is out of the office for a few weeks. In the meantime, I am forwarding it to you for any comments you may have that could be included in his response. Also I will include a review of this project as an agenda item for our April 23 Agent Orange Coordinating Council meeting.

Sincerely,

Priscilla Barry

Priscilla A. Barry
Secretary to Admiral Zumwalt

1000 Wilson Boulevard, Suite 3105
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Enclosure

UT-HOUSTON 25th ANNIVERSARY

School of Public Health

E. R. Zumwalt
Admiral, U.S. Navy(Ret.)
1000 Wilson Boulevard
Suite 3105
Arlington, Virginia 22209-3901

Dear Admiral Zumwalt:

We are writing to you at the suggestion of Ms. Betty Mekdeci, Executive Director of the Association of Birth Defect Children. We are researchers at the University of Texas, Houston School of Public Health. Dr. del Junco has been affiliated with the Houston VA Medical Center for the past 5 years. The U.S. Environmental Protection Agency (EPA) recently funded our study entitled, "Serum dioxin, cytochrome P450 genes and reproductive health in Vietnam veterans." Ms. Mekdeci telephoned last week to express her enthusiastic support of our "Agent Orange" study, having just read about it on an Internet News site. We enjoyed talking with her and look forward to working with her in the course of our research.

Ms. Mekdeci thought that you might be interested in our project. She strongly encouraged us to seek your endorsement. Please accept the enclosed complementary copy of our research proposal for your review. If the study's aims, proposed methods and research team meet with your approval, perhaps you would be willing to help draft and co-sign a letter of introduction to veterans who will be recruited to participate in our study.

As we discussed with Ms. Mekdeci, we are indeed fortunate that the EPA has given us the opportunity to conduct this important study. We originally wrote the proposal in response to a solicitation from the Department of Veterans Affairs (VA) aimed at establishing a Center for epidemiologic and toxicologic studies of the reproductive and developmental effects of military exposure to environmental agents. The VA told us that our Center proposal came in second (after the University of Louisville's). Though the EPA had never before funded a project focused on veterans, we took a chance and submitted our proposal. We believed that our proposal was strong and that the research needed to be done. We are proud and eager to pursue the first EPA-funded investigation of herbicide exposures in the Vietnam War. We hope that this is a convenient time for you to consider our request, and we look forward to hearing from you soon. Please feel free to call or e-mail Dr. Sweeney (713) 500-9471, asweeney@utsph.sph.uth.tmc.edu or Dr. del Junco (713) 500-9239, ddeljunco@utsph.sph.uth.tmc.edu.

Sincerely,

Anne M. Sweeney, Ph.D.
Assistant Professor of Epidemiology

Deborah J. del Junco, Ph.D.
Assistant Professor of Epidemiology

ABSTRACT

1. **Sorting Code**: **97-NCERQA-3B**
2. **Title**: Reproductive health, serum dioxin, and P450 genes in Vietnam veterans
3. **Investigators:** AM Sweeney, Principal Investigator, University of Texas-Houston School of Public Health;DJ del Junco, Co-Investigator, VAMC, Houston, Texas, SP Cooper, Co-Investigator, University of Texas-Houston School of Public Health, E. Symanski, Co-Investigator, University of Texas-Houston School of Public Health, M. Denison, Co-Investigator, University of California at Davis
4. **Project Summary:**

According to the Institute of Medicine's 1996 Update, past research relating adverse reproductive outcomes to Agent Orange exposure has suffered from ambiguous exposures measures and insufficient sample sizes. An equally important potential explanation for conflicting study results may be the lack of any genetic susceptibility component to identify high risk subpopulations. The proposed nested case-control study will test the hypothesis that the interaction between parental P450 genotype and dioxin exposure increases the risk of neural tube defects, using Vietnam era veterans as the study population.

a. **Objectives:**

1. To obtain the complete listing of NTDs that were diagnosed in the U.S. between 1965-1990 using birth certificates, fetal death certificates, and death certificates for older offspring. This interval represents the majority of childbearing years for Vietnam era veterans.
2. To select NTD cases and controls from pregnancies in which either parent was a Vietnam era veteran. This criterion is enforced to a) improve the likelihood of obtaining a wide variability in range dioxin exposure among parents, and b) to facilitate obtaining current addresses for the parents using the VA interagency agreement (described below).
3. To categorize study subjects likelihood of dioxin exposure using various indices found through linkage with military records.
4. To determine dioxin-like activity in the serum of a subset of cases and controls utilizing a bioassay method.
5. To determine the P450 genotype in a subset of cases and controls.
6. To evaluate the association between spina bifida and the interaction of P450 genotype and dioxin exposure.
7. To evaluate the association between anencephaly and the interaction of P450 genotype and dioxin exposure

b. **Approach:** From all NTDs diagnosed in the U.S. between 1965-1990, a total of 100 cases, which are parents of NTD infants who were Vietnam era veterans, as determined through linkage with military records, will be selected, along with 100 controls (parents of normal infants). Mailed surveys soliciting information on reproductive, medical and exposure histories and demographic characteristics will be obtained. Blood samples will be drawn and analyzed for dioxin-like activity by a bioassay. P450 genotyping will also be performed on all subjects. The major analysis is the evaluation of the association between NTDs and the interaction of P450 genotype and dioxin exposure.

c. **Expected results**: It is hoped that the results from this study will provide new information on the role of genetic susceptibility and the interaction with dioxin exposure resulting in an adverse health effect.

Reproductive Health, Serum Dioxin and P450 Genes in Vietnam Veterans

1. Objectives

Vietnam veterans' concerns about adverse reproductive and developmental effects from exposure to environmental and occupational hazards (e.g., herbicides like Agent Orange and its toxic contaminant, dioxin) remain unresolved despite a decade of intensive, expensive research. According to the March, 1996 findings of the National Academy of Sciences' "Committee to Review the Health Effects in Vietnam Veterans of Exposure to Herbicides," there is only "limited/suggestive evidence of an association" between paternal herbicide exposure and spina bifida in the offspring of male Vietnam veterans. Nevertheless, the federally mandated Committee has called for additional, more definitive studies to confirm the association as causal, and to uncover any other associations of herbicide exposure with adverse reproductive or developmental outcomes. In response to the Committee's findings, and at the request of the Secretary of the Department of Veterans Affairs (DVA), Jesse Brown, Congress has mandated precedent-setting legislation that provides medical and other benefits to Vietnam veterans' children who were born with spina bifida. {VA WWW http.va.gov posted July 29, 1996}.

Dioxin exposure is **not** limited to Vietnam veterans, however, and a "safe threshold" for dioxin levels in the U.S. general population has not been established. The contradictory findings of previous research relating dioxin exposure and adverse health effects may be due in part to the absence of a genetic susceptibility component to identify high risk subpopulations. The proposed case-control study is designed to investigate a gene-environment interaction by testing the hypothesis that the interaction between parental P450 genotype and dioxin exposure increases the risk of neural tube defects (NTDs), using Vietnam era veterans as the study population.

The study objectives are as follows:

1. To obtain the complete listing of NTDs that were diagnosed in the U.S. between 1965-1990 using birth certificates, fetal death certificates, and death certificates for older offspring. This interval represents the majority of childbearing years for Vietnam era veterans.
2. To select NTD cases and controls from pregnancies in which either parent was a Vietnam era veteran. This criterion is enforced to a) improve the likelihood of obtaining a wide variability in range of dioxin exposure among parents, and b) facilitate obtaining current addresses for the parents using the VA interagency agreement (described below).
3. To associate the participant' likelihood of dioxin exposure, derived from geographic, occupational, and military history, with the occurrence of NTDs offspring.
4. To determine dioxin-like activity in the serum of a subset of cases and controls utilizing a bioassay method.
5. To determine the association between cytochrome P450 genotype and NTDs.
6. To evaluate the interaction of cytochrome P450 and dioxin levels in association with spina bifida.
7. To evaluate the interaction of cytochrome P450 and dioxin levels in association with anencephaly.

Background

Neural tube defects (NTDs) are a group of malformations of the developing brain and spinal cord, the most common of which are anencephaly and spina bifida. The prevalence of spina bifida in the United States is 4.6 per 10,000 live births/stillbirths {Edmonds L 1990}. This figure has been declining for several years, with the decline preceding the widespread utilization of prenatal diagnosis and elective terminations, which began in the late 1970's . {Elwood M 1992 }.

The etiology of NTDs is unknown, although there is growing evidence that these defects are multifactorial in nature {Sever 1995}. There is also increasingly widespread support for the heterogeneity of the NTDs and the importance of examining individual NTDs separately to avoid masking associations with suspected etiologic agents. {Khoury MJ 1982; Seller MJ 1986; Dolk H 1991; Van Allen 1993}.

The need to examine the role of genetic heterogeneity in the susceptibility to environmental insults resulting in a NTD has also become apparent. It has been estimated that ingestion of a supplement containing 0.4 mg folic acid approximately three months prior to conception and in early pregnancy is associated with an approximate 50% decrease in NTDs {Oakley 1993}. This has not been demonstrated in all populations {Mills JL 1989}, and the mechanism of this effect is not understood.

Genetic heterogeneity in maternal metabolism of xenobiotics is strongly suggested by these investigations. In a recent paper discussing the potential role of environmental factors in the etiology of NTDs, Sever recommended four areas of focus for future studies of this issue, including: 1) the evaluation of specific birth defects; 2) improving exposure measurements; 3) exploring common mechanisms through which multiple agents could contribute to the etiology of NTDs; and 4) the evaluation of genetic variability in response to environmental exposures. {Sever 1995}. The proposed research addresses each of these areas through the assessment of spina bifida separately from anencephaly; through the analysis of serum for dioxin levels and the toxicokinetic modeling of these data to extrapolate to the time of the event; and through the examination of cytochrome P450 polymorphisms and the interaction with dioxin levels in the association with NTDs.

The total body burden of dioxin may be modulated by genetic polymorphisms in the enzymes responsible for activation and detoxification of aryl hydrocarbons. Cytochrome P450 (CYP1A1), the gene that codes for aryl hydrocarbon hydroxylase (AHH), initiates a multi enzyme pathway that activates and metabolizes polycyclic aromatic hydrocarbons (PAHs) and various carcinogens. AHH activity varies up to several thousand fold, is inducible, and induction of CYP1A1 mRNA is extremely sensitive to dioxin treatment {DeVito MJ, 1996; Vanden Heuvel JP, 1994}. Landi and coworkers {1994} have recently reported evidence of genetic susceptibility to dioxin toxicity involving *CYP1A1* polymorphism. Activation of toxic metabolites was greater in individuals with variant *CYP1A1* genotypes than in individuals with only wild-type alleles.

Two closely related polymorphisms at the *CYP1A1* gene locus have been identified. One is a restriction fragment length polymorphism (RFLP), *Msp*1. The other polymorphism is located in exon 7 and is responsible for an isoleucine-to-valine substitution in the protein catalytic region that affects the protein's function. The RFLP polymorphism has been associated with lung cancer in a Japanese study {Kawajiri J, 1990}. To date, there are no published reports associating reproductive outcomes (in animals or humans) with these polymorphisms. However, the most recent IOM review {1996}of the toxicology of dioxin has identified genetic

susceptibility (particularly through cytochrome P450 polymorphisms) as a key area of new research in the assessment of dioxin effects on health.

Dioxin (for purposes of this proposal defined as 2,3,7,8-tetrachlorodibenzo-p-dioxin or dioxin) has been dubiously hailed as "one of the most perplexing and potentially dangerous chemicals ever to pollute the environment." {Scientific Review Committee} It is a ubiquitous contaminant, produced as an unwanted by-product during the manufacture of many industrial and agricultural chemicals, as well as from incineration of municipal waste. It is believed that ingestion of contaminated food is the most likely source of dioxin exposure for the general population. {Scientific Review Committee, Rottluff W 1990}. Adverse reproductive effects associated with dioxin exposure have been evaluated in numerous studies, with often conflicting results. {For a comprehensive review of this literature, please see Sweeney AM 1994, IOM 1994,1996}. This review will focus only on the two studies that formed the basis for the IOM's conclusion that there is suggestive evidence of an association between paternal herbicide exposure in Vietnam and spina bifida.

The CDC Case-Control Study 1984

In 1984, the CDC released a report describing the results of the large case/control study that examined the relationship between service in Vietnam (not dioxin exposure per se) and risk of congenital malformations {Erickson JD 1984}. Case-group babies were infants with serious structural malformations born between 1968-1980 and registered with the Metropolitan Atlanta Congenital Defects Program (MACDP). Of the 7,133 eligible cases, maternal interviews were obtained for 4,929 (69%). Control babies were selected from Georgia vital statistics records, and were frequency matched to cases on race, and year and hospital of birth. Of the 4,246 eligible controls, maternal interviews were obtained for 3029 (71%). Paternal interview rates were 56% and 57%, respectively.

Among the children with congenital malformations, 428 (9%) were fathered by Vietnam veterans and 268 (9%) were fathered by non-Vietnam veterans. The odds ratios for service in Vietnam and birth defects of any type was 0.97 (95% CI, 0.83-1.14). Odds ratios were also calculated for 96 separate categories of birth defects with no significant associations observed.

The exposure opportunity index (EOI), developed for this study as described above, also was not associated with total birth defects. However, significant associations were observed for spina bifida, cleft lip $\pm$ cleft palate, neoplasms, and coloboma. With the exception of the last defect, all three also showed evidence of a dose-response relationship.

For the analysis that examined potential associations between self-reported Agent Orange exposure and birth defects, the frequency of exposure among fathers of each type of malformation was compared with the frequency among fathers with all other defects in an attempt to reduce recall bias. Twenty-five percent (n=74) of the Vietnam veterans reported that they believed they were exposed to Agent Orange during their service in Vietnam. The analysis of self-reported exposure and birth defects was negative on all counts, in contrast to the EOI analysis which found significant associations as described above. The small numbers of cases of many individual defects resulted in a virtual lack of power to detect any associations for these specific defects. In addition, the documented poor correlation between both self-reported exposure and the EOI without serum dioxin levels allows no inferences to be drawn from this study regarding the relationship between dioxin exposure and adverse reproductive effects.

The Baseline Ranch Hand Study - 1984

This initial report of the health of Ranch Hand personnel involved with the dissemination of herbicides during the Vietnam conflict utilized cohort status (Ranch Hands versus comparisons) as the basis of the analysis {Lathrop GD 1984}. The study is a matched cohort design with the comparison group selected from cargo mission personnel who also flew in Southeast Asia (SEA) but who were not directly exposed to Agent Orange. Controls were matched to the Ranch Hands for age, race, and occupational category.

The protocol consisted of a comprehensive personal and family health questionnaire and a physical examination, including an in depth laboratory analysis. The response rates for each phase of the protocol were quite different both within and between cohorts. Compliance with the questionnaire phase was 97% (n=1174) for the Ranch Hands and 93% (n=956) for controls. In the physical examination phase, compliance dropped to 87% (n=1045) for the Ranch Hands and 76% (n=773) for controls.

The reproductive outcomes evaluated in this phase of the study were ascertained through questionnaires obtained from both the veterans and their spouses/partners. A total of 7399 conceptions were analyzed in this preliminary report. There were 3293 conceptions among 1174 Ranch Hands, and 4106 among the 1531 controls.

Unadjusted analyses were conducted to examine the relationship between exposure and neonatal death, infant death, physical handicaps, birth defects, and learning disabilities. These analyses were stratified by pre- and post-SEA service periods. The results indicated that Ranch Hands were more likely to report physical handicaps (p=.07), birth defects (p=.08) and neonatal deaths (p=.02) in the post-SEA analysis. After adjustment for the maternal and paternal covariates described above, the relationship with birth defects achieved statistical significance (p=.04); the other relationships were not statistically significant.

Twelve of the 76 birth defects reported to have occurred among the Ranch Hands after post-SEA service were skin anomalies (ICD Code 757). Because these twelve anomalies represented a very broad range of conditions from mild birthmarks to serious conditions, they were excluded from subsequent analysis. When these anomalies are excluded, this relationship is no longer statistically significant (p=.14) although "still of interest."

Finally, semen samples from Ranch Hands (n=560) and controls (n=409) were analyzed for sperm count and morphology. The response rates for this parameter were 72.5% and 76.5%, respectively, although some of the samples submitted were ineligible for analysis because of prior vasectomies and orchiectomies. Linear regression techniques examined sperm count (as a continuous variable) and percentages of sperm with abnormal morphology as the dependent variables. Independent variables were age and exposure to industrial chemicals. No differences between groups in either parameter were identified.

This finding contrasts with the semen analysis results performed among 324 Vietnam veterans and 247 non-Vietnam veterans in the VES {CDC 1988}. That analysis indicated that Vietnam veterans had significantly lower mean sperm concentrations (p=.05) and twice the proportion with values below the clinical reference value (20 million cells/ml) than the non-Vietnam veterans. In addition, the mean percentage of morphologically normal cells was significantly decreased in the Vietnam veterans. These analyses were adjusted for twelve covariates, although exposure to industrial chemicals was not among them.

The Ranch Hand Study - Reproductive Outcomes

The marginally significant association between Ranch Hand status and birth defects

found in the previous study was sufficiently troubling to launch a massive project to verify all reported conceptions and pregnancy outcomes through medical record abstraction. In addition to outcome verification, individual dioxin exposure documentation via serum assay was undertaken in 1988 for Ranch Hands and controls. In 1992, the Air Force released the results of the first study that examined the relationship between direct measure of individual serum dioxin and verified reproductive outcomes {Wolfe WH 1992}. A total of 4,607 conceptions were examined in this study; 2,533 were contributed by 791 Ranch Hands, and 2,074 were contributed by 942 controls.

Ranch Hand personnel were shown to have significantly higher serum dioxin levels compared with the controls. The median values were 12.8 ppt and 4.2 ppt, respectively. The 98th percentile for Ranch Hands was 166.4 ppt; for controls, 10.4 ppt. Dioxin levels were determined in 1987. These results were used to estimate initial doses received during the veterans tour in Southeast Asia (SEA). However, no attempt was made to estimate dioxin level at the time of conception.

Several analyses of reproductive effects by serum dioxin level were trichotomized into: low (10-14.9 ppt; medium (15-$\leq$ 33.3 ppt; and high (> 33.3 ppt) dioxin groups. The potential association between cohort status and birth defects was examined for all defects combined, and an additional 12 categories of malformations. The only categories with sufficient numbers of verified post-SEA cases to detect a relative risk of 2 were total birth defects (229 cases among 1045 Ranch Hands and 289 cases among 1602 controls); and musculoskeletal deformities (132 cases among Ranch Hands and 180 among controls).

A significant variation was observed in the association between total birth defects (p=.03), defects of the respiratory system (p=.03), and urinary system abnormalities (p=.04), by Ranch Hand vs control status with time of conception (pre- or post-SEA). All of these findings were due to a lower rate among Ranch Hands in the pre-SEA conceptions and a higher rate among the post-SEA conceptions for the Ranch Hands.

Analyses of birth defects by dioxin level did not find any "consistent patterns" to support an association. For example, among children of flying enlisted and enlisted ground personnel, children of Ranch Hands in the low dioxin category had higher rates (433 per 1000 and 317 per 1000) than children of controls with background dioxin levels (229 per 1000). However, rates of children of enlisted ground personnel in the high dioxin group were not significantly elevated. Again, these analyses were not based on dioxin level at time of conception. Moreover, if high dioxin levels were related to early pregnancy loss, these results would make more biological sense. Most importantly, it was this study that provided what the IOM considers to be the most reliable evidence currently available. They found ***4 cases of NTDs (3 spina bifida and 1 anencephaly) among the offspring of 900 Ranch Hand veterans compared with no cases of NTDs among the offspring of 900 veterans ion the comparison group*** {Wolfe 1995}.

Finally, no association was detected between dioxin level and either sperm count or percentage of abnormal sperm. These analyses were based on semen samples that had been collected in 1982. The research to date has been successful in determining that exposure misclassification has had a profound impact on the studies of dioxin and adverse reproductive events in Vietnam veterans. This factor, in addition to selection bias, differences in case definitions, and small sample sizes have severely limited the power of these studies to resolve these issues.

2. Approach

Study Design, Population and Sample Selection

The study design can best be described as a case-control study nested within the cohort of Vietnam era veterans. We define Vietnam era veterans as members of the U.S. Armed Forces who served on active military duty anytime between January 12, 1962 (the date of the first U.S. Air Force herbicide mission in South Vietnam) and May 7, 1975 (the date of the presidential proclamation declaring the end of the Vietnam War era). We are focusing on the population of Vietnam era veterans to ensure a gradient of past dioxin (TCDD) exposure and current total body burden {IOM, 1994;1996}.

While the majority of aerial spraying missions involved Agent Orange and occured between 1967-68, the highest concentrations of dioxin were in the herbicide formulas sprayed between 1962 and 1965 (involving Agents Pink, Purple and Green) {Young et al, 1988}. These earlier formulations contained 16 times the average dioxin concentration in Agent Orange. Paradoxically, Vietnam veterans from the early years of the Vietnam Conflict (when the potential for dioxin exposure was especially high) have most likely been underrepresented in published studies to date. One of the problems in studies of Vietnam veterans (rarely, if ever, discussed) is an inability to follow-up veterans who separated from the military before 1971 because their records lack the social security numbers (SSNs) necessary for tracing {Bullman, 1990}. None of the published epidemiologic studies of Vietnam veterans have discussed what, if any, steps were taken to reduce this potential for serious selection bias. The investigators of the proposed study have taken the steps necessary (with the SSA and IRS) to acquire SSNs and contact information for the "early" Vietnam veterans based on longstanding as well as recent legislation authorizing the National Institutes of Occupational Health and Safety (NIOSH) and Department of Veterans Affairs to conduct studies of high risk occupational cohorts (personal communication, Chip Lehman, NIOSH, Pete Benson and Lucille Brown, SSA).

Veterans who served in South Vietnam between 1962 and 1969 and who frequently handled dioxin-contaminated herbicides (Agents Pink, Purple, Green and Orange) (e.g., Air Force Ranch Hands, Army Chemical Corps, etc.) are assumed to have the highest dioxin exposures, and many of these veterans have been shown to have high total body burdens of dioxin even 20 years after exposure {Wolfe et al, 1994 and 1995; Michalek et al, 1995 and 1996}. Other veterans who served in South Vietnam (including all women Vietnam veterans) are assumed to have moderate dioxin exposure, although the published data are inconsistent with regard to long-term, elevated dioxin levels among the few veterans in this group who have been tested {Kang et al, 1991; CDC 1988; Kahn et al, 1988; Schecter et al, 1986}. Veterans who never served in Vietnam are assumed to have the lowest average dioxin exposure, and Air Force veterans who did not serve in Vietnam have been reported to have lower average serum dioxin levels (in 1987) than their Ranch Hand counterparts {Air Force Health Study, 1991}.

This study will be the **first to include women veterans** in a biological assessment of dioxin exposure and its effect on offspring. Although women Vietnam veterans were neither directly engaged in combat nor in the spraying of herbicides, they did serve in areas of South Vietnam where herbicides were routinely used {IOM, 1994;1996}. The inclusion of **women veterans** is important for two reasons: 1) serum dioxin level and half-life have been shown to be positively correlated with increased percent body fat {Michalek et al, 1996}, and women have a higher average percent body fat than men; and 2) teratogenic effects would more likely result from maternal than paternal dioxin exposure {IOM 1994}. The National Registry of Women

Veterans currently includes 7,854 women known to have served in Vietnam. The Registry also includes over 220,000 women veterans who served during the Vietnam era, but never in South Vietnam.

A case is defined as a veteran parent (mother or father) of an offspring with a neural tube defect (NTD) born between January 1, 1965 and January 1, 1990. An NTD is defined as a birth defect in which the cranial and/or spinal portion of the neural tube has failed to close completely, leaving tissues of the central nervous system exposed and/or protruding through the protective bony skeleton. The individual NTD subtypes and the ICD-7 through ICD-9 codes are anencephaly (740) and spina bifida (741) and encephalocele (742.0). Cases occurring between the years 1965 and 1990 will be ascertained from U.S. birth certificates that list an NTD as a congenital anomaly, and from U.S. fetal and regular death certificates that list an NTD as an underlying or contributing cause of death. This method of ascertainment is estimated to include an average 50% to 90% of infants born with NTDs across the different states and calendar years {Hay, 1971; Edmonds et al, 1981;1990; Sever, 1982; Greenberg et al, 1983; Jorde et al, 1984; Boling et al, 1988; Hexter et al, 1990; Yen et al, 1992; Allen et al, 1996; }. Veteran parent will be linked to the birth and death records by parent's full name, age at delivery of the infant, and parent's state of birth {NCHS, 1995; 1996}. For the state of Michigan, birth certificates dated between 1970 and 1990 will be linked to veteran mothers by mother's SSN.

Virtually complete databases of male Vietnam theater and era veterans are available from the DOD Defense Manpower Data Center (personal communication Mike Dove) and the Department of Veterans Affairs Austin Automation Center. The National Registry of Women Veterans is networked to the DVA Austin Automation Center with full FTP capability. The National Registry of Women Veterans, DOD and DVA veteran databases include veterans' full name, maiden name, birth dates, state of birth, and SSN as well as branch of military, rank, and military occupational specialty codes, and dates of service. According to DOD estimates, there are approximately 2.6 million male Vietnam veterans and 6.1 million male Vietnam era veterans who did not serve in Vietnam {IOM, 1994}.

The study design has evolved from numerous conversations with Robert Bilgrad at the National Center for Health Statistics (NCHS) National Death Index and from a recent meeting with George Gay, Stephanie Ventura and Mike Kogan of the NCHS Vital Statistics Branch. The investigators are indebted to the staff of NCHS and especially Robert Bilgrad for assisting with the design of this study. To link offspring and parent records, we will use the same matching algorithms developed by NCHS for linking records to the National Death Index. Review of natality and mortality reports for 1965, 1975, 1985 and 1991 has revealed that mortality rates were high for spina bifida in the earlier years of the proposed study period. Although the sensitivity of birth certificates for NTD ascertainment has been shown to be somewhat low before the 1989 revision, many if not most of these cases are expected to be ascertained by fetal, infant or adult death certificates.

False positives (due to error in completing or coding the birth/death certificate) will be determined from subsequent parent surveys and authorized review of medical records and will be excluded from the study. Although there may be substantial underascertainment of NTDs for some states and some calendar years, there is no *a priori* reason to expect selection bias (i.e., selection into the study should be relatively unbiased with respect to dioxin exposure and total body burden). Assuming an average annual rate of 5 per 10,000 live and stillbirths, an average 3 million U.S. births per year, 25 years of follow-up, and a 50% ascertainment rate, we estimate

18,750 cases of NTDs will be ascertained by review of national and state-specific vital statistics (computerized databases and older, microfilm-based record systems). For comparison, we note that 15,503 cases of spina bifida and anencephaly were identified among 20 million live- and stillborn infants in the nationwide Birth Defects Monitoring Program between the years 1970 and 1989, at a rate of 7.75 per 10,000 live and stillbirths using hospital discharge diagnoses for more complete ascertainment than birth and death certificates (Yen et al, 1992). We assume 9% of NTD cases will have a Vietnam veteran parent (Erikson et al, 1984) which should yield 1,688 cases for study. We estimate another 1,688 to 3,376 NTD cases (9-18%) will have a veteran parent who did not serve in Vietnam {IOM, 1994}. Assuming that we will correctly match only 70% of true NTD-case/veteran parent pairs, and that only 70% of the necessary birth and death certificate copies will be acquired from the individual states, we should have approximately 800 Vietnam veteran cases and between 800 and 1600 non-Vietnam veteran cases.

For controls, we estimate between 3,376 and 5,064 total Vietnam era veterans will be linked (as a parent) to a random sample of 18,750 U.S. birth certificates representing the 25-year study period. The control selection strategy is intended to be roughly equivalent to incidence density sampling for nested case-control studies {Lubin, 1986; Langholz and Thomas, 1990; 1991; Robins et al, 1986}. Again, assuming a 70% success rate linking veteran parents to liveborn offspring and a 70% success rate of acquiring copies of valid birth/death certificates, we expect between 1600 and 2400 controls. A control is defined as a veteran parent (mother or father) of a liveborn offspring free of NTDs. False negatives (failure to indicate a true NTD on birth or death certificate) will also be determined from parent surveys and authorized review of medical records, however, very few are expected owing to the rarity of NTDs. Any false negatives will be re-classified and retained for study.

Exposure Index Development

In order to test the hypothesis of an interaction between P450 genotype susceptibility and dioxin toxicity resulting in a NTD, the bioassay developed by Dr. Denison (described below) to measure dioxin and dioxin-like activity in the serum will be utilized. In order to determine the sensitivity and specificity of the assay, variability in serum dioxin levels among the subjects is required. Therefore, in order to optimize the likelihood of obtaining this variability, an exposure index has been developed based on military occupational specialty (MOS), early years of service in Vietnam, and service in Vietnam for women. The three possible categories of exposure are as follows:

High Exposure Probability - includes Ranch Hand personnel, Army Chemical Corps personnel, veterans with service in Vietnam between 1962-69, and specific MOSs that are considered to carry a high risk of dioxin exposure, based on maps generated by the HERBS tapes and surveys of veterans regarding dates and locations of service in Vietnam.

Medium Exposure Probability - includes all women Vietnam veterans, and specific MOSs considered to be at moderate risk for dioxin exposure.

Low Exposure Probability - includes only Non-Vietnam veterans.

Each of the 1600-2400 cases and 1600-2400 controls will be classified into one of the three exposure categories, based on the information obtained through linkage with the military databases. Then, while maintaining the observed proportions of cases and controls within each exposure category, 67 persons will be randomly selected from each stratum for a total of 200

individuals for the case-control phase of the project. By preserving the true sampling fractions within each exposure category for back-calculation, this strategy is expected to yield valid stratum-specific odds ratios.

A similar approach to sampling has been utilized by Shantz, et al, in their study of PCBs and dioxin-like chemicals and neuropsychological functioning in elderly Great Lakes fisheaters and controls {ATSDR H75/AT H5 98339}. In that study, all subjects were categorized into high, medium , and low exposure categories, based on their reported Great Lakes fish consumption histories and other factors. The subjects were then randomly selected from within each tertile, and their serum samples were sent to Dr. Denison for analysis.

Recruitment and Data Collection Procedures

Once cases and controls have been selected, current addresses will be obtained through an **existing interagency agreement with the National Institute of Occupational Safety and Health (NIOSH), the Internal Revenue Service (IRS), the Social Security Administration (SSA) and the Department of Veterans Affairs (VA).** For veterans more difficult to locate, we will rely on the services of Equifax, which has a considerable track record in locating Vietnam veterans. A packet will be mailed to all the selected individuals, containing a letter explaining the nature of the study, a consent form, a survey detailing reproductive and military history; demographic and lifestyle history; medical history; occupational history; a short "refuser" post card, and a self-addressed, stamped envelope. If the subject refuses to participate, the "refuser" post card will attempt to ascertain the reason for nonparticipation as well as the veteran's self-perceived probability of dioxin exposure, from service-related as well as civilian-related activities. Other pertinent data will already be available to allow for a comparison of refusers to study subjects for those characteristics associated with both NTDs and dioxin exposure, including age, race, parity, branch of service, and military rank and occupation.

For each subject who agrees to enroll, the research team will make arrangements to have a blood sample collected at the VAMC or outpatient clinic nearest to their home. Each participant will be asked to donate a 50 ml blood sample for dioxin and P450 genotyping analysis. The VA's mobile clinics will be used if the veteran is unable to make the trip to the VA site. The subject will be reimbursed for any travel expenses incurred during this visit. As an additional courtesy, participants will be offered a complete blood chemistry profile and a copy of the results will be sent to the physicians of their choice. The research team will monitor the storage and shipment of the specimens to the laboratory at the University of Texas-Houston School of Public Health.

The questions concerning both reproductive history as well as military history will be adapted from the questionnaires used by the CDCs Vietnam Experience Study, the Air Force's Ranch Hand study, the on-going CDC study of reproductive health in women Vietnam veterans, and Dr. del Junco's study of reproductive health related to military experience and post-traumatic stress disorder. A critical difference between these studies and the proposed research is that ***no data exist on biological measures of dioxin exposure in women Vietnam veterans,*** which will be obtained in this project, **as well as P450 genotyping.**

Additional questions from the recently completed study of early pregnancy loss in the semiconductor industry {Schenker M 1996}, as this survey contains validated questions regarding reproductive histories and occupational exposures. These questionnaires also solicit data regarding potential confounders and other risk factors for adverse reproductive events,

including history of smoking and alcohol use, other job-related toxic exposures, physical activity levels, illicit drug use, etc. This approach will allow us to examine potential dioxin exposures occurring after Vietnam service, which will be included in the exposure assessment models ***to extrapolate serum dioxin levels back to the time of conception.***

Data on current height and weight, as well as weight at 18,25,35,45 and 55 years of age will be ascertained to serve as a surrogate for percent body fat of subjects over time, which has recently been shown to be associated with dioxin half-life {Pirkle 1995}. This proxy measure will also be incorporated into the toxicokinetic modeling of dioxin levels over time and estimates of dioxin levels at the time of conception. Dr. Wun has had considerable experience in this area, most recently working with Dr. Sweeney on examining the factors associated with changing PCB levels over time in women of childbearing age.

Dioxin Bioassay for Dioxin-like Activity

Dr. Michael Denison will conduct the bioassays in his laboratory at UC-Davis. Dr. Dension has been working for a number of years to develop these assays for detection of dioxin and dioxin-like chemicals in environmental and biological samples {El-Fouly 1994, Garrison 1995, Denison 1996, Aarts 1996, Murk 1996, Balaguer 1996}.The bioassay to be used in this study utilizes a sensitive and easily measurable reporter gene (firefly luciferase (LUC)), present in a recombinant dioxin-inducible expression plasmid vector. This vector contains the LUC gene under dioxin-inducible control of four dioxin
responsive DNA enhancer elements {Denison 1993}. These recombinant cell bioassays are quick and sensitive (minimal detection limit of 0.1 pM TCDD/assay) and have been used for detection and relative quantitation of dioxin-like chemicals present in biological and environmental samples {Anderson 1995}. Initially small aliquots of serum from all 200 of the study participants will be screened using the cell bioassay in order to identify individual samples which contain detectable levels of dioxin-like chemicals. Confirmation that the increase in luciferase activity is due to the presence of dioxin-like chemicals will be accomplished using an AhR-dependent DNA binding assay {Denison 1991, Helerich 1991}. Serum from positive individuals will be further analyzed using the bioassay system to determine the relative serum concentrations of dioxin-like chemicals.

Cell Culture Screening Bioassay

For this study, we will utilize a TCDD-responsive mouse hepatoma (Hepa1c1c7) cell line we have developed which contains a stably integrated pGudLuc1.1 expression vector. These cells will be maintained in culture as we have previously described {Denison 1988}. Our previous studies have demonstrated the utility of this assay for detecting TCDD and it's related chemicals present in whole serum. In addition to determining the quantitative magnitude of response due to HAHs in these cells, serum samples will also be spiked with an Ah receptor antagonist which will block the induction by HAHs present in the serum. The difference in induction between samples with and without antagonist provides a quantitative measure of the amount of HAH in the serum sample. The expression of reporter gene activity occurs to a level proportional to the amount of dioxin-like chemical present in the sample.

To confirm that an increase in luciferase activity in a specific sample is due to the presence of AhR-receptor binding, dioxin-like chemicals, rather than to some AhR-independent mechanism (such as stabilizing luciferase mRNA or protein and/or direct effects on other regulatory factors), we will utilize gel retardation analysis {Denison 1991, Denison 1991a}. This assay provides us

with an appropriate second tier analysis to confirm the presence of dioxin-like chemicals in the samples. It will be carried out as we have previously described in detail (Helerich 1991, Dension 1991}. Serum samples from individuals determined by these analyses to contain dioxin-like chemicals will be further examined in dose-response experiments described below.

Acid Denaturation Extraction.

Although both HAHs and PAHs can induce in this system, the PAHs are significantly weaker. Given that the focus of this project is an assessment of the amount of dioxin-like activity HAHs we need to eliminate the contribution of PAHs to the luciferase induction response. This will be accomplished by differential destruction and inactivation of PAHs using an acidic denaturation step (HAHs are resistant to acid denaturation, unlike PAHs). Thus, any luciferase induction by samples which have been subjected to the acid denaturation step will be due to dioxin-like HAHs remaining in the sample. We have previously demonstrated the utility of this procedure for the detection of HAHs (Parts 1996, Murk 1996}.

Relative quantification of dioxin-like chemicals in positive serum samples

For these studies, cells will be plated out into 96 well microtiter culture plates, and grown as described previously, incubated with 20-50 *u*l of 5-8 serial dilutions of serum from the study participant for 4-6 hours, followed by measurement of luciferase activity. Concurrent with each assay, TCDD will be added to another series of wells (containing uncontaminated serum) for the determination of a standard TCDD dose-response relationship; uncontaminated serum will also be added to several wells as a negative control. Luciferase activity in cell extracts will be measured as described previously and the EC50 (estimated concentration to half maximal induction) values will be determined. Comparison of the EC50 values between the TCDD-spiked serum and unknown serum samples will allow estimation of the relative TCDD-equivalents of the particular test samples (equation 1). EC50 values (reported as the estimated dose for half-maximal induction) will be calculated by probit analysis with TCDD-treated cells serving as positive controls {Sawyer 1984, Tillett 1991}.

Equation 1

$$\text{TCDD-TEQ} = \frac{\text{EC50 (Chemical Standard TCDD))}}{\text{EC50 (Unknown Sample Extract)}}$$

GC/MS Analysis for Serum Dioxin Levels

The bioassay results will be quantitative, but they will only provide a measure of total dioxin-like activity. Therefore, serum from a subset of the study participants , including all of those individuals with readily dateable dioxin-like activity and a random sample of the remaining subjects (n=25) will be sent to the Michigan Department of Public Health's (MDPH) laboratory for quantitative serum dioxin analysis. The MDPH laboratory has been trained in the CDC dioxin analysis methodology, and is currently serving as the laboratory for dioxin measurement in the Schantz study described above.

Cytochrome P450 Analysis

For the genetic studies, blood will be collected in 10 ml sodium heparinized tubes. Tubes without anticoagulant will be used for the toxicology studies. All blood samples will be shipped to Dr. Wu's laboratory at the UTSPH for processing. Two procedures for CYP1A1 genotyping

will be utilized. One method reveals the MspI polymorphism; the second detects an A/G substitution on exon 7 of the CYP1A1 gene, the genetic basis of an isoluceine/valine substitution. Homozygous wild type individuals show only the 339-bp parent band, whereas heterozygotes show three bands. Individuals with the homozygous mutant allele have only the 134-bp and 205-bp bands.

Data Analysis

The initial examination of the data on the total 18,750 birth certificates purchased from the State Health Departments will include a comparison of the proportion of Vietnam and non-Vietnam and civilian subjects in the case and control groups. This represents a crude measure of the association of Vietnam service, and military service in general, and NTDs.

The next analysis will examine differences in estimated probability of dioxin exposure between cases and controls, using the exposure index described above. These sampling fractions will be preserved in the subset of 200 individuals selected for the case-control study. The agreement between the exposure index and the bioassay for dioxin-like activity will be measured using the Kappa statistic. It is noted that this test will indicate only the agreement between these two measures of exposure, and does not indicate the sensitivity or specificity of the bioassay. However, the bioassay itself will be analyzed for sensitivity and specificity using the MDPH dioxin analysis as the gold standard. If the bioassay demonstrates good sensitivity and specificity, the results can then be used to examine the validity of the exposure index. Crude and adjusted odds ratios for NTDs and high versus low serum dioxin levels as determined by the bioassay will be calculated. Odds ratios for NTDs and each type of P450 allele will be calculated and compared to test the hypothesis that among NTD- affected pregnancies, there will be a higher proportion of subjects with variant alleles when compared with NTD pregnancies in the wild type group. The most important analysis will consist of testing for the interaction between P450 genotype and high and low serum dioxin levels and association with NTDs.

3. Expected Results

The proposed research will utilize a case-control study to test the hypothesis that spina bifida and anencephaly are associated with a P450 mutant allele and exposure to dioxin. This will provide the first assessment of dioxin exposure among female Vietnam veterans and permit the determination of a ***maternally-mediated effect*** on these defects. In addition, this study ***will include a sample of Vietnam veterans who served in the earlier years of the conflict (1962-1966), an important group previously under represented in earlier studies of Agent Orange exposure and adverse health effects.*** These early Vietnam veterans were present when the herbicides with higher concentrations of dioxin were being utilized. Because social security numbers were unknown for these veterans at the time the previous studies of Vietnam veterans were being conducted, they were not selected for inclusion in these studies Finally, this will be ***the first attempt to examine an underlying genetic susceptibility to dioxin toxicity, resulting in an adverse health outcome.***

4. General Project Information.

Project Personnel ***Dr. Sweeney*** has had extensive experience conducting large-scale epidemiologic studies of environmental exposures and reproductive effects. Currently, she is the

Principal Investigator on an NIEHS-funded project that is examining PBB and PCB exposure and reproductive outcomes, via linkage with birth certificates, in two well-characterized cohorts established by the Michigan Department of Public Health. She is also a consultant on two projects funded by the Agency for Toxic Substances and Disease Registry in the Great Lakes Basin area. One of these, the New York State Anglers Cohort Study, is assessing reproductive events in fisheaters exposed to PCBs. Dr. Sweeney was a member of the EPA's 1994 Dioxin Peer Review Panel, contributing to the resulting EPA document on dioxin toxicity.

Dr. del Junco is developing the National Registry of Women Veterans (SDR#93-115) to facilitate the inclusion of women veterans in potentially beneficial health and health services research and to provide researchers access to a universal sampling frame. The Registry currently identifies 1.1 million of the estimated 1.4 to 1.8 million women separated from the military since 1942. She has identified all women separated from the military since 1965 (including all Vietnam era veterans and all known Vietnam theater veterans). Dr. del Junco is using the resources of the National Registry to recruit a nationally representative sample of women veterans for her study entitled, "Reproductive Health in Women Veterans Related to Posttraumatic Stress Disorder and Military Experience" (SDR#93-108). The Reproductive Health Study will establish the feasibility and validity of self-administered questionnaires to ascertain complete military and reproductive histories.

The area of exposure documentation in this proposal is greatly enhanced by the expertise of Drs. Cooper and Wun and Symanski.. ***Dr. Cooper*** is an occupational epidemiologist with considerable experience in developing lifetime occupational exposure histories for toxic exposure assessments. She is the Principal Investigator on an NCI-funded project to examine the feasibility of following migrant farmworkers in studies of pesticide exposure and health effects in this population. The determination of factors associated with serum dioxin and changes in those levels over time is the responsibility of the biostatistician, ***Dr. Wun***. She has had extensive experience in this area and is currently developing the toxicokinetic models for serum PCBs in Dr. Sweeney's project. She will work closely with Dr. ***Symanski,*** who has made toxicokinetic modeling the major focus of her research.

A major strength of this proposal is the examination of metabolic enzyme genetic polymorphisms in relation to serum dioxin, spina bifida and other adverse outcomes. ***Dr. Wu*** has extensive experience with the analysis of metabolic enzyme polymorphisms and with studies associating human cancers with mutant alleles. ***Dr. Denison*** is a pioneer in the development of bioassays to measure dioxin-like activity in biologic and environmental samples.

This research will draw on the expertise of ***Dr. Lowell Sever,*** an eminent perinatal epidemiologist who is regarded as a leading researcher in the epidemiology of NTDs. He is a former Assistant Director for Science, Division of Birth Defects and Developmental Disabilities at the Centers for Disease Control and is currently serving on advisory committees on birth defects surveillance for Texas and Washington State. His vast number of publications in this area attests to the invaluable contributions Dr. Sever will make to this effort. Ware also fortunate to have Major Kevin Grayson, Ph.D., from Brooks Air Force Base, to serve as a consultant on this project. Dr. Grayson's experience with the Ranch Hand study will provide important insights into the conduct of this study, particularly with regard to issues of exposure assessment.

Resources

The physical space and sophisticated computer facilities needed for this study are in place at the NRWV located within the Houston VAMC. These facilities are being used for construction and maintenance of the National Registry of Women Veterans. The NRWV is directly linked with VA's Austin Automation Center. Interagency agreements are in place for the use of DOD, NCHS, NIOSH and the IRS data facilities. The VA system of hospitals and outpatient clinics will be used for obtaining blood samples from participants and the Houston VAMC will provide complementary blood chemistry profiles.

The physical facilities and equipment provided by the School of Public Health include an extensive library, office and laboratory space, a -70 degree freezer for storage of blood samples, a centrifuge and other equipment and supplies to separate blood into serum and cellular components and to extract DNA, one PCR analyzer and the non-disposable laboratory supplies. To accommodate the additional workload proposed in this investigation, an additional lab technician and a PCR engine will be required.

The UTSPH molecular genetics laboratory will be responsible for shipping properly separated and labeled serum specimens to the dioxin lab at the MDPH. The UTSPH will also provide necessary administrative, statistical, data management, and clinical support (for blood collection). The ability to coordinate and standardize blood collection and shipment is a special strength of the VA system. The ability to offer veterans a complementary blood chemistry profile is also a unique capability of the VA system.

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