DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Defining the range of expression, risk factors for, and incidence of FASD in children born to women who drink varying amounts of alcohol during pregnancy is of vital importance in terms of prevention, intervention and treatment. The proposed study entails a collaboration with the Moscow Region Ministry of Health to screen 26,000 pregnant women over two years. From these, 640 moderate to heavy drinkers and 640 controls will be selected for longitudinal follow-up including standardized physical exams and neurobehavioral testing of infants through twelve months of age. The specific aims of the project are: 1) To measure the birth prevalence and range of alcohol-related physical features and neurobehavioral impairment among children born to pregnant women in the Moscow Region who report consuming moderate to heavy amounts of alcohol by utilizing methods designed to permit earlier diagnosis of alcohol-related effects, and 2) To evaluate the contribution of maternal nutritional factors to increased risk for prenatal growth deficiency, neurobehavioral impairment, and alcohol-related physical features in infants prenatally exposed to moderate to heavy amounts of alcohol by conducting a randomized trial of a micronutrient supplementation intervention and measuring micronutrient levels in maternal blood. The proposed study will contribute to a better understanding of the incidence and range of FASD based on early diagnosis in a cross-cultural environment, and will for the first time specifically test a nutritional intervention that may have widespread applicability should undernutrition prove to be a modifiable risk factor for FASD.

PERFORMANCE SITE(S) *(organization, city, state)*

University of California, San Diego Medical Center, San Diego, California

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KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. *Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.*

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A. Specific Aims

As a component of the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD) we propose to conduct a prospective study in the Moscow Region of Russia. In keeping with one of the primary goals of the consortium, which is to definitively outline a diagnostic schema for detecting the full range of effects from prenatal exposure to large and moderate amounts of alcohol utilizing cross-culturally generated data, we believe that a prospective study in Russia can contribute significantly to the consortium effort as well as benefit from the exchange of information with other projects in the CIFASD. The Specific Aims of this proposal are:

Specific Aim 1: To measure the birth prevalence and range of alcohol-related physical features and neurobehavioral impairment among children born to pregnant women in the Moscow Region who report consuming moderate to heavy amounts of alcohol by utilizing methods designed to permit earlier diagnosis of alcohol-related effects. The hypothesis to be tested is that moderate to heavy alcohol consumption during pregnancy will lead to an increased prevalence of alcohol-related structural features, growth deficiency, and neurobehavioral impairment that is recognizable in infants ≤1 year of age when compared to infants prenatally exposed to lesser amounts. This research will be accomplished by the following:

a. Prospective ascertainment of a group of pregnant women who are receiving prenatal care at one of 4-6 selected hospitals in the Moscow Region and who report moderate to heavy alcohol consumption during the first trimester of pregnancy.

b. Prospective ascertainment at the same 4-6 hospitals of a similar group of women who do not report moderate to heavy alcohol consumption during the first trimester of pregnancy.

c. Collection of exposure data by maternal interview regarding dose, timing, type and pattern of alcohol consumption during pregnancy, other exposures, medical history, and demographics, and validation of maternal alcohol history by measurement of selected biomarkers of alcohol abuse.

d. Collection of outcome data by prenatal ultrasound examination, postnatal maternal interview, medical record review, photographic analysis, dysmorphological examination, and neurobehavioral testing of children up to 12 months of age.

Specific Aim 2: To evaluate the contribution of maternal nutritional factors to increased risk for prenatal growth deficiency, neurobehavioral impairment, and alcohol-related physical features in infants prenatally exposed to moderate to heavy amounts of alcohol by conducting a randomized trial of a micronutrient supplementation intervention and measuring micronutrient levels in maternal blood. The two hypotheses to be tested are that a) additional supplementation with certain micronutrients during pregnancy will improve prenatal growth as well as neurobehavioral performance in infants ≤1 year of age, and that the effect size will be significantly greater among infants whose mothers drank moderate to heavy amounts of alcohol compared to infants whose mothers drank lesser amounts, and b) that blood levels of certain micronutrients as measured during the first trimester of pregnancy will be predictive of the prevalence of alcohol-related structural abnormalities in the offspring of women who drink moderate to heavy amounts of alcohol and blood levels of micronutrients measured over the course of pregnancy will be predictive of prenatal growth and neurobehavioral performance in infants ≤1 year of age.

B. Background and Significance

1. Fetal Alcohol Spectrum Disorder

The Fetal Alcohol Syndrome (FAS), initially described by Jones et al in 1973, is a specific pattern of malformation seen in the offspring of women who drink alcohol heavily during their pregnancy.[1] The principal features of the disorder include prenatal onset growth deficiency; microcephaly; and a characteristic pattern of

minor malformations which is made up of subtle but distinct facial features including short palpebral fissures, maxillary hypoplasia, and a long smooth philtrum with a thin smooth vermilion border of the upper lip; joint anomalies; and alterations in neurobehavioral development. In the U.S. the birth prevalence of recognized FAS is estimated to be 1-2/1000 live birth;[2] however, among alcoholic women, the birth prevalence of FAS may be as high as 44%.[3] Furthermore, children who have the full-blown syndrome are thought to represent only a small fraction of those prenatally affected by alcohol. It is estimated that the combined birth prevalence of FAS and or Alcohol-Related Neurodevelopmental Disorder approaches 1/100 live births in the U.S.[2] In order to better conceptualize the entire set of structural and developmental problems associated with prenatal alcohol exposure, the umbrella term Fetal Alcohol Spectrum Disorder (FASD) has been coined.

Defining the range of expression, key diagnostic criteria, risk factors for, and incidence of FASD in the offspring of women who drink varying amounts of alcohol during pregnancy is of vital importance in terms of primary and secondary prevention, intervention and treatment. The difficulties inherent in conducting research on FASD, including barriers to making an early diagnosis, inability to appropriately validate maternal alcohol history, and the relatively rare occurrence of the most severe end of the spectrum, require that multiple approaches to study design be employed in a variety of settings.

Retrospective case series of children with FAS or heavy prenatal exposure are an efficient approach to developing a full set of criteria for diagnosing FASD. However, these samples may involve significant selection and referral biases so that children with certain types of structural features and/or developmental problems are more likely to be recruited, and therefore may not accurately represent the full spectrum of the disorder. In addition, retrospective samples usually lack the advantages afforded by the prospective involvement of both the mother and the child in the research effort. For example, clinic-based prospective studies of FASD conducted in the U.S., such as those of Jacobson et al,[4] Day et al,[5] and Stoler et al,[6] include more detailed measures of maternal drinking patterns and timing of exposure and can link these measures to the full spectrum of alcohol effects.

Furthermore, prospective studies generally provide the best opportunity for developing techniques that allow for earlier diagnosis of FASD. By identifying fetuses and newborn infants with significant levels of alcohol exposure, methods for prenatal and early postnatal identification of physical features can be tested and refined. This information is vital to implementation of early intervention and treatment efforts both on behalf of the mother and the exposed fetus or infant. However, in the U.S., prospectively ascertained samples often suffer from small numbers of heavy drinking women and therefore few children who actually meet the criteria for a diagnosis of FAS. These factors taken together lend support to the potential contribution of studies conducted in settings where frequent alcohol consumption among pregnant women is relatively common and is concentrated in certain geographic areas, such as among some Native Americans, in certain sections of South Africa, and in the Moscow Region of the Russian Federation.

2. The Moscow Region

The Moscow Region comprises an area approximately 200 km in diameter with an estimated population of 11,000,000, both urban and rural communities, and diverse economic conditions. There are 56 birthing hospitals in the Region that are responsible for prenatal care and the delivery of approximately 50,000 infants per year. Each of these hospitals comes under the direct supervision of the Ministry of Health Maternal Child Health Deputy Minister Dr. Gaiane Tamazian who is a collaborator on the proposed study and has served in an advisory capacity in developing the study design.

The proposed Moscow Region study provides the rare opportunity for implementing a prospective study in a population where heavy drinking is relatively common. The combined advantages of the site and study design will permit evaluation of additional maternal risk factors such as nutritional status and biomarkers of maternal exposure, as well as early measures of effects including prenatal ultrasound, newborn and infant physical examinations, early childhood neurobehavioral testing, and photographic analysis. In addition, ascertainment of a prospective sample of heavy-drinking nutritionally-challenged women provides the unique opportunity to

incorporate a nutritional intervention. Thus at the conclusion of this study, we will not only be able to describe the correlation between nutritional deficiencies and some features of FASD but also be able to measure the effect of modifying those risk factors on some outcomes associated with FASD.

There is evidence to support the assertion that moderate to heavy alcohol use among women is relatively common in Russia. In a 1993 study by Skosyreva et al,[7] among 250 women interviewed, 95% reported current drinking, and 10% were considered alcohol abusers. In data representing female deaths in 1984, Nemtsov found that 19.9% of the total number were alcohol-related compared to 5-9% of total deaths of both males and females in the U.S.during approximately the same time period.[8] Grjibovski et al reported data from 1404 prenatal interviews conducted in municipal prenatal care centers in northwestern Russia in 1999.[9] In that sample, approximately 18% of women reported drinking during pregnancy compared to 12.8% of women in the U.S. based on the most recent BRFSS survey data. Similarly, in unpublished data collected in St. Petersburg, (Wilsnack & Kristjanson) among 208 pregnant women interviewed, 37% reported any drinking and 4.3% were considered moderate drinkers (averaging between 3 and 12 standard drinks per week).[10] In a preliminary survey of women presenting at a genetics center in Moscow in 2002, approximately 2.5% of women reported drinking between 4 and 7 days per week during pregnancy.[11] Although not directly comparable, recent BRFSS data from the U.S. indicate that only 0.6% of pregnant women reported drinking 7 or more drinks per week in the previous 30 days.[12] Finally, as a measure of the extent to which drinking by Russian women translates into children with the full-blown FAS, in orphanages and boarding schools in Moscow, the prevalence of the disorder has been estimated at 8-15% (see preliminary studies). This is in contrast to the prevalence in foster-care settings in the state of Washington in the U.S. where the prevalence of FAS has been reported as 1-1.5%.[13]

In addition to the relatively high prevalence of drinking during pregnancy, the feasibility of conducting a prospective study in the Moscow region is supported by the highly centralized infrastructure for the provision of prenatal and postnatal medical care and the fact that the coordination of these services is under the direction of Deputy Minister Tamazian. In addition, Moscow Region collaborators include Dr. Lela Kavtaladze who is the Chief of the Department of International Scientific Cooperation for the Moscow Region and an obstretician from the Institute of Obstetrics and Gynecology, and Dr. Ludmila Joutchenko who heads the Genetics Institute for the Moscow Region as well as the Moscow Region Birth Defects Registry, both of whom have committed full support to implementing the proposed project. This study will also build upon relationships with other key personnel in Russia that have been established in the context of other research efforts over the last four years and will utilize consultants (Drs. Wilsnack and Kristjanson) who have developed successful techniques for eliciting alcohol exposure information from pregnant Russian women and can assist in the appropriate training of interviewers for this study. Furthermore, this study will draw upon the expertise of pediatricians, psychologists and other staff who have previously been trained by investigators who are now consortium core members to perform some of the diagnostic procedures that will be included in this study.

3. Risk factors for FASD

Although the developmental pathogenesis of FAS is unknown, a number of maternal risk factors have been identified which seem to confer susceptibility including African-American or Native-American ethnicity,[14] lower socioeconomic group,[15-16] and alcohol-metabolizing enzyme genotype.[17-19] Poor nutrition including specific micronutrient deficiencies may also be important but has not yet been adequately examined in humans. In addition, a pattern of binge drinking has been suggested as conferring higher risk than similar quantities of alcohol consumed in a more evenly distributed fashion.[20]

a. Nutritional deficiencies. While pregnancy complications can be attributed to numerous factors, recent reports have underscored the fact that a major contributor can be suboptimal nutrition. As examples, in developing countries, Vitamin A supplementation has been reported to result in large (>40%) reductions in maternal mortality;[21] and reports of reductions in the incidence of preeclampsia with magnesium supplementation,[22] as well as with vitamin C and vitamin E supplementation[23] suggest that micronutrient deficiencies contribute to the high frequency of maternal mortality in developing countries, and potentially in developed countries. Given that impaired immune system function is a common result of malnutrition,[24]

coupled with the observation that perinatal infection is a significant contributor to maternal, fetal, and infant mortality [25,26] the observation that two positive outcomes of micronutrient supplementation in undernourished populations, i.e., reductions in the frequency of maternal and infant mortality, is not surprising given that, in principle, some repair of the immune defense system will occur with supplementation, assuming the rate limiting nutrient(s) are provided. Similarly, given the roles that multiple micronutrients have in energy metabolism and anabolic processes, reductions in the frequency and severity of intrauterine growth restriction would be predicted to occur in the above populations.[27]

Less predictable is the effect micronutrient supplementation might have on the frequency and spectrum of congenital defects. However, with the above said, it has been long recognized that maternal diet can influence pregnancy outcome even when caloric intake is adequate. Starting with the classic study of Ebbs in 1941, numerous investigators have reported that women who consume "poor" diets are characterized by a high frequency of pregnancy complications.[27, 28] Importantly, in several prospective studies, the incidence of pregnancy complications, including the number of infants with malformations, have been reported to be reduced with maternal diet supplementation. [29-34] The above findings are important as they suggest that an association between "poor" maternal diets and poor pregnancy outcome are largely due to nutrition per se, rather than other lifestyle factors. It is important to note that this does not imply that lifestyle factors such as smoking and excessive alcohol consumption do not represent significant risk factors to the conceptus, but rather that the reproductive toxic effects of these insults is in part mediated by their influence on the nutritional status of the maternal-fetal dyad. Complementary to the above is the hypothesis that a compromised nutritional status of an individual increases the risk of adverse reactions to potential reproductive hazards.[35] Illustrative of the above, the teratogenicity of several compounds is increased in animals fed marginal protein diets,[36, 37] and the teratogenicity of acetazolamide and methanol are exacerbated by concomitant marginal zinc and folate status.[38, 39]

The possible contribution of nutritional factors to risk for FASD in humans is not yet known. However, the overall nutritional status of alcoholics is generally thought to be inadequate.[40] This may be due to poor dietary composition, limited food intake, and possibly reduced absorption and utilization of many nutrients. Given the association of FASD with lower socioeconomic status, African-American and Native-American ethnicity, and older maternal age which may represent long-term alcohol abuse, the role that malnutrition may play in susceptibility to this disorder is of critical importance to define.

Of particular interest in the pregnant alcoholic is status for those micronutrients which are critical for normal neurulation and development of the central nervous system.[41,42] For example, vitamin A (retinol), both in excess and deficiency, has been demonstrated to have an influence on central nervous system development in humans. The possible relationship between retinol metabolism and alcohol intake has been suggested by Duester.[43] Retinol dehydrogenase is an enzyme needed to convert retinol to retinoic acid, a metabolite that is crucial in the normal embryonic development of the central nervous system and limbs. Alcohol acts as a competitive inhibitor of the retinol dehydrogenase activity attributed to mammalian alcohol dehydrogenase (ADH), an enzyme that uses both retinol and ethanol as substrates. Therefore, if high levels of alcohol act as a competitive inhibitor of ADH-catalyzed retinol oxidation in the embryo or fetus, the result might be a reduction of retinoic acid synthesis in the embryonic tissues in which adequate levels are essential for normal development. Furthermore, if available circulating concentrations of retinol are decreased in poorly nourished alcoholic women, presumably the potential for insufficient availability of retinoic acid during embryogenesis could be magnified.

Maternal thiamin deficiency during pregnancy may occur due to increased requirements, inadequate dietary intake, hyperemesis gravidarum, or malabsorption. In addition, chronic alcoholism results in a high incidence of thiamin deficiency even when dietary sources are adequate. Animal and human data have associated thiamin deficiency in pregnancy with intrauterine growth restriction in the offspring. Impaired fetal growth is a feature of FASD;[44] therefore, maternal thiamin status in alcohol-drinking pregnant women may be relevant to pregnancy outcome with respect to FASD.

Similar to vitamin A and thiamin, folate, zinc and copper deficiencies in pregnancy can result in abnormal central nervous system development, and deficiencies of these nutrients are common in alcoholics.[40,45-47] While deficits of any of the above nutrients can result in serious malformations, it is important to note that there can be interactions between these nutrients. For example, Bremert reported that the frequency of dysmorphogenesis in litters from rats given diets low in zinc and folate were markedly increased when compared to dams who were given diets low in only one of the two nutrients. Significantly, the frequency of dysmorphogenesis in the zinc and folate deficient diet group was higher than an additive effects model would predict.[48] Similar to folate and zinc, the teratogenicity of vitamin A deficiency is increased when marginal zinc diets are fed.[49,50] Given that there is a high occurrence of deficiencies of all of the above nutrients in alcoholics, it is reasonable to suggest that a combination of these multiple deficiencies in pregnant alcoholic women may be related to risk for FASD. Significantly, the teratogenicity of alcohol is thought to be due, in part, to oxidative damage. Thus, deficits of zinc, copper, vitamin C and vitamin E would all be predicted to increase the sensitivity of the developing conceptus to alcohol, given that these nutrients all contribute to the oxidative defense system. [51]

In the evaluation of the hypothesis that suboptimal fetal nutrition contributes to the teratogenicity of alcohol, it is important to recognize the multiple ways a nutritional deficiency can arise. First, a deficiency can occur as a consequence of an inadequate dietary intake of an essential nutrient. An insufficient amount of any essential nutrient in the diet will eventually result in a primary deficiency of the nutrient, with concomitant adverse effects on reproductive capacity.

A secondary, or conditioned deficiency may occur even if the dietary content of the essential nutrient seems to be adequate.[51] Conditioned deficiencies can arise through several mechanisms. First, genetic factors can create a higher than normal requirement for a nutrient. Examples of the above would be acrodermatitis enteropathica and Menkes disease which are genetic disorders in zinc and copper metabolism respectively,[47,52,53] and gene polymorphisms associated with folate metabolism that increase the risk for neural tube defects.[54] Second, nutritional interactions can result in conditioned deficiencies. For example, dietary-binding factors, such as fiber and phytate, can form a complex in the gut with essential minerals that limit their absorption. Illustrative of this interaction is the occurrence of zinc deficiency syndromes in individuals who consume diets rich in phytates.[55] Interactions between two or more essential nutrients can also result in deficiencies. For example, iron supplements can inhibit the absorption of zinc, while zinc supplements can inhibit the uptake of copper. [51] A third mechanism by which a conditioned deficiency can arise is through an effect of drugs or chemicals. Broadly speaking, drug-nutrient interactions can be separated into two categories; one composed of drugs that act via the chelation of a nutrient, and the second composed of drugs that indirectly affect the metabolism of a nutrient. Representative of the first class is the chelation of copper by D-penicillamine, [47] while representative of the second class is the influence of diuretics on magnesium excretion, and the increased turnover of folate and ascorbic acid which occurs via oxidative damage secondary to smoking and alcohol consumption,

A fourth mechanism by which a conditioned deficiency can arise is through physiological stressor-induced changes in micronutrient metabolism. For example, diabetes, hypertension and AIDS can alter the metabolism of several minerals including zinc, copper , iron and selenium.[47,55-57] To a significant extent, stressor-induced changes in nutrient metabolism are secondary to an acute phase response (APR) which can be triggered by a diverse set of cytokines that are released following tissue injury. It has been hypothesized that the developmental toxicity of a wide variety of drugs and environmental insults is mediated in part through the induction of the APR. According to this hypothesis, one consequence of the APR is an increased synthesis of the zinc-binding protein metallothionein (mt) in maternal liver. The excessive production of this protein results in a sequestration of zinc in maternal liver, with a subsequent reduction in maternal plasma zinc concentrations, and reductions in zinc transfer to the conceptus which if severe enough can result in abnormal development. [28,58,59] In rat models, the teratogenicity of several developmental toxicants including a-hederin and TNF-a can be influenced by maternal dietary zinc intakes. Relative to dams fed control zinc diets, dams fed marginal zinc diets show an amplified response to these teratogens, while dams fed high zinc diets show a reduced response.[58,60] Important to the current proposal, in both mouse and rat models, acute alcohol exposure is associated with APR-induced reductions in fetal zinc uptake [61-63] Significantly the teratogenicity of

acute alcohol exposure has been shown to be reduced in mt knockout mice (alcohol does not induce hypozincemia in these animals), compared to mt wild-type mice.[59] Similarly, the teratogenicity of acute alcohol exposure has been reported to be lessened when animals are given an injection of zinc prior to the alcohol exposure.[63] Over 20 years ago Flynn and co-workers[64] presented data that suggested an etiologic role for zinc deficiency in human FAS. One of the outcomes of the current proposal will be an evaluation of the hypothesis that a nutrient supplement containing zinc will reduce the frequency and severity of FASD in a high risk population. In addition to the mounting evidence that certain micronutrients affect normal and abnormal structural development, it is clear that prenatal malnutrition is an important factor both in prenatal growth and in postnatal cognitive performance.[65,66]

In the Moscow Region, these factors may be of particular importance due to poor economic conditions limiting access to food and supplemental vitamins. Food fortification with folic acid is not yet in place in the Moscow Region, and although women are advised to take prenatal vitamins once a pregnancy is recognized, these supplements are not provided free of charge to all pregnant women. Although the exact frequency of use is not certain, the chief obstetrician for the Moscow Region has estimated that a very small proportion of women follow advice to purchase these supplements, and given the nutritional challenges in this population, even complete compliance may be insufficient to insure appropriate levels of essential micronutrients during pregnancy, and particularly during the pregnancy of a woman who drinks moderate to heavy amounts of alcohol.

b. Genetic susceptibility. Only recently have studies been conducted in humans suggesting that polymorphisms in alcohol-metabolizing enzyme genotype represent risk factors for FASD. Chernoff initially raised this possibility in studies comparing the incidence of fetal anomalies in three different inbred strains of mice.[67] CBA, C3H and C57BL females maintained on a similar diet of 20 percent ethanol-derived calories prior to and throughout gestation were mated in a diallele cross. Maternal blood alcohol levels varied with maternal strain (CBA > C3H > C57BL) and were inversely related to maternal alcohol dehydrogenase activity. Fetal death, malformation and fetal weights were correlated with maternal blood alcohol levels; fetal blood alcohol levels were similar to maternal values, regardless of fetal genotype. These results indicate that liability for the pattern of malformation was dependent on maternal blood alcohol levels, which were determined by alcohol consumption and the genetically influenced rate of maternal alcohol metabolism. Studies in twins have suggested that genetic factors confer susceptibility to FAS in humans as well.[68] Among 16 twin pairs prenatally exposed to high levels of alcohol, both members of 5 monozygotic twin pairs were affected. However in 11 dizygotic twin pairs there was discordance for FAS in 4 of the 11 suggesting that the fetal genotype plays a role in development of FAS. It should be emphasized that differing environments within the same uterus cannot be ruled out as the cause for this discordance.

Most recently McCarver et al[18] studied ADH2 genotypes in maternal-child pairs to determine if particular polymorphisms conferred susceptibility for FAS in the offspring of alcohol drinking mothers. Two hundred and forty-three maternal–infant pairs were chosen based on maternal alcohol intake during pregnancy and all mothers were genotyped for ADH2. Infant outcome was based on performance on a Bayley Scale of Infant Development Mental Index (MDI) at one year of age. Drinking during pregnancy was associated with a lower MDI but only in the offspring of women without the ADH2*3 allele. Thus, the maternal ADH2*3 allele in drinking mothers afforded protection against alcohol-related impairment in test performance in their offspring. McCarver has recently suggested that this protective effect may be secondary to ADH2*3 allele's increased efficiency for ethanol metabolism in these women.[69] The protective effect of the maternal ADH2*3 allele for alcohol-related performance in the offspring of African-Americans was recently confirmed by Jacobson et al.[70]

The ADH2*2 allele, also associated with increased efficiency for alcohol metabolism, has recently been shown by Viljoen to be protective for the diagnosis of FAS in a South African sample.[17] In contrast, Stoler et al[19] showed that the ADH2*2 allele was a risk factor for alcohol-related physical features in a sample of moderate to heavy drinking women in the U.S. As women in this sample who carried the ADH2*2 allele tended to report higher rather than lower amounts of alcohol consumption, these data suggest that drinking pattern in combination with genotype is the critical factor.

In the Moscow area, it is of particular interest that the prevalence of the ADH2*2 allele seems to be higher than in other white populations sampled, with an allele frequency of 41%.[71] Given the estimated high prevalence of risky drinking in this population, DNA banked from subjects in this sample for future analysis of alcohol-metabolizing enzyme genotype could help clarify the seemingly contradictory results reported by Viljoen and Stoler. Further defining the relationships among alcohol quantity and pattern of consumption, maternal and fetal genotype, and features of FASD in this population will enhance our understanding of the role of genetic susceptibility in risk for this disorder.

c. Amount and pattern of alcohol consumption

Binge drinking as a pattern of alcohol consumption may pose the highest risk.[20] Therefore, data collection techniques are critical in eliciting the best measures to represent variations in patterns of alcohol consumption. Because maternal report may be an unreliable measure of true alcohol use, a number of different approaches to enhancing the accuracy of recall and validating maternal history have been tested. Interviewing technique, framing the time period in questioning, and the use of drink models to better estimate drink size are some of these.[72-74] In addition, the use of a consistent core set of questions modified only for cultural sensitivity will allow for comparability of measures to others derived both within and outside the consortium.

Attempts at validation of self-report have been made through the exploration of a variety of biomarkers of exposure. These include breath or blood alcohol, carbohydrate deficient transferrin (CDT), mean cell volume (MCV), gamma glutamyl transferase (GGT), and others.[75] Although none of these biomarkers has proven to be sufficiently sensitive and specific to serve as an adequate screening tool for drinking during pregnancy in the general population,[76] at least one study has shown that the combination of 2 or more positive biomarkers is significantly associated with abnormal physical findings in alcohol-exposed infants.[77] Furthermore, the combination of MCV and GGT has been noted by Sarkola et al to be the most efficient laboratory markers for excessive alcohol consumption in pregnant women as well as the adverse effects of alcohol on the fetus.[78] In a research setting these markers taken individually and in combination can provide some corroboration of maternal drinking history and add to our understanding of the utility of these markers in validating maternal exposure derived by self-report.

d. Early diagnosis

The exact timing in gestation that growth deficiency is initiated has not been demonstrated in alcohol-exposed pregnancies. The use of serial prenatal ultrasound at specified gestational ages can help elucidate the sequence of events in the context of varying patterns of drinking while at the same time abnormal findings can be explored as a possible early predictor of infants who should be carefully followed after birth. Wass et al have demonstrated using prenatal ultrasound that reduced size of the frontal lobe can be demonstrated on prenatal ultrasound in fetuses of women who drink heavily in pregnancy.[79] In unpublished data from South Africa, somatic growth restriction has not been demonstrated in prenatally exposed fetuses scanned at up to 28 weeks gestation; however, only a limited number of late third trimester scans were able to be performed, a period when the most dramatic effects on growth might be expected. [80]

Because pediatricians are frequently unaware that a newborn has been prenatally exposed to alcohol and because the physical features are difficult to recognize in the newborn period, the typical age at which full blown FAS is diagnosed is at 4 years of age or older.[6] Even at this age, developmental delay and not structural abnormalities is the primary reason that children are referred for evaluation. Thus, most children with FASD do not benefit from early intervention and treatment that might have been provided had the diagnosis been made at an early age. Furthermore, early recognition of this disorder can lead to counseling and intervention with women at risk of having a subsequent affected child. [81]

There is some evidence that early diagnosis of structural features is feasible given an adequate level of training and expertise on the part of the examiner. For example, Hanson et al identified 11/163 infants with some features compatible with FAS when performing a blinded examination of the newborn children of moderately drinking mothers.[82] Similarly, Chambers et al [83] found that in infants examined under one year of

age, a dose response curve could be demonstrated associating number of drinks per day with a small number of very specific FASD-related structural features (see preliminary studies). Another avenue recently explored with some success is the use of photographic analysis to improve diagnosis.[84] The application of these analytic methods to images of very young children has not yet been systematically explored as a tool aiding in earlier diagnosis, but is an objective of the proposed study.

For the same reasons that early diagnosis of structural abnormalities is desirable, early recognition of neurobehavioral abnormalities in children with prenatal alcohol exposure, particularly those who lack the physical features, can benefit the child by leading to earlier intervention and treatment. Furthermore, the development of a behavioral phenotype in very young children that is specific for prenatal alcohol exposure and how this relates to subsequent neurobehavioral development, particularly in a prospective sample that is followed longitudinally, can define one end of the spectrum of FASD. This can enhance earlier diagnosis and contribute to the design of more effective interventions and treatments. Finally, early neurobehavioral evaluations are imperative to properly distinguish between the extent to which prenatal alcohol exposure versus postnatal environmental effects contribute to the neurobehavioral abnormalities in FASD.

C. Preliminary Studies

Clinical Recognition of FAS: As with the majority of known human teratogens, prenatal exposure to alcohol at the most severe end of the spectrum of FASD is associated with a subtle pattern of minor malformations referred to as the fetal alcohol syndrome (FAS) as opposed to a single major structural defect.[85] The principal features that permit recognition of that disorder were first delineated by one of us and include prenatal onset growth deficiency, developmental delay and a characteristic craniofacies that includes short palpebral fissures, a hypoplastic midface, and a smooth philtrum[1,86] The lack of obvious major malformations, the need for expertise in documenting the length of the palpebral fissures and the subjectivity of features such as maxillary hypoplasia and a smooth philtrum lead to significant difficulties in diagnosis by all but the most well-trained pediatricians.[6] However, as noted below, following a relatively short training period, it has been demonstrated that pediatricians can reasonably differentiate between school-age children with FAS and those without. With respect to the potential for using similarly-trained pediatricians to make an earlier diagnosis, there is evidence that this is possible. We have reported on 234 prospectively ascertained children with varying levels of prenatal alcohol exposure, 90% of whom were examined at less than one year of age by a dysmorphologist who was blinded to alcohol exposure history. Using short palpebral fissures, smooth philtrum, head circumference <10th centile and small for gestational age (<10th centile) on height or weight as the specific features representative of FASD, 28.6% of the children in the heaviest exposure category had at least one of the target features, while 14.3% had at least three and were diagnosed with FAS.[83]

FASD-Related Structural Defects of Brain Development: Of greatest significance relative to prenatal alcohol exposure is the effect of alcohol on brain development. Extensive brain anomalies including microcephaly, migration anomalies and callosal dysgenesis were documented on neuropathologic evaluation of one of the initial children evaluated with this disorder[87] and subsequent neuropathologic studies have extended and confirmed these observations. Recently we have begun studies correlating structural anomalies of brain development documented on magnetic resonance imaging (MRI) with the neurobehavioral deficits seen in this disorder.[88,89] Selective defects of the corpus callosum have been documented including agenesis in two of thirteen alcohol-exposed children accessed and in the remaining children significantly smaller overall callosal areas, as well as smaller regional areas of four of the five callosal regions when compared with control children. With respect to the basal ganglia, when it was divided into the caudate and lenticular nuclei, both of these regions were significantly reduced in children with FAS. In addition, when the overall reduction of brain size was controlled, the proportional volume of the basal ganglia, and more specifically, the caudate nucleus, was reduced in children with FAS. With respect to the cerebellar vermis, brain MRI's of 9 children and young adults with prenatal alcohol exposure were performed. The anterior region of the vermis was significantly smaller in subjects with prenatal alcohol exposure, whereas the posterior region and the remaining vermal area did not differ from controls. Our findings suggest that regionally specific Purkinje cell death may occur in

humans with prenatal exposure to alcohol as has previously been documented in an animal model of neonatal alcohol exposure.[90]

Additionally, we have documented neurobehavioral deficits in individuals with FASD, including those with full-blown FAS as well as those with prenatal exposure to alcohol who do not have the characteristic clinical features of FAS.[91,92]

FAS-Related Craniofacial Anomalies: Their Relationship to Defects In Brain Development: In addition to the neurobehavioral deficits resulting from defects in brain development, a number of the unique craniofacial features characteristic of FAS are secondary to these anomalies.

Short palpebral fissures: Based on ultrasonographic studies of the eyes of 104 normal individuals as well as eighteen children with full-blown FAS, we concluded that the palpebral fissure length is dependent on ocular size early in life, but that as we age other factors become more influential. These data indicated that the palpebral fissures in full-blown FAS are short due to shorter ocular diameters. In that the optic vesicle is an out pouching of the frontal region of the brain, we concluded that the short palpebral fissure in full blown FAS is reflective of abnormal brain development.[93]

Long smooth philtrum which lacks lateral philtral ridges (LPRs): Based on the gross and histologic evaluation of 20 therapeutically aborted human fetuses between 9 weeks and 20 weeks gestation, as well as fetuses with holoprosencephaly, bilateral cleft lip and full-blown FAS, 3 disorders in which LPRs are not present, indicate that the LPRs develop as a result of a dynamic relationship between lip musculature which derives from cells which migrate medially from the maxillary processes and the frenulum of the upper lip, a midline structure which derives from the medial nasal processes and as such is dependent on central nervous system development. Thus we suggest that the long smooth philtrum seen in children with full blown FAS is indicative of an underlying defect in brain development.[94]

Epidemiological Studies in South Africa and Russia: Although early studies by our group documented a 44% risk for full-blown FAS in the offspring of chronic alcoholic women,[3] a number of factors are now emerging that clearly influence the magnitude of that risk. Maternal age >30 or alternatively number of drinking years, low socioeconomic status, African-American and American-Indian ethnicity, nutritional deficiencies, and genetic factors are all being considered. We have recently completed an epidemiological study of FAS in a South African community in the Western Cape Province sponsored by the National Institute on Alcohol Abuse and Alcoholism (NIAAA). In that community, many of these risk factors are present. In this study, the highest full-blown FAS rate in an overall community population in the world was documented.[95]

A 2-tiered screening system was used. Four 2-physician teams (1 expert dysmorphologist and 1 South African physician training in diagnosis of FAS) worked independently but simultaneously. Four hundred and six of the 992 first grade children in that community were examined by two different teams using standardized assessment. This included a complete dysmorphology examination to determine growth parameters and if the child had FAS. Thereafter, the remaining 586 first graders were assessed with respect to length, weight, and head circumference. Children whose measurements were below the 10th percentile on head circumference or on both height and weight were referred for the complete examination (tier 2) by the dysmorphology teams. Two hundred and twenty-two of the remaining 586 children met these criteria and were referred for complete examinations. Therefore, 626 children received full dysmorphology examinations. Based on the dysmorphology examinations, a child was assigned a preliminary diagnosis of FAS, deferred, or not FAS. In the deferred category were children who had some features of FAS with growth deficiency but for whom further information was required for a final diagnosis. For children in that category, subsequent documentation of maternal alcohol exposure and results of neuropsychological evaluation were used to determine if the diagnosis was changed to FAS or not.

It is important to recognize that a significant educational component was built into this study. During the evaluation of the initial 406 children, a total of 12 South African physicians worked one-on-one for a 2-day period with each of the expert dysmorphologists. During that time, each child was examined by both the South

African physician and the dysmorphologist; palpebral fissure length, innercanthal distance, ear length and philtral lengths were measured and compared and the South African physician's final diagnosis was reviewed for accuracy. This exercise resulted in minimal inter-rater variability and demonstrated the fact that physicians with little or no expertise in diagnosing FAS can, through intensive training over a 2 days period, be educated to do so.

We are involved in a similar study also funded by NIAAA, in Moscow, Russia at the present time. Four pediatricians in Moscow were trained using the same techniques as were employed in the South African study. As of July, 2002, these pediatricians have evaluated 2,922 children between the ages of 5 and 18 at 30 boarding schools or orphanages in Moscow. During three one-week visits to Moscow, the 384 children who the Russian pediatricians had diagnosed as having FAS or who they deferred for further evaluation were separately examined by the two dysmorphologists, each of whom were blinded to the diagnosis of the Russian pediatrician as well as the other dysmorphologist. One hundred and forty-three of the 155 children (92%) diagnosed as FAS by the Russian pediatricians were confirmed to have FAS by both the dysmorphologists. This supports the viability of this method for training pediatricians in a relatively short period of time to diagnose with good specificity the extreme end of the spectrum of FASD.

California Teratogen Information Service and Clinical Research Center. In 1979, Kenneth Jones started the California Teratogen Information Service and Clinical Research Program as part of the Department of Pediatrics at the UCSD School of Medicine. This program has two goals 1) a service goal: to provide information to pregnant women regarding the effect of drugs, chemicals and environmental agents on fetal development and 2) a research goal: to gain new information about the prenatal effect of various drugs for which little or no data are available. With respect to our service goal, we receive telephone contacts from approximately 1000 pregnant women and/or physicians each month from throughout the State of California regarding the prenatal effects of agents about which they are concerned. Regarding our research goal, when a pregnant woman contacts us regarding the prenatal effects of an agent about which little or no information is available, we ask that woman if she would be willing to enroll in our follow-up program. If she agrees, a designated follow-up counselor contacts that woman at prearranged times during the remainder of her pregnancy to gain information about other exposures, illnesses, family history and demographics. Following the birth of the prenatally exposed child, a dysmorphologic examination performed by a single observer blinded to the exposure is scheduled and completed. These methods have been used to study pregnancy outcome in women with exposure to a variety of different agents, including heavy alcohol and illicit drugs. For substance-abusing women, who are often difficult to ascertain and retain in follow-up, special interviewing and follow-up techniques have been developed to elicit information and encourage completion of the study requirements. Data from approximately 100 mother-child pairs exposed to the same agent and evaluated in this manner provides sufficient power to determine whether a drug represents a serious human teratogen. Using this methodology, we have gained new information regarding the prenatal effects of a variety of agents.[96-99]

Since 1995, we have also been conducting an assessment of the neurobehavioral teratogenicity of a variety of exposures as a collaborative study between the California Teratogen Information Service and Clinical Research Program (UCSD) and the Center for Behavioral Teratology (San Diego State University). Over 600 children throughout the State of California have been evaluated using a battery of neurodevelopmental measures. In June, 1999, we added infant development to our assessment protocol, and over 100 children from the San Diego area have been evaluated using the Bayley Scales of Infant Development - second edition (BSID-II). Using this protocol, we have completed an examination of the neurobehavioral effects of maternal varicella infection,[100] the interaction of maternal tobacco use and a variety of agents,[101] and are near completion of studies of maternal exposure to fluoxetine.[102]

At present we are the coordinating center for two large four-year, multi-center prospective cohort studies with participants ascertained through over 20 Teratology Information Services in the U.S. and Canada (www.otispregnancy.org). The first is a study of a number of medications used to treat asthma and involves 1100 pregnant women. Outcome measures include data from an outcome interview, and obstetrical, birth, pediatric, physician, and other hospital records. The second is a study of Arava (leflunomide), a drug used to

treat rheumatoid arthritis, and involves 300 women located throughout North America. In this study, dysmorphologic examinations are being performed on each of the prenatally exposed infants by one of four trained dysmorphologists.

Thus, we have gained extensive experience using a methodology similar to that outlined in this grant proposal, both through our efforts to identify new structural and behavioral human teratogens through the California Teratogen Information Service and as the coordinating center for two large multi-center prospective cohort studies.

D. Research Design and Methods

A multi-center, prospective cohort study design incorporating a randomized clinical trial will be used to ascertain and follow up pregnancies of women in the Moscow Region with and without moderate to heavy alcohol exposure during pregnancy. The patient population from which the cohort will be assembled will be accrued from approximately 4-6 hospitals in the Region selected for their representation of the socioeconomic variability in the Region and for yielding an annual projected pool of 13,000 pregnant women eligible for screening.

1. Subject selection, maternal interviews and maternal and newborn medical record review

All women presenting for a first prenatal visit at one of the clinics feeding into the participating hospitals will be administered a short screening instrument by designated staff assessing alcohol consuming behaviors in the early weeks of pregnancy. Based on answers to the screening questions, prenatal care staff will provide information regarding the risks of alcohol and advice regarding continued drinking, and referrals will be offered to women who are abusing alcohol. Potential subjects will be asked to speak to a specially trained study interviewer about the study. Those who agree will be referred to the interviewer, qualified for study participation, and administered informed consent sometime prior to the next prenatal visit. The interviewer will follow each subject with an extensive intake interview, a third trimester interview and a postpartum interview using the interview questions and techniques previously established and validated by Jacobson et al and meeting the criteria set by the dysmorphology core.[38,70] All interviews will be conducted preferably in person, and by telephone only if necessary, using a structured interview technique. The interviews will include questions on the following: maternal age, pregnancy history particularly with respect to a previous child with birth or learning problems; current health history; pre-pregnancy weight and height; socioeconomic and demographic information including maternal and paternal occupation, education, income category and ethnicity; current medication use; other environmental or occupational exposures; alcohol, tobacco, caffeine and illicit drug use; vitamin/mineral supplements; the use of vitamin or mineral fortified food products such as breakfast cereals; herbal products; current pregnancy complications including illnesses and results of prenatal tests. Three-day dietary recall questionnaires will also be administered at the time of each prenatal interview.

The interviewer will abstract data from prenatal ultrasound reports generated from scans that are routinely performed in the first, second and third trimesters of pregnancy. These standard measurements will include the occipital frontal diameter, biparietal diameter, measurements of the humerus, tibia, abdominal circumference, and amniotic fluid index, as well as other data as specified by Dr. Hull, the project consultant for ultrasonography. Prior to the beginning of subject enrollment, Dr. Hull will observe ultrasonographic procedures and techniques at each of the participating hospitals and set the protocol for data collection from routine ultrasounds. In addition, a sample of routine scans at each trimester of pregnancy from each of the participating hospitals will be archived and reviewed by Dr. Hull in order to validate the measurements as recorded in the medical record. Specific problems will be addressed early on in the data collection period.

Additional data will be abstracted from medical records for each subject by the interviewer. The data collected will include birth size, birth complications, results of any routine or specialized prenatal tests, and maternal weight gain. Interviews and medical record review will be completed for all enrolled pregnancies regardless of pregnancy outcome, i.e., spontaneous abortion, stillbirth or neonatal death.

2. Nutritional intervention trial

Prior to the study initiation, participating hospitals will be matched based on similar numbers of annual deliveries and similar social/community settings so that the hospitals receiving the intervention will be expected to result in approximately 640 study eligible deliveries and the hospitals not receiving the intervention will be projected to result in a similar number of study eligible deliveries. Women in the treatment group setting will be given free of charge by their obstetrician a vitamin/mineral supplement provided by the study team, while women in the control group setting will be given routine advice to take prenatal vitamins. The content of the intervention pill will include all ingredients in a typical prenatal vitamin available in Russia (Provit – Roche Laboratories) except for higher amounts of the following micronutrients none of which are considered harmful in pregnancy and all of which have demonstrated benefit in previous studies:

- 25 mg zinc[103]
- 365 mg elemental magnesium as magnesium aspartate hydrochloride[22]
- 1000 mg vitamin C[23]
- 400 IU vitamin E[23]

Because it would be unethical to assign the control condition to "no prenatal vitamins " and because in both the treatment and control conditions there is no sure way to confirm compliance, the use of blood markers of micronutrient levels both early and late in pregnancy will serve as a measure of both compliance and the effect of the intervention supplementation.

3. Biological sample collection and distribution

Maternal samples:

At intake and again at the third trimester interview, the following samples will be collected and distributed

1. 25 ml of blood drawn by venipuncture. These samples will be divided into aliquots and a portion used for the measurement of MCV and GGT in the hospital laboratory. The remainder will be transported, labeled with subject identification number, and distributed by the study interviewers for the nutritional analyses. A portion of the first blood draw will be transported to the laboratory of Dr. Pavel Ogurtsov for DNA extraction and banking.
2. A sample of maternal saliva will be collected and applied to a commercially available test strip to estimate maternal blood alcohol concentration.

Newborn samples: collected prior to hospital discharge:

- 2 ml of blood from heel stick will be collected prior to hospital discharge and transported to the laboratory of Dr. Pavel Ogurtsov for DNA extraction and banking.

4. Nutritional analyses

Collected blood samples will be processed as described below. All samples will be split. One aliquot of each sample will be analyzed as described, and a second aliquot will be frozen back in the event that additional, or repeat, analyses are required. It is envisioned that the majority of the assays will be conducted at the laboratory site in Russia. At the beginning of the grant the investigators will meet in Russia to go over the analytical techniques that will be used. Blood samples will be collected from 20 non-pregnant healthy adult women at the time of the initial visit. Each plasma and erythrocyte sample will be split. Samples will be analyzed and the results from the two laboratories (Russian laboratory site and Dr Keen's laboratory at the University of California, Davis) will be compared. Causes for any differences in analytical values between the two laboratories will be identified and corrected. Throughout the actual study, a subset of the collected samples will be transferred to the University of California, Davis and analyzed. Results from the two laboratories will be compared on an ongoing basis. If there are significant analytical differences between the two sites, actions will be taken to immediately correct the cause of the differences.

The blood samples will be evaluated using the following methods:

Plasma Extracellular SOD. The analysis of plasma extracellular SOD will provide us with an indication of the individual's zinc and copper status, as well as an indication of their level of oxidative stress. Activity will be determined using methods developed in our laboratory modified from the method described by Prohaska,[104] in which the sample's ability to inhibit the autoxidation of pyrogallol will be assessed. Samples will be incubated in buffer (50 mM TAPS/0.1 mM DTPA, pH 8.2) with 2 mM pyrogallol for 2 minutes. Five different volumes of sample will be used along with a blank (no sample; no inhibition of pyrogallol oxidation). A regression analysis will be used to determine the amount of sample needed to obtain 50% inhibition of pyrogallol oxidation. One unit of SOD activity will be defined as the amount of sample needed to obtain 50% inhibition of pyrogallol oxidation, expressed as units/ml plasma.

Plasma Ceruloplasmin. The analysis of plasma ceruloplasmin (CP) activity will provide us with an indication of the individual's copper status, as low CP activity is indicative of copper deficiency. A finding of high CP activity would be suggestive of an acute phase response. C-reactive protein concentrations will be used to assist in the identification of individuals with active inflammatory conditions. CP Activity will be measured as described by Schosinsky et al,[105] which follows the oxidation of o-dianisidine dihydrochloride. o-Dianisidine dihydrochloride will be converted into a yellowish-brown reaction product in the presence of ceruloplasmin and oxygen in acetate buffer (ionic strength 0.2) at pH 5. Acidification with 9 M sulfuric acid stops the reaction and a stable purplish red solution will be formed that absorbs maximally at 540 nm. Results will be expressed as units of activity, defined as the difference in absorption at 540 nm at 5 and 15 minutes.

GSH-Px and GSH-Red. The assessment of GSH-Px and GSH-Red activities will give additional information on an individual's level of oxidative stress. As low GSH-Px activities can be reflective of poor selenium status, blood selenium concentrations for the subjects will be determined if a high percentage of the population (>10%) are found to have low GSH-Px activity. Activities in plasma and erythrocytes will be determined by the method of Lawrence and Burk,[106] which measures the GSSG formed in the GSH-Px reaction by coupling it to the oxidation of NADPH. Selenium-dependent GSH-Px will be specifically measured by utilizing 5 mM hydrogen peroxide in the assay system; total GSH-Px will be measured using 1.5 mM cumene hydroperoxide. The activity of GSH-Red in tissues and erythrocytes will be measured via the formation of GSH from GSSG as catalyzed by the amount of GSH-Red present in the sample, by coupling it to the oxidation of NADPH.[107] Activities of tissue GSH-Px and -Red will be expressed as mol NADPH oxidized per minute per mg protein. Activities of erythrocyte GSH-PX and -Red will be expressed as mol NADPH oxidized per minute per mg hemoglobin.

Total Antioxidant Capacity (TAC). While the interpretation of plasma antioxidant measurements can be fraught with difficulties, they can represent a useful assessment of an individual's overall oxidant defense system. Given that alcohol-induced oxidative damage is thought to contribute to the occurrence of FASD, the measurement of plasma TAC can be of value. Plasma samples will be assayed for their ability to inhibit the chemiluminescence produced by a mixture of 2,2'-azp-bis(amidinopropane) in phosphate-buffered saline, pH 7.4, and luminol. The chemiluminescence will be measured in a bioassay reader. The antioxidant capacity value will be calculated as the lag time before an increase in the chemiluminescence will be observed.[108] This lag time will be proportional to the cumulative amount of antioxidants present in the sample. A reference lag time will be obtained by using a known amount of 6-hydroxy-2,5,7,8-tetramethoxychroman-2-carboxylic acid (Trolox), and plasma total antioxidant capacity will be expressed as Trolox Equivalents.

Protein Carbonyls. These markers for protein damage will be determined by a modification of the procedure described by Levine et al,[109] using dinitrophenylhydrazine (DNPH) dissolved in HCL, accompanied by a blank (HCL). After the DNPH reaction, proteins will be precipitated with an equal volume of 20% (w/v) trichloroacetic acid. Pellets will be washed once with 10% (w/v) trichloroacetic acid and three times with ethanol/ethyl acetate (1:1). Washings will be achieved by mechanical disruption of the pellets in the washing solution using a small spatula, and re-pelleting by centrifugation at 6,000 g for five minutes. Finally, precipitates will be dissolved in 6 M guanidine-HCL solution and the absorbance peak at 320-400 nm will be determined on the HCl blank pellets using a BSA standard curve in guanidine-HCl.

Plasma TBARS. Plasma TBARS will be measured as an indication of lipid peroxidation. TBARS will be measured using a modification of the method described by Yagi.[110] One hundred uml of whole blood will be added to one ml of physiological saline and centrifuged at 1700 g for 15 minutes. The supernatant will be collected and frozen at -70°C until analyzed. Lipids will be isolated by precipitating them with a

phosphotungstic acid/sulfuric acid system. The lipid fraction will then be reacted with thiobarbituric acid, and the resulting adducts measured fluorimetrically, using 1,1',3,3'-tetraethoxypropane as the standard. Results will be expressed as nanomoles of MDA per milliliter plasma and as a function of the concentration of plasma polyunsaturated fatty acids.

Trace Element Analysis. Plasma will be wet-ashed with 16N nitric acid (Baker's Instra-analyzed, J.T. Baker Co., Philipsburg, NJ), evaporated and diluted with 0.1 nitric acid (Baker's Instra-analyzed). Mineral concentrations will be determined in the diluted ashed samples by inductively coupled plasma optical emission spectrometry (ICP) (Trace scan; Thermo Elemental, Franklin, MA). Certified reference solutions (QC 21, Spec Centri Prep, Metuchem, NJ) will be used to generate standard curves for each element. Natation Bureau of standards reference samples will be included with each run to ensure accuracy and reproducibility.[111]

Vitamins Plasma vitamin C. This antioxidant will be measured following oxidation to dehydroascorbic acid with cupric sulfate and a subsequent reaction with 2,4 dinitrophenylhydrazine to form dinitrophenylhydrazone.[112] The red color of this latter compound will be measured spectrophotometrically at 520 nm; concentrations will be determined using a standard curve containing known amounts of ascorbate. Tocopherol and vitamin A. Tissue and plasma levels of these antioxidants will be measured simultaneously by HPLC by the method of Driskell et al.[113] For tissue tocopherol and vitamin A determinations, tissue will be homogenized in nine volumes 0.15 M Na phosphate/1% (w/v) Na ascorbate, pH 7.4. Concentrations will be quantitated by comparing heights of retinol or tocopherol peaks to the height of the retinyl acetate internal standard peak. Folate will be analyzed using a selected ion monitoring gas chromatography with mass selective detection protocol.[114] Retinol and B-carotene concentrations will be determined by reversed-phase high-performance liquid chromatography using the methods described by Clifford.[115] The amount of total plasma cobalamin vitamin B_{12} bound to the carrier protein transcobalamin II will be determined with an indirect assay that uses anti-transcobalamin II antibodies.[116]

Other Methods. The cyanmethemoglobin colorimetric procedure (Sigma Chemical Co., St. Louis, MO) will be used to measure hemoglobin concentrations. Protein concentrations will be determined using the method of Bradford.[117]

Results of all analyses will be reported to the study Coordinating Center where they will be entered into the study database. Data from the 3-day dietary recalls will also be tabulated and entered into the study database.

5. Outcome evaluation for the physical features of FASD

Routine ultrasounds to evaluate growth are performed at standard gestational ages in the first, second and third trimesters of all pregnancies in the Region. The reports for these scans will be collected and abstracted for each enrolled pregnancy and sent to the Coordinating Center. Before hospital discharge of live born infants, the study site interviewer working with the study coordinator will schedule all infants for an examination by a specially trained pediatrician identified for each study site. This dysmorphological evaluation will be focused on the physical features of FASD using the checklist and protocol designated by the dysmorphology core. Pediatricians will be blinded as to the prenatal exposure history of each infant at the time of the physical exam. All completed standardized examination forms will be labeled with the subject identification number and will be sent to the Coordinating Center in Moscow.

At 12 months of age, the study coordinator will schedule a second pediatric physical examination using the same protocol and preferably by the same pediatrician. At the time of the second exam, the pediatrician will take a set of photographs of the face and hands using digital cameras that are already available at a standard distance and angle. Photographic files will be labeled with the subject identification number and transmitted to the study Coordinating Center.

Any child who receives a diagnosis of FAS or deferred by one of the study pediatricians will be referred for a validating diagnosis by a member of the dsymorphology core at the earliest opportunity. The dysmorphology core will review all photographs for evidence of features of FASD and employ the Astley/Clarren screening tool developed for photographic analysis as a second method of review.

6. Neurobehavioral outcome evaluation

The study coordinator will schedule all study infants for a standard neurobehavioral assessment battery at 6-6.5 months and again at 12 months of age in conjunction with the second pediatric evaluation. For infants born at less than 37 weeks gestational age, age-at-testing will be corrected accordingly so that the actual testing will take place at the correct conceptual age. The testing battery designated by the neurobehavioral core is appropriate for this investigation of prenatal alcohol exposure and nutritional supplementation. Testing will be done by one of four psychologists on the study team who will be trained by Dr. Coles. The composition of the core testing battery will be determined based on the resources available from the neurobehavioral core. The battery will include the Bayley Scales of Infant Development, Second Edition (BSID-II) at 6 months and 12 months. Additional tests (see Table 1) will be performed, as determined by the core. By selecting measures that have demonstrated their effectiveness in identifying alcohol-related differences in development during the first two years of life and which have been related to later alcohol-related deficits in the domains being assessed at other sites in the consortium, we will be able to document the pattern of neurodevelopmental compromise associated with alcohol exposure in our Moscow sample at 6 and 12 months and relate these outcomes to those seen in older children collected at different consortium sites. Testing time for infants will not exceed 60 minutes at 6 months or 90 minutes at 12 months.

Table 1. Neurobehavioral Core Recommendations for Infant Assessment

Test	*Age (months)*	*Type*	*Priority*	*Testing Time*
BSID-II (MDI & PDI) with the BRS	6 and 12	Standard Scores	1	6 mo: 30 min 12 mo: 45 min
Fagin Test of Infant Intelligence	6.5 and 12	Experimental	3	20 minutes
Infant Numerosity	6.5 and 12	Experimental	3	30 minutes
A-not-B	12	Experimental	2	15 minutes
Spontaneous Alternation Delayed Non-Matching to Sample	6 12	Experimental Experimental	2 2	15 minutes
Belsky complexity of play	12	Experimental (Video)	3	30 minutes
Teller Acuity Cards (Tests Vision)			Have to do if do FTII	10 mintues
Achenbach CBCL (18-36 mo) (translate)	24 (subset)	Standard scores	1	Mother completes

In addition to the Infant testing battery, study team psychologists will administer a standardized questionnaire to the mother/caregiver regarding caregiving environment and the baby's eating, sleeping habits and temperament. This questionnaire will be an adaptation of that used by Platzman et al[118] modified and translated for use with a Russian population. Test results will be scored, labeled with subject identification number and sent to the Coordinating Center. A subset of mothers of infants enrolled in the first or second year of the study whose children reach 24 months of age within 6 months of the study completion will be mailed the Russian translation the Achenbach CBCL for ages 18-36 months. This is a measure of infant behavior and behavior problems that is completed by the caregiver.

All mothers of children who either receive a diagnosis of FAS or who show evidence of developmental delays will be offered referrals to appropriate services for further evaluation and care.

7. Training of study personnel and retention of subjects

The study personnel in Russia will include four interviewer/pediatrician/psychologist teams each of whom will be assigned to 1 –2 hospitals. The interviewers will be trained by the principal and co-investigators and consultants Sharon Wilsnack and Arlinda Kristjanson who have designed and administered similar interview training protocols previously in St. Petersburg. The pediatricians will receive the necessary training and supervision by the dysmorphology core team. Dr. Coles will be responsible for training the psychologists on the testing battery as selected by the neuropsychological core. The day to day oversight of the study activities will be conducted by a study coordinator who will be trained and directly supervised by Dr. Kavtaladze and Dr. Joutchenko in Moscow and Dr. Chambers in the U.S. If there is turnover in study personnel, additional training will be provided as necessary.

Little data exists in Russia on the success of retaining subjects in longitudinal studies. The University of North Carolina longitudinal household survey in the Russian Federation provides some location-specific data over the last ten years, and indications are that between survey phases, usually consisting of a two-year interval with no interim contact, approximately 65% of households in Moscow City maintain the same residents over the time period. Furthermore, urban attrition based solely on this measure of household stability is greater than in rural areas. Therefore, it is expected that with interim contact with study participants, the use of incentives, and focus on the health of the child, retention rates will be far better for this study. The study coordinator will be responsible for overseeing attrition rates and enhancing retention if necessary by increasing the frequency of contact and or instituting other measures as necessary.

8. Criteria for alcohol exposure classification

a) The minimum criteria for enrollment in the alcohol exposed group will be an average of at least 1 standard drink (equivalent to 1 four-ounce glass of wine, 1 single shot or single shot in a mixed drink of spirits, or 1 twelve-ounce beer or the Russian equivalents) on average per day for the two week period prior to the intake interview date
b) The maximum criteria for enrollment in the control group will be no more than 1 standard drink per week in any of the two weeks previous to the interview date.

9. Criteria for FASD classification

The following criteria for classification are based on Institute of Medicine guidelines[119] as well as the diagnostic algorithm developed for studies previously and currently being conducted in South Africa and in Russia. The use of comparable diagnostic criteria will enhance comparisons with other study results.

FAS: subjects who have the constellation of the following features will be classified in this group

- head circumference less than or equal to the 10th centile, plus growth deficiency defined as weight or height less than or equal to the 10th centile for sex and age using National Center for Health Statistics current growth charts or Russian norms (and adjusted for prematurity for growth measurements for infants less than 12 months of age).
- at least two of the following facial features:
- palpebral fissure length unilaterally or bilaterally less than or equal to the 10th centile for age (and adjusted for prematurity for measurements at the first evaluation for infants less than 12 months of age).
- philtrum smoothness using the Astley/Clarren Likert scale[84] valued at 4 or 5
- thin smooth vermilion border of the upper lip using the Astley/Clarren Likert scale[84] valued at 4 or 5
- hypoplastic midface

Deferred Classification for FAS: subjects who meet all of the criteria for FAS as above except for head circumference less than or equal to the 10th centile. These children will ultimately be reclassified as either FAS or Some Alcohol Related Features (below) following the neurobehavioral testing and validation of the physical

exam by a member of the dysmorphology core team. The criteria for classification of FAS on the basis of neurobehavioral testing among those in the deferred group with validated structural features will be a test score of at least 1 standard deviation below the mean of the control group on either the MDI or PDI measures of the BISD II at 6 or 12 months.

Some Alcohol-Related Features: subjects who have one or more facial features as defined in FAS criteria above, or additional features such as ptosis, joint contractures, aberrant palmar creases as identified by the dysmorphology core, or who have head circumference less than or equal to the 10th centile but who do not meet the criteria for FAS will be classified as having some FASD-related features. Therefore, deferred children who do not subsequently become classified as FAS will ultimately be included in this group. This classification, although not FAS, is analogous to the Alcohol Related Birth Defects (ARBD) classification as outlined in the Institute of Medicine Report.[119]

10. Analysis and interpretation of data

a) Data from screening instruments, interviews, medical records, ultrasound, and lab reports will be gathered at the Coordinating Center in Moscow and entered into a database. All other reports and records associated with the study will also be pooled at this site. All sources of data will be reviewed for completeness and validity by the study investigators working at frequent intervals during the study period. Problems in data collection will be addressed with retraining measures.

b) When recruitment and first physical examination data are complete, at approximately 36-40 months after study initiation, an analysis of the intervention trial with respect to birth size will be conducted (see below). An interim analysis for physical features of FASD will also be conducted. Frequencies and distribution of variables will be examined. Crude comparisons will be made between the proportions of all FASD classification categories separately (i.e., FAS, deferred, and Some Features) and collapsed in the alcohol-exposed group relative to the control group.

c) Following completion of the final physical and neurobehavioral examinations, and after final classification of subjects with respect to FASD the following analyses as they relate to the Specific Aims will be conducted.

Specific Aim 1: To measure the birth prevalence and range of alcohol-related physical features and neurobehavioral impairment among children born to pregnant women in the Moscow Region who report consuming moderate to heavy amounts of alcohol by utilizing methods designed to permit earlier diagnosis of alcohol-related effects. The proportion of children classified as FAS and the proportion with some FASD-related features both separately and combined will be compared between the group of newborns in the alcohol-exposed group to the group of newborns in the control group using Fisher's Exact Test or chi-square tests as appropriate. The prevalence of each of these diagnostic categories within exposure groups categorized by alcohol consumption quantity and pattern will be estimated and confidence intervals generated. Multivariate analyses including logistic regression will also be employed to characterize factors that alone or in combination appear to influence risk for these outcomes. These analyses will be repeated with data from the 12 month exam and both the overall prevalence and individual change in classification group will be compared using the newborn and 12 month exam results.

Analogous comparisons will be made utilizing the neurobehavioral outcome data at 6 and 12 months using both continuous measures and categorical measures of neurobehavioral impairment. The primary endpoint in the testing battery will be the Bayley Infant assessment. Based on the publisher's recommendations, BSID-II scores 1 standard deviation below the mean will be characterized as "mildly delayed" and 2 standard deviations, as "significantly delayed". BSID-II mental (MDI) and psychomotor (PDI) outcome will be examined in relation to prenatal alcohol exposure, including dose (oz AA/day) duration of exposure (weeks in pregnancy) and pattern of use (e,g,. binging), and in relation to micronutrients levels (see plan for analysis relative to Specific Aim 2 below). Initial analysis of outcomes will evaluate gender, conceptual age, exposure to tobacco, caregiving, and other control factors. Factors found to be related to outcomes

will be controlled for in subsequent analyses. In addition, the relationship of alcohol-related structural features and growth factors to neurobehavioral outcomes will be examined. If other measures in the neurobehavioral core are included in the study, similar analyses will be carried out.

Repeated measures analysis will be used to evaluate growth measurements on prenatal ultrasounds in relation to alcohol exposure category and adjusted for important confounders. The prenatal ultrasound growth percentiles at each gestational age timepoint both continuously and categorized as SGA/no SGA will be examined as predictors of FASD classification group using binary or polychotomous logistic regression and loglinear analytic techniques.

Review of photographs by the dysmorphology core team will be performed subjectively to help validate the sensitivity of the pediatrician's diagnosis. In addition, formal analysis using the Astley/Clarren method will be conducted to determine the level of correlation by kappa statistic for this method versus the dysmorphology classification at 12 months as the gold standard.

Specific Aim 2: To evaluate the contribution of maternal nutritional factors to increased risk for prenatal growth deficiency, neurobehavioral impairment, and alcohol-related physical features in infants prenatally exposed to moderate to heavy amounts of alcohol by conducting a randomized trial of a micronutrient supplementation intervention and measuring micronutrient levels in maternal blood.

Hypothesis a) Intervention Trial: Additional supplementation with certain micronutrients during pregnancy will improve prenatal growth as well as neurobehavioral performance in infants ≤1 year of age, and the effect size will be significantly greater among infants whose mothers drank moderate to heavy amounts of alcohol compared to infants whose mothers drank lesser amounts.

We will use a two-factor analysis of covariance to test the hypothesis that size at birth and neurobehavioral performance of infants are affected positively by additional micronutrient supplementation, and negatively affected by alcohol. The two factors will be alcohol intake during pregnancy (initially evaluated at three levels derived from average consumption over the entire pregnancy: heavy, moderate, and light to none, but with additional alcohol variables constructed reflecting gestational timing of exposure, binge drinking pattern, etc.), and micronutrient supplementation (treatment vs control group). We will consider the interaction between the two factors, as we hypothesize that heavier drinkers may respond better to supplements.

The outcomes of primary interest are weight, length, and head circumference at birth expressed as a sex- and gestational-age adjusted percentile using Russian growth norms. Additionally, we will consider the following outcomes: Bayley Motor and Mental subtests at 6 and 12 months of age and prenatal growth as estimated by specific ultrasound measurements taken during the first, second and third trimesters.

The following covariates will be entered into the analyses to clarify the effect of the main factors on the outcomes of interest:

- maternal pre-pregnancy BMI and pregnancy weight gain
- maternal smoking
- maternal other drug use
- parity (1 or more previous children vs no previous children)
- maternal age
- socioeconomic status

Additional variables will be tested in the models and included if important predictors or modifiers of the main effects.

Hypothesis b) Blood Levels of Micronutrients: 1) Blood levels of certain micronutrients as measured during the first trimester of pregnancy will be predictive of the prevalence of alcohol-related structural abnormalities in the offspring of women who drink moderate to heavy amounts of alcohol, and 2) blood

levels of micronutrients measured over the course of pregnancy will be predictive of prenatal growth and neurobehavioral performance in infants ≤1 year of age.

1) As the intervention trial will typically not be initiated until most or all of embryonic development is complete, blood levels of micronutrients taken in the first trimester of pregnancy will be utilized to address the hypothesis that nutritional deficiencies are associated with increased risk for alcohol-related structural abnormalities. We will perform a logistic regression analysis, with the two predictors of primary interest being the alcohol consumption variables and the level of micronutrients (lower quartile vs. above) as measured in the first trimester, and the outcome variable being the presence vs. absence of FAS or FAS-related structural features with additional covariates being tested in the model. The interaction between alcohol and micronutrient level will be tested to determine if separate models for alcohol-exposed and unexposed groups will need to be constructed.

2) We are aware of the potential problem of the treatment vs. control factor being an imperfect identifier for supplementation with micronutrients. It is possible that some women in the treatment group will not take the supplement, while women in the control group who receive advice only will acquire supplements and take them. Since we will use an intent-to-treat analysis for the primary analysis of the intervention trial, one approach that we plan to take in looking at this issue is to conduct a 3 group analysis of covariance comparing women in the treatment group to women in the control group who report taking standard prenatal vitamins as advised and women in the control group who report taking no vitamins. However, the addition of early and late pregnancy blood marker data will also help address this potential problem as well as the issue of subject compliance, while at the same time allowing for another method of independently testing the hypothesis in question. Therefore, we will repeat the analyses described in hypothesis a), but instead of using the treatment/control factor, we will use binary factors derived from direct measurements of micronutrients in the blood: (lower quartile vs. above) for each of the micronutrients measured both early in pregnancy and in the third trimester. To address change in micronutrient status over the course of pregnancy, a three group categorical measure for the independent variable will also be used reflecting "good" nutritional status throughout, "improved" status over the course of pregnancy, or "poor" status throughout.

Additionally, for all of these analyses, we will also express the level of micronutrients in the blood as a continuous predictor rather than a binary factor. As these nutrients tend to be highly correlated with one another, an aggregate or summary measure of nutritional status will be constructed for both the categorical and the continuous approaches, using a standardized measure of each micronutrient in the blood for a given time point of measurement. Finally, data derived from the periodic 3-day dietary recall questionnaires will be incorporated into the analyses to help validate data derived from the blood markers and also as an independent predictor of outcome.

11. Inclusion and exclusion criteria

(1) Inclusion criteria - women who meet the alcohol consumption criteria for either the exposed or control groups and who agree to the study requirements and are capable of giving informed consent.
(2) Women who report toluene abuse during the current pregnancy will be excluded at the time of enrollment.
(3) Although not likely to occur with high frequency, subjects who meet the following criteria, in separate analyses, will be both included and excluded:

- women who take a medication that places them at increased risk of having a child with learning difficulties, e.g., an anticonvulsant
- women enrolled in the control group who subsequently report drinking that exceeds the criteria for control or who are above the cutpoint for a positive test on one or more biomarkers of exposure
- women who deliver a child with a chromosomal anomaly or genetic condition or other abnormality which may be associated with mental deficiency, e.g., Down Syndrome, or may hinder the child's ability to perform on cognitive tests

12. Sample size and power

The following sample size calculations are based on the alcohol-related physical features component of the Specific Aim 1 of the study, i.e., to compare the birth prevalence of FASD-related physical features in children born to women who consume moderate to heavy amounts of alcohol compared to children born to women who do not. For purposes of this initial estimate, the sample size is projected based on numbers of subjects recruited and followed up to the time of the first physical examination.
These estimates are based on the following assumptions:

- A screened sample of 26,000 women over two years
- A 2.5% prevalence of subjects who meet the alcohol consumption criteria and agree to enroll
- A 0.6% prevalence of subjects who meet the alcohol consumption criteria and who fall into the heavy consumption end of the range (14 or more standard drinks per week in the previous 2 weeks)
- An estimated recruitment period of approximately 24 months yielding 640 subjects enrolled in the alcohol-exposed group and 640 in the comparison group
- Following enrollment, attrition with respect to the newborn physical exam will be estimated based on:
  - 10% lost to follow-up or withdrawals from the study
  - 15% spontaneous pregnancy loss after enrollment
- Due to chance, errors in diagnosis, or to the possibility of severe underreporting of maternal alcohol use, a 1% figure will be used as the estimate of the baseline prevalence of children in the control group meeting the criteria for full blown FAS.

Under these assumptions, the net yield of live born infants is estimated to be 480 in each group with 120 of those in the alcohol-exposed group being classified as heavily-exposed. Overall, this sample is sufficient to detect a 4-fold relative risk, or approximately a 4% birth prevalence, for full-blown FAS using a two-tailed test, 80% power, and alpha of 0.05 using exact methods. Within the heavily-exposed subgroup, relative to the complete low-or-no-alcohol comparison group, there will be sufficient power to detect a relative risk of about 6.3 or a 6.3% birth prevalence of full blown FAS. This birth prevalence is estimated to be an adequately sensitive target proportion based on prospective data on infants born to women with similar levels of alcohol exposure who have enrolled in a pregnancy outcome study through the California Teratogen Information Service and Clinical Research Program[83] and on the composite estimate developed by Abel.[15] Furthermore, the recruitment strategy will be evaluated frequently as the study progresses to insure complete sampling of all eligible and agreeable participants with heavy exposure so that the proportion of high exposure infants in the study is maximized.

The following sample size calculations apply to the nutritional intervention component of Specific Aim 2 of the study. The key comparison for the first hypothesis under this Specific Aim involves comparing birth size across four groups with the expected trend in lower birth weight proceeding from the alcohol-exposed-no-intervention-group (lowest mean birth weight) to the alcohol-exposed-intervention-group, followed by the low-or-no-alcohol-no-intervention-group and lastly the low-or-no-alcohol-intervention-group (highest mean birth weight).

These estimates are based on the same assumptions regarding attrition as above, that approximately equal numbers of women will be randomized to the intervention or control condition, and that approximately equal numbers of women in each group will deliver liveborn infants. Using adjusted mean birth weight as a representative endpoint, the net sample size of 240 in each of the exposure/treatment conditions will provide sufficient power at alpha of 0.05 and 1-beta of 0.80 to detect as small as a 100 gram difference between any two groups.

The primary instrument in the neurobehavioral testing battery will be the BSID II. With an estimated 80 infants in the heavily exposed group who complete follow-up to 12 months and a comparison group of 320 controls (33% attrition at 12 months), sufficient power at alpha of 0.05 and 1-beta of 0.80 will be available to detect the effect sizes on MDI or PDI as shown in Table 2 below.

Table 2. Sample Size and Power for Neurobehavioral Endpoints at 6 and 12 months of Age

Age at Testing	Heavy Exposed	No-Low Exposed	Mean Difference	Effect Size MDI or PDI	Power
6 months	100	400	4.8 points	0.32	81%
12 months	80	320	5.3 points	0.35	80%

13. Study timetable

As shown in Table 3, following a six-month startup period, enrollment is expected to continue for 24 months from initiation of recruitment based on the projected number of qualified subjects and the participation rate. All subjects will have completed pregnancies by approximately 36 months from the study start date. Results of the intervention trial with respect to birth size and complications will be analyzed first, followed by early physical exam results, early neurobehavioral evaluation results, and finally the 12 month physical and neurobehavioral evaluations will be analyzed. A second analysis of the intervention trial will involve the neurobehavioral endpoints as the data become available. All analyses will be complete by 5 years from the study start date.

Table 3. Study Timetable

	Oct-Mar Yr1	Apr-Sept Yr1	Oct-Sept Yr2	Oct-Sept Yr3	Oct-Sept Yr4	Oct-Sept Yr5
Startup	X					
Recruitment/Interviews/Blood Sampling		X	X	X		
Physical Exams 0 and 12 months		X	X	X	X	
Neurobehavioral Evaluations 6 and 12 months			X	X	X	X
Interim Analysis Intervention Trial				X		
Analysis Newborn Physical Exams/Ultrasound					X	
Final Analysis of Intervention Trial						X
Analysis of Neurobehavioral Findings						X
Analysis of 12 month Physical Findings						X

14. Flow of information and assignment of responsibility

The Principal Investigator, Investigators and collaborators, as the protocol team for this project, will develop with input from the various consortium core groups the instruments, tools, forms, and operations manual for the study. The protocol team will be responsible for training the study coordinator, the screeners, the interviewers, and supporting the training of the pediatricians and the psychologists by the cores. The progress of the study during the startup period for training of personnel and selection and qualification of laboratory and technical facilities, as well as monitoring for quality and pacing of data collection after enrollment begins will require a significant amount of oversight by the study investigators, and therefore travel costs constitute a relatively large percentage of the U.S. portion of the budget. Dr. Chambers will be primarily responsible for maintaining close contact with the study team in Russia during all years of the study and for coordinating efforts of the other team members to meet the study objectives within the specified time frame. The Russian portion of the budget, particularly in the first two years of the study, supports the cost of a large number of analyses of blood samples for micronutrient levels and for biomarkers of exposure. Oversight of the quality of the data generated from these samples will be the responsibility of Drs. Keen and Schuckit. The flow of funding to the Russian team members will go through the U.S. Civilian Research and Development Foundation (CRDF), a nonprofit charitable organization that promotes scientific and technical collaboration between the United States and the countries of the Former Soviet Union. This organization has been successfully utilized for previously funded NIAAA studies in Russia. The distribution of monies in Russia will be the responsibility of Dr. Kavtaladze.

Oversight of the physical follow-up and neurobehavioral testing will be the responsibility of Drs. Chambers, Jones and Coles, with the support of the cores. The flow of information from the Russian Coordinating Center to the IT core will be overseen by Dr. Chambers. Drs. Chambers and Jones will be responsible for working with Dr. Kavtaladze to communicate progress regarding the study to the Ministry of Health for the Moscow Region.

15. Strengths and limitations of research design and methods

The proposed study inter-relates with other projects in the consortium by the following:

1. The current study plan has drawn on the expertise of several members of the CIFASD, including Dr. Coles, in creating our neurobehavioral assessment protocol. We have selected measures that have demonstrated their effectiveness in identifying alcohol-related differences in development during the first two years of life, and which have been related to later alcohol-related deficits in the domains that have been assessed in older children. By doing so, we will be able to document the pattern of neurodevelopmental compromise associated with alcohol exposure in our Moscow sample at 6 and 12 months and relate these outcomes to those seen in older children collected at other consortium sites. These relationships will be particularly salient when comparisons are made with outcomes for older children at the other Moscow project.

2. The prenatal ultrasound component of this project can be compared to data collected from prenatal ultrasound in the consortium Ukraine pilot project and to that being collected in Cape Town, South Africa. In addition, if the Seattle pilot project results prove informative relative to the value of early postnatal ultrasound of the brain, that protocol could be implemented in the Moscow Region sample in years 2 and 3.

The proposed study will benefit the consortium by the following:

- produce a measure of the full range of FASD resulting from moderate to heavy prenatal alcohol exposure in a prospective cohort that is presumably less subject to ascertainment bias and that can be compared to other samples retrospectively and prospectively ascertained through the consortium
- contribute to the broad diagnostic schema which is the focus of this consortium by testing strategies for earlier detection of affected fetuses through prenatal ultrasound, early pediatric physical exams and photographs, as well as earlier detection of neurobehavioral abnormalities, thereby allowing for earlier intervention and initiation of treatment
- translate findings from animal studies to humans regarding the protective effect of certain micronutrients with respect to pregnancy outcome following exposure to alcohol, thereby suggesting promising interventions that could be applied in other settings in the consortium
- identify maternal risk factors, such as maternal age and genetic susceptibility, that can be compared to those identified through other members of the consortium, and explore new risk factors such as nutritional deficiencies and maternal conditions that might warrant testing in other samples generated through the consortium
- provide a cohort of alcohol-drinking mothers and their children with detailed exposure histories and validating biomarkers that can serve as the basis for translational research hypotheses generated through other members of the consortium. With the exception of the Ukraine pilot project, the Moscow Region project will provide at the end of the study period the only sample of preschool age children available to the consortium for the application of findings resulting from other consortium projects. In addition, maintenance of this prospectively ascertained cohort will allow future longitudinal assessment of the sensitivity of early diagnosis of FASD.

The proposed study will be benefited by participation in the consortium by access to the dysmorphology, neurobehavioral, imaging, and bioinformatics core teams to develop standard protocols that will facilitate cross-consortium comparisons and will enable efficient and appropriate training of study pediatricians and psychologists.

A limitation of the proposed study design is the inability to initiate a nutritional intervention prior to conception so that the impact on structural development in alcohol-exposed fetuses could be appropriately measured. However, if a significant effect on growth and neurobehavioral performance can be demonstrated through this study, this will provide strong impetus for future efforts to test the efficacy of a preconceptional intervention. On the other hand, the nutritional intervention trial is a major strength of this study. Unfortunately, given the current difficult economic situation, relatively poor nutritional status and under-utilization of prenatal vitamins by pregnant women in the Moscow Region is the status quo, and the limited resources available are not being utilized to address this problem. However, similar to the MRC vitamin trial which definitively resolved the long-standing controversy regarding the protective effect of folic acid with respect to neural tube defects, the proposed study in a relatively short period of time by using a randomized trial design can produce answers that may be definitive enough to support the effectiveness of providing nutritional enrichment for women who drink alcohol as well as for all women of reproductive age throughout the Russian population.

E. Gender and minority inclusion for research involving human subjects

Women will be the only subjects enrolled in this study because the area of research involves pregnancy. Both male and female children will be involved in the postnatal evaluations and testing. Although no ethnic group will be excluded, based on the racial/ethnic composition of the study sites, it is likely that almost all subjects recruited will be white.

Participation of children

Children, i.e., females under the age of 21 who are pregnant and who meet the alcohol consumption criteria will not be excluded from this study. Children will be the focus of the postnatal evaluations and testing with the consent of the parent.

Human Subjects

- The human subjects participating in this project will be recruited from pregnant women receiving care at a participating birthing hospital site in the Moscow Region. The study will involve approximately 1280 subjects. It is expected that they will range in age from 14 to 50, and it is expected that there will be a narrow ethnic distribution as described above. The health status of these subjects is expected to be variable given the fact that some women are expected to have heavy alcohol exposure. Pregnant women are the only subjects in this study because it is a prospective study regarding pregnancy outcome.
- All women who are screened for the study will be advised by the screener that they should discontinue drinking during pregnancy and breastfeeding.
- Research material will be collected from individually identifiable living human subjects; however, data in the final format for analysis will be stripped of personal identifiers. The data and specimens collected will be obtained specifically for research purposes. Physical and neurobehavioral assessments could be important to the health of the children involved in the study, and so results of these tests will be made available to the subjects if they so desire.
- Subjects will be recruited from existing hospital sites from among pregnant women who are already receiving care at these locations. The study interviewer will consent each subject at the time of enrollment at the site. The interviewer will explain the study objectives, requirements, benefits and risks, and will obtain written consent if the subject agrees to participate. A copy of the signed consents will be maintained at the Coordinating Center.
- There may be slight risk of pain, infection or fainting associated with the drawing of the blood sample from the mother; there could by psychological stress involved with discussing sensitive issues about exposures during pregnancy or being informed that her child has features of FASD; and there is the potential risk of loss of confidentiality. There may be social and/or legal consequences associated with loss of confidentiality in this study. This is an unlikely event; however, the consequences of such loss of confidentiality could be serious. For the live born infant or child of each subject, there may be some discomfort associated with the physical exam but no more than would be normally encountered during a routine well-baby pediatric examination.

- Blood sampling will be conducted at the hospital site and, if possible, in conjunction with other routine blood sampling procedures. The interviewer conducting maternal interviews, as part of the study training, will be sensitized to the guilt and privacy issues surrounding alcohol use during pregnancy. Appropriate referrals will be made for women who wish to receive additional alcohol-related services. In addition, mothers of children who are diagnosed with FAS or who have clinically relevant cognitive impairment will be offered appropriate referrals for follow-up. The risks to patient confidentiality are protected against by coding all data in final format by subject number and not personal identifiers. The study personnel will be responsible for maintaining restricted access to subject data through use of locked files, computer passwords and precautions when transmitting data.
- The anticipated potential risks to the subject in this protocol are far outweighed by the benefits both to the individual and to society. The benefits of participating in this protocol for the individual subject are specialized evaluation of her child with respect to the effects of prenatal alcohol exposure and appropriate referrals for affected children. Benefits to the public health of the community at large can result from the research goals of this study being realized. Among other benefits, knowledge regarding the prevalence of FASD among the offspring of alcohol exposed women will provide valuable information regarding the range and prevalence of abnormalities associated with FASD, risk factors including those that are modifiable for FASD, and projected numbers of children in the Moscow Region requiring future services related to this disorder.

Targeted/Planned Enrollment Table **This report format should NOT be used for data collection from study participants.** □**Study Title:** Risk Factors for FASD in the Moscow Region □**Total Planned Enrollment:** 2240 □**TARGETED/PLANNED ENROLLMENT: Number of Subjects**

Ethnic Category	Sex/Gender		
	Females	Males	Total
Hispanic or Latino	0	0	0
Not Hispanic or Latino	1,760	480	2,240
Ethnic Category Total of All Subjects*	1,760	480	2,240
Racial Categories			
American Indian/Alaska Native	0	0	0
Asian	90	30	120
Native Hawaiian or Other Pacific Islander	0	0	0
Black or African American	15	5	20
White	1,655	415	2,100
Racial Categories: Total of All Subjects *	1,760	480	2,240

*The "Ethnic Category Total of All Subjects" must be equal to the "Racial Categories Total of All Subjects."

F. Vertebrate Animals

There are no animals involved in this study.

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