Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD)

Progress Report
Winter 2011 Meeting
January 31 – February 2, 2011

Table of Contents

I. Principal Investigator: Edward P. Riley, Ph.D.

II. Title of Project: Administrative Core of the CIFASD - U24 AA014811

III. Objectives:

The CIFASD coordinates basic science and clinical investigators in a multidisciplinary research project to better inform approaches aimed at developing effective intervention and treatment approaches for FASD.

The **Specific Aims** of the **Consortium** are as follows:

1. Utilizing 1) novel animal models and 2) clinical samples of varying ages and demographics located at various sites, we will continue to establish procedures for better defining and characterizing the range of outcomes from prenatal alcohol exposure. These procedures are based on a multidisciplinary approach, involving both basic and clinical projects for the study of dysmorphia, neuropsychological and behavioral functioning, and brain imaging. Within this aim, new, innovative techniques will be assessed and implemented as indicated by the data. These innovative techniques will help in defining characteristics indicative of prenatal alcohol exposure that can be assessed easily and reliably.

2. Our consortium builds upon existing research programs within the previously funded CIFASD and new sites, to build a subject population large enough to adequately address many of the questions posed in our application. Some countries have much higher rates and levels of alcohol consumption during pregnancy and FASD than does the United States and we will utilize these resources. Our goal is to obtain concordance on a set of diagnostic criteria between sites, basing these criteria on the techniques established in Aim #1.

3. The Basic Science Component seeks to uncover mechanisms involved in alcohol teratogenesis and to design effective interventions to mitigate or prevent the effects of prenatal alcohol exposure. The basic science projects use models ranging from molecular analysis of alcohol antagonist actions to prenatal alcohol effects in mice, rats and sheep. Basic science projects will be integrated throughout by a common focus on neural systems that are vulnerable to ethanol in humans, new diagnostic procedures and effects of agents that prevent ethanol teratogenesis. The Basic Science and Clinical Components will work collaboratively to translate fundamental scientific observations into clinically useful diagnostic tools and treatments.

The **Primary Goals** of the **Administrative Core** are:

1. Provide scientific and administrative direction, leadership and oversight to the CIFASD. The PI ensures that CIFASD investigators adhere to its goals and mission, as well as assist and coordinate interactions between various projects. It also assists and provides necessary administrative support to the Science Advisory Board and the two Steering Committees and functions as the main liaison to NIAAA regarding the CIFASD.

2. Facilitate communication between the various projects using the CIFASD web site, scheduled monthly conference calls, the biannual meetings of the Steering Committees, and the preparation and distribution of annual progress reports. It also serves a liaison role for the Administrative Core, the Steering Committees, and the Science Advisory Board, facilitating the interaction and communication of consortium scientists.

3. Monitor the cores and individual research projects for progress.

4. Provide assistance where necessary with data collection and ensure that data from the projects are uploaded into the Central Repository of the Informatics Core in a timely fashion. It works with the Informatics Core to develop online interactive capacity among CIFASD investigators, and will eventually provide these databases to outside, interested scientists.

5. It will assist the Science Advisory Board and the Steering Committees in their annual evaluations of progress for each of the projects and the two major components. In conjunction with the Steering Committees, it will establish annual priorities and deal with issues related to allocation of resources. It will also provide a mechanism for the evaluation of the projects.

6. Administer the Exploratory/Developmental Projects of the CIFASD and oversee the administration of the Exploratory/Developmental Projects.

7. Facilitate the recruitment of new scientists and new technologies into the consortium.

8. Disseminate new data arising from the consortium.

IV. Methods:

1. Regular monthly conference phone calls conducted through the use of Accuconference.

2. CIFASD.org website maintenance and updates.

3. Biannual Meetings and Progress Reports.

4. Interaction and assistance in facilitating the goals of Central Repository with the Informatics Core.

5. Annual project evaluations and priority/goal-setting.

6. Exploratory/Developmental Projects.

V. Accomplishments and Results:

1. Since the last progress report in January of 2010, the Administrative Core has been fulfilling its role as liaison between the CIFASD PIs, the Scientific Director, Science Advisory Board members and the NIAAA staff. This was accomplished by:

    a. Monthly Conference Calls: Separate Basic Science and Clinical Research Component conference calls were held monthly and were moderated by the Consortium Coordinator/ Administrative Core PI. PIs reported on their monthly progress on each of these calls. Verbatim mp3 recordings of these calls were posted in the secure area of the CIFASD website (www.cifasd.org) upon call completion. Please contact Jill Vander Velde (vanderv@mail.sdsu.edu), if necessary, for login information.
    b. Special Conference Calls: The Administrative Core assisted in facilitating smaller special area group conference calls as needed. For example, the calls held to discuss and suggest the specific genetic markers to target on an administrative supplement to an existing CIFASD project grant.
    c. Biannual Meetings: The January 2010 CIFASD meeting held in Chapel Hill, NC and the June 2010 meetings for the Basic Science group and the Science Advisory Board held in San Antonio, TX convened. The Clinical group did not meet in the summer of 2010. At present, the Administrative Core is coordinating the final arrangements for the January 2011 CIFASD meeting for all CIFASD participants to be held in Rockville, MD. Coordination of these meetings included date selection, contracting sleeping room rates, reserving meeting space, completing travel arrangements for the Consortium Coordinator, Science Advisory

Board members and invited guests, collating and distributing meeting materials and researching and inviting outside experts to the meetings.

d. Progress Reports: Progress reports from all CIFASD PIs have been solicited and will be collated for the upcoming late January/February 2011 meeting. Progress report PDFs and PPT presentations are posted in the secure area of the CIFASD website. In addition to the annual beginning of the year CIFASD progress reports, the Science Advisory Board and NIAAA staff along with the Administrative Core PI and Scientific Director reviewed the project specific NIH progress reports at their June meeting.
e. Various Communications: The Administrative Core is in communication with PIs, the Scientific Director, Science Advisory Board members and the NIAAA staff as needed in the forms of phone calls, emails and face-to-face meetings.

2. The Administrative Core upheld its responsibility to update and maintain the CIFASD website. Latest news and upcoming events sections were updated on a monthly basis.

3. The Administrative Core facilitated the recruitment of new scientists and introductions to new technologies to the consortium through guest presentation invitations at the biannual meetings. These invitations were also extended to experts in fields of interest to members of the consortium.

    a. At the January 2010 meeting, Dr. So Hee Lee presented on women's drinking in Korea and Dr. Ken Warren discussed other potential international projects in FASD. Local Chapel Hill researchers also spoke at the meeting. Dr. John Gilmore presented on his work imaging early brain development in normal and high risk children, Dr. Clyde Hodge presented on neural mechanisms of alcohol-seeking behavior and Dr. Fulton Crews discussed adolescent brain development and underage drinking.
    b. For the upcoming January/February 2011 meeting, Drs. Hannah Kinney, an ad hoc member of the CIFASD, and Bill Fifer have been invited to attend and assist in brainstorming potential collaborations between PASS (Prenatal Alcohol, SIDS, and Stillbirth) and CIFASD. In addition, Dr. Peter Hammond from the Institute of Child Health, University College of London, UK, and his colleague Dr. Mike Suttie will be attending to share their findings on the CIFASD data they have been analyzing due to the funding provided by their Administrative Core subcontracts.

4. Dr. John Hannigan joined the Science Advisory Board for the Basic Science projects. In April 2010, Dr. Hannigan agreed to assist on the Clinical projects' SAB as well.

5. A review of current CIFASD projects was completed at the June 2010 Science Advisory Board meeting. The PI, Scientific Director and members of the Science Advisory Board discussed the performance and progress of the existing projects. Tables created by the Administrative Core using secured Google Docs spreadsheets to assist in keeping track of the monthly data collection progress on selected clinical projects were also used to assist in setting priorities. Graphs created to illustrate the actual progress versus the target for each research site were also provided to all attendees. These Google Docs are updated monthly and distributed to all on the clinical conference call. As collected data aren't useful to the CIFASD unless it is uploaded to the Central Repository, these data are also charted on the graphs to illustrate how many subjects per site have had their finalized data uploaded. See the latest progress reports at the end of this section and contact Jill if sign on information is needed for the actual Google Docs.

6. Through the oversight of the Administrative Core, the Informatics Core froze the Central Repository in early February 2010 so that cross-queries could be conducted for integrative analysis of the various data domains. It has been emphasized repeatedly to the projects that until the data are entered into the Central Repository, they are not counted as having been collected. Improvements continue to be made to the Dysmorphology and Demographics databases.

7. A special issue of *Alcohol* was edited by CIFASD's Dr. Charles Goodlett. The issue, Volume 44 Issue 7-8, published in November-December 2010 was entitled, "Special Issue on Fetal Alcohol Spectrum Disorders: Diagnosis and Intervention." This issue featured articles from CIFASD PIs Drs. Riley, Mattson, Foroud, Sulik, Zhou, Jones, Chambers, Barnett and their colleagues. Also contributing to this publication were Dr. Goodlett and SAB members Drs. John Hannigan and Dan Savage.

8. The Administrative Core PI has given several talks about the consortium educating the community and providing scientific outreach on behalf of the CIFASD. Dr. Riley represented and promoted the CIFASD at several international and national meetings over the last 12 months reporting on the projects and their progress. These presentations included reports to scientists and policy makers in Poland, Italy and South Korea. These sites are being considered for inclusion in the CIFASD through either administrative supplements or from their home countries or both. The Polish site will replicate, in part, the current Ukrainian project, and serve as a replication site for CIFASD. The South Korean site will assess FASD in an Asia population, something that has never been done on any scale. Ideas on how to get Italy involved in the CIFASD are still developing. In November of 2010, Dr. Riley attended the World Heath Organization WHO) meeting on Alcohol, Health and Development in The Netherlands. At this meeting, there was great enthusiasm for the work being completed by the CIFASD and interest in adopting our protocols and policies. Dr. Riley also attended international meetings in Italy (IMAG), The Netherlands (First European Converence on FASD) and France (ISBRA). On Thursday, March 3, 2011, the CIFASD (PIs: Riley, Jones, Sowell, Mattson, Chambers, and Tesche along with the CIFASD Scientific Director, Charness) will present at a plenary session at the 4th International Conference on Fetal Alcohol Spectrum Disorder entitled, "The Power of Knowledge: Integrating Research, Policy, and Promising Practice Around the World" in Vancouver, BC, Canada.

8:30am **PLENARY**

Remarks - Croatia
Giorgie Petkovic, MD, Pediatrician, Children´s Hospital Srebrnjak, Zagreb, Croatia

The Collaborative Initiative on FASD: An Overview of International Research
Moderator: **Sterling K Clarren,** MD, FAAP, CEO and Scientific Director, Canada Northwest FASD Research Network, Developmental Neuroscience & Child Health; Clinical Professor of Pediatrics, University of Washington School of Medicine; Clinical Professor of Pedatrics, Faculty of Medicine, University of British Columbia, Vancouver, BC, Canada

Introduction by **Edward P Riley,** PhD, Director, Centre for Behavioral Teratology, Department of Psychology, San Diego State University, San Diego, CA, USA

What We've Learned About the Clinical Phenotype of FAS Through CIFASD
Kenneth Lyons Jones, MD, Chief, Division of Dysmorphology/Teratology, Department of Pediatrics, University of California, San Diego, San Diego, CA, USA

Imaging the Brain, the Face, and Neurocognitive Function in Children with FASD
Elizabeth Sowell, PhD, Associate Professor, Developmental Cognitive Neuroimaging Laboratory, University of California Los Angeles, Los Angeles, CA, USA

Towards the Development of a Neurobehavioral Profile of FASD
Sarah N Mattson, PhD, Associate Director, Centre for Behavioral Teratology,

10:30am (Plenary Continued)
The Epidemiology of FASD and Update on Experience from a Longitudinal Study of Alcohol-Exposed Pregnancies in Ukraine
Christina Chambers, PhD, MPH, School of Medicine, Department of Pediatrics, University of California San Diego, La Jolla, CA, USA

Network Connectivity and Dynamics in FASD
Claudia D Tesche, PhD, Professor, Department of Psychology, University of New Mexico, Albuquerque, NM , USA

Diagnosis, Prevention, and Intervention in FASD: Insights from the CIFASD Laboratories
Michael Charness, MD, Chief of Staff, VA Boston Healthcare System; Professor of Neurology and Faculty Associate Dean, Harvard Medical School; Assistant Dean, Boston University School of Medicine, Boston, MA, USA

9. Dr. Michael Charness, the CIFASD Scientific Director, organized symposiums for CIFASD PIs to present their findings at the 2010 RSA meeting in San Antonio and at the ISBRA 2010 Congress in Paris. The RSA symposium is entitled, "Consortium Initiative on Fetal Alcohol Syndrome: From microRNA to Behavior," highlighted the research of Drs. Charness, Mattson, Miranda, Sowell and Zhou. The ISBRA symposium entitled, "Fetal Alcohol Spectrum Disorders: From Molecules to Man," and featured Drs. Charness, Sulik, and Thomas. In December 2010, Dr. Charness, with the assistance of CIFASD PI Dr. Tatiana Foroud, organized the submission of a CIFASD-focused symposium for the 2011 RSA meeting to be held in Atlanta, GA. Dr. Charness also moderated monthly conference calls as needed.

10. Dr. Jennifer Thomas, Administrative Specialist, maintained and updated the CIFASD.org website and assisted the PI in the facilitation of the monthly conference calls.

11. The Administrative Core developmental projects appointed in 2009 have data collection well underway. The current developmental projects are Dr. Rajesh Miranda's study microRNA biomarkers and Dr. Claudia Tesche's project on CNS biomarkers. Their second year subcontracts were executed in the fall of 2010. Drs. Jennifer Thomas and Andrew Hull's developmental projects came to a close at the end of July 2010. Their final progress reports follow this report.

12. The Administrative Core provided assistance and leadership to various CIFASD projects as needed. The continuation of an administrative supplement to Dr. Charness allowed for the completion of the project entitled, "Photolabeling of alcohol binding sites on L1." Dr. Riley spent considerable effort coming up with additional monies to improve the data collection efficiency at Dr. Phil May's UNM and Plains sites. ARRA funds from Dr. Sarah Mattson's CIFASD project provided support for coordinators at these sites, but that assistance alone wasn't enough. The carryover request approved on 1/26/10 authorized the allocation of additional monies to support additional psychometrist effort at these sites.

13. As is the nature of a U24, carryover of funds from one year to the next is not automatic. As developmental projects are facilitated under the Administrative Core, the PI has submitted carryover requests for these unobligated balances. In addition, the PI has requested that carryover funds be used to extend subcontracts to Dr. Peter Hammond and to potential new projects beginning in Poland and Korea. A carryover request was submitted on 12/22/10. Dr. Peter Hammond at the UCL Institute of Child Health in London, England received an administrative supplement for his project, "Dense surface models of facial dysmorphology in fetal alcohol," and for his contributions as a consultant on Drs. Sulik and Foroud's CIFASD U01 projects. For a few months prior to the formal April 2010 start date of his subcontracts, Dr. Hammond had been working unfunded with Dr. Sulik's lab on embryo MRI sequences. With the cooperation of Rob Lipinski, he has been determining optimal protocols for segmenting surfaces from MRI images of embryos. The initial results look very promising. Dr. Hammond's lab specializes in dense surface modeling (DSM) analysis and has previously established its efficacy in discriminating between the faces of controls and various genetic conditions where there is a facial gestalt. The same analytical approach combining DSM and pattern recognition techniques will be applied to ethnically matched groups of controls and FASD diagnosed children. Mike Suttie in Dr. Hammond's lab has been liaising with Drs. Foroud and Leah Wetherill in transferring 3D images. Preparatory landmarking of the images has begun.

14. Following discussions at the January 2010 meeting to increase the generation of integrative papers, Dr. Riley solicited a request to the group for ideas for the top five questions related to the collaborative data in the central repository. The goal was to identify the big problems that need to be addressed and ideas for their analysis utilizing multisite, multidisciplinary data to be published in the upcoming months. The following five question areas have been selected as the focus of the group:
    1. Dr. Ken Jones will select a relatively small subset of children (N = 15-20) with classic FAS features, who also have neuropsychological testing, brain imaging, and 3D facial imaging data and will conduct a clinical case report type of presentation. Input from Drs. Mattson, Foroud, and Sowell will be required.
    2. Dr. Sarah Mattson will conduct a multisite evaluation of the neuropsychological data to determine if a more concise profile can be identified than the one submitted for Phase 1 data publication. Behaviors found to be discriminating will be compared to dysmorphology data requiring input from Dr. Jones.
    3. Dr. Tatiana Foroud will apply the dysmorphology algorithm for diagnosing FAS (based on CIFASD criteria) to the 3D facial imaging data. The goal is to determine if the 3D data can be used to diagnose FASD in a similar (i.e., clinically-relevant) manner. Input from Dr. Jones regarding dysmorphology and animal data from Drs. Kathy Sulik and Feng Zhou will assist in meeting this aim.

4. Dr. Elizabeth Sowell will lead the group towards determining brain correlates of dysmorphology. Data will include dysmorphology and brain variables. This is a multisite study involving all sites with dysmorphology and brain imaging data and input from Drs. Jones and Sulik.
5. Drs. Mattson, Jones, Foroud and Sowell will be working towards answering the question of "What is the total spectrum of the effects of prenatal alcohol exposure?"

VI. Discussion:

The Administrative Core has helped to facilitate the goals of the consortium and the individual projects**.** The Administrative Core has assisted in the expansion of the consortium by funding developmental/ exploratory projects and carryover requests on existing projects. Additionally, CIFASD exposure has been generated by the many meetings and talks the PI has attended and provided. Through these endeavors, new contacts and collaborators for researchers within the CIFASD have been established.

VII. Interrelation with Aims of the Consortium and Other Projects:

By its very nature, the Administrative Core interrelates with each of the CIFASD projects.

VIII. Plans for the Next Year:

An ongoing focus of the Administrative Core is to ensure that the both the Basic Science and Clinical groups needs are met to execute their research goals, and to encourage and assist in coordinating collaborations between these research components. Additionally, it is the Administrative Core's responsibility to facilitate and oversee the progress of the developmental projects.

However, a top priority of the Administrative Core in the coming year is increase CIFASD's visibility within the alcohol research community and to get the multisite, multidisciplinary data being collected by the CIFASD projects into print in peer-reviewed publications. This goal will also be met through increased CIFASD presentations at scientific meetings.

In the upcoming year, the Administrative Core will also take the lead in developing a competitive renewal should an RFA be imminent.

IX. Publications (2010):

Administrative Core U24 AA014811

Books

Preece, P.M. and Riley, E.P. (Editors). Alcohol, drugs and medication in pregnancy: The Outcome for the Child. Clinic in Developmental Medicine #187. London: MacKeith Press, 2011.

Riley, E.P., Clarren, S., Weinberg, J., and Jonsson, E. (Editors) Fetal Alcohol Spectrum Disorder: Management and Policy Perspectives of FASD. Weinheim: Wiley-Blackwell, 2011.

Articles and Chapters

Barrow, M., and Riley, E.P. Diagnosis of the fetal alcohol syndrome: Emphasis on early detection. In Preece, P.M. and Riley, E.P. (Editors). Alcohol, drugs and medication in pregnancy: The Outcome for the Child. Clinic in Developmental Medicine #187. London: MacKeith Press, 2011, In press.

Kaminen-Ahola, N., Ahola, A., Maga, A.M., Mallitt, K.A., Fahey, P., Cox, T.C., Whitelaw, E. & Chong, S. Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. *PLoS Genetics*, 2010;6(1):e1000811. PMCID: PMC2797299

Keen, C. L., Uriu-Adams, J. Y., Skalny, A., Grabeklis, A., Grabeklis, S., Green, K., Yevtushok, L., Wertelecki, W. W. and Chambers, C. D. The plausibility of maternal nutritional status being a

contributing factor to the risk for fetal alcohol spectrum disorders: The potential influence of zinc status as an example. *BioFactors*, 2010;36:125-135. PMCID: PMC2927848

Mattson, S.N., Roesch, S.C., Fagerlund, Å., Autti-Rämö, I., Jones, K.L., May, P.A., Adnams, C.M., Konovalova, V., Riley, E.P., and the CIFASD. Toward a neurobehavioral profile of fetal alcohol spectrum disorders. *Alcoholism: Clinical and Experimental Research*, 2010;34:1640-1650. PMCID: PMC2946199

McGee, C.L., and Riley, E.P. The effects of prenatal alcohol exposure on brain and behavior. In Preece, P.M. and Riley, E.P. (Editors). Alcohol, drugs and medication in pregnancy: The Outcome for the Child. Clinic in Developmental Medicine #187. London: MacKeith Press, 2011, In press.

Nguyen, T.T., Coppens, J., and Riley, E.P. Prenatal alcohol exposure, FAS, and FASD: An introduction. In Riley, E.P., Clarren, S., Weinberg, J., and Jonsson, E. (Editors), Fetal Alcohol Spectrum Disorder: Management and Policy Perspectives of FASD. Weinheim: Wiley-Blackwell, 2011:1-13

Neurobehavioral Core U24AA014830 currently U01 AA014834
Mattson, S.N., Foroud, T., Sowell, E.R., Jones, K.L., Coles, C.D., Fagerlund, Å., Autti-Rämö, I., May, P.A., Adnams, C.M., Konovalovam, V., Wetherill, L., Arenson, A.D., Barnett, W.K., and Riley, E.P. Collaborative Initiative on Fetal Alcohol Spectrum Disorders: Methodology of Clinical Projects. *Alcohol*, 2010;44:635-641. PMCID: PMC2888656

Neurobehavioral (U24AA014830) and Pilot (U24AA014828) Cores
Klingenberg, C.P., Wetherill, L., Rogers, J., Moore, E., Ward, R., Autti-Rämö, I., Fagerlund, A., Jacobson, S.W., Robinson, L.K., Hoyme, H.E., Mattson, S.N., Li, T.K., Riley, E.P., Foroud, T. and the CIFASD Consortium. Prenatal alcohol exposure alters the patterns of facial asymmetry. *Alcohol*, 2010;44:649-657. PMCID: PMC2891212

X. Posters and Presentations (2010):
All presentations by the Administrative Core PI, Dr. Ed Riley.

"Effects of Alcohol on Brain Development: Understanding and Addressing the Impairments," presented at the 25th National Training Institute (NTI) Zero to Three conference, Phoenix, AZ, December 2010.

"The Foetal Brain & Alcohol – Defining Foetal Alcohol Spectrum Disorder (FASD). Including a Look at Bimanual Coordination and Interventions," presented at The Adolescent and Children's Trust's (TACT) Bruised Before Birth Meeting, Birmingham, England, November 2010.

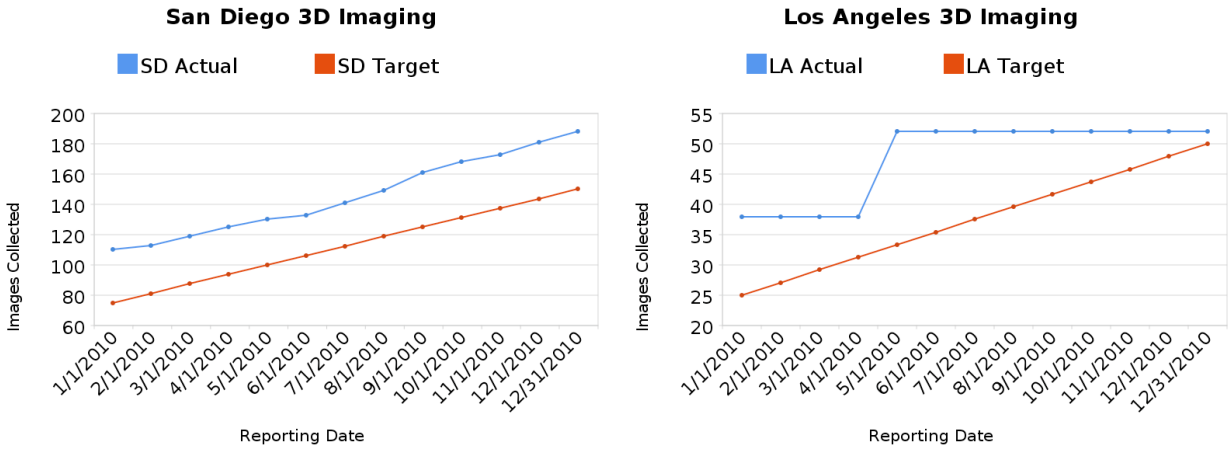
"Findings from CIFASD," presented at the International Workshop for Research on Fetal Alcohol Spectrum Disorder, Seoul, Korea, August 2010.

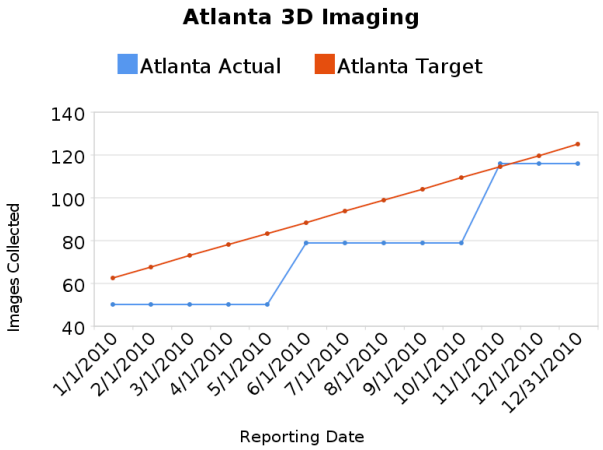
"Interagency Coordinating Committee on Fetal Alcohol Spectrum Disorders ARND Workgroup and FASD in the DSM-V," presented at the SAMHSA FASD Center for Excellence FASD Field Trainers Update Meeting, Orlando, FL, July 2010.

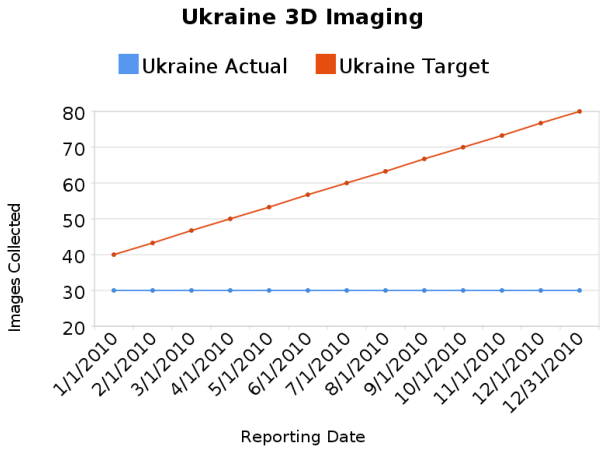
"International FASD Research Update," presented at the International Fetal Alcohol Spectrum Disorders (FASD) Conference: A Framework for FASD Diagnosis and Services, Sault Ste Marie, MI, April 2010.

"The Effects of Prenatal Alcohol Use on the Developing Brain," presented at the International Fetal Alcohol Spectrum Disorders (FASD) Conference: A Framework for FASD Diagnosis and Services, Sault Ste Marie, MI, April 2010.

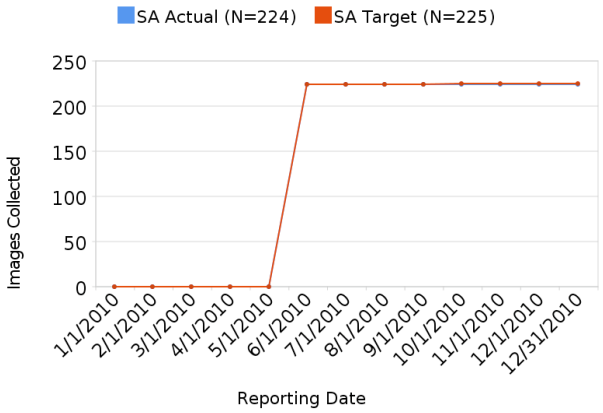
Foroud 3D Imaging Progress as of 12/31/10:

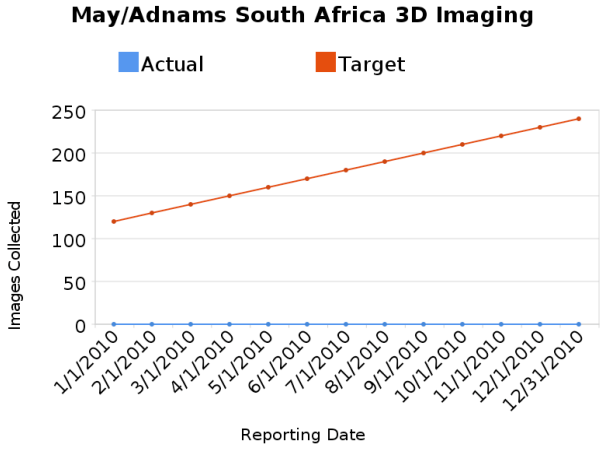


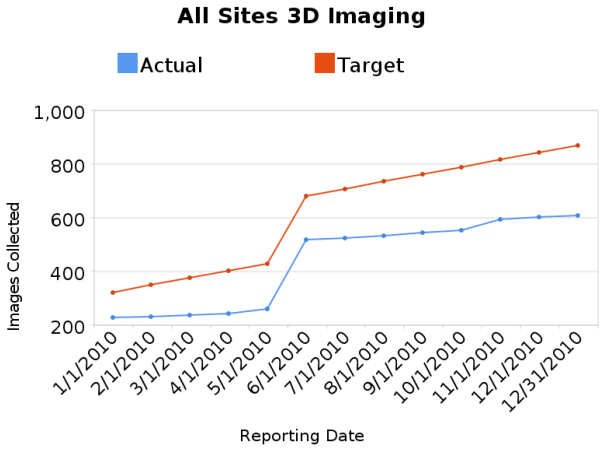


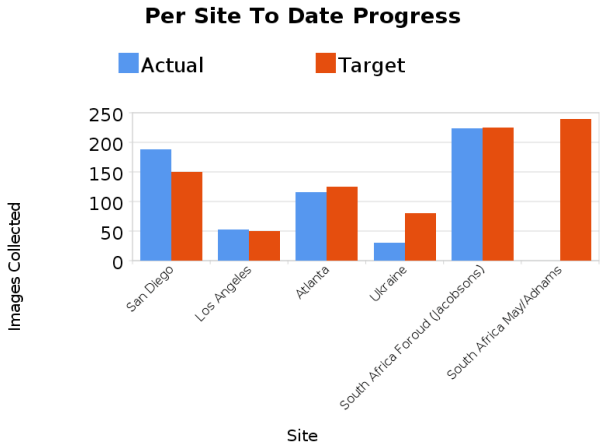


Foroud (Jacobsons) South Africa 3D Imaging (Lines are merged) Data is STATIC.





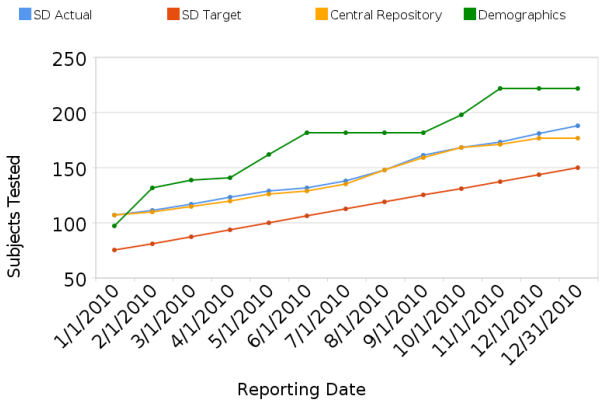




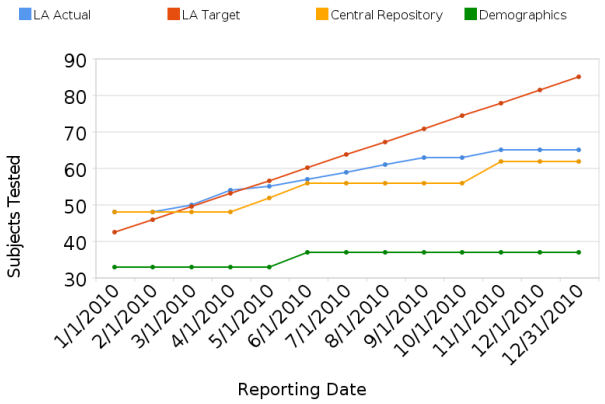
Note: Targets represent project end goals to be achieved by 12/31/10.

Mattson Neurobehavioral Progress as of 12/31/10:

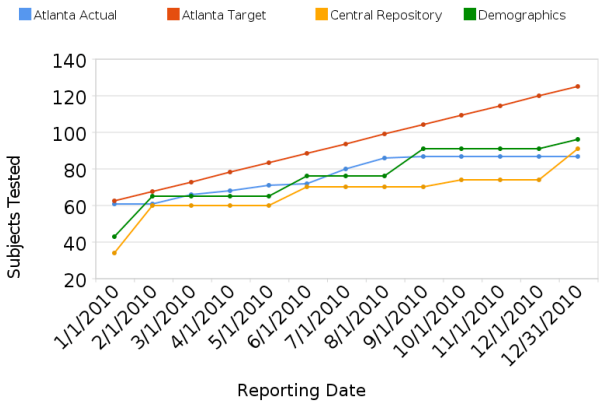
San Diego Neurobehavioral



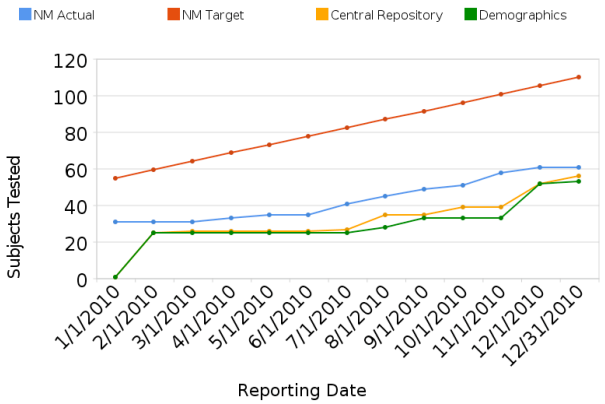
Los Angeles Neurobehavioral



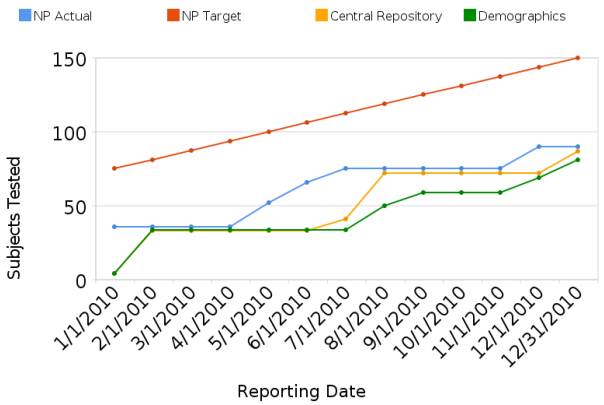
Atlanta Neurobehavioral



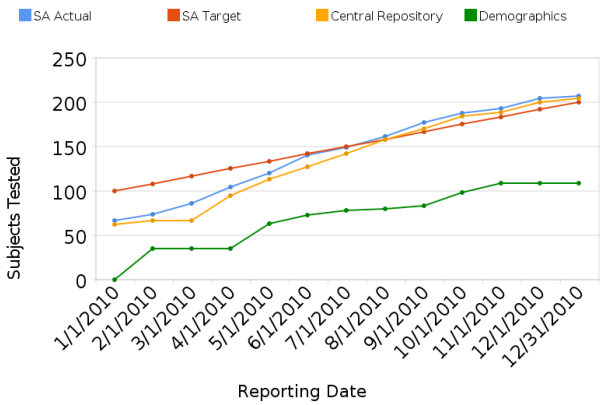
New Mexico Neurobehavioral



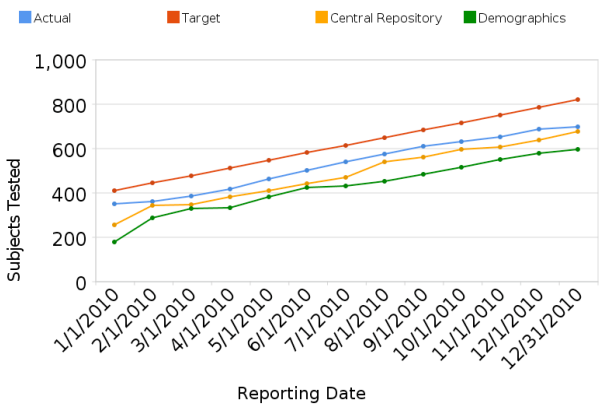
Northern Plains Neurobehavioral



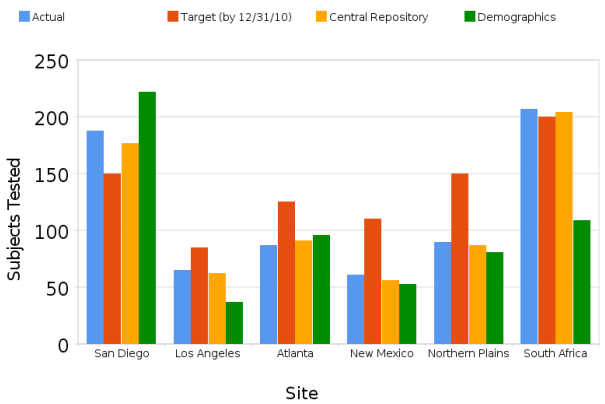
South Africa Neurobehavioral



All Sites Neurobehavioral

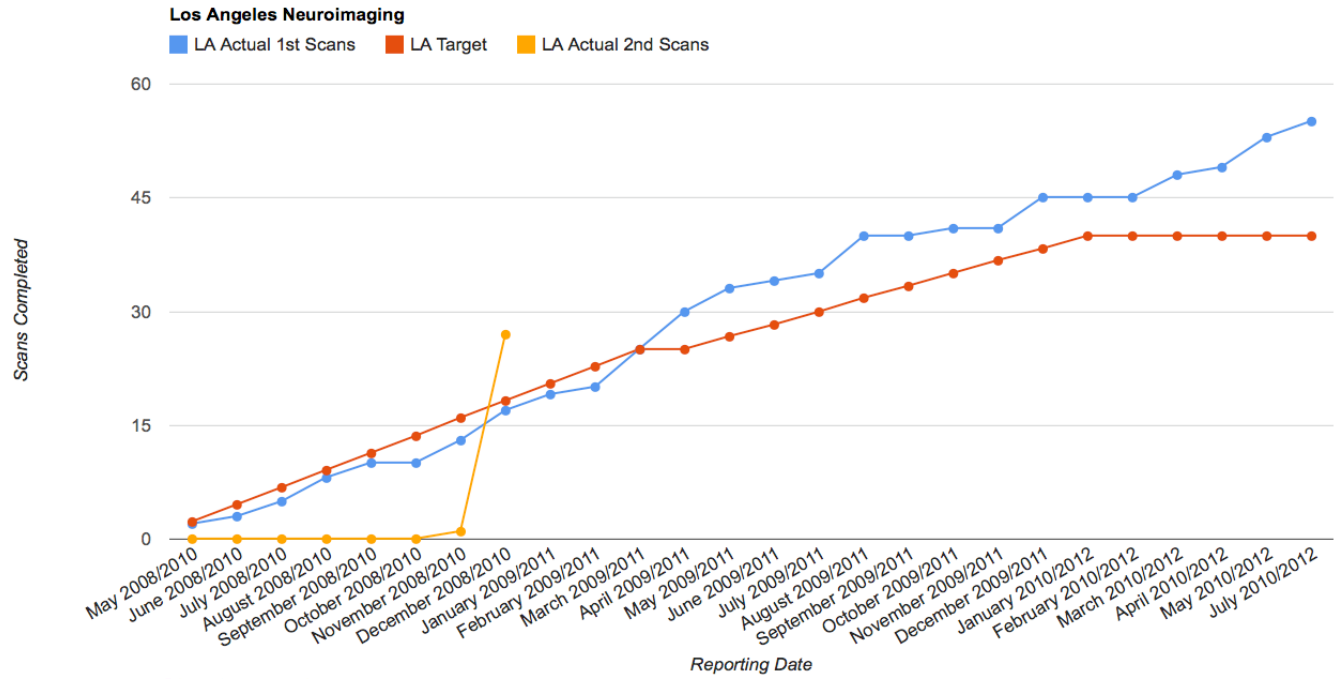


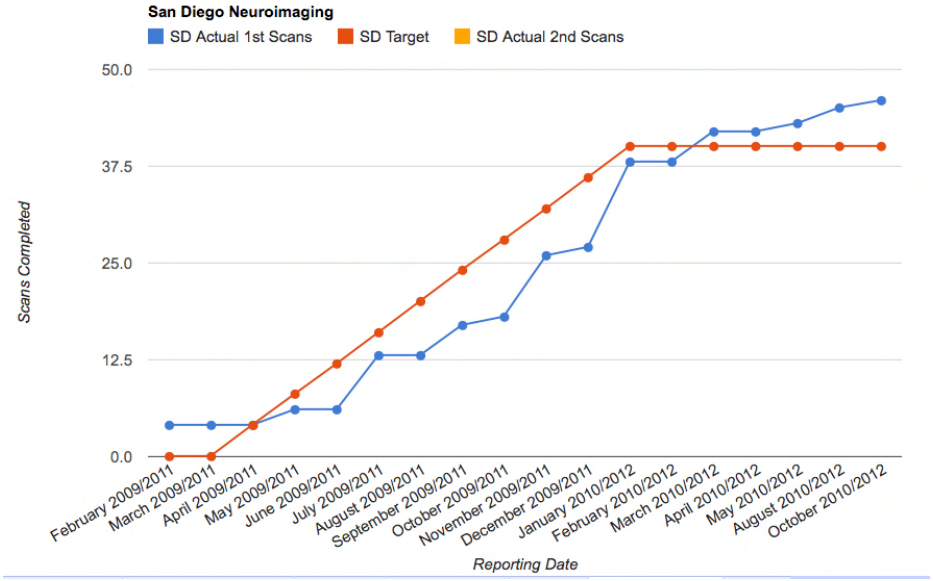
Per Site To Date Progress

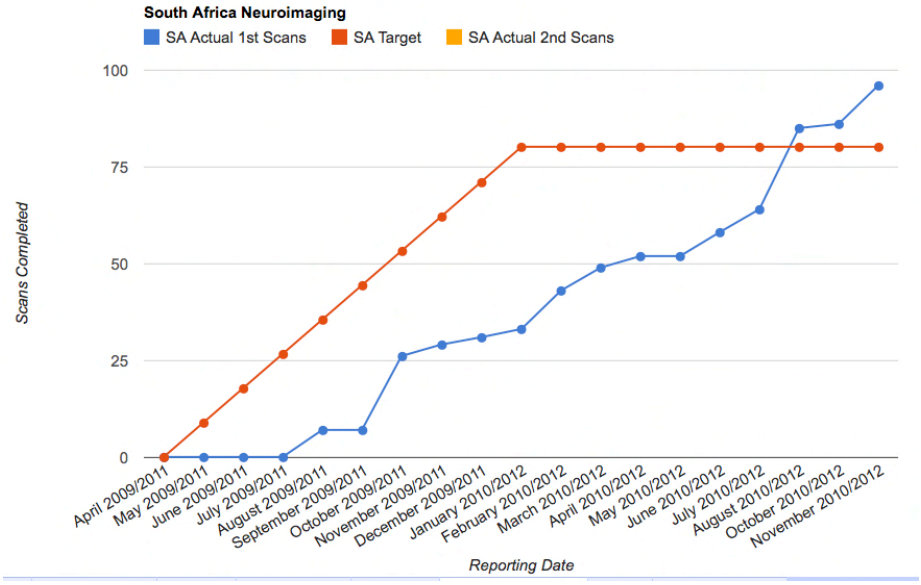


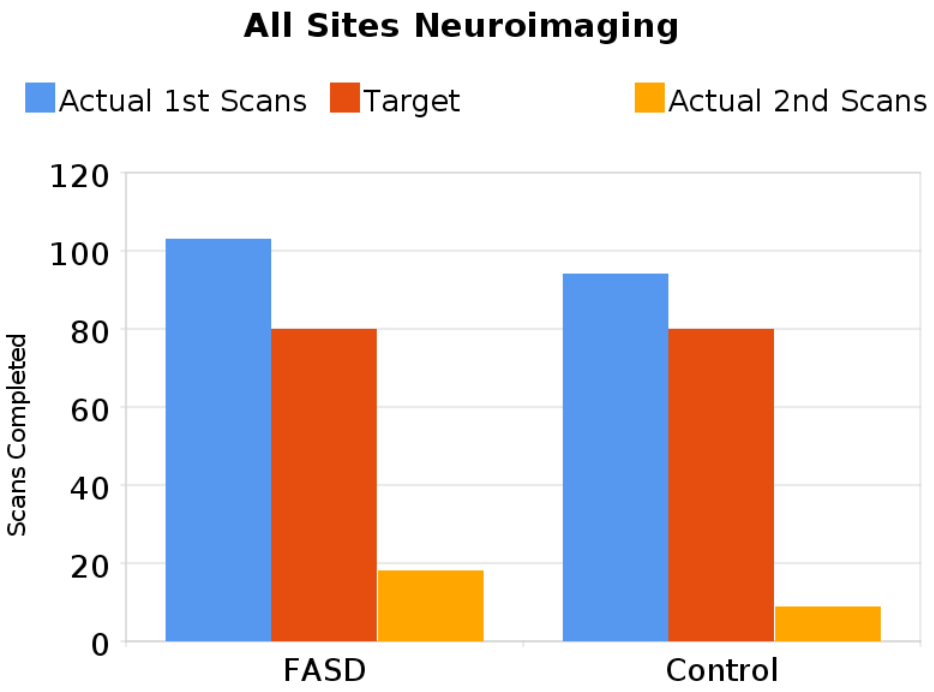
Note: Targets represent project end goals to be achieved by 12/31/10. When the Actual (blue), Central Repository (orange), and/or Demographics (blue) numbers are the same, only one line will be present to represent both/all.

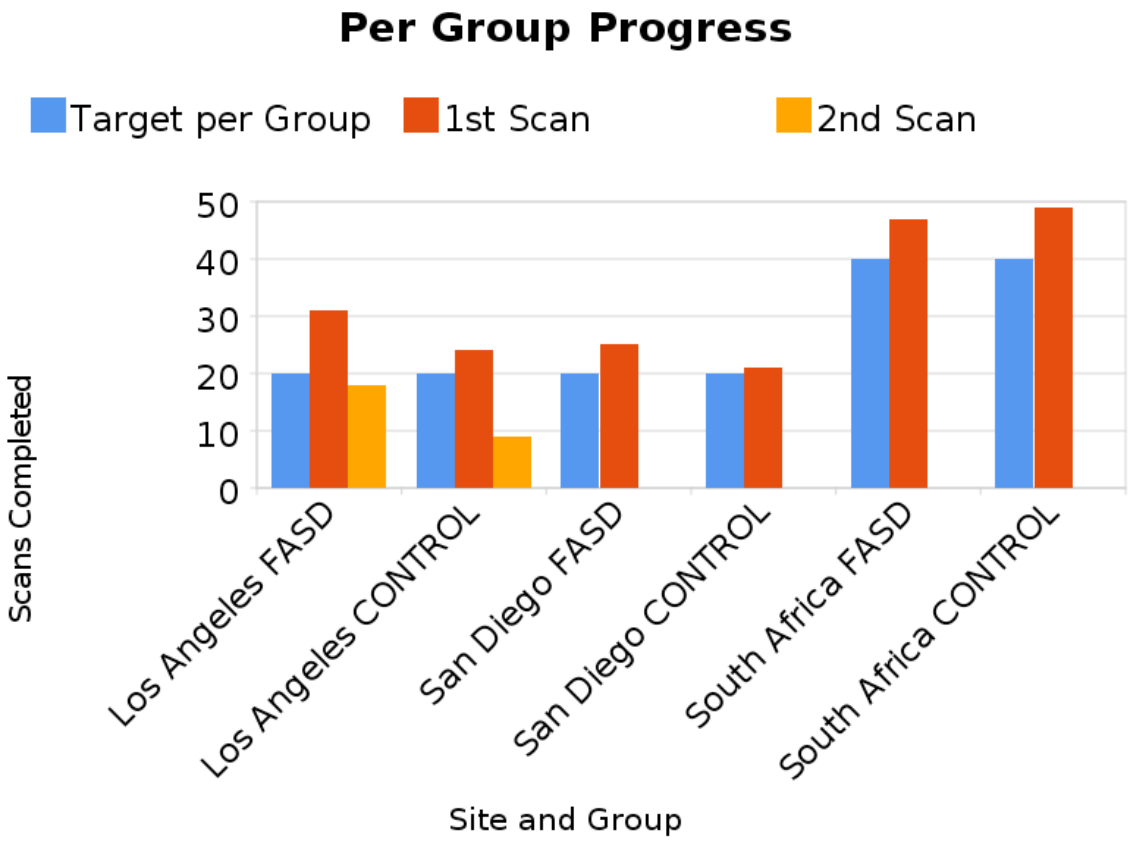
Sowell Neuroimaging Progress as of 12/31/10:











Note: The 12/31/09 goal for this project is to have all new (first) scans completed. 2nd scans are to be completed 2 years after the initial scan.

I. Principal Investigator: Jennifer D. Thomas, Ph.D.

II. Title of Project: Choline Availability and FASD, Developmental Project U24 AA014811

III. Objectives:

Choline is an essential nutrient, known to influence CNS development. Studies in otherwise typically developing rats indicate that perinatal choline deficiency impairs brain development whereas perinatal choline supplementation can alter CNS structural, neurochemical and electrophysiological development to produce long-lasting improvements in cognitive functioning. We have previously shown that perinatal choline supplementation can reduce the severity of some fetal alcohol effects, suggesting that choline levels can modulate ethanol's teratogenic effects. This developmental project examines how choline availability influences ethanol's teratogenic effects.

The Specific Aims are as follows:

1. Determine whether choline supplementation during late gestation will mitigate ethanol's teratogenic effects.

2. Determine whether choline deficiency exacerbates ethanol's teratogenic effects.

IV. Methods:

Specific Aim 1: We examined the effects of choline supplementation initiated during late gestation among alcohol-exposed subjects. Pregnant Sprague-Dawley rats were exposed to 6.0 g/kg/day ethanol from gestational days (GD) 5-20 and pups were exposed to 5.25 g/kg/day from postnatal days (PD) 2-9, to model alcohol exposure throughout gestation (all three trimesters). Pair-fed and ad lib lab chow controls were included. Following birth, ethanol-exposed subjects were treated with various doses of choline from PD 1-21 and tested on a series of behavioral tasks.

Specific Aim 2: To determine whether choline deficiency exacerbates ethanol's teratogenic effects, dams were fed a diet containing 40, 70, or 100% recommended levels of choline, levels that are consistent with those reported in epidemiological studies and are, thus, clinically relevant. Pregnant dams were intubated with 6.0 g/kg/day ethanol from GD 5-20. Pair-fed and ad lib lab chow controls were also included. Physical and behavioral development was examined in the offspring. Secondly, to determine if alcohol itself reduces choline metabolism and utilization, we collected brain lever and blood from pregnant dams (ethanol-exposed, pair-fed and lab chow controls) and fetuses, sending the tissue to the laboratory of Dr. Carl Keen for analyses of choline and associated metabolites. They have analyzed maternal and fetal samples for choline, and two of its metabolites, betaine and dimethylglycine, using a stable isotope dilution LC-MS/MS method in both the plasma and liver. Deuterated standards of analytes are used for quantification ("spiking solution"). Briefly, 5 µL of spiking solution are added to 40 µL of plasma followed by protein precipitation by addition of acetonitrile. After centrifugation, the clear supernatant fraction is analyzed using LC-MS/MS. Finally, to determine if there are interactions between prenatal alcohol exposure and dietary choline levels, we generated subjects exposed to 6.0 g/kg/day ethanol from GD 5-20 and fed a diet containing 40, 70 or 100% recommended levels of choline (in a 3 (ethanol, pair-fed, ad lib) x 3 (40, 70, 100%) design. Those tissues are generated and awaiting analysis.

V. Accomplishments and Results:

Specific Aim 1: We have found that administration of choline at the same time as prenatal alcohol exposure (GD 5-20) reduces the severity of a variety of alcohol-related alterations in physical and behavioral development. When alcohol exposure is extended through all three trimesters and choline supplementation is limited to PD 1-21, it does not reduce the severity of overactivity in the open field, but it does reduce the severity of Morris maze deficits and alterations in spontaneous alternation (Figures 1-2).

Specific Aim 2: We illustrated that the combination of prenatal alcohol exposure and 40% choline diet produced the most severe alterations in physical and behavioral development, even when neither prenatal alcohol nor suboptimal choline levels had effects by themselves (Figs. 3-5).

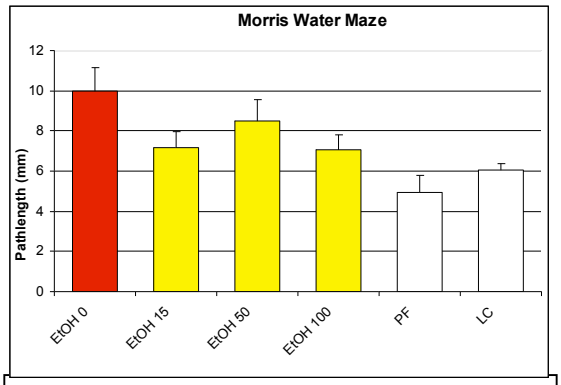


Figure 1. Ethanol exposure during all 3 trimesters significantly impaired spatial learning performance among males, an effect attenuated with 15 and 100 mg/kg choline supplementation.

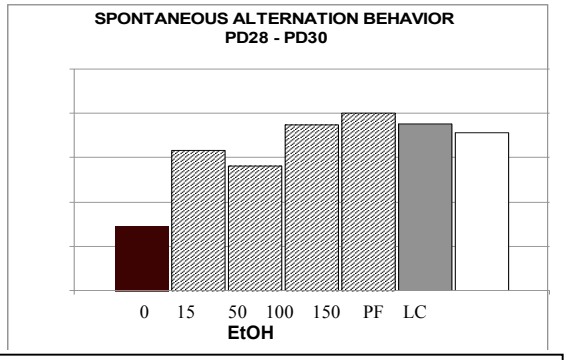


Figure 2. Ethanol delays development of spontaneous alternation, a behavior that depends on cholinergic development, an effect normalized by choline.

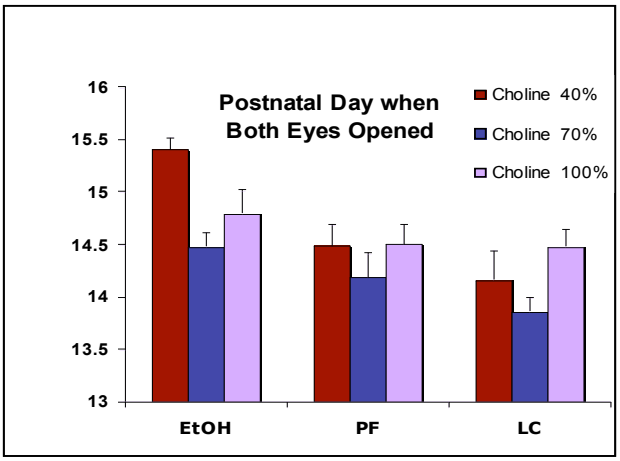


Figure 3. The combination of choline deficiency (40% recommended levels) and prenatal alcohol delayed eye opening.

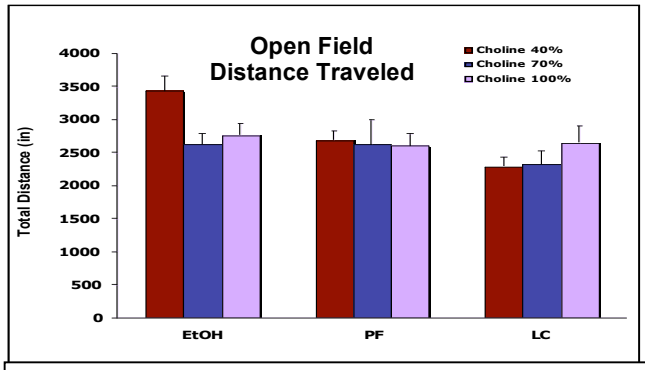


Figure 4. The combination of choline deficiency and prenatal alcohol exposure produce significant increases in open field activity levels.

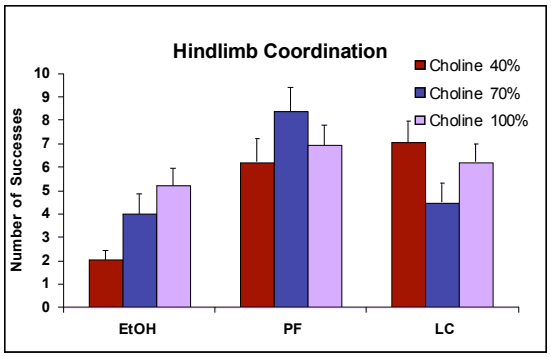
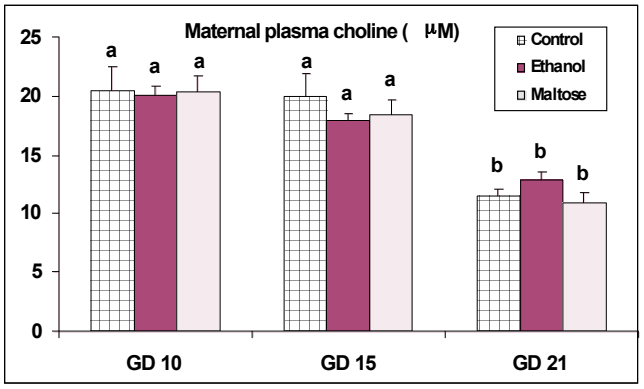


Figure 5. The combination of choline deficiency and prenatal alcohol exposure significantly impaired hindlimb coordination.

We also found that, consistent with clinical data from Dr. Chambers' study, prenatal alcohol exposure did not affect maternal plasma choline levels (nor did it affect fetal plasma choline levels independent of nutritional effects). However, prenatal alcohol exposure did lead to a transient reduction in maternal plasma dimethylglycine, which suggests that prenatal alcohol may interfere with various choline metabolic pathways (Figs. 6-9).



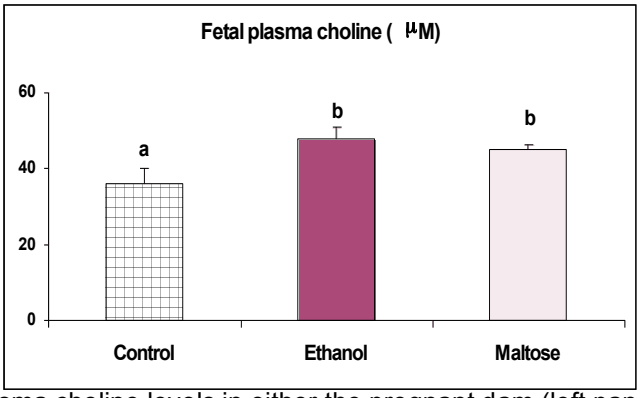


Figure 6. Prenatal alcohol did not significantly affect plasma choline levels in either the pregnant dam (left panel) or the fetuses (right panel), although reduced nutritional intake led to an increase in fetal plasma choline. Fetal plasma choline levels (pooled within a litter) were approximately 3-4 times higher than maternal plasma choline concentrations at the end of gestation indicating increased uptake of choline across the placenta.

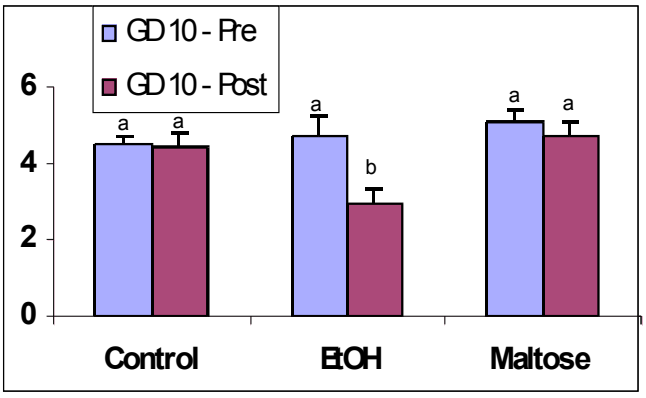


Fig 7. Maternal plasma DMG (µM) on GD 10, before and after saline, ethanol or maltose gavage.

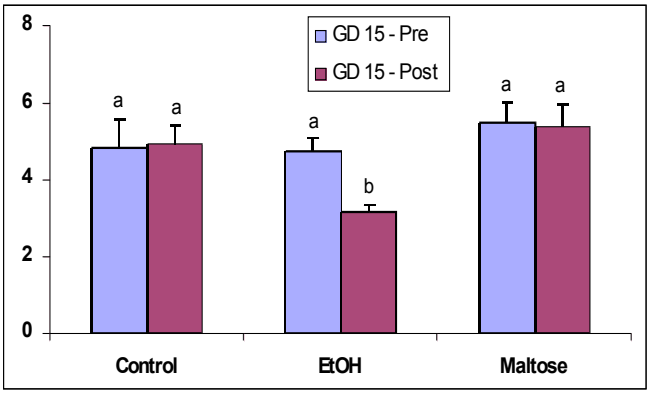


Fig 8. Maternal plasma DMG (µM) on GD 15, before and after saline, ethanol or maltose gavage.

Altered DMG concentrations may be related to alcohol-related changes in betaine levels, although the effects of alcohol on maternal plasma betaine depended on the developmental age and may also be related to changes in nutritional state (Figs 9-10). At the time of parturition,

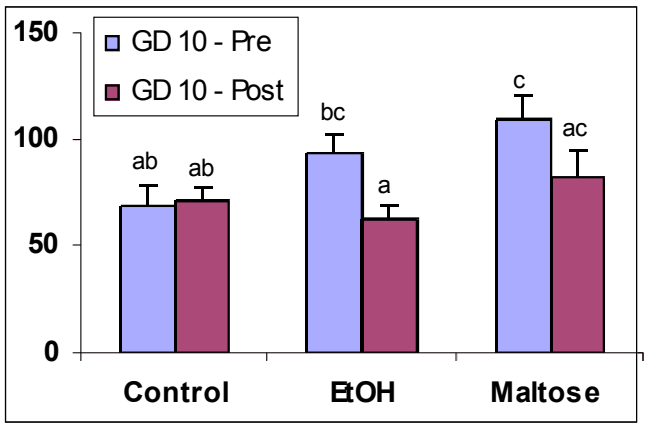


Fig 9 Maternal plasma betaine (μM) on GD 10, before and after saline, ethanol or maltose gavage.

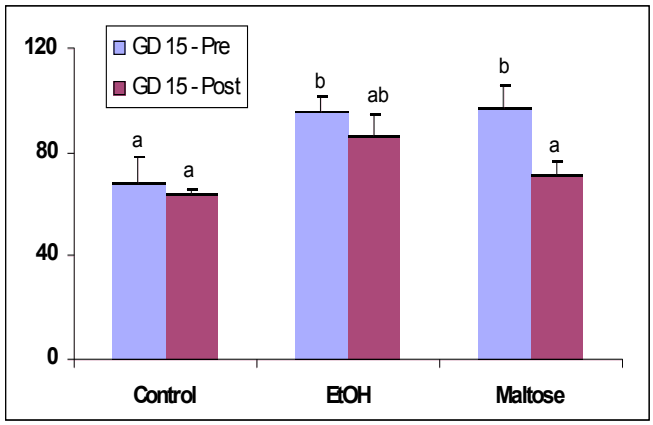


Fig 10. Maternal plasma betaine (μM) on GD 15, before and after saline, ethanol or maltose gavage.

betaine levels were reduced among the ethanol-exposed group compared to both control groups, although there were no differences in fetal plasma betaine concentrations among the three groups. Low betaine status has been shown to increase homocysteine concentrations in humans.

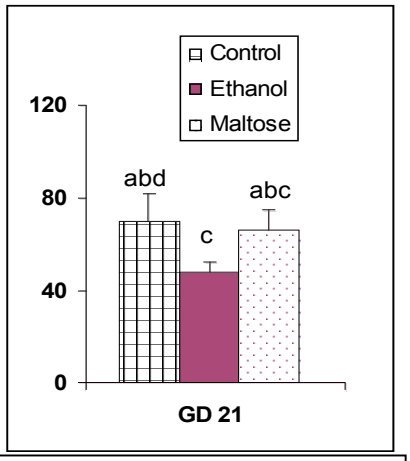
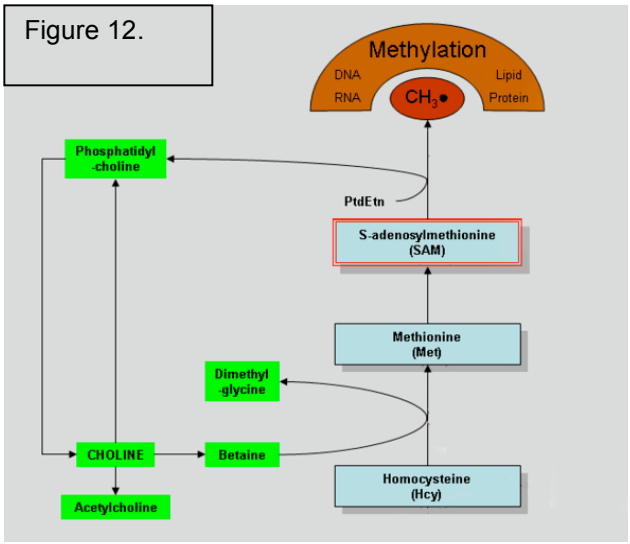


Figure 11. Prenatal alcohol exposure reduced maternal plasma betaine levels.

Figure 12.



Prenatal alcohol exposure tended to reduce levels of choline and dimethylglycine in the dam's liver and significantly reduced betaine levels (Fig. 13). However, the levels of choline and metabolites were not altered in the fetuses (Fig. 14). The reduction of choline stores in the dam's liver could reduce mobilization of choline postnatally, during suckling.

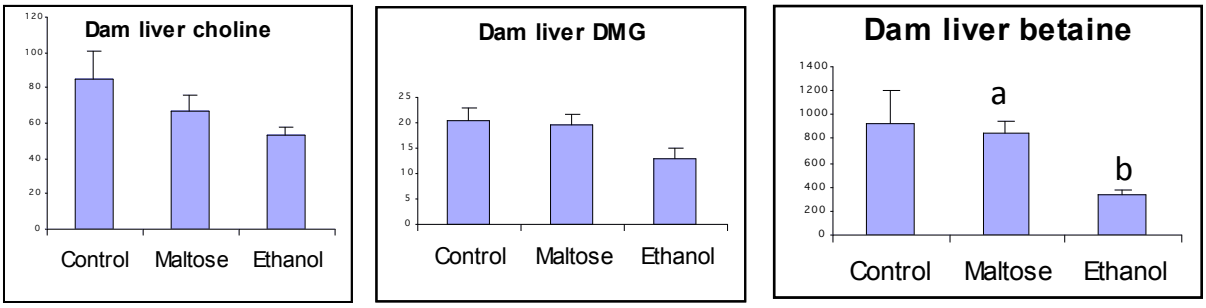


Figure 13. Levels of choline and metabolites in the dam's liver at GD 21.

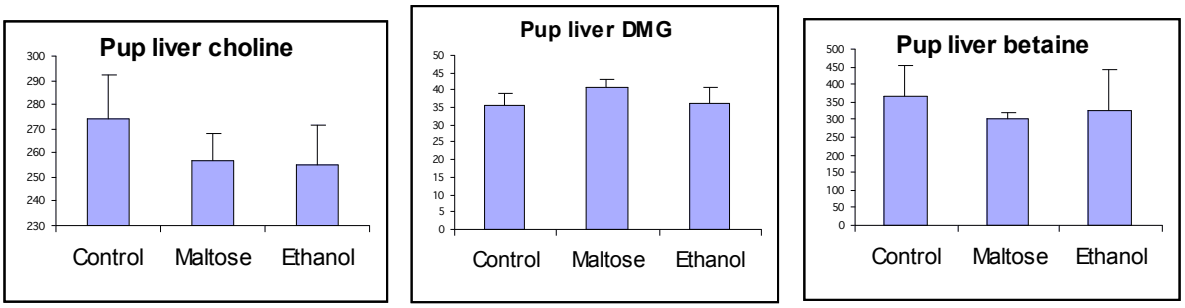


Figure 14. Levels of choline and metabolites in fetal liver at GD 21.

C. Significance

These data suggest that prenatal alcohol exposure does not lead to reductions in choline, indicating that choline is an effective treatment for fetal alcohol spectrum disorders even if it is not compensating for a choline deficiency. However, it is possible that choline metabolism is affected, particularly during acute periods of alcohol intoxication. Unfortunately, these measures are more difficult to determine with clinical populations and acute, transient changes in choline metabolism associated with alcohol exposure may not be as easily detected. Changes in choline metabolism could alter development, particularly by altering homocysteine levels and DNA methylation. Determining the levels of choline and metabolites in fetal brain tissue will address the question of how choline is distributed during prenatal alcohol exposure.

The data illustrating that the combination of prenatal alcohol and reduced dietary choline intake produces the most severe alterations in physical and behavioral development are consistent with the above findings. These data suggest that even in the absence of effects of either alcohol or suboptimal dietary choline levels by themselves, the combination is damaging. It is possible that the prenatally alcohol exposed subject is less able to modulate choline metabolism in the context of a diet low in choline and data from our final study which examines the combination of prenatal alcohol and varying dietary choline levels on choline and choline metabolites will be important addressing this issue. These findings have important implications for the understanding of how nutritional factors contribute to and protect against fetal alcohol spectrum disorders.

VI. Discussion:

Findings from Specific Aim #1 indicate that the dose levels of choline supplementation being used in Chambers' clinical study are adequate for reducing some fetal alcohol effects. Findings from Specific Aim #2 illustrate that nutritional factors influence alcohol's teratogenic effects.

First, our data indicate that suboptimal choline can exacerbate ethanol's teratogenic effects. Secondly, plasma levels from the dams and fetuses indicate that prenatal alcohol does not induce a choline deficiency. Of interest, ethanol acutely does reduce dimethylglycine, which could impact development. These data indicate that the timing of blood/tissue collection in relation to alcohol exposure may be important.

VII. Interrelation with Aims of the Consortium and Other Projects:

Specific Aim #1 examines the effects of choline supplementation during a period of development in the rat that models late gestation in humans, with the goal of informing the sheep studies of Cudd (U01 Translational Studies of FASD Using a Sheep Model) and the clinical nutritional intervention study proposed by Chambers (U01 Spectrum of and Nutritional Risk Factors for FASD in Russia and Ukraine), both of which include choline supplementation. Specific Aim #2 examines whether prenatal alcohol and/or choline deficiency exacerbates ethanol's teratogenic effects, complementing Chambers' clinical U01, which examines blood choline levels in the clinical population. Notably, they have not found any difference in choline or choline metabolite levels in maternal plasma associated with prenatal alcohol exposure. However, the animal data would suggest that data from the clinical study may not detect transient changes in choline metabolism.

VIII. Plans for the Next Year:

We hope to have the data on choline levels and metabolites from the study examining the effects of prenatal alcohol and varying dietary choline levels and complete manuscript publication.

IX. Publications (2010):

Thomas, J.D., Abou, E.J. & Dominguez, H.D. (2009). Prenatal choline supplementation mitigates the adverse effects of prenatal alcohol exposure on development in rats. *Neurotoxicology and Teratology*, 31, 303-311.

Thomas, J.D., Idrus, N.M., Monk, B.R., & Dominguez, H.D. (2010) Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats. *Birth Defects Research*, 88, 827-837.

Idrus, N.M & Thomas, J.D. Fetal alcohol spectrum disorders: Experimental therapeutics and strategies for intervention. *Alcohol Research and Health*, In press.

X. Posters and Presentations (2010):

Thomas, J.D. Evidence-based Treatments for Fetal Alcohol Spectrum Disorders, International Conference on FASD, Ste Sault-Marie, MI, April 2010.

Thomas, J.D. Choline availability and fetal alcohol spectrum disorders: Nutritional risk factors and potential treatments. *Alcoholism: Clinical and Experimental Research,* 34(6): S194; Research Society on Alcoholism, San Diego, CA, June 2010.

Thomas, J.D., Idrus, N. & Riley, E.P. Choline Availability and Fetal Alcohol Spectrum Disorders. International Society of Biomedical Research on Alcoholism, Paris, September 2010.

Thomas, J.D. Nutritional Interventions to Fetal Alcohol Spectrum Disorders. International FASD Conference, Atlanta, October 2010.

Nguyen, T.T., Chambers, C., Keen, C.L. & Thomas, J.D. The effects of prenatal alcohol exposure on choline metabolism: Implications for the role of choline in moderating FASD. Research Society on Alcoholism, June 2011.

I. Principal Investigator: Andrew D. Hull, M.D.

II. Title of Project: Prenatal Ultrasound and the Early Detection of FASD, Developmental Project U24 AA014811

III. Objectives:

This project performed in Ukraine examined the use of prenatal ultrasound in the assessment of fetuses exposed to alcohol in utero.

Specific Aim #1
To examine the utility of specific ultrasound measurements of fetal brain in the detection of fetuses who will go on to demonstrate features of FASD.

1. To correlate these ultrasound measures with postnatal assessment of growth and structural features as assessed by a standardized dysmorphological examination.
2. To correlate these ultrasound measures with altered neurobehavioral assessment at 6 & 12 months.
3. To assess the dose response relationship between these ultrasound measures and the timing and quantity of alcohol ingested prenatally.
4. To determine the best cut points and gestational age timing for ultrasound measures that produce the most sensitive and specific set of markers predicting 1 - 3 above and the set with the highest positive predictive value.

Specific Aim #2
To assess the role of second and third trimester ultrasound assessment of biophysical profile (BPP) and startle response in the detection of fetuses who will go on to demonstrate features of FASD.

1. To correlate BPP and startle responses with postnatal assessment of growth and structural features as assessed by a standardized dysmorphological examination
2. To correlate BPP and startle responses with altered neurobehavioral assessment at 6 & 12 months
3. To assess the dose response relationship between BPP and startle responses and the timing and quantity of alcohol ingested prenatally
4. To determine the best cut points and gestational age timing for BPP and startle responses that produce the most sensitive and specific set of markers predicting A - C above and the set with the highest positive predictive value.
5. To correlate BPP and startle responses with the ultrasound measures of brain growth in **Specific Aim 1** above.

IV. Methods:

The project continued the preliminary prospective cohort study design at one performance site – the Rivne Oblast in Ukraine. A second site, the Khmelnitsky Perinatal Center was added in 2010. Subject screening, selection recruitment, interviews and record review continued as previously established in the first phase of this project. Serial ultrasound measurements were performed at both sites utilizing previously trained ultrasonographers. Subjects were recruited from the cohort of individuals identified as exposed with an equal number of controls. Women in the exposed and control groups

participated in standard ultrasound measures in each trimester conducted by specially trained technicians. DVD recordings of all ultrasound examinations have been archived. A total of 310 women have been recruited to date in addition to those recruited in the pilot project.

Each of the three ultrasound examinations included routine measurements of fetal head circumference, biparietal diameter, abdominal circumference and femur length (BPD,HC,AC,FL). An estimated fetal weight (EFW) was calculated using a standard method according to Hadlock. A detailed fetal anatomic survey was performed at the initial study and targeted anatomic imaging at subsequent follow up studies. All systems were evaluated according to AIUM guidelines for targeted obstetric imaging. Particular attention was paid to evaluation of CNS and cardiac anatomy. All anomalies were noted and recorded. Additional assessments of CNS anatomy and biometry were made to include fronto-thalamic distance (FTD), caval-calvarial distance (CCD), transverse cerebellar diameter (TCD) and orbital measurements.

Biophysical Profile and Startle responses were also obtained. Study sonographers were taught how to obtain the BPP and use the standard scoring system. Spontaneous and evoked startle responses were recorded .

Live born infants received the standard CIFASD dysmorphological examination at either site, and all infants receive a neurobehavioral evaluation using the BSID II at 6 and 12 months of age.

V. Accomplishments and Results:

A total of 845 ultrasounds have been performed and entered into the database. Ultrasound data continues to be obtained from new subjects at both sites. Since the pilot study did not include a micronutrient intervention the pilot data are not included in this first pass analysis.

310 subjects had data available for analysis for the second trimester studies (Table 1). There were 157 exposed and 153 unexposed subjects. Preliminary analysis suggests significant or borderline significant effects of alcohol as marked with *

245 subjects so far have data available in the third trimester suitable for analysis (Table 2). There were 129 exposed and 116 unexposed subjects. Preliminary analysis suggests significant or borderline significant effects of alcohol as marked with *

Biophysical Profile (BPP) and Startle Response data are shown (Table 3). 216 subjects are currently available for analysis. There are no differences in BPP score or time taken to acquire BPP between exposed and unexposed groups at any GA. In the third trimester fewer evoked startles occurred in the exposed group whilst spontaneous startles occurred more frequently.

Table 1. Second trimester ultrasound brain and somatic measures by Alcohol Group, Intervention Group, and alcohol*intervention interaction

Measure*	Alcohol Group P - value	Micronutrient Intervention Group P-value	Alcohol*Intervention Interaction P-value
TCD	0.613	0.007*	0.994
OFD	0.498	0.851	0.101*
CCD	0.873	0.547	0.462
FTD	0.194	0.092*	0.843
OOD	0.300	0.740	0.495
IOD	0.936	0.513	0.169
OD	0.094*	0.854	0.586
BPD	0.602	0.386	0.438
HC	0.904	0.382	0.098*
AC	0.698	0.533	0.085*
FL	0.735	0.587	0.140
TL	0.953	0.335	0.538
HL	0.930	0.799	0.110

*brain measures adjusted for gestational age at scan

Table 2. Third trimester ultrasound selected brain and somatic measures by Alcohol Group, Intervention Group, and alcohol*intervention interaction

Measure*	**Alcohol Group P - value**	**Micronutrient Intervention Group P-value**	**Alcohol*Intervention Interaction P-value**
TCD	0.417	0.985	0.143
OFD	0.070*	0.375	0.874
CCD	0.537	0.551	0.162
FTD	0.056*	0.296	0.922
OOD	0.612	0.793	0.278
IOD	0.778	0.611	0.188
OD	0.641	0.914	0.017*
BPD	0.547	0.255	0.620
HC	0.539	0.201	0.608
AC	0.884	0.491	0.002*
FL	0.640	0.371	0.407
TL	0.751	0.703	0.056*
HL	0.702	0.869	0.238

*brain measures adjusted for gestational age at scan

Table 3. BPP and startle responses in exposed at unexposed subjects in second trimester and two timepoints in third trimester

Measure	**<24 weeks GA Mean 20.8wk) E 31 UE 27**	**≥24 weeks GA (Mean 31.1wk) E 67 UE 52**	**<24 weeks GA (Mean 35.5wk) E 19 UE 20**
Time to obtain BPP (min) mean ±SD Exposed Unexposed	17.58 ± 2.53) 17.70 ± 1.82) p = 0.834	18.48 ± 2.99) 18.38 ± 2.62) p = 0.859	18.32 ± 2.21) 18.55 ± 2.21) p = 0.743
BPP Score mean ±SD Exposed Unexposed	7.68 ± 0.91) 7.85 ± 0.53) p = 0.370	7.97 ± 0.24) 7.88 ± 0.62) p = 0.343	7.89 ± 0.46) 7.80 ± 0.62) p = 0.591
Evoked Startles Exposed Unexposed	32.3% 51.9% p = 0.131	52.2% 61.5% p = 0.310	36.8% 45.0% p = 0.605
Spontaneous Startles mean ±SD Exposed Unexposed	4.48 ± 1.81) 4.37 ± 1.50) p = 0.797	3.46 ± 1.64) 3.19 ± 1.64) p = 0.403	3.47 ± 1.22) 2.90 ± 1.59) p = 0.215

VI. Discussion:

Clearly data analysis is in the preliminary stages as far as almost all the data points are concerned. Subjects continue to be recruited and as current subjects progress through their pregnancies further data becomes available.

As yet no comparison of ultrasound findings and postnatal evaluation have been completed although some comparisons with postnatal neurobehavioral studies are in the early stages. Ultrasound findings have yet to be correlated with postnatal dysmorphology results.

VII. Interrelation with Aims of the Consortium and Other Projects:

The PI has continued to work closely with the Nutritional Risk Factor Study, the Dysmorphology core and the postnatal neurodevelopmental study all of which will provide significant interactions with the ultrasound data for subsequent analysis

VIII. Plans for the Next Year:

Recruitment is likely to continue into 2011. All new subjects will be incorporated into the database for analysis. Several publications are planned.

IX. Publications (2010):
None

X. Posters and Presentations (2010):
None

A. Specific Aim: To characterize an ethanol binding site on L1.

B. Studies and Results: Ethanol may damage the developing and adult nervous systems by blocking L1-mediated cell adhesion. We have shown that ethanol inhibits L1-mediated adhesion in human L1-cDNA gene-transfected cells, and molecules that prevent this action of ethanol also prevent ethanol teratogenesis in mice. The L1 cell adhesion molecule consists of six Ig domains, five FnIII domains, one transmembrane domain and one cytoplasmic domain. There is evidence that the Ig1 domain folds against the Ig4 domain to form a horseshoe configuration that favors L1 homophilic binding.

An alcohol binding site has been identified on the L1 molecule using 3-azibutanol and 3-azioctanol photolabeling (Arevalo et al; PNAS 08). Two residues of L1, E33 in domain Ig1 and Y418 in Ig4, were clearly photolabeled by those alcohol analogues. Protein homology modeling of human L1 Ig1-4 domains (residues 33–422) with axonin-1 suggests that E33 and Y418 are only 2.75 Å apart, allowing them to form a strong hydrogen bond that stabilizes the horseshoe configuration. We proposed that ethanol inhibits L1 adhesion by binding to these two residues and breaking the hydrogen bond, thereby disrupting the domain interface between Ig1 and Ig4.

Our model predicts that the E33-Y418 bond is necessary for stabilizing Ig1-Ig4 interactions, and that breaking this bond will reduce L1 adhesion. To test this prediction, we generated a double mutant L1-E33C/Y418C. Under non-reducing conditions, this molecule should demonstrate increased adhesion compared to wild type L1 because a disulfide bond is stronger than a hydrogen bond. Likewise, under non-reducing conditions, L1-E33C/Y418C should be less sensitive to ethanol, because ethanol cannot disrupt a disulfide bond as easily as a hydrogen bond. Finally, under reducing conditions, the disulfide bond should break, allowing us to evaluate the effect of this bond on cell adhesion.

When measured under non-reducing conditions, L1 adhesion was significantly higher in cells transfected with L1-E33C/Y418C than in cells transfected with wild-type L1. The homology model predicted that under these conditions there would be a strong disulfide bond between the Ig1 and Ig4 domains of L1. The effect of this E33-Y418 bond was then tested by measuring cell adhesion in the presence of β-mercaptoethanol (BME) to promote the reduction of the disulfide bond to two S-H bonds, thereby separating the two sulfur groups by almost 7 Å. BME decreased L1 adhesion by more than 50% in cells expressing L1-E33C/Y418C, approximately the same extent that ethanol inhibited wild type L1 adhesion. In contrast, BME had no effect on cell adhesion in cells expression wild type-L1 or single Cys mutations that could not form a disulfide bond between residues 33 and 418. Importantly, the potency and efficacy of ethanol was significantly reduced in cells expressing L1-E33C/Y418C compared to those expressing wild-type L1.

These data are consistent with the prediction that the alcohol binding sites at E33 and Y418 form a functional bond at the domain interface between Ig1 and Ig4. Experiments with L1-E33C/Y418C indicate that the integrity of this bond is important for L1 homophilic binding. Our data also provide further evidence that ethanol inhibits L1 adhesion by disrupting a hydrogen bond between E33 and Y418.

Genetic heterogeneity may account for some of the variability in the incidence and severity of fetal alcohol spectrum disorders among children exposed to alcohol during gestation. Identifying susceptibility genes for FASD may assist in the targeting of prenatal prevention programs for mothers who are at higher risk for bearing children with FASD. Ethanol causes FASD in part by inhibiting the functions of the L1 neural cell adhesion molecule. We have shown that ethanol inhibits L1-mediated cell adhesion in NIH/3T3 fibroblasts transfected stably with the gene for human L1. Drugs that block ethanol inhibition of L1 adhesion also prevent ethanol teratogenesis in mouse embryos. Interestingly, clonal cell lines produced during a single transfection of a non-clonal NIH/3T3 stock with human L1 have maintained a consistent ethanol-sensitive or ethanol-

insensitive phenotype for more than a decade. This finding suggests that a host cell factor either endows L1 with ethanol sensitivity or blocks the intrinsic sensitivity of L1 to ethanol.

To determine whether ethanol sensitivity is an intrinsic property of L1, we coated microspheres with the purified extracellular portion of L1. L1-coated microspheres showed markedly increased adhesion compared to uncoated microspheres or microspheres coated with Fc or bovine serum albumin. Ethanol did not inhibit L1-mediated adhesion in the L1-coated microspheres. These experiments demonstrate that ethanol does not inhibit L1 adhesion in a cell-free system, and that presentation of L1 by an intact cell is required for ethanol to inhibit L1 adhesion. The observation that some clonal L1-transfected fibroblasts are insensitive to ethanol indicates that cellular expression of L1 is necessary, but not sufficient, for ethanol sensitivity. We therefore sought to identify host cell factors that mediate ethanol sensitivity.

We showed previously that L1 sensitivity to ethanol can be reduced or abolished by the MAP kinase inhibitor PD98059 and by expression of an ERK2 siRNA, suggesting that MAP kinase activity is necessary for ethanol sensitivity. There are two ERK2 phosphorylation sites within the intracellular domain of L1 at residues S1204 and S1248. To determine the role of these two residues in ethanol sensitivity, we transiently transfected NIH/3T3 cells with cDNA constructs in which either one or both residues were mutated from Ser to Leu. The double mutant S1204L/S1248L was the most informative, because levels of adhesion were comparable to WT L1, yet ethanol had no effect on S1204L/S1248L-mediated adhesion. These data suggest that phosphorylation at S1248 or both ERK2 sites is necessary for ethanol inhibition of L1.

If increased phosphorylation of ERK1/2 accounts for the ethanol-sensitive phenotype of L1-expressing NIH/3T3 cells, then the stable phenotype of ethanol-insensitive L1-expressing cells should be modifiable by measures that increase the phosphorylation of ERK1/2. Ethanol-insensitive NIH/3T3 cells were transiently transfected with a constitutively active human MEK1 cDNA. Transfection increased ERK1/2 phosphorylation, as determined by immunoblot analysis. MEK1-transfection transformed the phenotype of ethanol-insensitive NIH/3T3 cells to an ethanol-sensitive phenotype after 15 years of consistently demonstrating insensitivity to ethanol. In contrast, treatment of ethanol-insensitive cells with the transfection reagent alone had no effect on ethanol sensitivity.

These data demonstrate that MAP kinase activity is a critical host factor that renders L1 sensitive to ethanol in NIH/3T3 cells. Our findings raise the possibility that ethanol teratogenesis in humans might be regulated by genetic polymorphisms that modify the activity of signaling molecules in the MAPK pathway. New studies are being conducted to examine the molecular basis for differences in the sensitivity of two C57Bl/6 mouse substrains to ethanol-induced teratogenesis. Preliminary data indicate that MAP kinase phosphorylation is constitutively lower in the more ethanol resistant C57Bl/6N strain than in the more sensitive C57Bl/6J strain.

C. Significance: The structural characterization of an ethanol binding site on L1 will provide a road map for the rational design of ethanol antagonists that might reduce ethanol teratogenesis. Identifying molecular mediators of ethanol sensitivity may prove valuable in identifying individuals or populations at risk for ethanol teratogenesis.

D. Plans. NA

E. Publications:

1. Dou X., Shanmugasundararaj S., Menkari C., Miller K. and Charness ME Alcohol Binding Sites on L1 Regulate L1-Mediated Cell Adhesion ACER 33(6) suppl. p. 165A, 2009 and manuscript in preparation
2. Dou X., Menkari C., and Charness ME. L1 sensitivity to ethanol is regulated by the MAP Kinase pathway. RSA annual meeting 2010, San Antonio, TX. and manuscript in preparation

I. Principal Investigator: Rajesh C. Miranda, Ph.D.

II. Title of Project: Circulating microRNA Biomarkers of Fetal Alcohol Exposure, Developmental Project U24 AA014811

III. Objectives:

The first specific aim of this proposal was to identify circulating microRNAs (**miRNAs**) in fetal and maternal sheep circulation that serve as biomarkers for fetal alcohol exposure. The second specific aim was to identify functions associated with ethanol-sensitive circulating miRNAs in placental trophoblasts. As mentioned in our previous, mid-year report, we are now focusing on the analysis of sheep trophoblast cells.

IV. Methods:

In our previous report, we showed that we were able to identify ethanol-sensitive miRNAs in maternal ovine plasma. We used plasma samples obtained by Dr. Cudd 24 hours following the last ethanol or vehicle exposure period of his binge ethanol experimental protocol, where dams were administered ethanol through all three trimester-equivalent periods. Since that previous project report, we have been able to use three different protocols, an ABI low-density PCR array platform, an Exiqon miRNA microarray platform, and an Exiqon low density PCR array platform to detect changes in maternal circulating miRNAs following ethanol exposure.

V. Accomplishments and Results:

(a) Maternal ethanol sensitive miRNAs. As shown in **Figure 1**, we were able to identify 19 ethanol-sensitive maternal miRNAs that were convergently validated by all three detection platforms, and an additional three that were validated in two out of three platforms. However, there was also a substantial number of ethanol-sensitive miRNAs/small RNAs that were detected in one platform (e.g. 24 that were only detected in the Exiqon microarray platform) but not replicated elsewhere. This lack of cross validation is mainly due to the presence of unique content on one platform that is not replicated on other platforms. However, in the case of at least three miRNAs (miR-197, Let-7g and miR-21), we did obtain wildly divergent platform-specific results (ethanol-induced in one or two platforms, and ethanol suppressed on the others). **Table 1** lists all of the miRNAs that were cross-validated on all three platforms. Coincidently, all of the cross-validated miRNAs were down-regulated following ethanol exposure. The unique stem-cell related Mir-plus small RNAs that we discussed in our previous report are not represented here because that content was unique to the Exiqon miRNA microarray platform. We are in the process of separately validating those data by real-time RT-PCR.

(b) Pregnancy-specific circulating miRNAs. A question that arose in our presentation of our data at the CIFASD/RSA meeting was whether circulating miRNAs were affected by pregnancy. In response to that question we have conducted preliminary

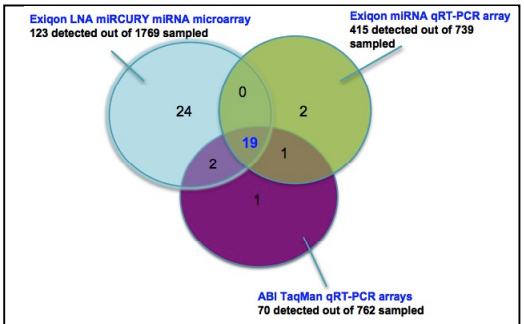


Figure 1: Venn diagram showing cross validation of ethanol-sensitive miRNAs using three different detection platforms. As shown in the diagram, 19 miRNAs were identified as differentially regulated using all three platforms

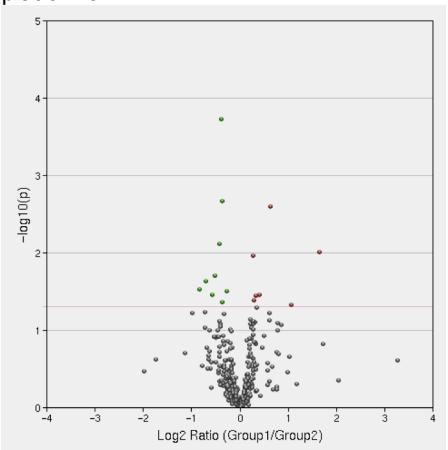


Figure 2: Volcano plot comparing expression of plasma miRNAs in non-pregnant vs. pregnant sheep (Exiqon PCR array, plate A, median normalized, n= 4). Red dots indicate significant up-regulation (raw p<0.05) and green, significant down-regulation in non-pregnant relative to pregnant sheep.

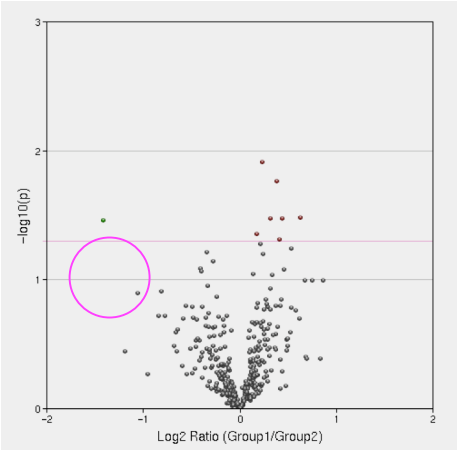


Figure 3: Volcano plot; Plasma miRNAs in non-ethanol exposed (Group 1) *vs. in utero* ethanol exposed (Group 2) lamb (Exiqon PCR array, plate A, median normalized, n= 4). Circled spot indicates miR-331-3p (potentially growth promoting), down-regulated 2.67-fold, raw p<0.034.

experiments using the Exiqon miRNA Q-RT-PCR platform. Our data (**Figure 2**) presented in the form of a volcano plot indicates that tentatively, we can indeed detect differences between plasma content in pregnant and non-pregnant dams. While several miRNAs reached raw statistical significance, our sample size (n=4 in each group) does not currently have enough power to control for false discovery rates (FDR). Only one miRNA, miR-487b comes close to FDR-controlled significance (adjusted p<0.067). Moreover, the changes observed are not large in general, suggesting unexpectedly, that pregnancy itself does not have a major impact on circulating miRNA content (at least the content represented in the measurement platform). We plan to increase our sample size and use at least one other platform to cross validate these data.

Table 1 Fold-change relative to control

miRNA	Exiqon Microarray	ABI qRT-PCR	Exiqon qRT-PCR
hsa-miR-25/25*	0.20	0.10	0.57
hsa-miR-93	0.26	0.33	0.44
hsa-miR-375	0.39	0.33	0.60
hsa-miR-192	0.44	0.33	0.69
hsa-miR-30e*	0.46	0.01	0.99
hsa-miR-20a	0.50	0.29	0.41
hsa-miR-215	0.60	0.43	0.63
hsa-miR-19a	0.63	0.24	0.96
hsa-miR-509-3p	0.68	0.72	0.94
hsa-miR-222	0.68	0.30	0.75
hsa-miR-17	0.68	0.37	0.94
hsa-miR-186	0.68	0.34	0.53
hsa-miR-223	0.71	0.21	0.40
hsa-miR-26b	0.72	0.31	0.78
hsa-miR-328	0.76	0.78	0.65
hsa-miR-128	0.80	0.62	0.88
hsa-miR-877	0.82	0.01	0.47
hsa-miR-30c	0.83	0.36	0.82
hsa-miR-92a	0.87	0.36	0.54
hsa-miR-142-3p	0.88	0.95	0.63
hsa-miR-378	1.08	0.01	0.61

(c) Persistent imprint of maternal ethanol exposure on circulating miRNAs in the offspring. We have now initiated a third series of experiments, also using the Exiqon Q-RT-PCR platform, to determine the extent to which we can detect postnatal effects of *in utero* ethanol exposure on plasma miRNAs in the lamb (again, samples are from Dr. Cudd's three trimester exposure paradigm). As shown in our preliminary analyses (**Figure 3**), we can identify sensitive miRNAs in lamb plasma that have changed as a consequence of *in utero* ethanol exposure. Again, while the sample size is too small (n=4) to reach significance when controlling for FDR, two interesting cancer related miRNAs stand out. MiR-331 (whose expression is directly correlated with cancer severity, shown **circled in Figure 3**) was down-regulated in the lamb following *in utero* ethanol exposure. In contrast, miR-518a (a feature represented on Exiqon Plate-B, data not shown), whose expression negatively correlates with cancer, was induced by ethanol. If these cancer-related miRNAs direct cell proliferation and growth, it is possible that the differential regulation of these miRNAs represent a growth retardation imprint due to in utero ethanol exposure. Again we need to increase our sample size and validate these data.

(d) Additional experiments. miRNAs are secreted by cells as a part of a vesicle, termed an exosome. We have now succeeded in a series of preliminary experiments, in isolating exosomes from cell culture conditioned medium, and have been able to amplify miRNAs from this secreted fraction. This step is important for our studies on the endocrine functions of circulating miRNAs in trophoblasts and in other cells.

VI. Discussion:

(a) These data expand on our initial finding indicating that circulating maternal and neonatal miRNAs do change following ethanol exposure during gestation. Consequently, they are a promising source of biomarkers for maternal alcohol consumption during pregnancy. We now have preliminary data showing that there is a persistent circulating miRNA imprint of maternal ethanol exposure in the lamb. Perhaps not so surprisingly in retrospect, the ethanol-sensitive miRNAs in the lamb are different from those in the mother. Further studies will be needed to sort out both the causes and consequence of the changes in these miRNAs. (b) In these most recent studies, we have focused on using the Exiqon miRNA Q-PCR array platform because it can be adapted to detecting miRNAs from a small amount of starting material (e.g. 500 microliters of plasma). We hope that this increase in sensitivity will enable us to adapt this technology to assaying for miRNA changes in samples from human patients. (c) Finally, the focus on exosome biology will be important. To understand the significance of alterations in circulating miRNAs, we will need to further study and understand the biology of

these exosomes, i.e., what chaperone proteins shuttle miRNAs from one cell to another, and what cell surface recognition molecules permit specific exosome-cell interactions.

VII. Interrelation with Aims of the Consortium and Other Projects:

This proposal interacts extensively with the component entitled, “Translational Studies of FASD Using a Sheep Model”, (Dr. Timothy Cudd, PI). Additionally, with the development of more sensitive miRNA screening assays, it is hoped that this project will interact with the human studies encompassed within CIFASD.

VIII. Plans for the Next Year:

We plan to continue with our analysis and validation of maternal and neonatal miRNA profiles following first trimester and all three trimesters of ethanol exposure. With the adoption of the Exiqon RT-PCR array platform, we are now in a position to assay miRNAs from human plasma samples as well. Finally we expect to continue with our exosome analysis and our experiments on sheep trophoblasts.

IX. Publications (2010):
None

X. Posters and Presentations (2010):

We presented the results of our analyses at the 2010 Research Society on Alcoholism meeting at San Antonio, TX, and at the 2010 Canadian Society for Toxicology meeting, Montreal Canada.

I. Principal Investigator: Claudia D. Tesche, Ph.D.

II. Title of Project: Network Connectivity and Dynamics in Fetal Alcohol Spectrum Disorders, Developmental Project U24 AA014811

III. Objectives:

The objective of this study is to initiate the use of magnetoencephalography (MEG) to characterize brain dynamics in adolescents with FASD. MEG is a non-invasive neuroimaging methodology which detects ongoing brain activity with exquisite temporal resolution. This effort will build on existing electroencephalographic (EEG) studies which reveal differences in brain dynamics between adolescents with FASD and normal children. The specific aims include ideintification of potential abnormalities in cortico-cerebellar network dynamics, particularly those that involve midline structures. Predictive capability and plasticity are characteristic features of cortico-cerebellar networks. Moreover, cerebellum has been suggested to play an important role in adaptive control of behavior through the development of models for mental representations that are critical to effective cognitive and affective processes.

IV. Methods:

We hypothesize that abnormalities in cortico-cerebellar dynamics will be found in adolescents with FASD. We use aversive conditioning and entrainment of movement to visual cues to probe the integrity of these networks. Whole-scalp (306-channel) MEG and 3 T structural MRI data will be recorded from 23 adolescents (12-18 years old) with FASD and 23 normal controls over a three-year period. Abnormal dynamics observed both during entrainment and aversive conditioning will support to the notion that abnormal cortico-cerebellar network dynamics in adolescents with FASD are pervasive, and may contribute to a broad spectrum of cognitive and behavioral disabilities.

Aversive conditioning:

The conditioning paradigm utilizes CS- visual images that are presented in isolation and CS+ visual images that are paired on 50% of trials with aversive sounds.

a) We will utilize MEG to characterize the conditioned auditory C50m evoked response to the unpaired CS+ images. This response in auditory cortex is elicited by visual stimuli even though no sounds are presented. We hypothesize that the C50m evoked response will develop more slowly within training blocks and will occur with smaller amplitude in adolescents with FASD.

b) We will quantify evoked-response measures of activation of medial prefrontal cortex and cerebellum elicited by the conditioned stimuli. We hypothesize that these responses will show differences in timing and amplitude in adolescents with FASD.

c) We will quantify wavelet, coherence and Granger causality measures of network activity. We hypothesize that abnormalities in brain dynamics will occur on the network level in adolescents with FASD.

d) We will assess potential correlations between differences in MEG measures of aversive conditioning and psychometric measures of emotional and social intelligence as measured by the Emotional Quotient Inventory (EQ-i).

Entrainment of Movement to Isochronous Visual Cues:

Subjects are requested to respond to the presentation of a sequence of visual images with button presses. Control subjects learn to recognize the temporal pattern of the presented stimuli, and thus entrain their responses to the anticipated time of presentation rather than the actual presentation of the visual stimuli.

a) We will quantify response times (RT) to isochronous visual cues. We hypothesize that adolescents with FASD will show a slower rate of entrainment and more variability in RT.

b) We will assess potential correlations between RTs and MEG measures of cortico-cerebellar dynamics during the entrainment process. We hypothesize that cortico-cerebellar plasticity will show reduced activation in cerebellum. Moreover wavelet, coherence and Granger-causality measures of brain dynamics will differ for adolescents with FASD.

c) We will assess potential correlations between differences in brain dynamics and psychometric measures of emotional and social intelligence as measured by the Emotional Quotient Inventory (EQ-i).

V. Accomplishments and Results:

Recruitment of subjects is ongoing. We have recorded MEG/MRI, response time and neuropsychological data from 10 adolescents with FASD and 11 adolescent control subjects for the entrainment and aversive conditioning paradigms. MEG data has been cleaned and averaged evoked responses extracted for all subjects. Source analysis of the evoked response data is ongoing.

VI. Discussion:

Results will be presented at The 4th International Conference on Fetal Alcohol Spectrum Disorder, March 2-5, 2011, Vancouver, BC, Canada.

VII. Interrelation with Aims of the Consortium and Other Projects:

The principal strategic goal of CIFASD is to develop better ways to diagnose fetal alchol effects. The development of MEG-based measures of abnormal brain dynamics using paradigms which probe elementary brain processes has the potential of contributing to the diagnosis of FASD, especially in the absence of dysmorphia. Importantly, MEG imaging of brain dynamics has the potential to provide a link between physical and brain-based abnormalities and specific behavioral deficits. Neuronal population dynamics are an emergent property of the underlying brain morphology and drive behavioral responses. We will use MEG to explore the hypothesis that abnormalities in cortico-cerebellar dynamics are present in individuals with FASD. This effort may lead to further investigations at other sites of the CIFASD collaboration. Sites which also may obtain access to a VectorView MEG laboratory include: Harvard University, University of California, San Diego, San Diego State University and Folkhälsän Research Center-Helsinki. We welcome the opportunity to develop, in collaboration with members of CIFASD, a RO1 proposal that combines MEG measures of brain activation explored in the present proposal with structural MRI measures of brain volumes and DTI connectivity, and with an evaluation of dysmophia in adolescents for submission to NIH.

VIII. Plans for the Next Year:

We anticipate measurement of 8 additional adolescents with FASD and 8 additional normal controls. The MEG data analysis will be extended to include wavelet, coherence and Granger-causality measures of brain dynamics.

IX. Publications (2010):
None

X. Posters and Presentations (2010):

"Network Connectivity and Dynamics in Fetal Alcohol Spectrum Disorders", The 4th International Conference on Fetal Alcohol Spectrum Disorder, March 2-5, 2011, Vancouver, BC, Canada (submitted).

I. Principal Investigator: William K. Barnett, Ph.D.

II. Title of Project: Informatics Core for the Collaborative Initiative on Fetal Alcohol Spectrum Disorders, U24 AA014818

III. Objectives:

The objective of the Informatics Core is to support the goals of the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD) by enabling the collection, sharing, and analysis of consortium data. In particular, we aim to:

1. facilitate the creation of data dictionaries to ensure that information of different types and from different locations can be shared and integrated,
2. develop a reliable and HIPAA-compliant central repository
3. provide other tools to facilitate data entry, data storage, and data retrieval, and
4. enable both hypothesis-driven and data mining methods for analyzing data.

IV. Methods:

CIFASD is currently collecting several different types of data: Dysmorphology, Neurobehavior, 3D facial images, Demographics, Alcohol and Control, Ultrasound, Infant Neurobehavior, and Brain images. For each of these categories of data, the Informatics Core worked with the rest of the consortium during 2010 to accomplish the following:

- Create data dictionaries that precisely describe data to be collected.
- Create one or more input tools that allow projects to record their data.
- Expand the ability of the central repository to store data and create methods to transfer data from the input tools to the central repository.
- Investigate new methods for retrieving data to include the ability to retrieve each type of data in turn, and new tools for data browsing directly by researchers.

The result is a set of software tools that allows project members to store each of the types of data being collected for the consortium, upload/submit those data to a central repository, and access this central repository for results obtained across the projects in the consortium.

Querying the central repository provides data sets that researchers can download and use for hypothesis-driven analysis. Additional flexibility in choosing datasets of interest and online visualization methods are being considered to enhance support for hypothesis-driven analysis. Supervised and unsupervised data mining algorithms, techniques, and software are also being considered to enable researchers to find patterns and meaning in the data.

V. Accomplishments and Results:

The development of data dictionaries is a particularly useful part of the Informatics Core services to the consortium as a whole. Data dictionaries are critical aspects of information repositories, as they provide the necessary consistency to ensure that data from different projects can be compared. The creation of the data dictionary facilitates the process of the consortium agreeing on the meaning of the data being collected, and the definitions in the data dictionary are programmed into the tools used by the consortium in order to enforce that the data entered and submitted conform to the data dictionary. The degree of detail required to create shared data input tools has repeatedly helped us discover areas in which definitions were

almost consistent rather than completely consistent. We have been very careful to leave it to CIFASD and subcommittees appointed by the CIFASD leadership to actually define the data items. This interplay has been very productive. Ultimately, these data dictionaries could become a set of standard terms for FASD research in general.

The Cross Query tool realizes the value of the integrated, consortium-wide repository by enabling researchers to select data of interest from across the range of data types and the range of participating sites and populations. The Cross Query tool provides data in multiple formats to enable researchers to use the provided data with the analysis tools of their choice. The Cross Query tool cannot provide every possible dataset that CIFASD collaborators may be interested in, so we supplement the Cross Query tool with a consulting service to provide customized datasets as needed.

The status of the development of particular tools follows. An asterisk (*) represents status that has changed since the last report in January, 2010.

Data Area	Task	Status
Cross Query	Cross Query Tool	**Finished ***
Dysmorphology	Data Dictionary	Finished
	MS Access Input Tool	Finished
	Central Repository Schema	Finished
	Upload Tool	**Updated ***
	Report Tool	Finished
Alcohol & Control	Data Dictionary	Finished
	MS Access Input Tool	Finished
	Central Repository Schema	Finished
Neurobehavior	Data Dictionary	Finished
	MS Access Input Tool	Finished
	Central Repository Schema	Finished
	Upload Tool	Finished
	Report Tool	Finished
Neurobehavior, phase II	Data Dictionary	**Updated ***
	MS Access Input Tool	**Updated ***
	Central Repository Schema	**Updated ***
	Upload Tool	**Updated ***
	Report Tool	**Updated ***
Demographics	Data Dictionary	**Updated ***
	MS Access Input Tool	**Updated ***
	Central Repository Schema	**Updated ***
	Upload Tool	**Updated ***
	Report Tool	**Updated ***
	Data Dictionary	**Updated ***
3D Facial Images	Data Dictionary	Finished
	Central Repository Schema	Finished
	Upload Tool	Finished
	Report Tool	Finished
Ultrasound	Data Dictionary	Finished
	MS Access Input Tool	Finished

Screener	Data Dictionary	Finished
	MS Access Input Tool	Finished
Follow-up/Outcome	Data Dictionary	Finished
	MS Access Input Tool	Finished
Infant Neurobehavior [Bayley, Maternal Questionnaire, and Heart Rate Monitoring]	Data Dictionary	Finished
	MS Access Input Tool	Finished
Brain Imaging	Data Dictionary	Finished
	Central Repository Schema	Finished
	Upload Tool	Finished
	Report Tool	Finished

Consortium researchers may download MS Access input tools from https://cifasd.uits.iu.edu:/downloads/.

Example screen images of data entry and retrieval tools we have created are shown in Figures 1 and 2.

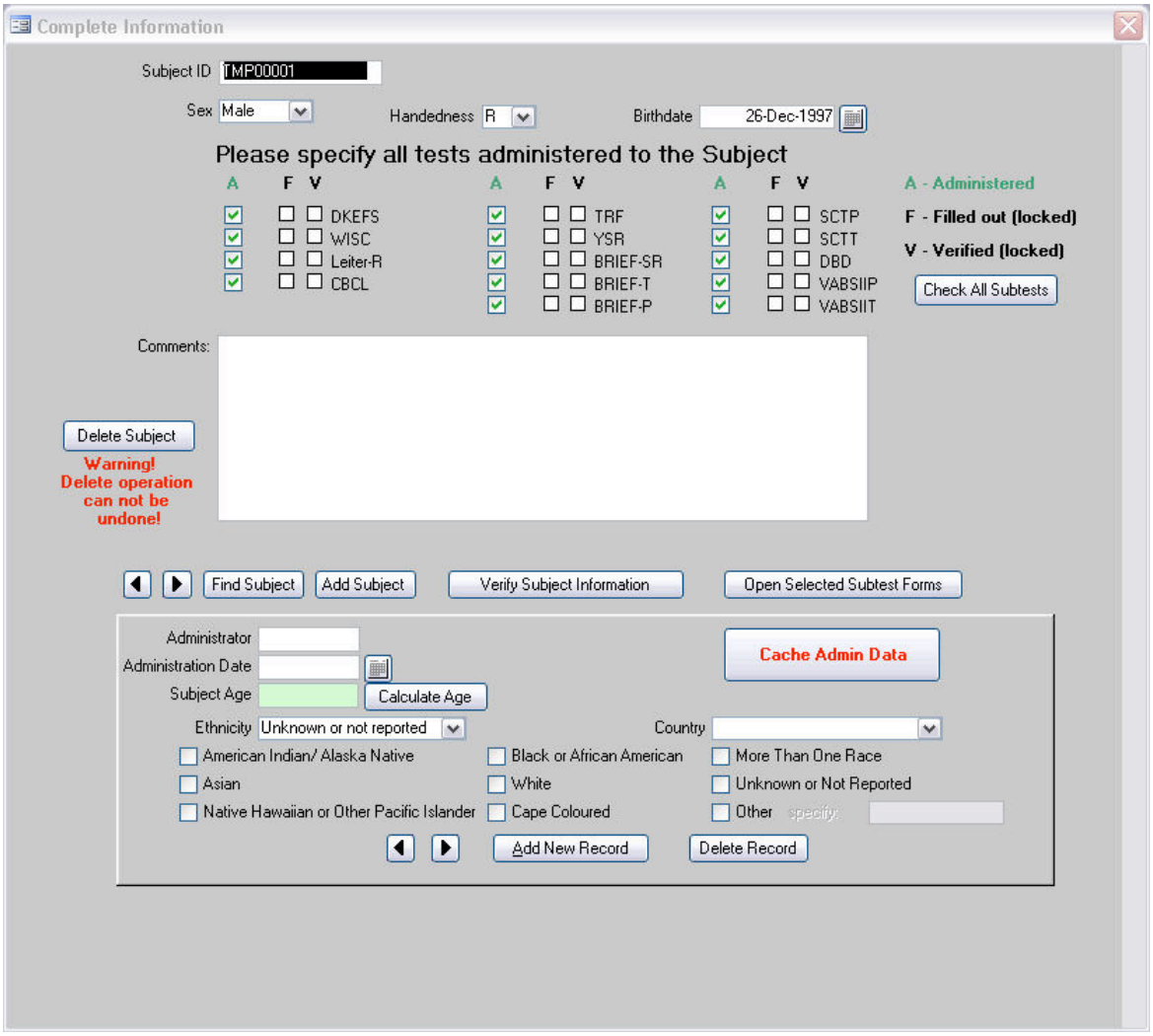


Figure 1: Neurobehavior Phase II Data Entry Input Tool. This data entry form includes double-entry data validation functionality to decrease data entry errors as well as range checking that makes it impossible to enter values outside the permissible ranges identified in the data dictionary.

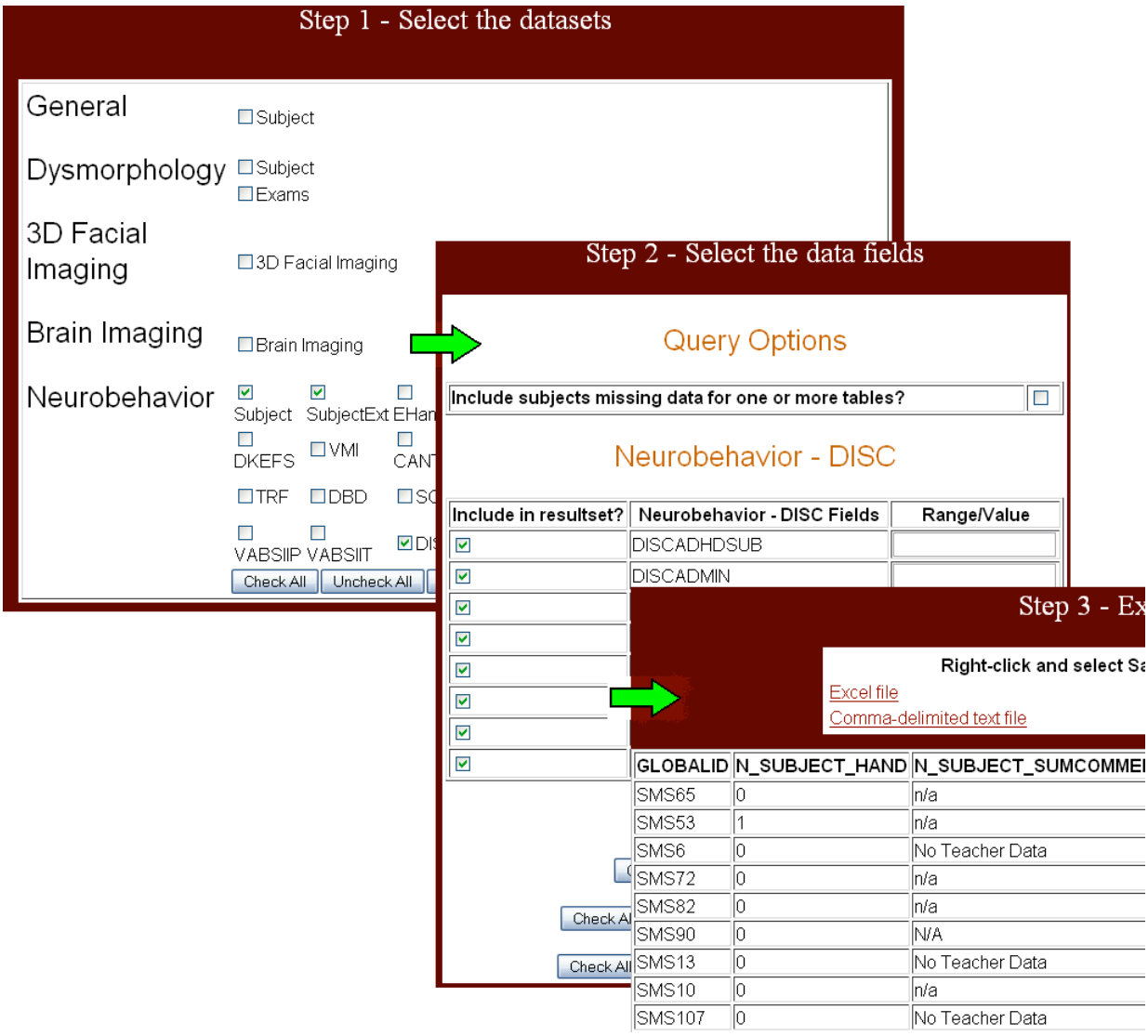


Figure 2: Web-based CrossQuery data access and retrieval tool. Researchers use a series of three screens to choose the dataset of interest for browsing or download.

Data for the consortium are stored in duplicate – one copy in a robotic tape storage system in Indianapolis, IN and a second copy in a robotic tape storage system in Bloomington, IN. This ensures that the consortium's valuable data will be kept reliably even in the event of a disaster at one of Indiana University's two computer rooms.

VI. Discussion:

The initial Informatics mandate for CI-FASD had been the creation of tools to enable the capture and submission of data. At the start of 2010, these tools were largely created. The emphasis of effort this year was thus on collaborating with the sites to update upload capabilities based on changing consortium needs, insure data integrity, and to provide useful sets of data for analysis.

The emphasis of the Informatics Core's work will continue to migrate from creating tools that capture and submit data to facilitating more and better methods for analysis of the data that has been collected.

VII. Interrelation with Aims of the Consortium and Other Projects:

The Informatics Core is essential infrastructure for CIFASD as a whole. The structure of a separate Informatics Core has facilitated the collaborative processes that have enabled the consortium's researchers to come to consensus on data definition and measurement issues that are essential to the broader goals of the CIFASD as a whole. We believe that this can be a model for FASD research programs in the future. The work of the Informatics Core has led to the creation of data dictionaries that will ensure that the common data collected by the consortium are usable and understandable indefinitely, and the suite of computer tools we have created will ensure that the data are accessible indefinitely.

VIII. Plans for the Next Year:

The Informatics Core will continue to work with the entire consortium to provide data input, management, and output services, understand the ongoing needs for software tools and their development, provide better tools for data analysis, and assist with data quality. Although most data input or upload tools are completed, there will be a continued need for Informatics Core staff to assist researchers with data uploads. There will be an ongoing need for tool modification as data upload needs, methodologies, or contributing sites change.

The focus of the consortium is moving from an emphasis on collecting to analyzing data. The Informatics Core is shifting its activities to support these changing needs. Software tools needed to enter and submit the most pressing data – Dysmorphology, Neurobehavior, 3D Facial images, and Brain images – are in place. Although further data entry and Central Repository submission tools, such as Upload tools for Alcohol & Control and Infant Neurobehavior data and adding biophysical profiling variables to the Ultrasound data dictionary are needed, we expect priorities for the next year to focus more on providing new and expanded methods for both hypothesis-driven analysis and data mining.

Currently, we provide a CrossQuery system that supports integrated queries across multiple data sets. Whereas this is a powerful tool that is absolutely required to meet Consortium goals for integrated research, it has the limitations of not supporting data browsing for quality control, and of having a steep learning curve. Because of this, researchers do not always trust data from the CrossQuery tool are correct or complete. We will be actively pursuing tools that allow researchers to browse and correct the data sets they have uploaded to the Central Repository.

By providing a relatively easy to use interface into those data, we will improve confidence and also hopefully enable greater engagement with CIFASD data, thus promoting its use. For those researchers who wish to use the CrossQuery tool, we will dedicate Informatics Core staff time to directly assist researchers with designing and executing queries.

The informatics core will also provide ongoing support for data quality improvements. We will not only provide better 'data browsing' tools so researchers can directly examine the data sets they have uploaded to the Central Repository, but we will also allocate time, as needed, to directly support the improvement of data quality in the Central Repository (for example, creating specific reports such as DemGroupClass).

IX. Publications (2010):

Arenson A.D., Bakhireva L., Chambers C.D., Deximo C., Foroud T., Jacobson J.L., Jacobson S.W., Jones K.L., Mattson S.N., May P.A., Moore E., Ogle K., Riley E.P., Robinson L.K., Rogers J., Streissguth A.P, Tavares M., Urbanski J., Yezerets Y., Surya, R., Stewart C.A, and Barnett, W.K. Implementation of a shared data repository and common data dictionary for fetal alcohol spectrum disorders research, *Alcohol*, 2010;44:643-647. PMCID: PMC2888879

Mattson, S.N., Foroud, T., Sowell, E.R., Jones, K.L., Coles, C.D., Fagerlund, Å., Autti-Rämö, I., May, P.A., Adnams, C.M., Konovalovam, V., Wetherill, L., Arenson, A.D., Barnett, W.K., and Riley, E.P. Collaborative Initiative on Fetal Alcohol Spectrum Disorders: Methodology of Clinical Projects. *Alcohol*, 2010;44:635-641. PMCID: PMC2888656

X. Posters and Presentations (2010):

n/a

I. Principal Investigator: Kenneth Lyons Jones, M.D.

II. Title of Project: Dysmorphology Core, U24 AA014815

III. Objectives:

The objective of the Dysmorphology Core is to support all sites contributing to the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD) by implementing a standard comprehensive protocol for physical examination of all children. In addition, we propose to address hypotheses regarding the relationship between FASD-related structural features identified through standard physical examinations and neurobehavioral development as well as complimentary diagnostic methods that are being tested in other Consortium sites.

The specific aims are as follows:

1. To assure consistency as well as accuracy in recognition of FASD at all CIFASD project sites where children are being evaluated.
2. To delineated the full range of structural anomalies in children prenatally exposed to alcohol in order to determine the boundaries that encompass FASD in prospective as well as retrospective studies in the Consortium.
3. To identify specific structural defects or clusters of features that are predictive of or correlated with deficits in neurobehavioral development across development ages spanning from infancy to adolescence.
4. To correlate the specific structural features or clusters of features identified on the CIFASD standard physical examination with alternative or complimentary diagnostic methods that are being tested in other CIFASD projects.
5. To better understand the extent to which structural features of FASD are related to specific defects in brain development.

IV. Methods:

We have performed a standard comprehensive physical examination of all children at all consortium sites. These children were examined blind to alcohol exposure in all cases. Based exclusively on the standard physical examination, each child was categorized as FAS, No FAS or Deferred. The latter category will be used to better understand the full spectrum of defects resulting from prenatal alcohol exposure.

V. Accomplishments and Results:

Specific Aims 1 and 2: Over the last year we have provided accurate recognition of FASD at two sites in Ukraine, the Plains, San Diego, CA, Atlanta, GA, Albuquerque, NM, and Los Angeles, CA. In 2010, 50 subjects have been evaluated in Atlanta, 14 in Los Angeles, 21 in Albuquerque, 13 in the Plains, and 25 in San Diego. During trips to Ukraine we have expended a considerable amount of time interacting with and retraining pediatricians and geneticists in the Rivne Oblast. In the six full day clinics that we have conducted in Rivne in 2010, we have examined 103 children during which time we confirmed the diagnosis that had previously been made by the pediatrician in the newborn period. Each of those examinations was done with the pediatrician who had done the initial examination in attendance. We have been very pleased to note that our examination of children has been completely consistent with their examination performed at the time of birth. In addition, we have had five full day clinics in Khmelnitsky, Ukraine, a second site in Ukraine that was added one year ago. Those clinics involve examining babies who have been evaluated by the geneticists in Khmelnitsky in the newborn period and are used as training and

re-training exercises. Each child that is seen by the expert dysmorphologist at those clinics is seen with the geneticist from Khymelnitsky to confirm his/her evaluation that had previously been done in the newborn period. Thirty-two babies have been born at that site in 2010.

Specific Aims 2 and 3: With respect to delineating the full range of structural anomalies in children prenatally exposed to alcohol, we have completed a study documenting the full spectrum of defects associated with FASD. We examined 831 children from the Collaborative Initiative on Fetal Alcohol Spectrum Disorders using a structured protocol for diagnosis of FASD using the cardinal facial and growth features, and assessment of additional structural defects thought to occur more often in children with prenatal alcohol exposure. Subjects were classified as FAS, Deferred or No FAS, Groups were compared on prevalence of additional features and number of additional features observed, stratified by diagnostic category, sex, race and age. Prevalence of most additional features was greatest among subjects with FAS and least among No FAS. A higher frequency of additional features was observed among FAS and Deferred subjects ≥12 years of age than among those under 12. This study shows that prenatal alcohol exposure may produce a broad spectrum of structural defects that goes beyond FAS with implications regarding the impact of alcohol on the developing fetus, a prerequisite for ultimate prevention of FASD. (see Section IX).

Specific Aim 3: As more individual children in the Consortium receive both neurobehavioral assessment and evaluation by the Dysmorphology Core, it is becoming possible to correlate features identified on the physical examination with neurobehavioral deficits. These analyses are beginning in conjunction with the Multisite Neurobehavioral Assessment of FASD. One paper has been published in which data was used from two sites, San Diego, CA and Helsinki, Finland. (see Section IX). A neuropsychological battery was used to determine a profile that could be used to accurately identify children affected by prenatal exposure to alcohol. Of particular importance, the data suggest that children with FAS based on the physical examination are similar based on a specific set of neuropsychological tests to children prenatally exposed to alcohol who do not fulfill criteria for FAS based on the physical examination. These represent the first data indicating that a behavioral phenotype may allow identification that a child's neurobehavioral abnormalities even without the characteristic physical phenotype of FAS is the result of prenatal alcohol exposure and further our understanding of the broad spectrum of abnormalities associated with prenatal exposure to alcohol.

Specific Aim 4: We have completed a study correlating maternal drinking during pregnancy with prenatal ultrasound findings. (see Section IX) We demonstrated significant differences in selected somatic and brain measurements between alcohol-exposed and comparison fetuses, suggesting these markers may be further explored for clinical utility in prenatal identification of affected children. In addition, we have completed a study examining the ability to recognize FAS from 3D facial imaging (see Section IX). The results demonstrate that computer algorithms can be used to automatically detect facial features that can discriminate FAS and control faces.

Specific Aim 5: We have completed a study the purpose of which was to examine whether short palpebral fissures are independent of or determined by the OFC. We concluded that palpebral fissure length is predominately independent of occipitofrontal circumference in children with and without features of FAS. Short palpebral fissures may well reflect a defect in forebrain development rather than being proportionally reduced in size as a reflection of microcephaly (see Section IX). In addition, one study has been accepted for publication relating regional brain volume reductions to facial dysmorphology and neurocognitive function in children with fetal alcohol spectrum disorders; an abstract has been submitted analyzing the extent to which

structural features of FASD are related to specific defects in brain development noted on MRI scans; and another abstract has been submitted to this years RSA meeting.

VI. Discussion:

The Dysmorphology Core has helped to facilitate the goals of the Consortium and some of the individual projects primarily through traveling to Consortium sites and performing standardized physical examinations on children ascertained at the individual sites. Overall the Dysmorphology Core has performed 2588 standardized physical examination at consortium sites throughout the world. 258 of those children have been examined in 2010. That has included 25 children at San Diego State University; 50 in Atlanta, Georgia; 103 children in Rivne, Ukraine; 32 in Khmelnytsky, Ukraine; 21 in New Mexico; and 14 at UCLA.
In addition, a number of talks and training sessions have been done in Ukraine.

VII. Interrelation with Aims of the Consortium and Other Projects:

The Dysmorphology Core has interrelated with a number of other projects in the Consortium including the 3D Facial Imaging Project, the Spectrum of and Nutritional Risk Factors for FASD in Ukraine Project, the Multisite Neurobehavioral Assessment of FASD Project and the Prenatal Ultrasound studies in Ukraine.

VIII. Plans for Next Year:

We will continue to see children ascertained at all CIFASD sites throughout the world. Plans are underway to become involved in studies in Poland and South Korea. We have visited both countries over the last year and plan to return to help with development of plans to start prevalence studies. We are planning to complete the analysis of data regarding the relationship between FASD-related structural features identified through standard physical examination and 3D facial imaging. We also will begin correlating the relationship between FASD-related structural defects identified through the standard physical examination and neuro-imaging studies. Finally we have started and will continue the correlation of the FASD-related structural features with the neuro-behavioral findings and structural brain studies.

IX. Publications and Abstracts (2010):

Jones, K.L., Hoyme, H.E., Robinson, L.K., del Campo, M., Manning, M.A., Prewitt, L.M., and Chambers, C.D. (2010). Fetal Alcohol Spectrum Disorders: Establishing the broad range of structural defects. *Amer J Med Genet*, 152A: 2731-2735.

Mattson, S.N., Roesch, S.C., Fagerlund, A., Autti-Ramo, I., Jones, K. L., May, P.A., Adnams, C. M., Konovalova, V., Riley, E.P. and the CIFASD. (2010). Toward a neurobehavioral profile of fetal alcohol spectrum disorders. *Alcohol Clin Exp Res, 34*(9):1640-1650. PMCID: 2946199.

Klingenberg CP, Wetherill L, Rogers J, Moore E, Ward R, Autti-Rämö I, Fagerlund A, Jacobson SW, Robinson LK, Hoyme HE, Mattson SN, Li TK, Riley EP, Foroud T; CIFASD Consortium. (2010). Prenatal alcohol exposure alters the patterns of facial asymmetry. *Alcohol* 44(7-8):649-657.

Roussotte, F., Sulik, K.K., Mattson, S., Riley, E.P., Jones, K.L., Adnams, C., May, P., O'Connor, M., Narr, K., Sowell, E. and the CIFASD. (2011). Regional brain volume reductions relate to

facial dysmorphology and neurocognitive function in fetal alcohol spectrum disorders. *Human Brain Mapping*, In press.

X. Posters and Presentations (2010):

Roussotte, F., Kan, E., Mattson, S., Riley, E.P., Jones, K., Adnams, C., May, P., O'Connor, M., Narr, K., Sowell, E. and the CIFASD. Cortical thickness is related to measures of facial dysmorphology in FASD. 2010 Society for Neuroscience abstract.

Robinow Lecture, University of Virginia, April 22, 2010.

Harvard Medical School Department of Continuing Medical Education, Human Teratogens: Environmental Factors Which Cause Birth Defects, April 26, 2010.

Grand Rounds Lecture, University of Pittsburgh Department of Pediatrics, June 10, 2010.

I. Principal Investigator: Timothy A. Cudd, Ph.D.

II. Title of Project: Translational Studies of FASD Using a Sheep Model, U01 AA017120

III. Objectives: The goals of the CIFASD consortium are to identify sources of variable phenotypes (facial dysmorphology, structural brain damage and neurobehavioral functional deficits) resulting from prenatal alcohol exposure (Aims 1-4), to improve diagnosis and early identification of FASD (Aims 1 and 5), and to develop early interventions that may limit adverse outcomes in at-risk pregnancies (Aim 6). This project will address many of these aims utilizing a unique translation model. This project has two long-term objectives. The first is to use the sheep model to compare the phenotypic effects of binge-like alcohol exposure during the period of brain development comparable to that of the human first trimester (1^{st}-trimester model), with similar binge-like exposure that extends over the stages of brain development encompassing all three human trimesters (3-trimester model). We will compare the two patterns of exposure in terms of effects on growth, facial dysmorphology and brain and behavioral development—the phenotypes used to diagnose fetal alcohol syndrome—to test the hypothesis that more pervasive effects on brain and neurobehavioral development will result from binge exposure that continues after the first trimester. We will use measures and methods derived explicitly from, and collaboratively linked directly to, approaches that have been developed or applied in the human components of the Consortium (e.g., facial morphometrics; eyeblink conditioning and spatial learning to test neurobehavioral function). The second goal is to test the hypothesis that choline supplementation initiated periconceptually will attenuate or prevent the adverse effects of alcohol exposure in the 3-trimester model. This goal will inform and complement the two other choline-supplementation projects in the consortium, a basic science project using rats (Thomas) and a prospective human clinical study (Chambers/Keen project).

Specific Aim 1: We will evaluate whether the sheep model expresses facial dysmorphology and microcephaly. If so then the sheep will be uniquely valuable to the field as a translational model of FAS that effectively models human pregnancy.

Specific Aim 2: We will test the hypothesis that deficits in cerebellar-dependent and hippocampal-dependent learning and memory will be more severe in the 3-trimester model than in the 1^{st}-trimester model, and that these effects will be associated with more severe cerebellar and hippocampal neuronal loss (from Aim #3). Pavlovian “delay” eyeblink condition will be used to assess cerebellar-dependent learning; Pavlovian “trace” eyeblink conditioning will be used to assess learning that requires both cerebellar and hippocampal function; spatial delayed alternation will also be used to assess hippocampal-dependent spatial working memory.

Specific Aim 3: We will test the hypothesis that both the 1^{st}-trimester and the 3-trimester models will produce deficits in neuron numbers in the cerebellum and hippocampus, but that loss of hippocampal and cerebellar neurons will be more severe in the 3-trimester model. Stereological counts will be obtained for cerebellar Purkinje neurons, deep cerebellar nuclei neurons, hippocampal pyramidal neurons of CA1 and CA3, and granule cells of the dentate gyrus, for analysis of group differences and for correlation with behavioral performance from Aim 2. Based on previous findings in mice that gestational alcohol exposure produces deficits in serotonin neurons, counts of serotonergic neurons in the raphe will be included (Dr. Zhoug).

Specific Aim 4: We will test the hypothesis that choline supplements in the 3-trimester model, beginning shortly after mating, will significantly reduce the effects of alcohol exposure on behavioral and brain outcomes. Facial morphology, eyeblink conditioning, spatial learning, and

neurohistological analyses similar to Aims 1-3 will be used to assess whether choline supplements protects against this full 3-trimester exposure.

IV. Methods: These studies will utilize a sheep model whereby pregnant sheep of known breeding dates will receive alcohol three days in succession per week followed by four days without alcohol exposure repeated weekly for 1) the first trimester equivalent of human brain development only (first study only) or 2) all three trimester equivalent of human brain development. These studies will include normal control groups and saline groups that control for all conditions (except alcohol) to which alcohol treated subjects are subjected. This study will establish facial, all neuroanatomical and neurobehavioral measures. Specifically, we will measure brain weight, cerebellar weight and cerebellar Purkinje cell number and density, deep cerebellar nuclei neuron number, hippocampal pyramidal neurons of CA1 and CA3, and granule cells of the dentate gyrus. The acquisition of classical conditioning learning will be measured using eyeblink classical conditioning. Spatial learning will be measured using T-maze learning. The correlation of neuroanatomical and neurobehavioral measures will then be estimated. A second study will include groups that receive a choline intervention during the two exposure periods and these studies will include all appropriate control groups. All of the dependent measures for the first study will also be applied to the second study. ***Note that because the alcohol dose utilized in the first study, 1.75 g/kg, did not result in behavioral deficits, the second study was modified to include only a first trimester exposure period at 1.75 g/kg dose and a 2.5 g/kg dose.***

V. Accomplishments and Results: Facial Dysmorphology: Facial dysmorphology is one of the potential consequences of prenatal alcohol exposure and recognition of specific types of dysmorphic facial features is required for diagnosis of fetal alcohol syndrome (FAS). However, facial dysmorphology is variably expressed and is present only in a small proportion of cases with known heavy prenatal alcohol exposure. In order to try to improve our understanding concerning the variable expression and in order to try to develop improved methodologies to recognize facial dysmporpholoty, coordinated studies of mice, sheep, and humans in the Collaborative Initiative on Fetal Alcohol Spectrum Disorder (CIFASD) have been established. The purpose of our study was to assess whether prenatal alcohol exposure induces facial dysmorphology in the ovine model of prenatal alcohol exposure, by applying standard anthropometric facial measurements that have been shown to successfully classify FAS in a clinical sample.

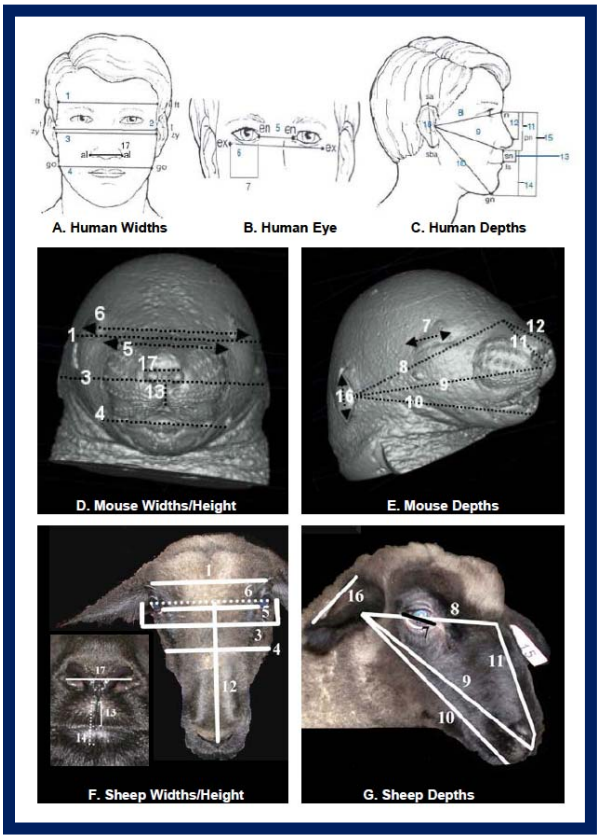


Figure 1: Comparable facial landmark measurements from morphometric analyses in human, mouse and sheep.

Ewes were individually housed and fed. Alcohol (1.75 g/kg as a 40% solution in saline) or a saline control intravenous infusion (of a volume equivalent to the alcohol infusion) was administered intravenously over one hour on three consecutive days per week (beginning on gestational day 4 [G4]). This pattern was repeated weekly throughout first trimester equivalent (ending on G41) or throughout all three trimesters equivalent (ending on G132). Lambs were born and the face was measured on postnatal days 1, 60 and 150. A method of quantitative morphometry of facial structure was developed in sheep that parallels those performed on humans (Moore et al., 2002; 2007) in consultation with Dr. E. Moore. It was determined that 15 of 17 anthropometric measures used in the human studies could be generated from comparable morphological landmarks on the sheep face, and could be objectively and reliably measured.

Data from first trimester exposure and the all three trimester exposure groups were combined as a single alcohol-exposed group to increase statistical power, since facial measurements did not differ between the two treatments. A multivariate analysis of variance (MANOVA) on the three groups (Alcohol, Saline, NC) demonstrated significant [Hotelling: $F(45,87) = 1.62$, $p=0.0055$] overall differences in the vector of 15 measurements. Univariate ANOVAs and Tukey-Kramer post-hoc pairwise tests for each measurement were used to test for mean group differences for each facial measurement mean: alcohol vs. saline; alcohol vs. NC; saline vs. NC. Logistic regression stepwise models were used to identify a set of anthropometric measurements that optimally classified the alcohol-exposed lambs from normal control lambs, as well as alcohol-exposed lambs from saline control lambs. Only two measures yielded significant differences between alcohol-treated and NC lambs (lower facial height was smaller and nasal bridge was longer in the alcohol-treated lambs). None of the measures differed significantly between the alcohol-treated and saline-treated lambs; saline lambs were significantly greater than NC lambs for six of the measures (though there were only 6 saline lambs).

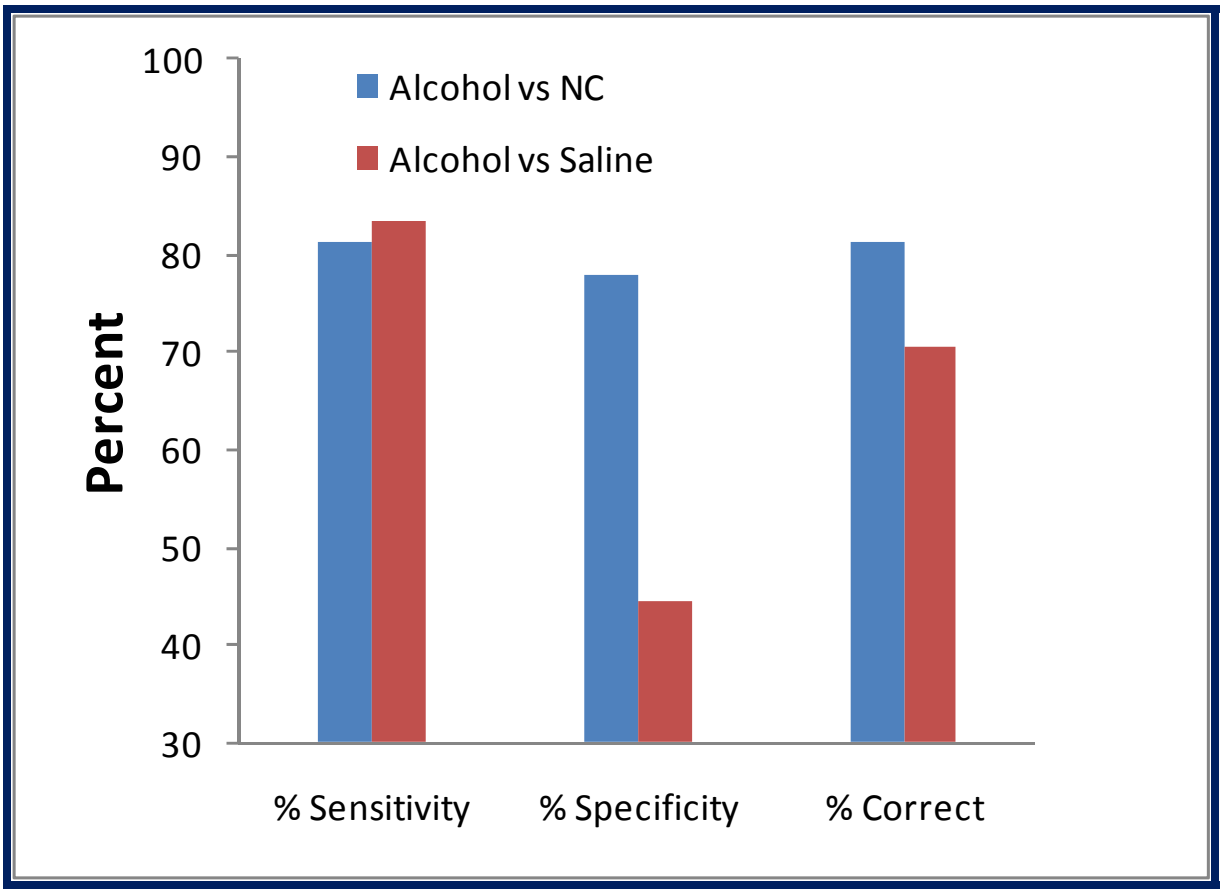


Figure 2: Sensitivity, specificity and % correct for a set of 3 measurements derived from logistical regression stepwise modeling.

Alcohol vs. Normal Control (NC)

Classification using 3 measures successfully identified the alcohol exposed lambs (sensitivity=81%) and NC lambs (specificity=78%)

Measure	Odds Ratio*	p-value
bigonial	1.61	0.0045
lower facial depth	1.37	0.0204
nasal bridge length	0.57	0.0006

Alcohol vs. Saline Control

Classification using 1 measure successfully identified the alcohol exposed lambs (sensitivity=83%) but classification of saline controls was not as good (specificity=44%)

Measure	Odds Ratio	p-value
outer canthal	1.21	0.054

*odds of being alcohol exposed per 1 unit decrease

We concluded that binge-like prenatal ethanol exposure in sheep beginning on gestational day 4 resulted in only limited group-wise effects on individual measures of facial features in newborn lambs. In contrast, a logistic regression model using 3 measures simultaneously (bigonial width, lower facial depth, and nasal bridge length) classified alcohol-exposed from normal control lambs with high sensitivity (81%) and specificity (78%). Multivariate approaches to facial

morphometry may provide sensitive and specific biomarkers of prenatal alcohol exposure in sheep, mice and humans, but measurements that best classify exposed offspring appear to differ across species and even across age. We expect to be able to report results from the 2 and 5 months of age subjects at the 2011 RSA conference.

Neuroanatomical dependent measures: Human magnetic resonance imaging (MRI) and autopsy studies reveal abnormal cerebellar development in children who had been exposed to alcohol prenatally, independent of the exposure period. Animal studies conducted utilizing the rat model similarly demonstrate a broad period of vulnerability, albeit the third trimester-equivalent of human brain development is reported to be the most vulnerable period, and the first trimester-equivalent exposure produces cerebellar Purkinje cell loss only at high doses of alcohol. The sheep model demonstrates a broad period of cerebellar vulnerability. We have previously reported significant cerebellar Purkinje cell deficits, regardless of exposure time period, suggesting multiple mechanisms are responsible for cell loss during late, early, and complete exposure periods. The present study differs from previous studies in that neuroanatomical assessments were performed on the 5 month old postnatal lamb after first trimester exposure or all three trimester exposure periods and after classical conditioning learning was assessed by eyeblink classical conditioning and special learning was assessed by T-maze. Four groups of pregnant sheep were used: all-3 trimester-equivalent pair-fed saline control group, first trimester-equivalent alcohol group (1.75 g/kg), all 3 trimester-equivalent alcohol group (1.75 g/kg), and a normal control group. The alcohol exposure regimen was designed to mimic a human binge pattern. Alcohol or saline was administered intravenously on 3 consecutive days beginning on day 4 and of gestation, and the treatment was followed by a 4-

	NC Male (3)	NC Female (6)	Sal Male (2)	Sal Female (6)	E1 Male (3)	E1 Female (6)	E3 Male (7)	E3 Female (1)
Brainstem	16.73 ± 0.94 (3)	15.84 ± 0.67	18.45 ± 1.63 (2)	15.946 ± 0.70	16.99 ± 1.11	16.522 ± 0.64	16.141 ± 0.59	11.86 ± 1.57
Cerebellum	11.79 ± 0.32	10.495 ± 0.32	12.203 ± 0.76	10.55 ± 0.54	12.005 ± 0.93	11.02 ± 0.54	11.881 ± 0.50	10.45 ± 1.31
Forebrain	79.147 ± 1.67	72.571 ± 1.67	78.907 ± 3.97	74.343 ± 2.81	75.42 ± 4.87	75.838 ± 2.81	78.587 ± 2.60	64.76 ± 6.88
Brain	108.324 ± 2.2	99.744 ± 2.15	118.535 ± 6.26	103.00 ± 3.96	106.39 ± 6.26	104.65 ± 3.61	108.80 ± 3.35	89.84 ±8.85

Table 1: Brain weights in gram ± SEM by group. N per group is given in parentheses.

day inter-treatment interval when the animals were not exposed. Such treatment episodes were replicated until gestational day 41 and 132 in the first and all-3 trimester-equivalent groups, respectively. All lamb brains were harvested on postnatal day 150 and processed for stereological cerebellar Purkinje cell counting.

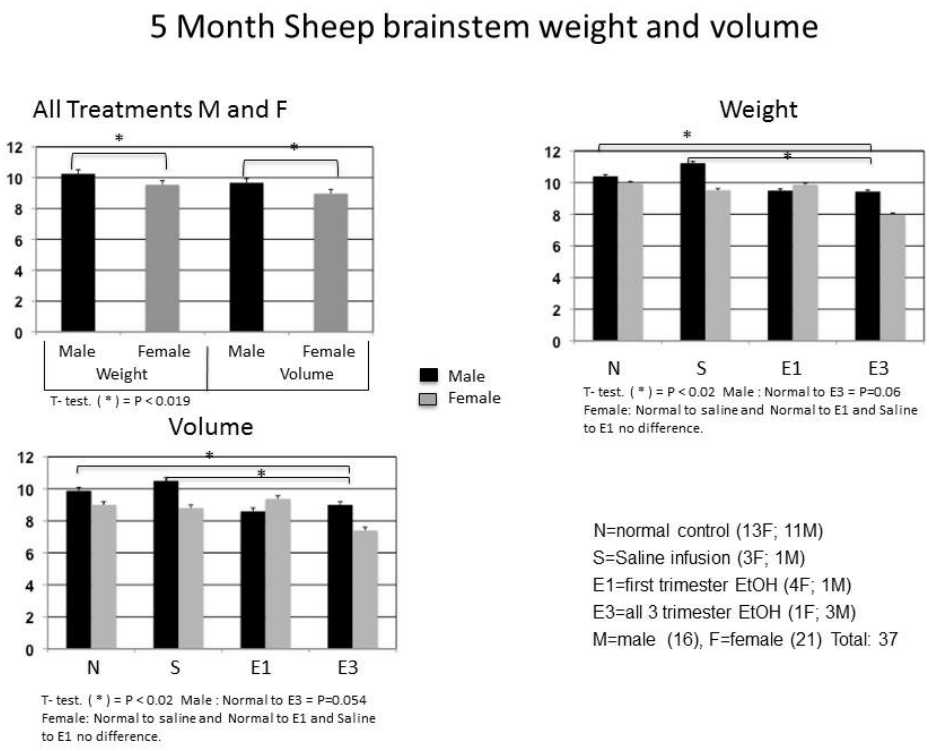


Figure 3: Brainstem weights in grams ± SEM and volumes ml ± SEM presented by group and by sex.

Whole brains and brain components, forebrain, cerebellum and brainstem, have been weighted and analyzed by two-way ANOVA with treatment and sex as factors. Sex was a significant factor for whole brain ($p = 0.009$), cerebellum ($p = 0.024$) and brainstem (0.007) and trended toward significance in the forebrain ($p = 0.067$). Treatment was a significant factor only in the brainstem in the E3 group compared to normal control and saline groups. Purkinje cell counts will be completed in the coming week in time for the RSA abstract deadline.

Choline: Choline is recognized as an essential nutrient and is necessary for fetal development (Zeisel, 2006). Several studies have demonstrated a beneficial effect on central nervous system development and behavioral measures with choline supplementation. Similarly, CDP-choline, composed of cytidine and choline, has been studied in numerous clinical trials in humans with beneficial effects reported for cerebral ischemia, traumatic brain injury, cognitive disorders, stroke, drug and alcohol addiction. Prenatal choline supplementation in rats has been shown to mitigate the adverse effect on development caused by prenatal alcohol exposure (Thomas et al., 2009). Collectively, these studies suggest that choline has important potential as a therapeutic or intervention strategy for fetal alcohol spectrum disorders. The human dietary reference intake for pregnancy is 450 mg/day for adequate intake and 3500 mg/day is the tolerable upper limit (Inst. Med., Natl. Acad. Sci. USA, 1998). This tolerable upper limit in humans would roughly equate to a dose of 60 mg/kg dose in a 60 kg woman. Animal studies in rodents have exceeded this range, with no apparent side effects at 200 mg/kg in mice (Mehta, et al., 2009) and amelioration of the adverse effects of prenatal alcohol exposure at 250 mg/kg in pregnant rats (Thomas et al., 2009). However, in baboons choline was supplemented at a dose of 500 mg per 1000 dietary calories to test it's ability to prevent alcohol induced hepatic

fibrosis. The choline not only failed to prevent the hepatic fibrosis but caused moderate but definite hepatotoxic effects (Lieber, et al., 1985). In rats, dietary deficiency in choline can produce a fatty liver whereas subhuman primates are far less susceptible. This species difference may be explained by differences in activity of choline oxidase, the enzyme that metabolizes choline. Unlike rat liver, human liver contains very little choline oxidase activity (Lieber, et al., 1985). Ruminants, like humans, also have a lower choline oxidase activity than rodents, and therefore both species likely have a lower tolerable upper intake level of choline than rats. We hypothesized that the ovine model would more closely model choline metabolism in humans compared to the rat model, and that sheep are a better model for investigating the safety, dosage, and potential toxicity of choline supplementation.

Non-pregnant ewes were divided into three treatment groups: normal control, high choline and low choline, with 6 ewes per group. The choline groups received choline by mouth once daily for 30 days at a dose of 10 mg/kg daily in the low group and 3.5 g daily in the high group. A microencapsulated form of choline (Reashure®, Balchem) was used to protect against ruminal degradation.

Liver to body weight ratios and liver wet to dry weight ratios were not significantly different between groups. There was no significant difference between groups in the following serum biochemistry measures: total protein, albumin, calcium, phosphorus, glucose, BUN, creatinine, total bilirubin, creatinine kinase, AST, globulins, A/G ratio, GGT and magnesium.

After 30 days, the animals were sacrificed and the livers harvested for histopathology. Upon removal, livers were infused and fixed in formalin. Tissue was then sectioned, paraffin embedded and histology sections stained with Hematoxylin and Eosin. Slides were evaluated independently by three veterinary pathologists blinded to treatment groups. They concluded that while all subjects had some hepatic pathology, no evidence of choline specific toxicity could be identified. However, it is important to understand that these subjects were not pregnant, did not receive concurrent alcohol administration and received the choline for a period shorter than the entire pregnancy. We will evaluate the livers of the sheep that are presently enrolled in the choline study.

Fetal Ultrasound: A pilot project to evaluate the feasibility of validating in the sheep model prenatal screening for neuoanatomical defects from prenatal alcohol exposure as presented in Kfir et al., Ultrasound Obstet Gynecol 33:683-689, 2009 is underway. A total of 21 76 day gestation sheep fetuses have undergone study and we have determined that the measurements given in the table below can be reliably obtained by transabdominal ultrasonography in unsedated animals in a study time of 5 to 15 minutes depending on fetal positioning. Preliminary findings based on limited N, of these eleven structures, the most promising appear to be the orbital diameter (OD) followed by the frontothalamic distance (FTD). The OD in high alcohol + choline group was 8% smaller compared to the low dose + choline group ($p = 0.117$, $n = 6$ per group). The FTD was 13% greater in the high dose + choline group compared to the low dose + choline group ($p = 0.149$, $n=6$ per group). Interestingly, mouse studies suggest that the orbit size would be a sensitive measure of prenatal alcohol exposure while human studies suggest that FTD would be a sensitive measure of prenatal alcohol exposure.

Table 2: Ultrasonograph measurements		
Abbreviation	**Measurement**	**Description**
BPD	Biparietal diameter	Head side to side
OFD	Occipitofrontal diameter	Head front to back
FTD	Fronto-thalamic distance	Front of head to back of thalamus
CTD	Caudo-thalamic distance	Back of thalamus to back of head
TW	Thalamic width	
IOD	Interorbital distance	
OOD	Outer orbital distance	Distance between lateral edges
OD	Orbital diameter	Left or right
LD	Lens diameter, long and short axis	Left or right
FL	Femur length	Left or right
HL	Humerus length	Left or right

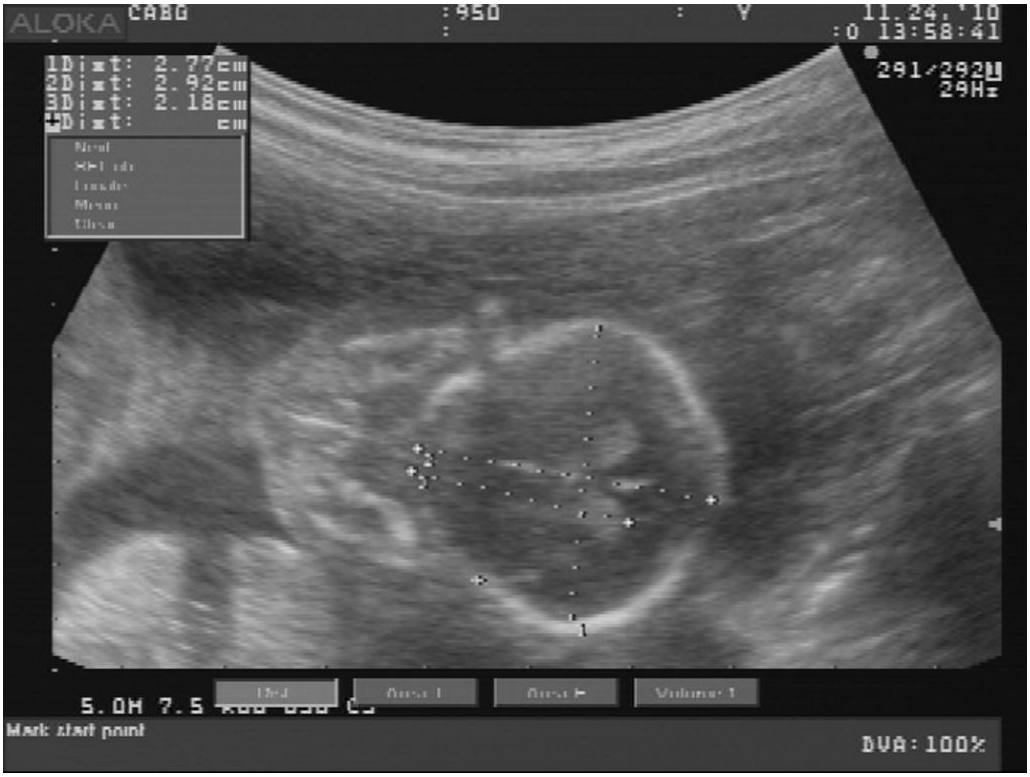


Figure 4: Depicted are the biparietal diameter (BPD), occipitofrontal distance (OFD) and frontothalamic distance (FTD) in a 76 day gestation fetal lamb.

We plan to present an abstract on prenatal ultrasound detection of FASD in sheep at the 2011 RSA meeting.

VI. Discussion: To date, our most interesting finding is the high sensitivity and specificity of facial measurements in sheep when subjected to logistical regression analysis. The specific measures that are useful in the sheep likely are species specific. Nevertheless, this finding suggests that the sheep is a model of FAS that could prove to be valuable in the evaluation of screening tools, mechanism and prevention.

The study to be completed in 2011 will be the first using a dose of 2.5 g/kg in sheep and we believe that functional as well as anatomical dependent measures will be sensitive to this dose.

VII. Interrelation with Aims of the Consortium and Other Projects: We collaborate and share tissues with Dr. Zhou (for the evaluation of raphe serotoneric neurons), Dr. Miranda (micro RNA biomarker pilot project) and Dr. Savage (placental proteomic biomarkers). Our choline project supports the choline work of Dr. Thomas and Dr. Chambers. Our ultrasound work supports the work of Dr. Hull. Our facial dysmorphology work interfaces with Dr. Sulik, Dr. Zhou and Dr. Jones. Our neurobehavioral work interfaces with the work of Dr. Thomas.

VIII. Plans for Next Year: During 2011, we will complete the second study on choline protection. We will complete the fetal ultrasound pilot project. Abstracts on the face, fetal ultrasound and neuroanatomical measures will be presented at the 2011 RSA meeting. A manuscript will be submitted reporting all of the findings from the first study.

IX. Publications (2010):

Johnson, T.B., Stanton, M.E., Goodlett, C.R., Cudd, T.A. T-maze learning in weanling lambs. Develop. Psychobiol. In review.

Facial dysmorphology in an ovine model of fetal alcohol spectrum disorders. In preparation.

X. Posters and Presentations (2010): The abstracts below were presented at the 2010 RSA meeting:

Johnson, T., Stanton, M. Goodlett, C.R., Cudd, T.A. T-maze learning deficits following first trimester equivalent binge alcohol exposure in weanling lambs. Alcohol. Clin. Exp. Res. Supp. 34:97A, 2010.

Goodlett, C.R., Wetherill, L., Moore, E.S., Johnson, T.B., Lunde, R.E., Cudd, T.A. Evaluation of facial dysmorphology in an ovine model of fetal alcohol spectrum disorders. Alcohol. Clin. Exp. Res. Supp. 34:100A, 2010.

Johnson, T.B., Torres, Z., Gonzalves, A., Goodlett, C.R., Cudd, T.A. Post-mortem corpus callosum digital morphology following prenatal alcohol exposure: ovine model. Alcohol. Clin. Exp. Res. Supp. 34:103A, 2010.

Wilson, S.E., Box, J., Lunde, E.R., Goodlett, C.R., Cudd, T.A. Maternal choline supplementation in the ovine model. Alcohol. Clin. Exp. Res. Supp. 34:213A, 2010.

Miranda, R.C., Balaraman, S., Sathyan, P., Lunde, R., Box, J., Cudd, T. Brain and circulating micro-RNA (MIRNA) biomarkers of fetal ethanol exposure. Clin. Exp. Res. Supp. 34:245A, 2010.

Balaraman, S., Lunde, R., Box, J., Cudd, T.A., Miranda, R.C. Circulating microRNAs as biomarkers for fetal alcohol exposure. Alcohol. Clin. Exp. Res. Supp. 34:13A, 2010.

I. Principal Investigator: Kathleen K. Sulik, Ph.D.

II. Title of Project: Magnetic Resonance and Diffusion Tensor Imaging of a Mouse FASD Model, U01 AA171204

III. Objectives: The primary objective of this work is to inform the establishment of improved diagnostic criteria for FASD and to make new discoveries regarding the impact of prenatal ethanol exposure on the brain and face. Utilizing a mouse FASD model, critical periods of ethanol-induced teratogenesis, with emphasis on embryonic and early fetal periods of development, are being assessed. Magnetic Resonance Imaging (MRI) and Diffusion Tensor Imaging (DTI) are being employed to allow detailed analyses of brain and facial structures and correlation of abnormalities of the brain and face within single individuals, as well as comparison among experimental groups. The results of this work will be compared to corresponding analyses of human subjects in addressing our overall hypothesis that ethanol induces structural abnormalities of the brain and face of mice that are consistent with and informative for those in human FASD.

In meeting this objective, our work is directed toward fulfilling the following specific aims:
1. To utilize high resolution Magnetic Resonance Imaging (MRI) and Diffusion Tensor Imaging (DTI) as a high throughput screening platform to provide comprehensive documentation and discovery of the ethanol-induced CNS dysmorphology that results from prenatal ethanol exposure at embryonic and early fetal stages of development.
2. To define, utilizing high resolution Magnetic Resonance Imaging (MRI) scans and 3-D reconstructions, the facial dysmorphology that results from prenatal ethanol exposure at embryonic and early fetal stages in mice and to relate the character and severity of these defects to accompanying brain abnormalities.
3. To identify, utilizing selected sections or 3-D reconstructions of MRI scans, regions other than the brain or face that may serve as diagnostic indicators of prenatal ethanol exposure.

IV. Methods: High resolution MRI and/or DTI methodologies are employed for all of our proposed work. Both individual scans and 3-D reconstructions derived from them are analyzed to provide data regarding developmental stage and dose-dependent dysmorphology of the brain and face of ethanol-exposed fetal mice. In order to efficiently utilize our resources, a subset of fetuses are being collected in our laboratory, while another subset is being collected in Dr. Zhou's laboratory. Pregnant mice are acutely administered alcohol via either intraperitoneal injections or via liquid diet on GD 7, 8, 9, 10, or 11, or are chronically administered alcohol over the period of GD7-11, 12-16, or 7-16. All fetuses are collected on GD 17, fixed in a Bouins + Prohance solution and delivered to Duke University's Center for In Vivo Microscopy (CIVM) for imaging. Imaging data is sent to our laboratory electronically for segmentation, reconstruction, and data analyses. For Specific Aims 1 & 2, linear, volume and/or area measurements for a number of selected brain and facial regions are made, with comparisons made between relevant control and alcohol-exposed specimens and potential correlations between facial and brain dysmorphology identified. For Specific Aim 3, regions other than the brain or face that may serve as diagnostic indicators of prenatal ethanol exposure are being similarly investigated.

V. Accomplishments and Results: Overall, our progress in the 3.5 years since this project was initially funded has been steady and substantial. Following is a review of our progress during 2010. During this year, our CIFASD- funded publications have included 5 full papers, a review article, and 9 abstracts. Two other full papers have been written, one of which has been submitted, and one of which is under revision. In addition, we have written 2 review articles, one of which is in press and the other of which is being finalized for submission. Our data has

also been presented by members of our laboratory in seminars, symposia, invited and course lectures, as outlined in section X. Notably, this included the TK Li plenary lecture and the FASDSG Merit Award talk at the 2010 RSA meeting, as well as a talk for the June, 2010 NIAAA Council meeting.

Progress toward each of the original specific aims follows:

Specific Aim 1: In 2010, papers describing MRI-based analyses of acute GD7 (Godin et al, 2010 a) and GD10 ethanol-induced brain abnormalities (O'Leary-Moore et al, 2010 a) have been published. The later included data regarding normal brain development in fetal GD 16-17 mice. Importantly, we have shown that the observed ethanol-induced changes in regional brain volumes are not the result of a simple developmental delay.

That the GD7 ethanol insult results in a spectrum of defects, some of which are within the holoprosencephaly (HPE) spectrum, was further described in an article published as part of an HPE review (Lipinski et al, 2010). Discussed is the developmental basis for HPE as caused not only by alcohol, but by other teratogens including cyclopamine. Genetic pathways that appear to be impacted by ethanol to yield median brain and facial deficiencies are highlighted. Of particular note is the sonic hedgehog pathway, which is involved in human HPE and also in facial clefting phenotypes (Lipinski et al, 2010b). Importantly, cleft lip and palate are recognized as part of the human fetal alcohol spectrum, but their genesis has received little research attention. These findings have provided the foundation/rational for future developmental and gene/environment interaction investigations in mice and for selection of potential candidate genes for analyses in FASD cases.

Following up on our MRI-based results, we have conducted immunohistochemical and in situ hybridization studies that have confirmed GD7 ethanol-induced ventro-median forebrain tissue loss (Godin et al, 2010b). This work has also illustrated reduction in the progenitors of cortical interneurons and oligodendrocytes. Thus, the primary ventro-median tissue loss yields a secondary effect involving the cortex.

In addition to the acute ethanol-exposure studies, for our chronic dietary ethanol exposure work we have collected, imaged, and segmented all of the fetuses for the GD7-16 group; collected and imaged all of those for the GD7-11 group and have nearly completed the segmentation for these fetal brains; collected and imaged approximately 1/3 of the fetuses for the GD12-16 group but have not yet begun the segmentation work on them. The total number of fetuses for the three exposure times will be 45.

Regarding DTI application, progress has been substantial with a first manuscript describing this work having been submitted (O'Leary-Moore et al, submitted). In addition to identifying changes in the corpus callosum, anterior commissure and fornix in fetal mice following GD7 ethanol exposure, fiber tract abnormalities have also been found in postnatal (adolescent) animals. As shown in Figure 1, in postnatal day 45 mice, corpus callosum deficiencies are readily apparent, with the morphology appearing strikingly similar to that published for children with FAS. Thus, it is clear that the defects caused by early prenatal ethanol exposure in mice are both persistent and pertinent to humans.

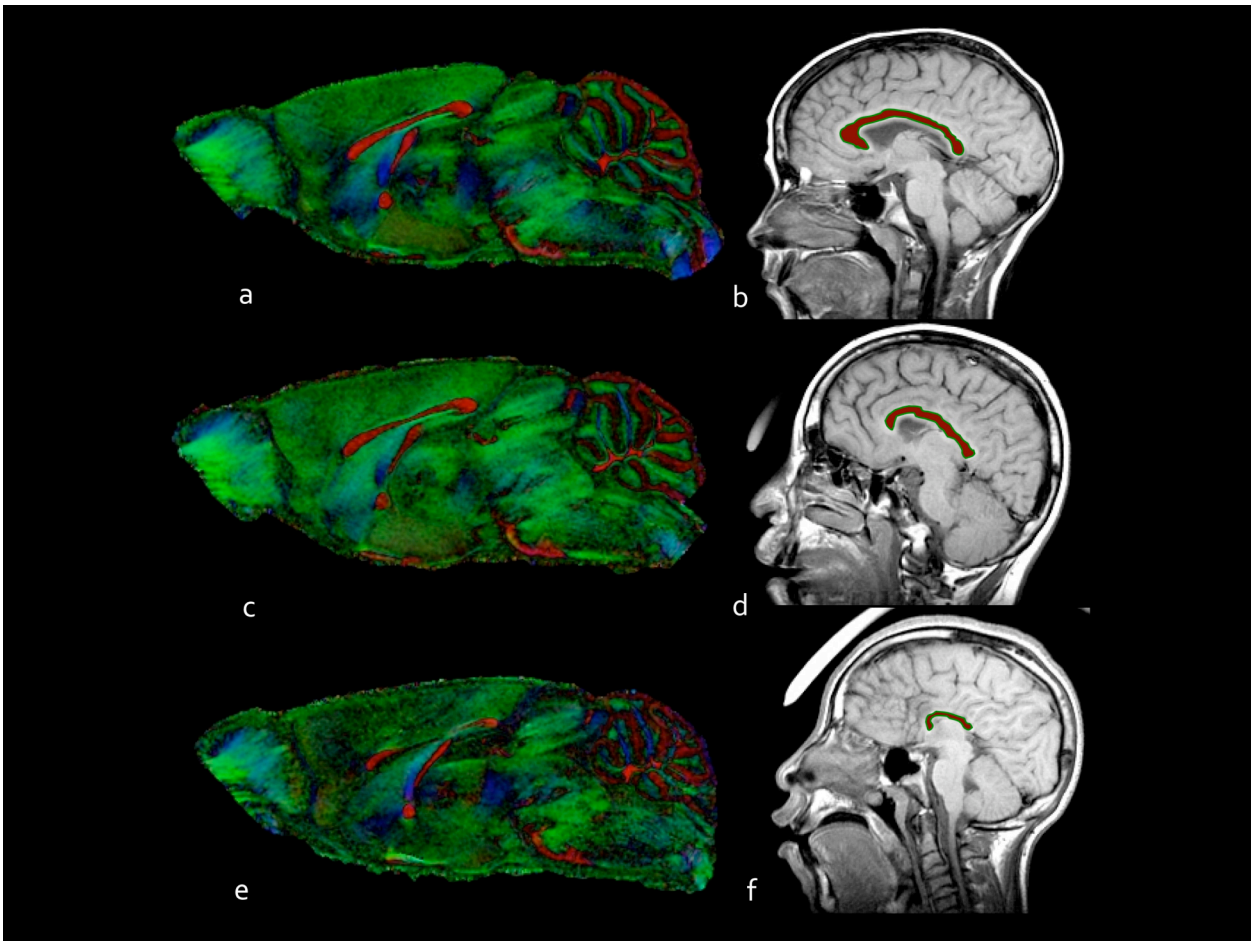


Figure 1. Magnetic resonance imaging (MRI) of prenatal alcohol-exposed 45 day old mice (c, e) and individuals with fetal alcohol syndrome (FAS) (d, f) illustrate comparable corpus callosum (CC) dysmorphology as shown in midsagittal scans. The normal form of the mouse CC is shown as the red structure between the cerebral hemispheres in a, and in a child in d. Subsequent to prenatal alcohol exposure on gestational day 7, moderate thinning, which is particularly notable in the mid-region of the CC is apparent in the mouse brain shown in the MRI in c. In an MRI from a more severely affected animal shown in e, the middle of the CC appears to be completely absent. In this animal, the anterior and posterior aspects of the CC, although reduced in size, remain evident. These defects appear remarkably similar to those in two variably affected FAS patients (d and f; human MRIs are courtesy of Dr. S. Mattson).

Specific Aim 2: In collaboration with Dr. Peter Hammond, we have begun analyses of 3-D MRI reconstructions of GD 16.5 and GD17 control and GD17 fetal mice that had been exposed to alcohol early in gestation. Images like those shown in Figure 2 are being analyzed to define ethanol-induced facial characteristics and to allow face/forebrain correlations for fetuses that had been acutely exposed to ethanol on either GD7 or 8. Entire litters are being examined so that a wide range of insult is included. To date, we have imaged 2 vehicle-treated control litters on GD 16.5 and 3 litters on GD17, for a total of 41 control fetuses. Additionally 5 litters (41 fetuses) from the GD7 ethanol exposure time have been collected on GD17 and imaged. Segmentation of the cerebral cortices, olfactory bulbs, diencephalon, hippocampus, striatum, septal region, pituitary, lateral and third ventricles is underway, with a total of 32 brains having been completed to date for this project.

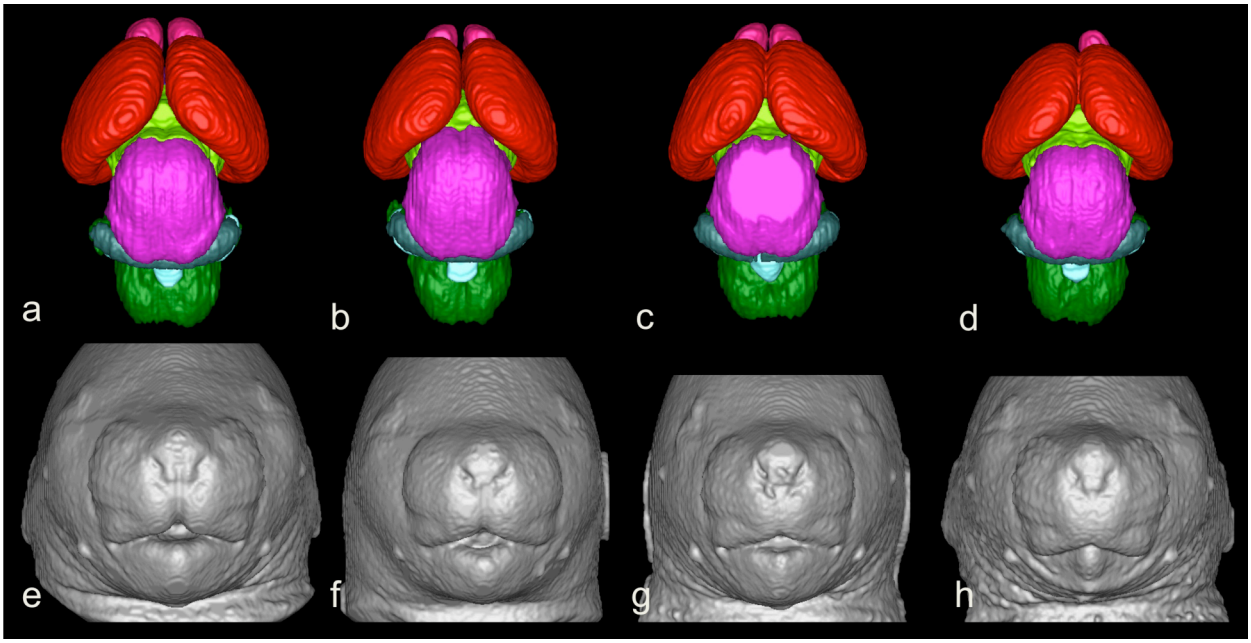


Figure 2. Shown are reconstructed magnetic resonance microscopy (MRM) images of the brain and face of a normal gestational day 17 fetal mouse (a, e), along with those from 3 fetuses that had been exposed to alcohol on their 7th gestational day (b, f; c, g; d, h). As compared to the normal fetus, the affected fetuses have varying degrees of olfactory bulb [bright pink structures at the top (front) of the brain] deficiency. Also notable in (c) is the abnormally close proximity of the two olfactory bulbs and of the cerebral hemispheres (red structures). The latter are not separated by a deep interhemispheric groove as in the normal brain. The faces of the alcohol-exposed mouse fetuses (f-h) show a characteristically small nose, with the nostrils being too closely approximated, accompanied by a long (from nose to mouth) central portion of the upper lip that is lacking its normal central groove. The animal in (h) also has an obviously small lower jaw (mandible). Brain region color codes are as follows: Pink = olfactory bulbs; Red = cerebral hemispheres; Light green = diencephalon; Magenta = mesencephalon; Teal = cerebellum; Dark green = hindbrain (minus cerebellum).

Specific Aim 3: This year we have focused on GD8 ethanol insult and its potential relationship to sudden infant death. We have found that the carotid body, a chemoreceptor that is innervated by the ninth cranial nerve is diminished in size or even absent in some of the alcohol-exposed fetal mice (Parnell et al, 2010; abstract). Currently, physiological studies are being conducted on newborn pups in an effort to identify altered response to hypoxia.

VI. Discussion: Our studies are providing important new data from which to draw for the study of human FASD subjects and to further our understanding of the range and critical periods for induction of defects that result from prenatal alcohol exposure. Importantly, during this reporting period, Dr. Sowell's analyses (Roussotte et al) have illustrated that in humans, as in our acute GD7-exposed mice, ethanol-induced facial changes are associated with ventromedian forebrain deficiencies. During 2010, we have extended our study of fetal specimens to newborn and adolescent animals. This work has shown that the dysmorphology resulting from very early insult persists into the postnatal period. Extending our work into the postnatal period also provides for correlation of morphological data to functional/behavioral alterations. In this regard, during this reporting period, Dr. Parnell, who was the primary author for our MRI studies of fetal

mice after acute GD8 ethanol-exposure, has received a K99 award to conduct behavioral analyses as a follow-up to his previous work. We have also begun behavioral studies on a GD7-exposed cohort, with inclusion of seizure threshold analyses.

VII. Interrelation with the consortium and other projects: Our high resolution MRI analyses of the CNS compliment those human MRI studies being conducted by Dr. Sowell and the ultrasound project of Dr. Hull, while the facial reconstruction work compliments that employing 3D images as are being prepared and analyzed by Dr. Faroud's group. Additionally, our mouse model work correlates with that of Dr. Cudd, which employs MRI analyses of a sheep FASD model. In testing our main hypothesis, we are working directly with Dr. Zhou's laboratory to produce and assess ethanol-exposed C57Bl mice.

VIII. Plans for the next year:

1. Submit manuscripts describing our MRI results from the acute GD 9, and 11 exposure groups
2. Complete the segmentations and analyses of all of the chronic ethanol exposure groups.
3. Work with Dr. Hammond to analyze and compare the mouse face/forebrain dysmorphology resulting from GD7 vs GD 8 ethanol exposure.
4. Conduct studies directed to further examining the genesis of GD7 ethanol exposure-induced hydrocephalus.
5. Submit 4-5 RSA abstracts, including one for a proposed symposium on the face of FAS
6. 2011 presentations will include a UNC seminar by Dr. Parnell, and participation by Dr. Sulik in an International FASD meeting in Vancouver, an Alcohol meeting in Volterra, Italy, and the Teratology Society Meeting with presentation of the Warkany lecture.

IX. Publications (2010):

Full papers

1. Godin EA, O'Leary-Moore SK, Khan AA, Parnell SE, Ament JJ, Dehart DB, Johnson BW, Johnson GA, Styner MA, Sulik KK, Magnetic Resonance Microscopy Defines Ethanol-Induced Brain Abnormalities In Prenatal Mice: Effects Of Acute Insult On Gestational Day 7. Alcohol Clin Exp Res, 2010 Jan 34:98-111 PMID: 19860813
2. Lipinski RJ, Godin EA, O'leary-Moore SK, Parnell SE, Sulik KK, Genesis of Teratogen-induced Holoprosencephaly in Mice, Am J Med Genetics, Part C 154C: 29-42, 2010 PMID: 20104601
3. Lipinski RJ, Song C, Sulik KK, Everson JL, Gipp JJ, Yan D, Bushman W, Rowland IJ. Cleft lip and palate results from Hedgehog signaling antagonism in the mouse: Phenotypic characterization and clinical implications. Birth Defects Res A Clin Mol Teratol. 2010 Apr; 88(4):232-40. PMID: 20213699
4. O'Leary-Moore SK, Parnell SE, Godin EA, Dehart DB, Ament JJ, Khan AA, Johnson GA, Styner MA, Sulik KK, Magnetic resonance microscopy-based analyses of the brains of normal and ethanol-exposed fetal mice Birth Defects Res A Clin Mol Teratol. 2010 Nov;88(11):953-64. PMID: 20842647
5. Godin EA, Dehart DB, Parnell SE, O'Leary-Moore SK, Sulik KK, Ventromedian forebrain dysgenesis follows early prenatal ethanol exposure in mice. Neurotoxicol Teratol. 2010 Nov 11. [Epub ahead of print] PMID: 21074610

Book chapters and review articles

1. Sulik KK, O'Leary-Moore SK, Godin E, and Parnell S, Normal and abnormal embryogenesis of the mammalian brain, In: Drugs in Pregnancy –The Price for the Child, Preece and Riley (Eds.), (in press)

2. O'Leary-Moore SK, Parnell SE, Godin EA, Sulik KK, Magnetic Resonance-Based Studies of FASD Animal Models, Alcohol and Health (sidebar article, in press)
3. Sulik KK, Images: Looking at FASD through the eyes of an embryologist, Fetal Alcohol Forum, NOFAS-UK, Nov, 2010

Submitted and in preparation
1. O'Leary-Moore SK et al, DiffusionTensor Imaging and tractography define a spectrum of fiber tract dysmorphology in the brains of prenatal ethanol-exposed mice. submitted to Neurotoxicol Teratol.
2. O'Leary-Moore SK et al Magnetic Resonance-based imaging in animal models of Fetal Alcohol Spectrum Disorder, Neuropsychology Review (in prep)
3. Parnell SE et al, Magnetic Resonance Microscopy Defines Ethanol-Induced Brain Abnormalities In Prenatal Mice: Effects Of Acute Insult On Gestational Day 9 (in prep)
4. O'Leary-Moore SK et al, Magnetic Resonance Microscopy Defines Ethanol-Induced Brain Abnormalities In Prenatal Mice: Effects Of Acute Insult On Gestational Day 11 (in prep)
5. Roussotte, F, Sulik KK, Mattson S, Riley E, Jones K, Adnams C, May PA, O'Connor M, Narr K, Sowell E. Brain volume reductions, facial dysmorphology, and cognition in fetal alcohol spectrum disorders (under revision)

X. Posters and Presentations (2010):
Abstracts
1. Lipinski RJ, Hammond P, Ament JJ, Jiang Y, Fubara B, Johnson GA, Sulik KK, Examination of face-brain dysmorphology patterns in a mouse model of hedgehog signaling antagonist-induced cleft lip and palate. Proc Greenwood Genetics Center (in press), 2011
2. O'Leary-Moore SK, Parnell SE, Godin EA, Johnson, GA, Styner M, Oguz I, Budin F, Jiang Y, Dehart DB, Sulik KK, Magnetic resonance microscopy and diffusion tensor imaging define stage-dependent alterations in brain morphology following prenatal exposure to ethanol in mice. Alcohol Clin Exp Res, 34:298A, 2010
3. Sulik KK. O'Leary-Moore SK, Parnell SE, Godin EA, Styner MA, Johnson, GA, Magnetic resonance imaging-based analyses of a Fetal Alcohol Spectrum Disorders mouse model, Alcohol Clin Exp Res, 34 suppl: 31A, 2010
4. Godin EA, Parnell SE, Dehart DB, Sulik KK, Acute ethanol exposure early in pregnancy affects medial ganglionic eminence-derived oligodendrocyte progenitor cells and interneurons, Alcohol Clin Exp Res, 34: 159A, 2010
5. Lu Z, Sulik KK, Chen S-Y, Acute ethanol exposure results in reduced FGF8 expression in the developing mouse forebrain, Alcohol Clin Exp Res, 34:208A, 2010
6. Parnell SE, O'Leary-Moore SK, Johnson GA, Jiang Y, Styner MA, Budin F, Oguz I, Sulik KK, Neuronal fiber pathway abnormalities in mice resulting from gestational day 8 ethanol exposure as demonstrated by diffusion tensor imaging Alcohol Clin Exp Res, 34:160A, 2010
7. Parnell SE, Dehart DB, Sulik KK, Hypoplasia of the carotid body in C57Bl/6J mice following early gestational ethanol exposure: Implications for sudden infant death syndrome, Alcohol Clin Exp Res, 34:160A, 2010
8. O'Leary-Moore SK, Godin EA, Parnell SE, Johnson GA, Jiang Y, Styner MA Dehart DB, Ament JJ, Pecevich S, Khan AA, and Sulik KK, Examination of ethanol-induced craniofacial and central nervous system dysmorphology in an animal model using high resolution magnetic resonance microscopy and diffusion tensor imaging. Proc Greenwood Genetics Center (in press), 2010
9. Sulik KK. O'Leary-Moore SK, Parnell SE, Godin EA, Styner MA, Johnson, GA, Magnetic resonance imaging-based analyses of a Fetal Alcohol Spectrum Disorders mouse model, ISBRA proceedings, 2010

Presentations

KK Sulik

"Fetal Alcohol Spectrum Disorder", UNC Medical School, Dec 15. 2010

"Imaging-based Advances in Basic Fetal Alcohol Spectrum Disorder Research"; Sanford Children's Hospital, Sioux Falls, SD, Nov, 2010

"Brain Structure and Function in an FASD Mouse Model"; UNC Bowles Center for Alcohol Studies seminar, Oct, 2010

"CNS Imaging and Behavior in a Mouse Model of FASD", UNC Carolina Institute for Developmental Disabilities, Oct 12, 2010

"Central Nervous System Abnormalities in FASD: New Insights from an Animal Model"; Addiction Professionals of North Carolina, Carolina Beach, NC, Oct, 2010

"Prevention of FASD: a "Hands on Science" Approach"; NC Substance Abuse Prevention Providers Mtg, Carolina Beach, NC, Oct, 2010

"Magnetic resonance imaging-based analyses of a Fetal Alcohol Spectrum Disorder mouse model"; ISBRA, Paris, France, September, 2010

"Normal and Abnormal Mammalian Embryogenesis", Reproductive Epidemiology, UNC School of Public Health, Sept 7, 2010

"Genes, Teratogens and Craniofacial Abnormalites"; Conference on Congenital Labiopalatine Anomalies, Bauru, Brazil, August, 2010

"Disruption of Normal Development with Exogenous Agents"; Society for Developmental Biology, Albuquerque, NM, August, 2010

"FASD: Research to Prevention"; TK Li Lectureship, Research Society on Alcoholism, San Antonio TX, June, 2010

"Application of MRM to Defining Toxicant-Induced Abnormalities in the Developing Brain"; Society of Toxicologic Pathology Mtg, Chicago, Ill, June, 19 2010

"Imaging-based Advances in Basic Fetal Alcohol Spectrum Disorders Research" NIAAA council Mtg, June 10, 2010

"Normal and Abnormal Mammalian Embryogenesis", NIH, Bethesda MD, April 20, 2010

"Congenital Disorders of the CNS", UNC, Pathology 715, April 16, 2010

"FASD: Research to Prevention"; Neurodevelopmental Disorders Research Center, April 7, 2010

"New Views on Normal and Abnormal Facial Morphogenesis" American Cleft Palate Association, D. Ralph Millard Jr., MD, Keynote Address, Fort Worth Texas, March 17, 2010

"FASD: Research to Prevention"; UNC School of Dentistry; Feb 12, 2010

"FASD: An Overview"; NC Court Improvement Program Training Conference, Concord NC, January 29, 2010

SK O'Leary Moore

"High-Resolution Neuroimaging Studies of Ethanol-Induced Teratogenesis in a Mouse Model"; UNC Bowles Center for Alcohol Studies Seminar Series. March 29, 2010.

"Functional and neuroanatomical consequences of exposure to ethanol in early gestation". *Fetal Alcohol Spectrum Disorders Study Group (Research Society on Alcoholism)* San Antonio, TX, June 2010. Merit Award Recipient Presentation.

"Magnetic resonance microscopy and diffusion tensor imaging define stage-dependent changes in brain morphology after prenatal ethanol exposure in mice. *Research Society on Alcoholism*, San Antonio TX, June 2010. Symposium: “You can measure that? Leading-edge neuronal techniques applied to rodent and human alcohol research”

EA Godin

"Magnetic Resonance Imaging, In Situ Hybridization, and Immunohistochemistry-Based Analyses of Early Prenatal Ethanol Exposure-Induced Central Nervous System Abnormalities" UNC Ph.D. Dissertation Defense, July 9, 2010

RJ Lipinski

"Examination of face-brain dysmorphology patterns in a mouse model of hedgehog signaling antagonist-induced cleft lip and palate", DW Smith Workshop on Malformations and Morphogenesis, August 2010

I. Principal Investigator: Feng C. Zhou, Ph.D.

II. Title of Project: Mouse Model Neuro-Facial Dysmorphology: Translational and Treatment Studies, U01 AA14819

III. Objectives:

Specific Aim 1. Determine the longitudinal 3D-facial dysmorphology as a function of the dose and developmental timing of alcohol exposure via liquid diet consumption in the C57BL/6 mouse model.

Specific Aim 2. Determine the brain's structural and neuro-facial abnormalities as a function of the dose and developmental stage of alcohol exposure. (parallel to Aim 1)

Specific Aim 3. Determine the extent to which neurotrophic peptide NAP/SAL, given concurrently with prenatal alcohol exposure, will provide long-term protection against alcohol-induced neuro-facial dysmorphology and neurobehavioral deficits.

IV. Methods:

Animal treatments

C57BL/6J dams were divided into three groups included Alcohol (ALC), Pair-Fed (PF), and Chow (C). Chow group received ad libitum chow and water diet throughout experimental period. Both ALC and PF groups received a pre-preganancy alcohol treatment with a 2.4% v/v liquid diet for two days, then 5 days 4.8% v/v alcohol prior to mating. During pregnancy, ALC group were treated with 4.8% alcohol, in optional 2 developmental periods, embryonic day 7 (E7)-E11 or E7-E16. The Pair-Fed dams were given volumes of an isocaloric diet (maltose-dextran replacing alcohol) matched to a respective alcohol-consuming dam. Ad lib chow and water were provided when no treatment procedure were involved. For embryonic analyses, all embryos were harvested at E17. For postnatal analyses, pups were tested at postnatal (P) 7 and P21 for microCT, or other ages for behavioral paradigms.

1. Embryonic Facial Measurement and Growth Analyses

1A. Embryonic Facial Measurement Analysis:
The images of craniofacial regions over 80 angles were captured for each embryo with microvedio under microscope. Fifteen facial measurements, paralleling those of the human anthropometric studies, were taken. Univariate ANOVAs were performed for each measure (litter nested within treatment) with the alpha level adjusted. Pair wise differences between treatments were examined in facial measurements with a treatment p-value less than the threshold utilizing a Tukey-Kramer correction for multiple testing. Discriminant analysis was used to identify which combination of facial measurements would best classify the embryos as being either alcohol exposed, non-exposed, or overall correct classification. To examine effects in dysmorphology due to nutrition, the same analysis was used to compare the PF to Chow controls. To ensure the best possible model, forward, backward and stepwise models were employed. Correct classification results are reported as sensitivity (% of alcohol exposed embryos correctly classified), specificity (% non-alcohol exposed (Chow or PF) embryos correctly classified), and overall correct classification (% all embryos correctly classified). Classification percentages were based on cross-validation results.

1B. Gross Embryonic Growth Analyses:
The Head circumference, Crown-rump length and Embryonic weights were measured at E17 embryos Crown-to-rump length is defined as the lateral distance between the crest of the skull (highest point) and the farthest rump. Cranial circumference is a circular measurement at the level at low edge of orbital lens and bridge of the snout. The embryonic weight was total body weight. ANOVA among treatment groups and t-test between treatments were used for statistical analysis.

2. Computational 3D embryonic facial analysis

Video Volume Algorithms: Collecting accurate 3D surface data from mouse embryos is difficult since the size of the embryo is too small for 3D surface scanners (e.g. laser scanners), and CT/MRI scans do not provide high quality surface representations. One alternative approach that has been developed in Computer Vision is to take multiple 2D images of the same object from different views and reconstruct the 3D shape using a 3D reconstruction algorithm. But, 3D reconstruction from photos is generally not very robust and accurate due to reconstruction errors and camera calibration errors. In a new approach, we developed a novel approach that explores a new video volume concept which avoids the 3D reconstruction process by directly extracting surface features from the video volume of multi-view 2D images. A video volume is a 3D image volume formed by stacking 2D video images taken from a sequence of angled steps. Since the micro-video images of all subjects were taken with the same camera system, 3D image features extracted from the stacked volumes represent the 3D shape properties of the subjects. In order to capture the facial features of each mouse embryo, the silhouette surface first needs to be extracted from the video volume images. Surface features were then be computed from this silhouette surface and analyzed using a 3D surface feature analysis algorithm.

Silhouette surface extraction: A silhouette surface is the surface formed by all the silhouette contours of the 2D images. Theoretically, an inverse radon transformation will transform this surface to the original 3D face. In a video volume, the silhouette surface can be constructed using an iso-surface extraction algorithm. The iso-value to be used should be a value between the background color and the lowest face image intensity. This ensures that the extracted surface will come from only the outer layer contours of each 2D image. A marching cube iso-extraction algorithm is applied to generate the polygon mesh surface. The Figure 1 shows the original volume (Figure 1a), the silhouette contour curves (Figure 1b), and the extracted silhouette surface (Figure 1c,d).

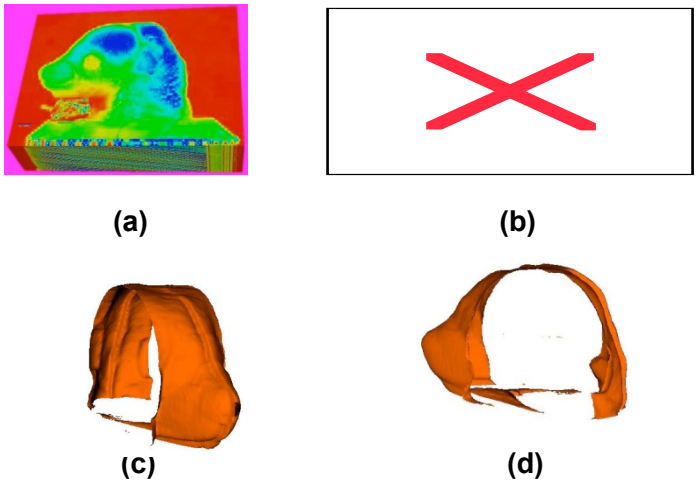


Figure 1. (a) The original stacked video volume; (b) The contour curves of the sequence of 2D images. (c)(d) The silhouette surface was formed by the contours

Surface alignment: Using a template surface as a reference, for each vertex, *P*, of the template surface, we iteratively computed the corresponding vertex, Q, on each of the other surfaces so that the distance between P and Q was minimum. This correspondence mapping allows us to compare the surfaces from different embryos at a vertex-by-vertex level.

Feature Selection and Analysis: At each vertex, the Gaussian curvature and a mean curvature were computed to represent the local surface property. Principal Component Analysis (PCA) is used to reduce the dimensionality of the image samples. Linear Discriminant Analysis (LDA) projection is performed in the space of the PCA projection for the optimal feature set that provides the most

discriminatory power. Radial Basis Function Networks classifier is used to analyze the selected feature vector.

*Validation***:** A Leave-One-Out cross validation approach is used for testing.

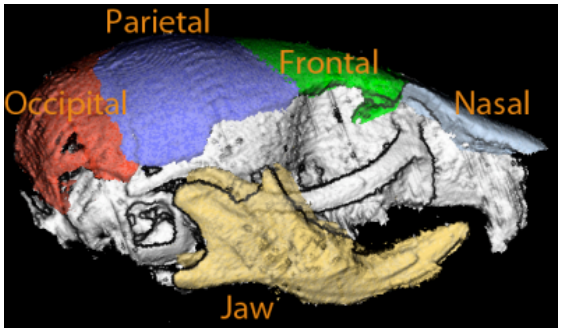


Figure 3. Segmented frontal, parietal, occipital, jaw and nasal bones shown on the whole skull.

*Human Comparison***.** In order to compare with human subjects, we employed a similar video volume analysis approach for human data. In this case, we used the scanned 3D human model (polygon mesh) and computer graphics rendering techniques to simulate the micro-video image collection process. For each 3D facial model, 100 2D images from 100 fixed angles were generated using OpenGL rendering operations with a given lighting condition. These 2D images were stacked together to form a video volume, which were then processed and analyzed the same way as the mouse images. The Figure 2 shows some examples of the silhouette surfaces of human facial data.

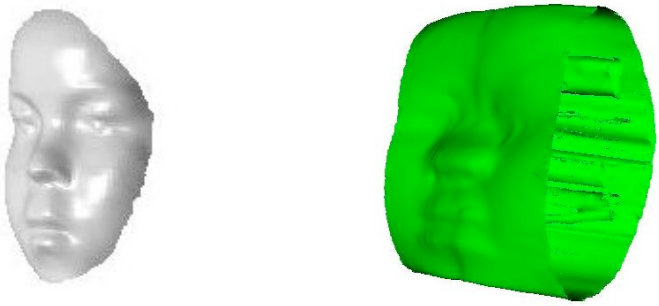


Figure 2. A 3D human facial model and its silhouette surface.

3. Micro-CT analysis in P7 and P21.
C57BL/6 dams were treated Chow, Pair-fed (PF), or alcohol (ALC, with 4.8%v/v alcohol pre-pregnancy and during pregnancy from E7-E16) as indicated above in Methods 1. At birth, Chow treated surrogate mothers were used for all the treated groups for nursing the offspring. The EVS-R9 micro-CT system was used for craniofacial bone image acquisition for this study. The Micro-CT scan yielded volumetric data for each mouse sample with 40x40x40 μm3 voxel spacing. Pups born to all treated dams were scanned at postnatal 7 day (P7) and at P21 for longitudinal studies of craniofacial bone development. Currently, two analyses were completed:

(a) *Facial anthropometric analysis* with measurements paralleled to CIFASD human study as described above in Methods 1A. Three additional measures, which are unique to the cranium, are inner orbital distance, bony mandible, and cranium circumference. The inner orbital is defined as the minimum separation (width) between orbital bones when viewed from the front. It is different from the inner canthal width since it measures the minimum orbital separation beneath the frontal surface of the orbital bones. Bony mandible length is the distance (depth) between the gonia and the tip of the menton. Circumference is the outer perimeter of the skull enclosure at the level of “nasion.” The results of the craniofacial anthropometric measures were compared to the treatments as shown in Table 1.

(b) *3D volume analysis of individual bones.* Craniofacial bone dysmorphology is an important but under explored diagnostic feature of Fetal Alcohol Spectrum Disorders (FASD). To further understand the developmental consequences of alcohol exposure, this study aims to examine the effect of alcohol exposure on underlying cranial facial bone growth in a mouse model. The craniofacial bone of pups born to three treatments of C57BL/6J dams, CHOW, PF and ALC groups were imaged with Micro-CT scan. Longitudinal studies were carried in pups at postnatal 7 day (P7) and at P21. Using Amira Software, five major skull bones, frontal, parietal, occipital, jaw and nasal bones were successfully

segmented (Figure 3) and 20 landmarks were determined on the Micro-CT scans. The head size was estimated as the cubic centroid size of 17 landmarks (the remaining 3 landmarks could not be determined on the Micro-CT scan for some P7 samples and thus were not included). The volumes of the four bones (frontal, parietal, occipital, jaw) were analyzed using general linear models with an IBM SPSS 18.0 to examine the effect of alcohol disposure on craniofacial bone development covaried for sex and head size. Discriminant analysis was performed using SAS 9.2 to distinguish ALC and Control groups.

4. Neuroprotective NAP treatments for C57BL/6 alcohol-induced neuro-facial and neurobehavioral deficits: Animals were treated as above using an alcohol dose of 4.8% v/v in both pre- and pregnancy periods. Both a yoked isocaloric pair-fed diet and chow diet were used for controls. Pregnant dams were treated with either control saline or NAP neuroprotective peptide treatment during the pregnancy period, timed parallel to a liquid diet administration (E7 through E16). Saline or Nap (20ug/animal/day) were injected i.p. (200ul total volume) at the beginning of the dark cycle. Pups from dams treated with alcohol during pregnancy were fostered to a chow only dam at P0-P1. All litters were culled to 6. At P21 one male and one female pup were scanned by Micro-CT and brains were harvested after scans. 4 additional pups were weaned at P28 and used in a neurobehavioral analysis at P50. N=7 for all groups. Behavioral analysis includes locomotor activity in the novel arena and special working memory in a Morris water maze. Additional analysis of brains from animals will include CNS dysmorphology and hippocampal neuron cell counts.

V. Accomplishments and Results:

1. Effect of Stage and Length of Alcohol Exposure**:** *4.8%(v/v) on E7-E16 vs E7-E11*

Effect on facial anthropometry*:* We have completed a number of comparisons on two different stages of alcohol exposure (E7-E16 vs E7-E11) at the dose of 4.8% v/v. We demonstrated that 4.8% E7-E16 treatment groups, using a MANOVA model, resulted in significant overall treatment effects, with significant individual measurement effects (for whisker pad, upper facial depth, mid-facial depth, and lower facial height, all $p<0.002$) when comparing alcohol to controls (Hotelling Lawley-Trace = 3.86, $(F30, 65)=4.92$, $p<0.0001$). Two measures were identified in which the Alcohol group differed significantly from both PF and the Chow Controls (upper facial depth and mid-facial depth both $p<0.0001$). Discriminant analysis demonstrated successful classification of the alcohol-exposed versus non-alcohol-Controls with 80% sensitivity, 78% specificity, and 79% overall correct classification. In addition, effects of pair feeding on facial measures shows the PF group differs from its respective Chow control only on the whisker pad measure ($P <0.0001$). These results are published this year [Pub 1].

For 4.8% E7-E11 treatments, results demonstrated an overall treatment effect, with significant individual measurements (for mid and lower facial depth, nasal length and minimal frontal height) comparing Alcohol group to both Chow and PF controls. Discriminant analysis between two stages of alcohol exposure is in progress.

Effect on Gross Growth Measurements: The E7-E16 alcohol exposure indicated that the ALC group differs from the Chow group in Crown-Rump length, embryonic weight, and head circumference (student t-test, Table 1), and differs from PF in Crown-rump length. The PF and chow groups are similar, except the embryonic weight is smaller in PF. These measurements indicated that alcohol plus nutritional disparity (perhaps including stress from a restricted liquid diet) lead to a major gross growth deficit. And, nutrition disparity can contribute to a partial growth deficit, i.e. embryonic body weight. In comparison, the earlier and shorter alcohol exposure (E7-E11) resulted in no discernable differences at E17 between the groups in the growth measurements. This indicates that long alcohol exposure throughout the second gestation caused major gross growth deficit while shorter and earlier alcohol exposure induced less gross growth deficit, or the deficit was diminished upon stopping alcohol intake. Similarly, in E7-E11 treatment, the effect of nutritional disparity (between liquid diet and Chow) was insignificant or was diminished after reinstating to ad lib Chow diet.

Table 1. Gross growth measurement						
Measurement	E7-E16	E7-E16	E7-E16	E7-E11	E7-E11	E7-E11
Comparison	ALC vs Chow	ALC vs PF	ALC vs Chow	ALC vs Chow	ALC vs PF	ALC vs Chow
Crown-rump length	0.001*	0.001				
Weight	0.01					
Circumference	0.05		0.03			

2. *Detecting alcohol exposure using 3-D facial surface analysis: Validating Method from mouse to human*

We have demonstrated that 3-D facial analysis Micro-video images of a total 30 mouse embryos (E17) were collected. For each subject, images were taken from a sequence of camera angles and computational *Silhouette surface extraction, Surface alignment, and Feature Selection* were performed. Using these computed images, the alcohol versus non-alcohol exposure subjects were validated using a Leave-One-Out cross validation approach. The overall classification rate is 86.75%. This result was submitted and accepted for publication this year [Pub 2].

Table 2. Classification results using		
A. Mouse Known classes	Prediction: 86.75%	
	Alcohol (+)	Alcohol (-)
Alcohol Treated	13	2
Control	2	13
B. Human Known classes	Prediction: 92.5%	
	Alcohol (+)	Alcohol (+)
Alcohol Treated	19	1
Control	2	18

Human Comparison: To demonstrate if this approach can be generalized for translational application, the above method is applied to human subjects to test its validity. The results of human data analysis in Table 2B are derived from 40 human subjects in CIFASD (3D polygon mesh models generated from laser scanner): 20 FAS subjects and 20 controls. 100 rendering images were generated from each 3D model. The classification rate for a human, 92.5%, is very similar to the previously reported in using 3D feature analysis. (Fang et al., Orthodontics and Craniofacial Research, 2008;11:162).

3. *MicroCT analysis of crananiofacial bone as a predictor for alcohol exposure*

3-1 Anthropometric measurement as a predictor

a. Predicting alcohol treatment: comparing ALC with PF suggests that alcohol exposure during pregnancy induced significant differences in minimal frontal width, mid facial depth, and circumference. Using logistic regression analysis, we demonstrate sensitivity of >78%, specificity >89% and overall correct >84% using minimal frontal and inner canthal widths when comparing ALC to PF controls, and >82%, specificity >91% and overall correct >87% using minimal frontal and inner canthal widths when comparing to chow controls. With two measures (minimal frontal width and circumference), the Alcohol group differed significantly from both the PF and the Chow groups ($p<0.0001$).

b. Nutritional / stress disparity: Comparisons between *Chow and PF* dietary controls demonstrated a significant dietary effect on bone dysmorphology in minimal frontal width, bitrigal widths, upper-facial and lower-facial depth.

c. Dietary & alcohol combination effect is identified through comparisons between *ALC and Chow* groups. Significant differences were seen in circumference, minimal frontal width, bigonial width, all depth measurements with the exception of upper facial depth and nasal bridge height.

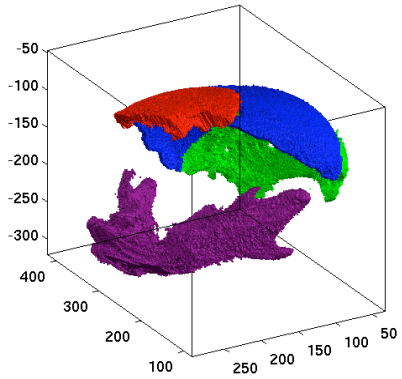


Figure 4. Skull bone volume analysis. Frontal (red), Parietal (blue), Occipital (green), Mandibular bone (purple).

3-2. Skull bone volume as a predictor

We next tested if the volume of the featured skull bones acquired through microCT would be a function of alcohol treatment. Four

craniofacial bones were segmented using Amira Software and 17 defined landmarks. The total head bone size and individual frontal, parietal, occipital, and mandibular (Jaw) bones (see example in the Figure 4) of P7 and P21 (longitunidally) were analyzed using general linear models (GLMs) with SPSS 17.0 software.

We found that alcohol exposure during pregnancy reduced the total skull bone volume, as well as individual bones, such as the occipital and parietal bones (Table 3).

The growth rate of these bone over P7 and P21 was also reduced.. These reductions were contributed solely by the alcohol (ALC vs PF; ALC vs Chow), disregarding the nutritional disparity (ALC vs Chow).

Table 3. Difference of Indivisual bone volume between treatment groups (in P value). N: ALC, 9; PF, 20; Chow, 26.

	Group 1	Group 2	Frontal		Occipital		Parietal		Jaw		Head size
P7 Volume[1]	ALC	PF	.240	.167*	.130	.129*	.004	.027*	.137	.055*	.004
	ALC	CHOW	.281	.005*	.010	.123*	.000	.001*	.116	.000*	.000
	PF	CHOW	.892	.071*	.227	.938*	.130	.097*	.951	.018*	.130
P21 Volume[2]	ALC	PF	0.002	0.819*	.000	.000*	.000	0.098*	0.02	0.389*	.000
	ALC	CHOW	.000	0.446*	.000	.000*	.000	0.001*	0.002	0.382*	.000
	PF	CHOW	0.145	0.224*	0.767	0.259*	0.39	0.038*	0.55	0.989*	0.39
Change[3]	ALC	PF	.068		.000		.040		.152		.002
	ALC	CHOW	.007		.004		.001		.248		.005
	PF	CHOW	.317		.165		.078		.632		.644
Rate of Change[4]	ALC	PF	.049		.000		.019		.116		.002
	ALC	CHOW	.004		.002		.000		.178		.004
	PF	CHOW	.327		.210		.091		.667		.573

Effect of alcohol disposure on bone development at P7[1] and P21[2] with* and without controlling for head (bone) size.

Effect of alcohol disposure on bone volume change[3] and bone rate of change between[4] between P7 and P21.

Discriminant analyses: The best result for P7 samples comparing ALC to Chow animals demonstrated 71.4% of the ALC animals and 93.5% of the Chow animals were correctly classified, using frontal, parietal, occipital and jaw bone volumes and the head size (overall cross validation accuracy 86.7%). For P21 samples, the best overall cross validation accuracy was 79.2%, which could be achieved by using any of the following combinations: (1) Jaw bone and head size (sensitivity 70.6%, specificity 83.3%), (2) occipital bone (sensitivity 64.7%, specificity 86.1%), and (3) occipital bone and head size (sensitivity 64.7%, specificity 86.1%).

4. *Alcohol Treatment using neuro-protective peptides*

Current treatments include alcohol +peptide, and alcohol saline controls. Neuroprotective peptide injections parallel to a 4.8% alcohol pregnancy treatment from E7-E16 are underway. Animals have begun microCT- scanning at P7 and P21, and additional litter-mates are presently staged for both immunohistochemistry of CNS and behavioral analysis.

VI. Discussion:

1. The facial anthropometric measurements predicted fetal alcohol exposure at a ~80% successful rate. A partial facial anthropometric alteration was also observed as a result of a nutritional effect in comparison to the liquid diet with Chow group.

2. The greatest gross growth deficit occurred in embryos exposed to alcohol and under nutritional disparity (between liquid diet and Chow). Nutrition disparity contributes to embryonic body weight reduction. Further, the longer alcohol exposure throughout the second gestation (E7-E16) caused major gross growth deficit, while shorter and earlier alcohol exposure (E7-E11) induced less gross growth deficit, or the deficit was diminished upon cessation of alcohol intake. Similarly, the growth deficit by nutritional disparity (between liquid diet and Chow) was observed in E7-E16 treatment but not

in E7-E11 treatment; or, the short-term deficit was diminished after reinstating the ad lib Chow diet.

3. The computational 3D analysis method of the Microvideo facial image is tested for the mouse and for the human to predict alcohol exposure. Improvement of this method may assist facial diagnosis of alcohol exposure.

4. The alcohol induced alteration on anthropometric measurements is demonstrated in the craniofacial bone.

5. The skull bone volumes (e.g. parietal, occipital, or entire skull) are sensitive to prenatal alcohol exposure, which may serve as a predictor to assist facial diagnosis.

6. We continue to find out whether nutrition disparity and/or the accompanying stress is/are (a) co-factor in alcohol induced facial dysmorphology, including the anthropometric measurement at the embryonic stage and at the post-natal stage, and the embryonic body weight. On the other hand, we found that the bone volume reduction is alcohol-specific, independent of nutritional disparity.

VII. Interrelation with Aims of the Consortium and Other Projects:

1. A cross-species anthropometry analysis including mouse, sheep, and human is being compared. The comparable parameter was analyzed.

2. A Computational 3D embryonic facial analysis, which predicted alcohol exposure in the mouse in our study, was extended to test human sample and is Dr. Foroud's U01 for translational study.

3. We have also completed a chronic treatment of the group embryos E7-16 for Dr. Sulik's U01 for MRI /DTI analysis in order to compare with her other groups with single-day treatment.

4. An effect of alcohol on sheep brainstem volume analysis was done in conjunction with Dr. Cudd.

VIII. Plans for the Next Year:

1. Determine the effect of alcohol dose and exposure duration on embryonic facial anthropometric dysmorphology.

2. Develop a Feature Analysis on 3-D images derived from Microvideo embryonic facial imaging and from MicroCT. The goal is to analyze features that are visually and geometrically on the faces to inform alcohol exposure.

3. Longitudinal analysis of alcohol effect on facial bone development (through MicroCT image) on anthropometric measurement and bone features.

4. Analyze the effects of prenatal alcohol exposure on postnatal brain structural and neuro-facial abnormalities and test whether the peptides NAP/SAL can provide long-term protection against alcohol-induced changes in behavioral activity and spatial learning in adulthood and in deficits of brain morphology.

5. Initiate a pilot study to develop MRI images in conjunction with MicroCT to help investigate brain and craniofacial correlation in C57BL/6J mice under influence of pregnancy alcohol exposure.

IX. Publications (2010):

1. Anthony B., Vinci-Booher S; Wetherill L, Ward R, Goodlett C, and FC Zhou. Alcohol Induced Facial Dysmorphology in C57BL/6 Mouse Models of Fetal Alcohol Spectrum Disorder. Alcohol, 2010.

2. Fang S, Liu Y, Huang J, Vinci-Booher S, Anthony B, and Zhou FC. Surface Feature Analysis using Video Volumes of Mouse Embryos for Fetal Alcohol Syndrome Classification, Proc. International Conference on Digital Image Computing: Techniques and Applications (DICTA), 2010, Sydney.

Manuscripts in submission:
Liang Y, Ai H, Anthony B, Shen L, and Zhou FC, MicroCT analysis of craniofacial dysmorphology after alcohol exposure in C57BL/6 mouse model". To be submitted in February, 2011.

Shen L, Liang Y, Ai H, B. Anthony B, and Zhou FC. Effect of Fetal Alcohol Exposure on Craniofacial Bone Development: A MoroCT Volumetric Study in C57BL/6J Mouse Models. To be submitted in March, 2011.

X. Posters and presentations (2010):

Effect of Fetal Alcohol on Craniofacial Bone Development in C57BL/6J Mouse Models. L. Shen, H. Ai, Y. Liang, B. Anthony, F.C. Zhou, and the CIFASD Consortium, , 2010 RSA, San Antonio, TX.

Surface Analysis from Video Volumes for 3D Facial Analysis of a Mouse Model of FASD. B. Anthony, B. Veen, S. Vinci-Booher, S. Fang, C. Goodlett, F. C. Zhou, and the CIFASD Consortium. , 2010 RSA, San Antonio, TX.

Longitudinal Study of craniofacial dysmorphology AT Postnatal stages IN C57BL/6J Mouse. Y. Liang, H. Ai, B. Anthony, L. Wetherill, R. Ward, F.C. Zhou, and the CIFASD Consortium, 2010 RSA, San Antonio, TX.

3D Surface Analysis by Video Volumes for Facial Dysmorphology of a Mouse Model of FASD. B. Anthony, Y Liu, B. Veen, S. Vinci-Booher, S. Fang, C. Goodlett, F. C. Zhou and CIFASD Consortium, 2010 RSA, San Antonio, TX.

Symposium presentation

Facial Measurements in Mouse as Model of Diagnosis for Fetal Alcohol Spectrum Disorders. F.C. Zhou, Y. Liang, H. Ai, B. Anthony, L. Wetherill, R. Ward, C. Goodlett, and the CIFASD Consortium, 2010 RSA, San Antonio, TX.

I. Principal Investigator: Christina Chambers, Ph.D.

II. Title of Project: Nutritional Risk Factors for and Spectrum of FASD in Ukraine, U01 AA014835

III. Objectives:

The principal goals of this CIFASD clinical project are to more fully delineate the range of expression of alcohol-related birth outcomes in relation to timing and dose in a prospectively ascertained sample of pregnant alcohol-consuming women and their children, to examine the role of nutritional factors in alcohol-related birth outcomes, to test the effectiveness of a multi-micronutrient intervention with or without choline in reducing alcohol-related effects, and to evaluate various methods of earlier diagnosis of affected children. The specific aims of this project are:

1. To measure the birth prevalence and range of alcohol-related physical features and neurobehavioral impairment among children born to women who report consuming moderate to heavy amounts of alcohol in pregnancy relative to children born to mothers who report consuming low amounts or no alcohol during pregnancy. Within this aim, we will:
    a. Evaluate alcohol quantity, frequency and timing in relation to growth, structure and neurobehavior
    b. Assess infant development using early infancy measures of processing speed and attentional regulation skills to measures from the BSID II

2. To evaluate the contribution of maternal nutritional status of specific micronutrients to risk for various features of FASD including growth deficiency, structural features, and neurobehavioral impairment. Within this aim, we will:
    a. Assess the relation of baseline nutritional status as measured in early pregnancy to alcohol exposure group
    b. Evaluate the impact of micronutrient supplementation with or without choline on change in nutritional status from baseline to third trimester in alcohol-exposed vs. comparison pregnancies
    c. Evaluate the predictive value of nutritional status for specific micronutrients with respect to alcohol exposure and specific pregnancy outcomes.
    d. Examine the relationship between indicators of oxidative stress and alcohol-related birth outcomes

3. To provide the performance site, local resources and human subjects for other current or proposed CIFASD projects.
    a. 2-D ultrasound imaging
    b. 3-D facial imaging

IV. Methods:

Pregnant women are screened for alcohol exposure at the time of first visit to the Rivne Diagnostic Center or the Khmelnitsky Perinatal Center. Women who meet the criteria for alcohol exposure are asked to participate. For each consented exposed woman the next woman who meets the low or unexposed criteria is recruited. After recruitment, women are randomized to vitamin supplementation group (no treatment, multivitamin supplement, or multivitamin plus 750 mg. of choline). Upon enrollment, completes a standard comprehensive interview about periconceptional and current alcohol exposure and other factors, each woman provides a blood

sample, and completes an ultrasound evaluation. All further routine ultrasounds are also included in the study data collection and at the Rivne site biophysical profiling and a startle response paradigm is employed. Women are re-interviewed about alcohol and other exposures in the third trimester, and a second blood sample is collected at approximately 32 weeks' gestation. Blood samples are shipped to UC Davis approximately every three months for nutritional analysis. Delivery outcome information is collected at the end of pregnancy, and liveborn infants receive a standard dysmorphology examination and digital photographs by one of the study pediatricians/geneticists at birth and at each return visit for neurobehavioral testing. At six and at twelve months of age mothers are asked to return for the BSID II evaluation; at six months of age at the Rivne site, infants also participate in the infant stimulus response evaluation. Periodically, infant examinations are validated by the Dysmorphology core.

V. Accomplishments and Results:

1. Recruitment and Maternal Interviews

Study recruitment continues at two sites: in Khmelnitsky and Rivne Oblasts. Cumulatively, we have enrolled 390 subjects in the new phase of the study in addition to the 161 in the pilot phase. We anticipate we will exceed the 600 subjects in the new enrollment period before the end of this year. Randomization to the micronutrient intervention has been proceeding as planned, with approximately 50% of the sample in the treatment as usual group, 30% in the multimicronutrient supplement group, and 20% in the multimicronutrient plus choline supplement group. To date <8% of the sample has been lost-to-follow-up or dropped out of the study.

2. Physical Examinations

280 physical examinations with 2D photographs have been performed by Ukrainian physicians in the new phase of the project, in addition to the 156 exams that were performed in the pilot for a total of 436 completed exams for unique individuals. Some infants have had multiple exams. All of the FAS and deferred children in the new phase are to be scheduled for the Dysmorphology Core to validate as many of these examinations as is possible. The distribution of diagnostic categories in the new dataset based on the blinded physical examination for those with entered records is shown in Table 1. There were no significant differences on these categorizations between the local examiners and the Dysmorphology core examiner, so the exam results are combined. Restricting the sample to those who have had the 6 month Bayley test, and reclassifying those children in the Deferred Group who had an MDI or PDI standardized score more than 1 standard deviation below the mean of the unexposed controls as affected, 25% of children in the exposed group are classified as affected compared to 4% who are false positives in the controls.

Table 1. Preliminary diagnostic category by exposure group; and revised diagnostic category incorporating 6 month Bayley

	Unexposed Control N = 74	**Alcohol Exposed N = 83**	**p-value**
FAS	0	7.2%	
Deferred	23.0%	24.1%	
No FAS	77.0%	68.7%	0.057
	N = 44	N = 48	
Affected*	4%	25.0%	
Unaffected	96%	75.0%	0.006

*reclassifying "deferred" as affected based on 6 month Bayley irrespective of alcohol exposure

3. 3D Images
A total of 31 images of infants enrolled in the study were obtained and sent to Dr. Foroud at Indiana University. The camera was returned to IU. There are no plans at this time to re-send the camera to Ukraine.

4. Nutritional Status
To date, 514 samples have been received from Ukraine. We have completed analyses of baseline blood samples and conducted statistical comparisons on 217 subjects (114 exposed and 103 unexposed).

With respect to the baseline samples analyzed to date, as reported previously, we are continuing to find that alcohol-exposed women have lower zinc (Zn; P=0.033) and borderline lower copper (Cu; P=0.075) concentrations in their plasma compared to non alcohol-exposed women (Table 2), and that these associations persist after adjustment for gestational age at blood draw, smoking, maternal age, socioeconomic status, parity and pre-pregnancy BMI. Furthermore, copper levels are dose related to the number of drinks per day and per drinking day around the time of conception and the number of drinks per drinking day at the time of enrollment. Deficiencies of Zn and Cu are highly teratogenic in all species studied to date. This finding supports the concept that suboptimal Cu and Zn delivery to the conceptus may contribute to developmental abnormalities in women who have high levels of alcohol intake.

It has been speculated that increased oxidative stress and damage can contribute to the teratogenicity of alcohol. Both Cu and Zn are required for optimal oxidant defense systems. Previously, we observed lower activity of ceruloplasmin, a Cu-binding protein that has antioxidant potential, in alcohol-exposed women. In samples analyzed to date, ceruloplasmin activity tended to be lower in alcohol-exposed women but this was not statistically different than controls (P=0.151).

Table 2. Baseline Maternal Samples: Analysis by Alcohol group

Measure	Unexposed Control Mean ± SD	Alcohol Exposed Mean ± SD	p-value
	N = 103	N = 114	
Cp activity (Units/L)	238.07 ± 51.29	228.12 ± 50.27	0.151
Zn (µg/mL)	0.65 ± 0.12	0.61 ± 0.10	**0.033**
Cu (µg/mL)	1.79 ± 0.29 [a]	1.72 ± 0.30 [b]	**0.075**
Mg (µg/mL)	16.35 ± 1.50	16.26 ± 1.46	0.656
Ca (µg/mL)	86.29 ± 9.60	84.45 ± 7.26	0.115
hsCRP (mg/L)	4.80 ± 7.39	3.18 ± 3.74	**0.047**
Fe (µg/mL)	0.87 ± 0.32	0.90 ± 0.38	0.523
Ferritin (ng/mL)	28.44 ± 23.13	36.60 ± 33.67	**0.037**
TfR	16.25 ± 4.78	15.88 ± 6.92	0.649

Concentrations of plasma choline, and two of its metabolites, betaine and dimethylglycine, were determined using a quantitative stable isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) method of detection, based on Holm (2003) and Innis (2006) with modifications. There continues to be no difference in concentrations of methyl-donor nutrients (choline, betaine, dimethylglycine, folate, Vitamin B12, and homocysteine) between the groups.

It has been reported that alcohol increases body Fe stores and is associated with a significant risk of Fe overload. Increased Fe can lead to the formation of reactive oxygen species through its involvement in Fe-catalyzed Fenton reactions and subsequent oxidative stress. To date, our data show that alcohol intake is associated with increased ferritin, indicating increased body Fe stores. This, coupled with the compromised Zn and Cu status observed in alcohol-exposed women may increase alcohol-induced oxidative damage and potentiate alcohol teratogenicity.

In the coming year, we have four baseline papers in preparation for mineral status, vitamin status, methyl donor status, and measures of oxidative defense system.

5. Neurodevelopmental Infant Outcomes - BSID

Developmental Assessment: Developmental assessments were carried out at two sites in the Ukraine, Khmelnitsky and Rivne. Examiners were trained by the investigators and reliability in both administration and scoring was evaluated by Dr. Coles and found to be acceptable (rescoring error rate <3%). 108 evaluations have been completed and 93 entered into the study database.

Analysis: For the 6 month assessment, developmental data on 93 children were available. Of those, 4 fell in the "questionable" category on the Alcohol Use variable and were not included in the subsequent analysis. For the 12 month assessment, developmental data on 58 children were available and 2 cases fell in the "questionable" category on the Alcohol use variable and were not included. At each age, one child did not complete both portions of the test.
For the analysis, there are two levels of the Alcohol use variable, None and Alcohol use. Preliminary analyses were carried out using the Vitamin Exposure variable, which had 3 levels, None, Prenatal Vitamins Only, Prenatal Vitamins plus Choline supplements. This preliminary analysis found no difference in outcomes between the Vitamin and Vitamin plus Choline groups and, for the subsequent analyses provided in this report, these categories were collapsed.
A number of potentially moderating factors were also available, including maternal smoking, socioeconomic status (SES) and maternal nutritional status based on measures taken in pregnancy. These factors also were examined in relation to observed outcomes.

6 Months: Outcomes are shown in Table 3. A 2-factor multivariate analysis of variance was done with Mental Development Index and Psychomotor Development Index as the dependent variables. Alcohol use (2 levels: None, Alcohol) and Vitamin/Mineral supplementation (2 Levels: None, Vitamins/Minerals) were the independent variables. Results suggest that alcohol affects developmental outcomes with a significant deficit in Motor performance noted at 6 months. Vitamin/mineral supplementation is associated with significantly better outcomes in cognitive performance at this age. There are no significant interactions. However, the sample size is still smaller than the final targeted planned enrollment; therefore, it is possible that some of the non-signficant trends noted in these data will demonstrate significant results when the rest of the sample is collected.

Table 3. Developmental Outcomes at 6 months (N=89) M (SD). Multivariate Analysis

Alcohol Exposure[1]	**None**		**Exposed**	
Vitamin/Mineral Group[2]	None (n=24)	Supplement (n=19)	None (n=20)	Supplement (n=24)
Mental Development (MDI)	94.46 (10.81)	97.89 (5.55)	89.60 (14.04)	96.50 (6.28)
Psychomotor Development (PDI)	98.83 (9.68)	99.21 (10.78)	88.65 (18.31)	96.71 (10.40)
[1] Alcohol, MDI: $F_{(1,83)}$=2.21, p=.14, NS; PDI: $F_{(1,83)}$=5.48, p<.02 [2] Vitamin/Mineral Supplement, MDI: $F_{(1,83)}$=6.04, p<.02; PDI: $F_{(1,83)}$=2.42, p=.12, NS Interaction: Alcohol Use and Vitamin/Mineral Supplement: MDI: F<1, NS; PDI: $F_{(1,83)}$=2.0, p=.16, NS				

Several exploratory analyses were carried out. When the SES variable was used as a covariate, this measure did not affect the pattern of outcomes for the Vitamin supplement variable but did attenuate the effects of alcohol use on motor performance. Inclusion of cigarettes smoked per day as a covariate, indicated that there was a significant effect of this variable on MDI (p<.05) and a trend for PDI (p<.07). When cigarettes were controlled, effects of alcohol on motor development were reduced ($F_{(1,81)}$=3.82, p=.054) while those of vitamin supplements were increased on both mental ($F_{(1,81)}$=7.19, p<.009) and motor development($F_{(1,81)}$=2.99, p=.09). There was no effect of maternal BMI (body mass index). Maternal use of prenatal vitamins before entry into the study was significantly related to MDI. Folic Acid use was not.

Figure 1.

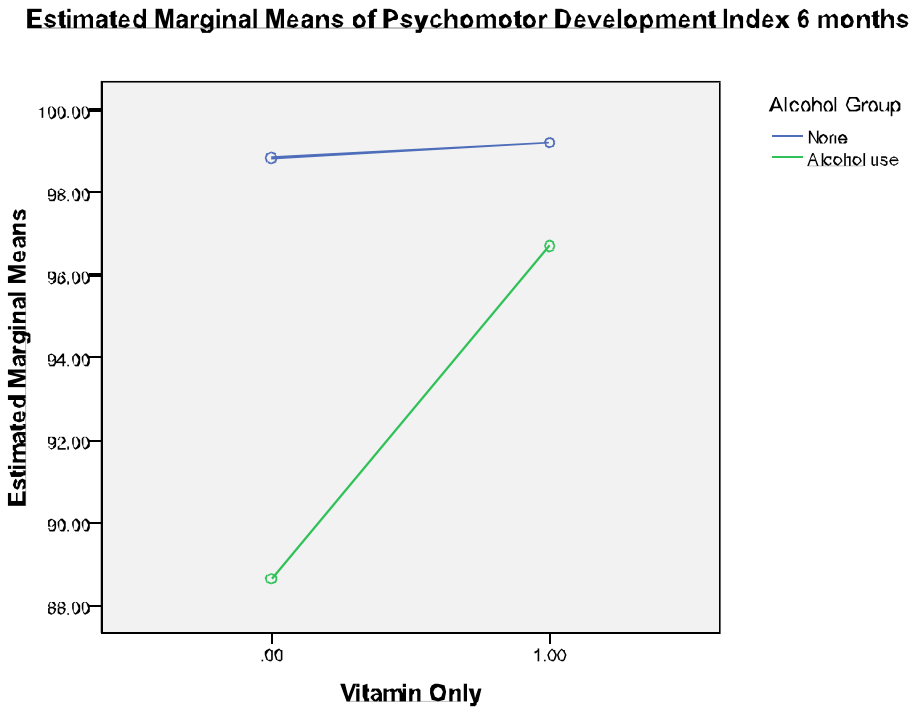
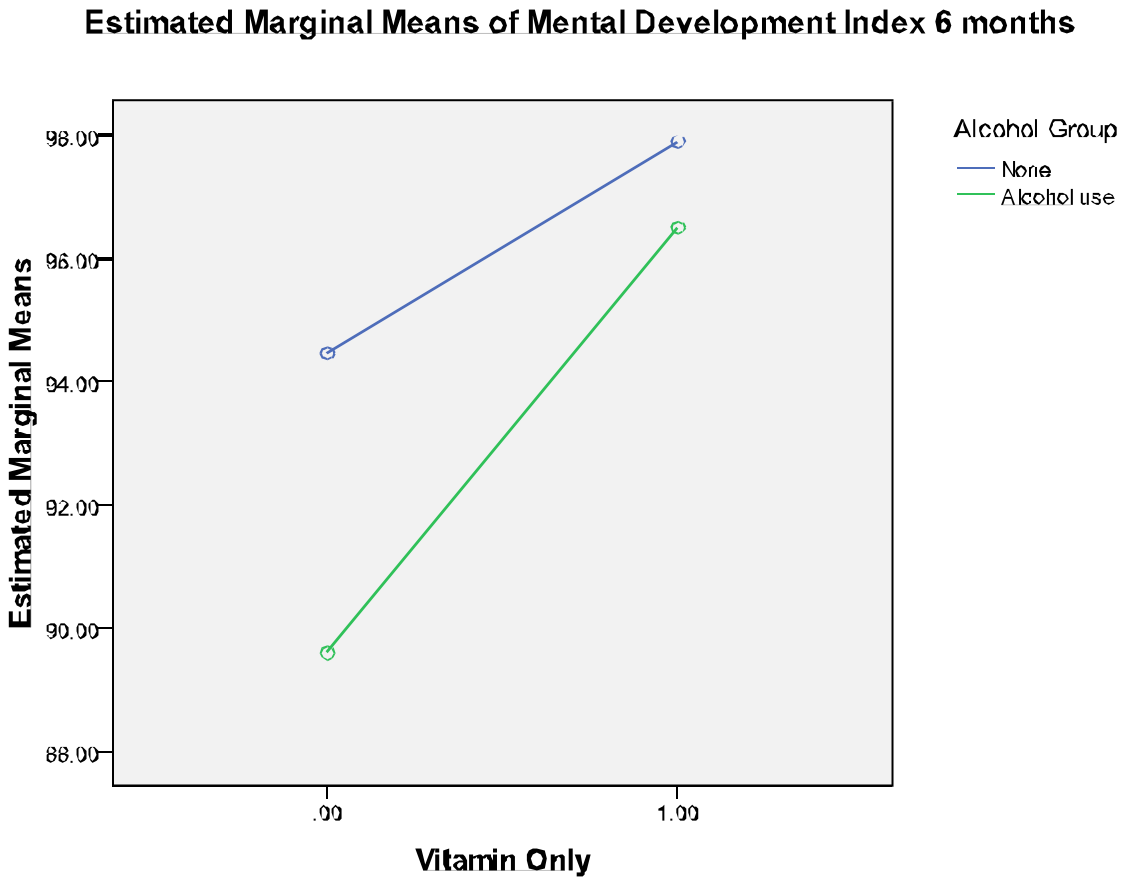


Figure 2.



12 Months: A similar analysis was carried out at 12 months. However, the final sample size (n=55) is smaller at this age. Results are shown in Table 4, below. In these results, the unsupplemented contrast group (1st column) is performing very well and there are no difference between this group and those who are receiving vitamin supplements. However, for the Alcohol exposed group, there does appear to be a decrement associated with exposure that is not observed in the alcohol-exposed group receiving vitamins. An interaction for mental development is observed between alcohol exposure and vitamin supplementation. As with the 6-month sample, several covariates were added to the analysis to examine their impact. In this small sample SES was highly significant, possibly reflecting a differential return rate for the different SES groups. When SES was included as a covariate, the effects of both Alcohol and Vitamins were not seen. Cigarettes per day, Maternal BMI, PNV or maternal use of vitamins independently and folic acid supplements were not related to outcomes variables at this age.

Table 4. Developmental Outcome at 12 months (N=55) M (SD), Multivariate Analysis

Alcohol Exposure[1]	**None**		**Exposed**	
Vitamin/Mineral Group[2]	None (n=15)	Supplement (n=12)	None (n=13)	Supplement (n=15)
Mental Development (MDI)	101.20 (14.87)	97.42 (10.13)	92.38 (13.69)	103.00 (9.73)
Psychomotor Development (PDI)	106.73 (8.15)	102.42 (11.12)	96.86 (18.94)	99.93 (11.91)
[1] Alcohol, MDI: $F_{(1,51)}$<1, NS; PDI: $F_{(1,51)}$=3.10, p=.084, NS [2] Vitamin/Mineral Supplement, : $F_{(1,51)}$=1.04, p=.313, NS; PDI: $F_{(1,85)}$ <1<NS Interaction: Alcohol Use and Vitamin/Mineral Supplement: MDI: $F_{(1,51)}$=4.61,p<.04; PDI: $F_{(1,51)}$=1.11, p=.29 NS				

These preliminary findings suggest that both exposure to alcohol and vitamin supplementation independently affect developmental outcomes. It is of interest that the alcohol effects are shown more strongly in the motor scores and the vitamin, in the cognitive. The addition of choline to the vitamin supplements does not seem to have improved outcomes in this sample. However, the numbers are still small and it will be important to reanalyze these data when there are additional subjects available.

In addition, it appears that it will be important to control for several factors in interpreting outcomes. Social Class appears to be related to a number of factors in this study including both the dependent variables and alcohol use. As there may also be a relationship between SES and attrition in this Ukrainian sample, this factor should be closely monitored. In addition, cigarette use appears to be having an effect on the outcomes of interest and should be evaluated further.

6. Infant Auditory and Visual Stimulation Outcomes

The following is a preliminary analysis of the cardiac orienting responses collected during the visual and auditory habituation/dishabituation paradigms. For each stimulus, infants are exposed for 10 trials of the initial habituation stimulus and five trials of a novel, dishabituation stimulus. Responses to the initial habituation stimulus reflect encoding and sustained attentional focus to the stimulus and responses to the dishabituation stimulus reflect detection of the novelty of the new stimulus after repeated exposure to a similar stimulus. This response requires both encoding and use of memory skills needed to discern that the novel stimulus is, in fact, novel. Analysis is done on the first three trials as a result of significant habituation of the cardiac response over trials.

For auditory trials, ORs, as defined by a deceleration in HR from baseline value by 9 seconds post-stimulus, were present on on 92.6% of the first and second trials of the initial habituation stimuli and 87.1 % of the third trial responses. On the auditory dishabituation trials, ORs were present in 63.1% of the first trial, 61.9% of the second trial, and 56.6 % of the third trial responses. There were no significant group differences in the presence or absence of ORs during the habituation trials but significant group differences were found on the presence or absence of ORs in the dishabituation trials. On trial one, among those without micronutrient supplements, the exposed group was less likely to detect the novel stimulus (χ=4.375, $p < .036$) and a significant effect was observed for intervention group within the exposed group (χ=7.710, $p < .021$) but not the non-exposed. On trial 3, a significant effect was observed for intervention

group among the exposed group (χ=8.280, p < .016) and a trend was found for those in the no-exposed group (χ=3.966, p < .138). Among those who received prenatal vitamins, the exposed group were less likely to detect the novel stimulus on dishabituation trial 3 (χ=4.701, p < .030). For the visual trials, ORs were present on 76.3% of trial 1, 69.1% of trial 2, and 72.3% of trial 3 on the habituation stimulus and 58.8% of trial 1, 58.8% of trial 2, and 59.5% of trial 3 on the dishabituation stimulus. Only a trend was found for an intervention effect among those that were non-exposed (χ=4.661, p < .097).

Table 5. OR by alcohol group and intervention group for auditory trials

Auditory Trial	Alcohol Group	Percent with an OR by Group		
		No Supplements	PNV	PNV +Choline
Hab Trial 1	Exposed	93.8	100.0	85.7
	Non-Exposed	92.0	91.7	100.0
Hab Trial 2	Exposed	93.8	100.0	100.0
	Non-Exposed	96.0	91.7	85.7
Hab Trial 3	Exposed	81.3	76.9	100.0
	Non-Exposed	92.0	83.3	85.7
DisHab Trial 1	Exposed	30.8	54.8	84.6
	Non-Exposed	66.7	81.8	83.3
DisHab Trial 2	Exposed	61.5	45.5	61.5
	Non-Exposed	66.7	72.7	83.3
DisHab Trial 3	Exposed	53.8	18.2	76.9
	Non-Exposed	56.5	63.6	100.0

Table 6. OR by alcohol group and intervention group for visual trials

Visual Trial	Alcohol Group	Percent with an OR by Group		
		No Supplements	PNV	PNV +Choline
Hab Trial 1	Exposed	87.5	83.3	85.7
	Non-Exposed	68.2	69.2	85.7
Hab Trial 2	Exposed	75.0	53.3	40.0
	Non-Exposed	77.3	46.7	60.0
Hab Trial 3	Exposed	81.3	61.5	71.4
	Non-Exposed	72.7	58.3	85.7
DisHab Trial 1	Exposed	75.0	66.7	50.0
	Non-Exposed	75.0	36.4	50.0
DisHab Trial 2	Exposed	66.7	41.7	40.0
	Non-Exposed	75.0	45.5	66.7
DisHab Trial 3	Exposed	66.7	50.0	50.0
	Non-Exposed	70.0	45.5	83.3

For baseline HR on the auditory stimulus, a significant intervention group was found (F (2, 81)=5.276, p < .007) and an exposure by intervention group interaction effect was found (F (2, 81)=3.104, p < .05). The PNV+ Choline group had higher overall HR than did the other two intervention groups and this effect was strongest among the non-exposed groups. For baseline HR on the visual stimulus, a significant exposure group * intervention group effect was found (F (2,78)=3.06, p < .05) with those in the non-exposed group that received PNV and Choline having higher baseline HR than any other group. Caution should be used when interpreting this effect, however, as a result of the small sample size in that cell (n=7) in the non-exposed, PNV+Choline group. Difference values (baseline HR –OR HR) will be used to evaluate group differences to control for pre-existing values.

Table 7. Mean baseline HR by stimulus, alcohol, and intervention groups

Stimulus	Alcohol Group	Mean Baseline HR (bpm) by Group		
		No Supplements	PNV	PNV +Choline
Auditory	Exposed	143.0 (11.2)	142.0 (8.9)	145.6 (17.6)
	Non-Exposed	135.9 (15.4)	140.2 (13.6)	157.7 (9.5)
Visual	Exposed	151.5 (19.3)	146.5 (12.9)	145.1 (16.2)
	Non-Exposed	142.3 (17.0)	141.4 (10.4)	158.9 (23.0)

For auditory habituation OR responses, there was a significant linear trend effect across trials (F (1,80)=4.421, p < .039) indicating that the magnitude of the ORs diminished over the first three trials. This response is typical and indicative of the habituation that occurs over repeated exposure. A significant exposure by intervention by trial effect was found (F (4, 160)=2.58, p < .0399). Figure 1 displays the interaction. Those in the exposed group who received PNV + choline showed a greater magnitude of deceleration relative to those who were exposed and received no micronutrients or PNV alone on trials 2 and 3.

For the auditory dishabituation ORs, there was a trend for a linear effect across trials (F (1,71)=3.4, p < .069). A significant vitamin group effect was found (F (2,71)=3.688, p < .03) with the greatest magnitude of deceleration in the choline intervention group. A trend was also found for a significant effect on exposure group (F (1, 71)=2.384, p < .127) with those in the exposed group having lower magnitude of ORs than those in the non-exposed group.

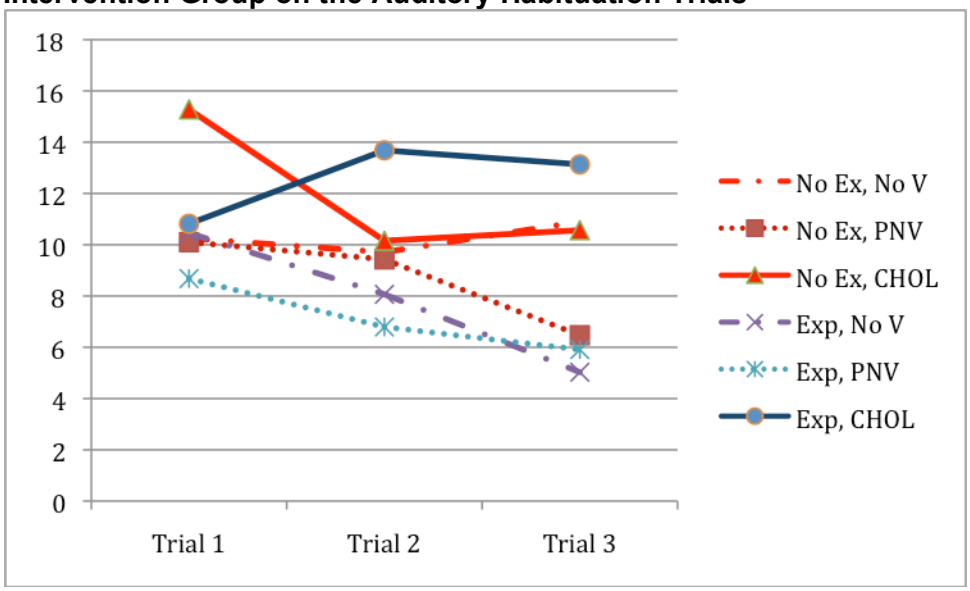
On the visual habituation trials, only a significant trial * intervention group effect was found (F (4,156)=2.352, p < .056). Post hoc comparisons indicated that on trial one, those in the choline group had greater magnitude of ORs than did those who received no micronutrients . On trial two the choline group had greater magnitude ORs than did both other intervention groups. Finally, on trial 3, the prenatal vitamin group had lower magnitude of ORs than did the group who received no micronutrients. There were no significant effects for exposure.

On the visual dishabituation trials, a significant linear trend was found over the trials (F (1,64)=5.257, p < .025) and a trend for an exposure group by trial interaction was found (F (2,128)=2.685, p < .072) with those in the alcohol exposed group demonstrating diminished ORs on trial 3 but not differing from the non-exposed group on trials 1 and 2.

Table 8. HR deceleration by stimulus, alcohol, and intervention groups

Stimulus	Alcohol Group	HR Deceleration (bpm)		
		No Supplements	PNV	PNV +Choline
Habituation Auditory	Exposed	-7.9 (7.8)	-7.1 (6.8)	-12.4 (9.9)
	Non-Exposed	-10.3 (7.6)	-8.7 (6.8)	-12.0 (11.9)
Dishabituation Auditory	Exposed	3.4 (11.0)	2.0 (6.0)	-4.3 (14.6)
	Non-Exposed	-0.0 (8.7)	-2.8 (5.7)	-7.8 (10.1)
Habituation Visual	Exposed	-3.5 (8.4)	-1.6 (3.7)	-2.6 (8.1)
	Non-Exposed	-3.4 (8.1)	-0.7 (5.4)	-7.6 (13.8)
Dishabituation Visual	Exposed	-4.1 (12.0)	1.8 (7.3)	-4.1 (12.6)
	Non-Exposed	-4.1 (17.0)	-0.6 (6.2)	7.0 (9.9)

Figure 3. Mean Change (magnitude of deceleration*) in HR over Trials by Exposure and Intervention Group on the Auditory Habituation Trials



*the deceleration or magnitude of change is reflected as a positive value (Baseline HR-OR HR).
Dose-Response Relationships with ORs and AA/Day in pregnancy

Within the entire sample, relationships were found between alcohol dose and OR responses but were not consistent across all estimates of AA/Day. Average OR decelerations for the visual habituation trials were significantly related to AA/Day reported during pregnancy (AADXP) (r=-.238, p .025) and the OR decelerations during the visual dishabituation trials were significantly related to AA/Day reported in the week around conception(r=-.242, p < .035).

7. Prenatal Ultrasound, Biophysical Profiling and Startle Response

Prenatal ultrasounds continue to be performed at both sites and at the Rivne site, biophysical profiling and startle response data are being collected. Brain and growth measures have been obtained. To date 845 ultrasounds including the pilot data have been entered into the database. Because the pilot data did not involve a micronutrient intervention, analyses are proceeding utilizing only the new data. Examining the 310 unique subjects (157 unexposed and 153 exposed) for whom a second trimester ultrasound is available, intervention group and alcohol* intervention group were significant or borderline significant predictors of selected measures.

Table 9. Second trimester ultrasound brain measures by Alcohol Group, Intervention Group, and alcohol*intervention interaction

Measure*	Alcohol Group p-value	Micronutrient Intervention Group p-value	Alcohol*Intervention Interaction p-value
TCD	0.613	**0.007**	0.994
OFD	0.498	0.851	**0.101**
CCD	0.873	0.547	0.462
FTD	0.194	**0.092**	0.843
OOD	0.300	0.740	0.495
IOD	0.936	0.513	0.169
OD	**0.094**	0.854	0.586

*Adjusted for gestational age at scan

In addition, in the second trimester, alcohol group was associated with shorter femur length, as was intervention group (p=0.012, and 0.073 respectively) but there was no evidence of interaction.

In the third trimester, among 245 scans (129 exposed; 116 unexposed) there was evidence of interaction with measures of growth, and frontothalamic distance was significantly associated with alcohol group.

Table 10. Third trimester ultrasound selected brain and growth measures by Alcohol Group, Intervention Group, and alcohol*intervention interaction

Measure*	Alcohol Group p-value	Micronutrient Intervention Group p-value	Alcohol*Intervention Interaction p-value
TCD	0.417	0.985	0.143
OFD	0.070	0.375	0.874
CCD	0.537	0.551	0.162
FTD	**0.056**	0.296	0.922
AC	0.884	0.491	**0.002**
TL	0.751	0.703	**0.056**

*Brain measures adjusted for gestational age at scan

Biophysical profiling measures did not differ between exposure groups in the second or the third trimesters. However, there were fewer evoked startles in the alcohol-exposed group in both trimesters, and a greater increase in heart rate in the exposed group after the evoked startle. This persisted after adjustment for smoking, and was associated significantly with absolute ounces of alcohol per drinking day in pregnancy.

VI. Discussion:

The overall progress of the study is promising with respect to addressing the first specific aim regarding the prevalence of FASD related physical and neurobehavioral outcomes across a range of specific quantities and timing of alcohol exposure. Continuous measures of quantity and timing are beginning to show some significant associations. With respect to our second specific aim, we have demonstrated baseline differences on some key micronutrients by alcohol group, and also by continuous measures of quantity and gestational timing of exposure. We are

beginning to see the impact of the intervention groups on neurobehavioral endpoints and on some ultrasound measures.

Based on the recent change in government leadership in Ukraine, we have been attentive to issues related to ensuring the smooth continuation of this project because it operates in the context of the health care system in the two performance sites in Ukraine. Our scientific liaison, Dr. Wladimir Wertelecki, met with officials in November of 2010 in both regions and in Kyiv to ensure that the study would continue to proceed unimpeded, and that the officials involved are kept apprised of the study progress.

VII. Interrelation with Aims of the Consortium and Other Projects:

We have worked closely with Jennifer Thomas' developmental project and with Andy Hull's developmental project this year, and consulted with others regarding additional uses of blood samples that have been banked. We have consulted with potential new members of the consortium on methods, and provided materials.

VIII. Plans for Next Year:

In 2011, we should complete recruitment, and have the full complement of blood samples for analysis. Our major challenge in the coming year is to retain the cohort especially as it relates to differential loss of women from villages who must come a great distance, and where drinking tends to be heavier. We plan another visit in April of 2011 to continue validation of the physical exams.

IX. Publications (2010):

1. Keen CL, Uriu-Adams JY, Skalny A, Grabeklis A, Grabeklis S, Green K, Yevtushok L, Wertelecki WW, Chambers CD. The plausibility of maternal nutritional status being a contributing factor to the risk for fetal alcohol spectrum disorders: the potential influence of zinc status as an example. *Biofactors*, 2010;36:125-35.

2. Jones KL, Hoyme HE, Robinson LK, Del Campo M, Manning MA, Prewitt LM, Chambers CD. Fetal alcohol spectrum disorders: extending the range of structural defects. *American Journal of Medical Genetics*, 2010;152:2731-5.

3. Uriu-Adams JY and Keen CL. Zinc and reproduction: effects of zinc deficiency on prenatal and early postnatal developments. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, 2010;89:313-325.

4. Bakhireva LN, Wilsnack SD, Kristjanson A, Yevtushok L, Onishenko S, Wertelecki, WW, Chambers CD. Paternal Drinking, Intimate Relationship Quality, and Alcohol Consumption in Pregnant Ukrainian Women. *Journal of Studies of Alcohol and Drugs*, under review

5. Four papers in preparation on baseline nutritional status: minerals, vitamins, methyl donors and measures of oxidative defense.

X. Posters and Presentations (2010):

Poster Presentation: Teratology Society Meeting, June 2010
Uriu-Adams, J.Y., **Chambers, C.D.**, Gross, H.B., Ensunsa, J.L., Green, K., Le, A., Yevtushok L., Zymak-Sakutnya, H., Wertelecki, W. "Alcohol Drinking Patterns and Nutrient Status in Ukrainian Pregnant Women." *Birth Defects Research (Part A): Clinical and Molecular Teratology*, 88:382, 2010.

Meeting Presentations or Symposia

1. Global Health Seminar, UCSD, April 2010.

2. Chambers CD, Uriu-Adams JY, Gross HB, Ensunsa JL, Green K, Le A, Yevtushok L, Zymak N, Wertelecki WW, Keen CL. Altered maternal nutritional factors may confer risk of fetal alcohol spectrum disorders (FASD). ACER 2010; 34 Supp: 293A. RSA, June 2010.

3. Visiting Professor – University of Pittsburgh – Magee Women's Research Institute – Works in Progress Seminar, October 2010.

4. Taking the Next Step: Innovative Interventions for FASD, conference, Emory University, October 2010.

5. First European Conference on FASD Fetal Alcohol Spectrum Disorder: Growing Awareness in Europe, Kerkrade, Netherlands, November 2010.

I. Principal Investigator: Tatiana Foroud, Ph.D.

II. Title of Project: 3D Facial Imaging in FASD, U01 AA014809

III. Objectives:

Goal 1: Improve understanding of the dysmorphic features in FAS.

1.1 Collect 3D facial imaging data from an ethnically diverse sample of prenatally alcohol exposed individuals and controls who span a wide age range.

1.2 Analyze the 3D facial images using novel analytic techniques to test whether there are unique facial features that best discriminate FAS and control individuals.

1.3 Collaborate with members of a clinical project (C. Chambers, PI) to determine how known quantities and frequencies of prenatal exposure affect facial features found in infants studied longitudinally during their first 2 years of life.

Goal 2: Enhance the capability of definitive diagnosis of fetal alcohol syndrome (FAS) and the broader spectrum of fetal alcohol spectrum disorders (FASD) at different stages of the lifespan.

2.1 Test whether the same facial features can discriminate FAS and FASD from controls in ethnically diverse samples.

2.2 Test whether the same facial features can discriminate FAS and FASD from controls at different stages of the lifespan.

2.3 Test whether the same facial features discriminate FAS and FASD from controls in ethnically diverse samples when longitudinal data are analyzed in the same subjects over a 2-5 year interval.

Goal 3: Establish whether there is a relationship between FAS dysmorphic features and the specific underlying impairments in brain function.

3.1 Collaborate with members of two clinical projects (E. Sowell, S. Mattson, PIs) to identify a set of variables which best discriminate FAS/FASD and controls and to determine if in combination they might provide greater classification sensitivity and specificity.

3.2 Collaborate with the members of the animal imaging projects (F. Zhou, K. Sulik, T. Cudd, PIs) to delineate the underlying embryological impairments resulting from prenatal alcohol exposure.

IV. Methods:

Facial Imaging

We are currently focusing on the collection of a sufficient number of 3D facial images to allow us to perform analyses with the 3DMD camera system. During the past year, data collection has occurred at 3 sites (UCLA, San Diego, and Atlanta; see Table 1). To ensure high quality images, we have reviewed with each site the most efficient means to collect images. We have developed several models:

San Diego – Members of this project trained staff at the San Diego site to collect images. San Diego collects images as the subjects are seen for the Neurobehavioral Assessment of Fetal Alcohol Spectrum Disorders study.

UCLA, Atlanta, – Rather than train staff at each site in the imaging protocol, members of this project travel to each site to collect images. Travel is coordinated to coincide with the visit of members of the Dysmorphology Core who are completing their evaluation. This model was selected since large numbers of subjects are seen on these days and it decreased the burden on site staff.

We are currently ahead of scheduled with regard to the collection of imaging data at several sites and close to target at the remaining sites.

Table 1: Summary of data/sample collection through January 3, 2011 (3dMD only)

Site	3D Images (% of target)	DNA
San Diego	188 (125%)	157
UCLA	52 (104%)	80
Atlanta	116 (93%)	54
Ukraine	35 (37%)[1]	0 (no approval)
Cape Town (Jacobson)	224 (100%)	225[2]
Totals	**599**	**516**

[1] The images collected in the Ukraine were not of sufficient quality to be analyzed. It was decided that the camera that would be sent to the Ukraine for the collection of additional images would instead be sent to South Africa for the collection of subjects in the PASS study as well as Phil May's sample.

[2] 218 DNA samples also obtained from the mothers and 52 DNA samples obtained from the father. RNA also collected at this site

DNA Collection Supplement

A supplement to U01 AA014809 was approved for the collection of DNA. DNA is collected as either saliva or a blood sample. Members of this project worked with each site to obtain IRB approval and saliva is now routinely collected at the San Diego, UCLA, and Atlanta sites as well as DNA in South Africa in a project led by Sandra and Joseph Jacobson (see Table 1). The collection of saliva is performed at the same visit as the 3D facial imaging in San Diego and is collected during a different study visit at UCLA and Atlanta. The saliva kits are shipped back to Indiana University and extraction of the kits is up to date.

Genomewide Association Study Supplement

A supplement to U01 AA014809 was approved for the completion of a genomewide association study (GWAS) at the Center for Inherited Disease Research (CIDR). This option was more cost-effective than initiating a series of candidate gene studies. A data sharing plan was recently approved and CIDR has provided the necessary paperwork to initiate this project. A GWAS will be performed in 235 unrelated individuals from the 3 US sites. The Atlanta site will reconsent subjects so that their data can be placed into dbGaP. The consent for the other two sites is appropriate for sharing in dbGaP. We anticipate that samples will be sent to CIDR for genotyping in March of 2011.

V. Accomplishments and Results:

GOAL 1: IMPROVE UNDERSTANDING OF THE DYSMORPHIC FEATURES IN FAS.

Analyses performed by Peter Hammond, Ph.D.

Background

2D images have been employed for many years in the study of facial dysmorphism in children affected *in utero* by alcohol exposure. 3D photogrammetry has also been used for the automated recognition of FAS facial features (Fang et al, 2008; Mutsvangwa et al, 2010). Recently, within CIFASD, morphometric techniques have been used to detect differences in facial asymmetry between controls and children with a diagnosis of FAS (Klingenberg et al, 2010). These studies commonly employ measurements derived from landmarks in their shape analyses. A major drawback of this approach is that differences can only be detected in regions where homologous landmarks can be placed reliably. This short report illustrates recent work employing dense surface modeling techniques where a set of sparse landmarks is employed to induce a dense correspondence of 25,000+ points on facial surfaces. With more points available, there is more opportunity to detect and visualize subtle shape differences, especially in regions more distant from the original landmarks.

Previously, dense surface modeling (DSM) has proven successful in delineating facial characteristics in a variety of conditions involving impaired development, especially those caused by genetic anomalies. When combined with pattern recognition techniques, the models discriminate well between controls and affected individuals (Hammond et al, 2005, 2007, 2008). The same techniques can be applied to fetal alcohol syndrome (FAS) to screen children for the well-documented facial characteristics.

Preliminary results

3D facial photographs captured by the CIFASD consortium have been transferred to London for analysis. Identifying appropriate ethnically matched controls and subgroups with sufficient numbers, as well as establishing common interpretation of exposure classifications, delayed the analysis for a short while. More recently, the number of images has increased significantly. The analysis described below of a relatively small number of images from the Cape Coloured community has demonstrated the advantages of the DSM-based approach for both the visualization of face shape differences and for discrimination testing. Two types of analyses are described below, one comparing controls to individuals exposed to alcohol *in utero* but without facial dysmorphology, and one comparing controls to individuals with FAS.

For the comparison of control and affected subgroups, average face surfaces give a useful visualization of shape difference. In Figure 1, the mean face of a male control group is shown on the left. Its green shading is used for comparison with the mean face of a male group with known heavy exposure to alcohol *in utero*. Where the faces on the right are green they agree with the control surface. In contrast, red/blue indicates the surface of the exposed group mean is smaller/larger than that of the control mean. For example, the red regions show underdevelopment and the small blue region just above the center of the upper lip identifies expansion of the philtral groove and hence increased smoothness.

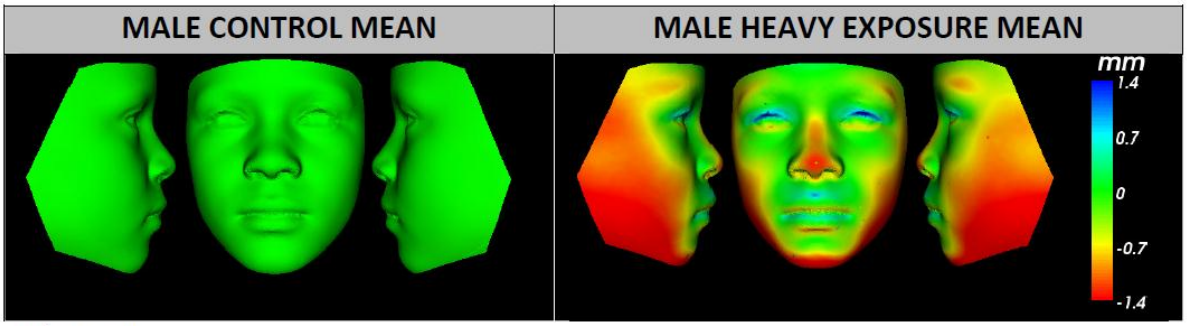


Figure 1

Relative similarity to an average control or average affected face can also be used to discriminate between subgroups. Figure 2 shows the relative scatter of control and FAS subgroups when compared horizontally to the normalized positions at -1 and +1 of the control and affected subgroup mean faces and vertically to dissimilarity from both means. The same comparison carried out on unseen randomly sampled test examples in 20 cross validation trials gives a mean discrimination of 0.95. This can be interpreted as an estimate of the probability of correctly classifying a pair of faces selected randomly, one from the control subgroup and one from the affected subgroup.

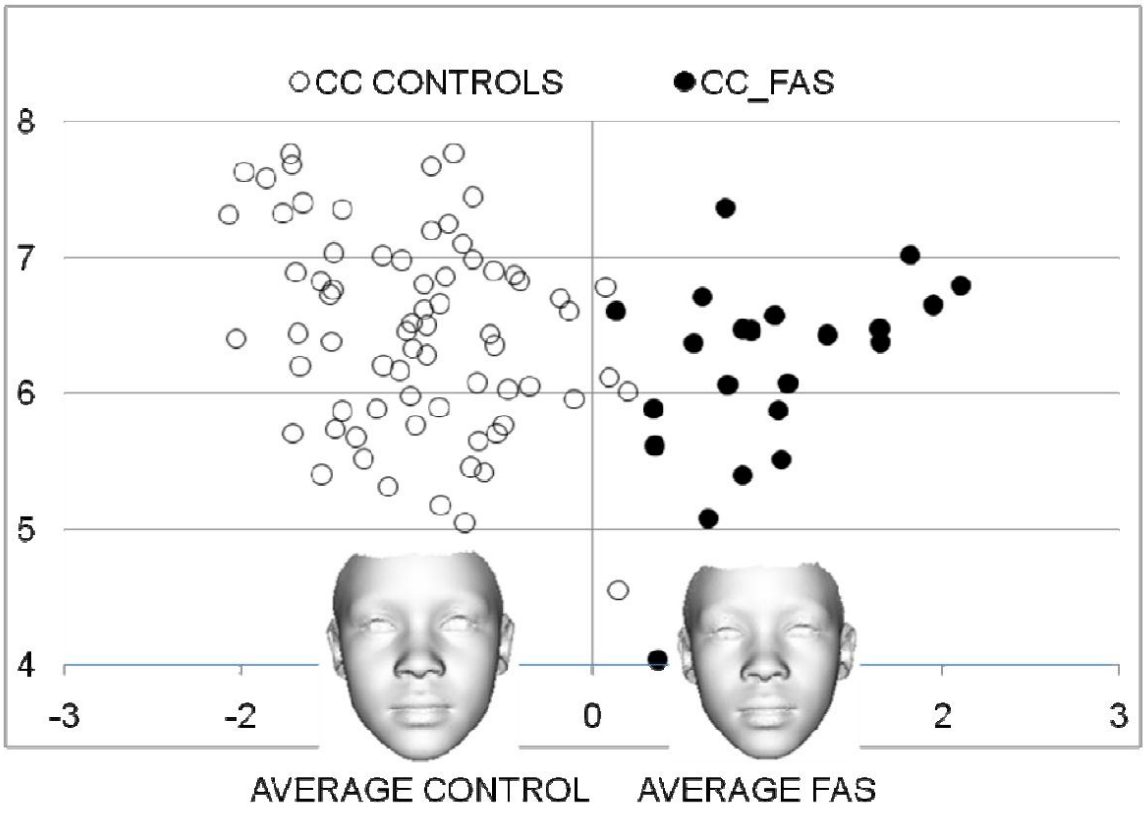


Figure 2

Discrimination testing tends to require reasonably large subgroups, at least of say 60 individuals. When sex and ethnic origin matching is required this can be demanding on recruitment. Therefore, more recently, we have been developing methods for analyzing small groups of, or even individual, affected children. Provided a control sample is available, it is possible to normalize the face shape differences of an individual using the densely corresponded points on the face surface.

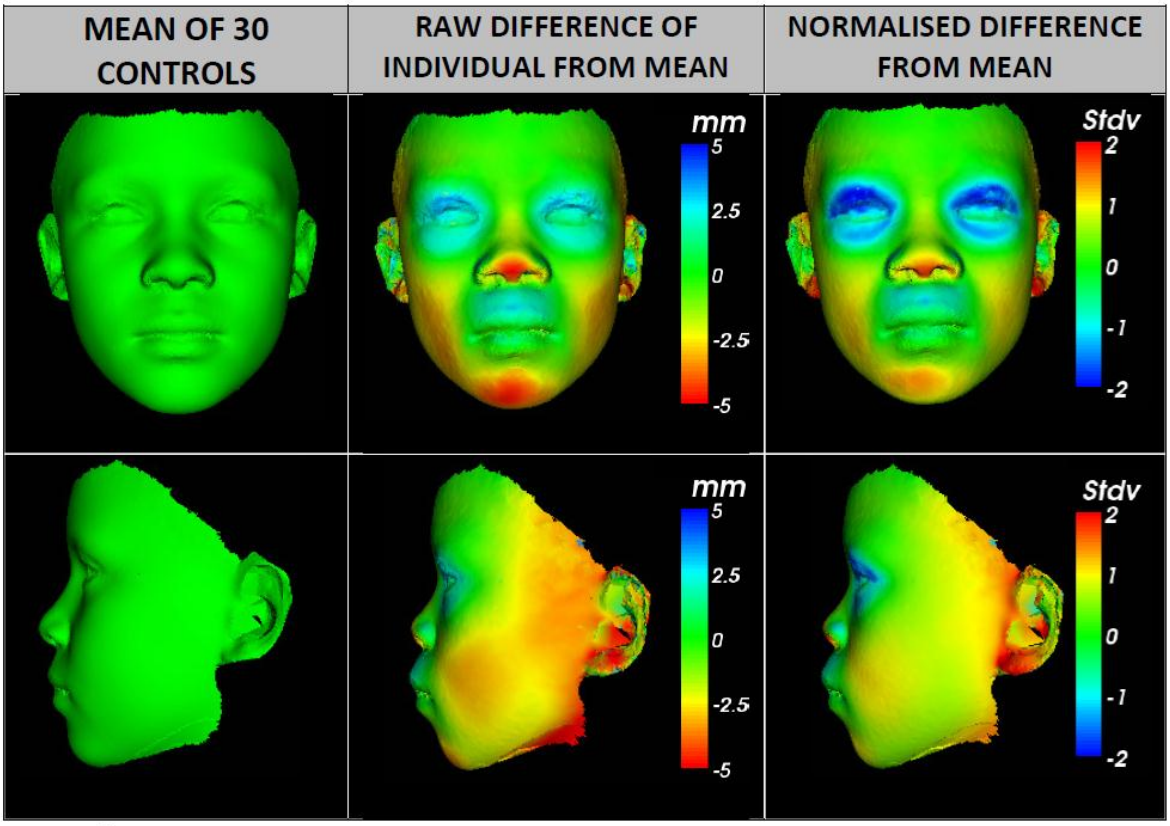


Figure 3

Figure 3 (column 1) shows portrait and profile views of the mean face of 30 controls of the same sex and with a matching mean age as the individual shown in the second and third columns. Column 2 shows the raw differences of the individual from the control mean on a scale where red means at least 5 mms smaller and blue means at least 5 mms larger. By calculating the mean and standard deviation of these raw differences at each densely corresponded surface point with respect to the control set, we can compute the normalized difference for an individual. For example, for the individual in Figure 3, the raw differences of reduced mandible size and periorbital prominence (column 2) are reversed in relative magnitude when normalized (column 3). Therefore, this individual's periorbital prominence is his strongest or most dysmorphic feature. By comparison, the smoothness of the philtrum, indicated by blue on the upper lip, remains at the same level.

Normalization enables facial dysmorphism to be studied at an individual level. We are currently developing a graph theoretic representation of normalized dysmorphism in sets of faces that identifies clusters of individuals with similar dysmorphism. This has already proven effective for

a number of syndromic groups including autism spectrum disorder but has yet to be tested on the FAS data.

Analyses performed by Li Shen, Ph.D.

Background

The collection of the adverse effects of alcohol on the developing fetus is known as fetal alcohol spectrum disorders (FASD). The ongoing clinical challenge is to expand the recognizable facial features so more individuals with prenatal alcohol exposure that do not exhibit the classic facial phenotype can be identified, allowing for early interventions. Craniofacial anthropometry has been used to accurately identify individuals with FAS. However, anthropometric assessments can be time consuming and usually require an experienced anthropometrist to obtain the measurements. So there is a need for newer techniques, which in combination with a clinician's assessment, would provide rapid and accurate pre-screening and early diagnosis of children with a FASD. Three-dimensional (3D) surface-based morphometry offers the opportunity to solve the problem. Surface-based morphometry (SBM), widely used in biomedical imaging to study various structures of interest, is used to identify morphometric abnormalities associated with a particular condition, assisting with diagnosis and treatment. Here we propose a novel computational framework that performs SBM on 3D facial imaging data for understanding the dysmorphic features affected by fetal alcohol.

Methods

We developed a unified surface-based morphometry (SBM) processing pipeline that integrated a set of effective surface registration (iterative closest point, conformal mapping and landmark-based thin-plate spline) and analysis (surface-based generalized linear model) methods (Wan et al 2010). We applied this framework to localize facial dysmorphology in FAS patients using the 3D data obtained previously in the Finnish sample using the Minolta camera. The analysis examined the surface deformations along the horizontal direction from back to front as well as surface curvatures. Results identified FAS dysmorphology patterns that were consistent with prior findings, including regions around the palpebral fissures, upper lip, and philtrum.

Based on the above SBM framework, we then applied this approach to a second data set, which was obtained using the newer 3dMD system in the South African sample from Cape Town. This sample was composed of 68 healthy controls (HC), 68 heavy alcohol exposure (HE), 23 partial FAS (PFAS), and 21 FAS individuals. Analyses had two goals. One was to localize morphological changes among the different groups. The second goal was to build a classifier to predict the group status of a new subject.

A series of group comparisons were made to address our first goal. These included: HC vs. FAS (the most extreme comparison), HC vs. FAS+PFAS (expanding the FAS group to include PFAS), HC vs. FAS+PFAS+HE (the broadest comparison including subjects with a range of effects due to alcohol exposure), HC vs. PFAS+HE (by removing the FAS, this comparison removes those with the most extreme dysmorphology), and HC vs. HE (the least extreme comparison). Group analysis of registered facial surfaces was conducted by employing a surface-based general linear model with random field theory addressing multiple testing issues. We compared the surface signals among the different group comparisons, with and without age and gender as covariates. We have examined a variety of surface features including deformations along the X, Y, Z axes as well as curvatures. For the second goal, we applied Support Vector Machine (SVM) methods to estimate accurate prediction and compared their performance on the dataset.

Results

Figure 4 shows a sample pipeline of our framework for 3D image processing and registration. Since the orientation of the original facial images was different, a least square rigid body transformation was used to align all the faces to a selected template using a predefined set of landmarks (Figure 4(a)). Next, each face was chopped to preserve the major frontal facial area (Figure 4(b)) using an automatic method in a consistent fashion. After that, conformal mapping and 2D thin-plate spline method were performed to build surface correspondence among all the faces (Figure 4(c)) for extracting surface signals and facilitating the subsequent analysis. After registration, the average faces can be calculated for the affected and unaffected groups as well as for the total sample (Figure 5), so that visual evaluation can be performed. In addition, the average face of the unaffected group can be used as the template for extracting the surface signals (e.g., deformation fields along X, Y, Z axes) to be used in subsequent analyses.

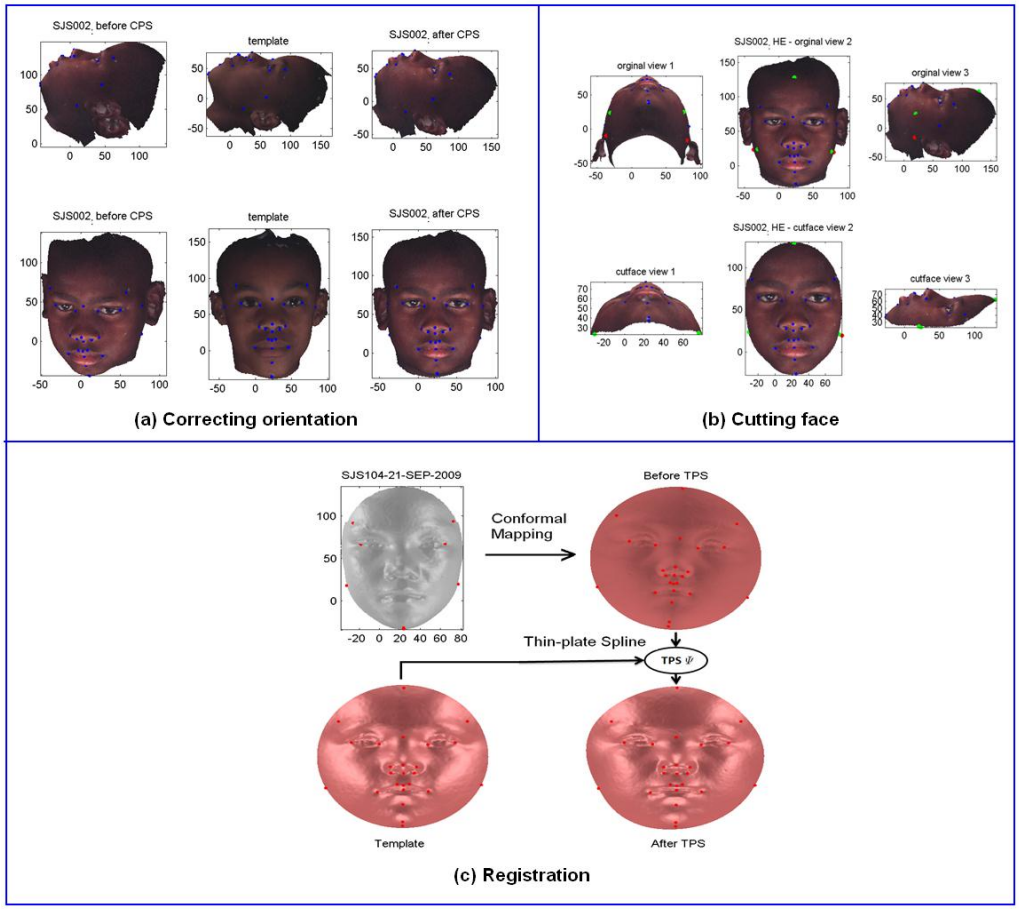


Figure 4. Processing pipeline of our surface-based morphometry framework

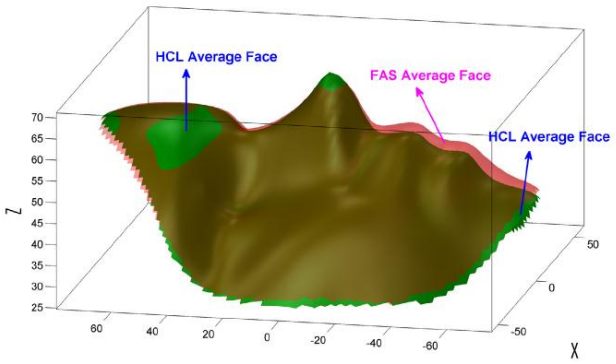


Figure 5. HC average face and FAS average face of the Finnish data set.

Shown in Figures 6-7 are example statistical maps of fetal alcohol effects on several surface measurements, including deformations along the X, Y and Z axes, as well as curvatures. The surface deformation along the Z direction (from back to front) were significantly larger ($p<0.05$) in the HC group than in the FAS group (Figure 6(a, b)) and in the FAS+PFAS group in the regions of nasal bridge, nose, mid-face, and jaw. When compared to the FAS+PFAS+HE group, the HC group showed significantly taller surface only in the region of nose (Figure 6(c, d)). The result of the surface deformation along the X direction (from left to right) indicated the HC group had a wider face than the FAS group and the FAS+PFAS group, and a wider bottom part of the face than the FAS+PFAS+HE group (Figure 7(a)). For surface deformation along the Y direction (from bottom to top), the HC group showed longer face than the FAS group, the FAS+PFAS group, and the FAS+PFAS+HE group (Figure 7(b)). For the fetal alcohol effect on surface curvatures, although no significant patterns were identified with a threshold $p<0.05$, the regions of the philtrum and mid-face detected in the t-map indicated that they were smoother in the FAS group and the FAS+PFAS group than the HC group (Figure 7(c)).

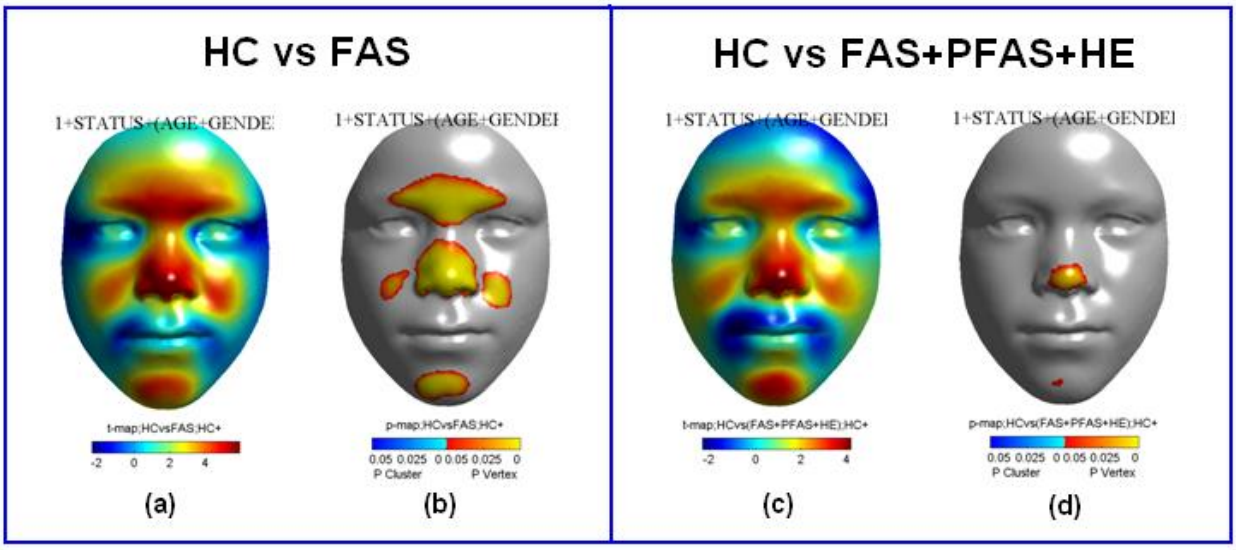


Figure 6. Fetal alcohol effect on surface deformation along Z direction (from back to front) while controlling for Age and Gender. The maps of the t statistics are shown in (a) and (c), where the red region indicates surface deformation going outward for the unaffected group and blue going inward. Shown in (b) and (d) are the maps of corrected P values for peak and cluster with $p<0.05$.

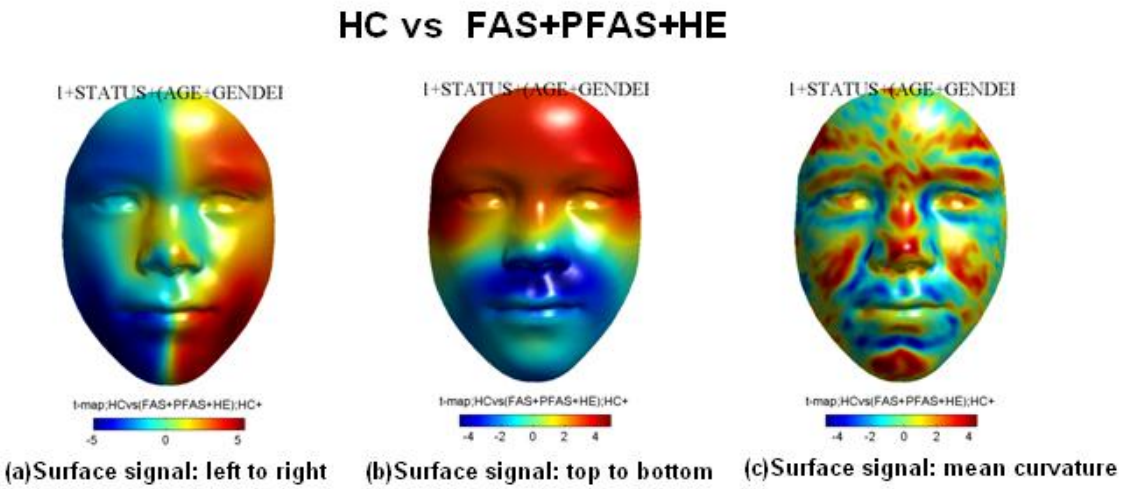


Figure 7. Fetal alcohol effects on three surface measures while controlling for Age and Gender. Here only t-maps are shown. (a) X-deformation: red indicates surface deformation going rightward for the unaffected group and blue going leftward. (b) Y-deformation: red indicates surface deformation going upward for the unaffected group and blue going downward. (c) Mean curvature: red corresponds to a convex shaped (∩-shaped) pattern for the unaffected group and blue to a concave shaped (∪-shaped) pattern.

As to classification using SVM, the leave-one-out cross-validation results for predicting fetal alcohol exposure status are shown in Table 2. As expected, the prediction rates consistently drops from the most extreme comparison (92.14% for HC vs. FAS) to the least extreme comparison (55.15% for HC vs. HE).

Table 2: The classification results

Group Comparison	Overall Accuracy	Sensitivity	Specificity
HC vs FAS	92.14%	85.71%	94.12%
HC vs FAS+PFAS	77.68%	72.73%	80.88%
HC vs FAS+PFAS+HE	61.67%	66.07%	54.41%
HC vs PFAS+HE	57.23%	60.67%	51.47%
HC vs HE	55.15%	52.94%	57.35%

Discussion and Plans

These results validate our hypothesis that alcohol exposure during pregnancy has a significant impact on facial surface morphometry. Facial surface dysmorphometry can be detected in the regions of the nose, nasal bridge, mid-face, philtrum, and jaw.

Future plans using SBM include: (1) develop methods to account for face size in facial imaging analysis; (2) examine additional surface signals (e.g., deformation fields, tensors) and test their discriminative power; (3) use dimensionality reduction methods (e.g., principal components analysis) to characterize the facial images in a low dimensional shape space and facilitate visual exploratory analysis; (4) apply our SBM framework to other CIFASD data sets to explore the generalizability of our results; (5) build predictive models for computer aided diagnosis and

sensitive marker discovery; and (6) detailed and focused analyses on regions of interest such as lip, philtrum, palpebral fissure, etc.

GOAL 2: ENHANCE THE CAPABILITY OF DEFINITIVE DIAGNOSIS OF FETAL ALCOHOL SYNDROME (FAS) AND THE BROADER SPECTRUM OF FETAL ALCOHOL SPECTRUM DISORDERS (FASD) AT DIFFERENT STAGES OF THE LIFESPAN.

Analyses performed by Leah Wetherill, M.S. and Tatiana Foroud, Ph.D.

Methods and Results

We focused our initial analyses on the sample with the largest number of subjects evaluated longitudinally, the South African, Cape Colored sample. A core set of ~140 subjects were seen twice, once at age 5 and again at age 9. At the first time point, the facial images were collected using the Minolta system while at the second time point, the facial images were collected using the 3dMD system. The data from the second time point were also analyzed by Dr. Hammond and Dr. Shen.

To examine longitudinal changes in facial features, we performed 3 types of group comparisons. First, we compared those with histories of prenatal alcohol exposure to controls. Then, we divided the alcohol-exposed children into two groups: those with facial dysmorphology who were classified as either FAS or partial FAS and those without dysmorphology but with heavy alcohol exposure (HE). These staged comparisons allowed us to explore whether a unique set of variables could accurately identify those with heavy exposure who do not have the traditional physical markers of FAS.

Results indicated that few common variables distinguished children with histories of heavy prenatal alcohol exposure from controls at the two time points. At age 5, the variables that best predicted group membership (any of the 3 alcohol-exposed groups vs. controls) were upper and lower facial depth, palpebral fissure length, minimal frontal length width and nasal bridge length. No measure was a significant predictor in all comparisons of 5 year olds. At both ages 5 and 9, lower facial depth was associated with prenatal alcohol exposure. As expected, in alcohol-exposed children with traditional markers of FAS, palpebral fissure length was smaller in the dysmorphic group than in the controls. In the comparison between controls and alcohol-exposed children without traditional physical markers of FAS, longer outer canthal length was associated with alcohol exposure at both ages. At each age and in each comparison, inclusion of additional variables in the logistic regression model improved classification accuracy.

Given the different models developed to predict group membership at ages 5 and 9, these data suggest that the rates of growth in the face vary during childhood development, and that the patterns of growth may mask some of the most characteristic features of FAS, making it more difficult to identify those who have been exposed prenatally. These data suggest that additional facial measures may be needed when assessing older children or adolescents for prenatal alcohol exposure. We will be replicating these analysis results in subjects from San Diego who have had facial images collected at two time points, approximately 4 years apart. However, the age range of the subjects from this site is substantially greater than for the South Africa sample, which should yield important insights regarding the generalizability of our results in the South Africa sample.

Analyses performed by Elizabeth Moore, Ph.D. and Richard Ward, Ph.D.

Background

Multiple approaches to diagnosing FAS and the less definitive manifestation of intrauterine ethanol exposure (fetal alcohol spectrum disorders) have been published (Chudley AE et al, 2005; Hoyme HE et al, 2005; Rosett HL, 1980; Stratton K et al, 1996; Astley SJ and Clarren SK, 1997; Centers for Disease Control and Prevention, 2004). However, forty years after its initial description by Lemoine et al. (1968), the "gold standard" for diagnosis remains the clinical judgment of a trained dysmorphologist. There is a clear need for a diagnostic aid that can be applied by less experienced observers in the frequent cases where dysmorphologists are not available to make the diagnosis. In addition, even trained clinicians may differ in their judgment of who has the necessary diagnostic criteria, a fact that has also argued for the development of automated or standardized diagnostic approaches (Astley SJ, 2006).

At the previous CIFASD meeting, we decided that a goal for this project was to determine whether the camera could be used instead of a trained dysmorphologist to make the diagnosis of FAS using the same algorithm employed in CIFASD. To address this goal, we compared three methods for identifying the presence of the key facial features required for the diagnosis of FAS: 1) direct clinical examination by a trained dysmorphologist using widely accepted revised (IOM) standards of diagnosis (Hoyme et al, 2005); 2) the Facial Analysis Software (Astley SJ, 2003) that uses the 4-Digit Diagnostic Code developed by Astley et al. (1997) and using two dimensional (2D) pictures assessed by a non-clinical observer as the program input; and 3) the revised (IOM) standards of diagnosis were applied by a non-clinical observer using the three dimensional (3D) images.

Methods

The same population of 45 young subjects drawn from the Cape Coloured, South Africa sample was used in all three assessments. This allowed for comparison of diagnostic outcomes from trained dysmorphologists with those determined indirectly by non-dysmorphologists. The 45 subjects included: 15 FAS, 15 heavy alcohol exposure (HE) but no FAS diagnosis and 15 healthy controls (HC) individuals with no alcohol exposure. This is a subset of the sample used by Drs. Hammond and Shen in their analyses described above under Goal 1 as well as the analyses performed by Dr. Foroud in Goal 2.

The key features assessed in each method were: palpebral fissure length (PFL), smoothness of the philtrum, and thinness of the upper lip. PFL measurements were obtained directly from the subject using a clear ruler, in the case of the dysmorphologist, or digitally from 2D photographs (Astley method) or digitally from 3D photos (3dMD method). In each case, measurement values for the left palpebral fissure were compared to published norms to establish normal/abnormal values, although as will be discussed below, the Astley method uses a different set of standards than do the CIFASD dysmorphologist and the 3D analysis. Assessment of philtrum smoothness and lip thinness was accomplished using a five-point photographic Likert scale published by Astley (Astley SJ, 2003; Astley SJ and Clarren SK, 1996).

The CIFASD criteria (based on the revised IOM criteria) were used for the assessment made by the dysmorphologist and the assessment of the 3D images. For the CIFASD assessment, two of three of the following features had to be present in order for the face to meet the FAS diagnostic criteria: short palpebral fissure (PFL <10^{th} percentile), smooth philtrum (5-point Likert score of 4 or 5), and thin upper lip (5-point Likert score of 4 or 5). For Astley's method, each feature was assigned a summary score of A, B, or C, representing the following: A: PFL > -1 standard

deviation (SD), a score of 1 or 2 on the five-point Likert scale; B: PFL > -2 SD and ≤-1 SD and a lip and philtrum score of 3; or C: a PFL ≤ -2 SD, a score of 4 or 5 on the Likert scale. The summary score was used to determine the 4-Digit Diagnostic Code Rank and the level of expression of FAS facial features as severe, moderate, mild, or absent. The FAS face was considered severe if all three features were assigned a summary score of C, moderate if two features were assigned a C and the other feature a B. Any other combination of summary scores that included at least one C was classified as mild and any summary score that did not have one C was designated as mild.

For purposes of this research, we dichotomized the subjective scale. Thus, Likert scores on both scales of 4 or 5 were considered affected. Scores of 3 or less were not assigned to the affected group. Results were considered concordant across methods if the same individual fell into the affected or not affected groups in all three methods.

Results

The subjective measures of philtrum smoothness and lip shape gave better agreement across the 3 methods than did assessment of PFL. Overall, the three methods demonstrated strong agreement in assessment of the philtrum (97.8% concordance) and good agreement on the lip shape (77.8% concordance).

PFL was assessed as abnormal if it fell below the lowest 10^{th} percentile on the CIFASD normative standards chart. This chart is derived from a version published by Thomas IT et al, (1987*). Astley uses a more conservative cut off of -2 SD (<5^{th} percentile), but her chart of normative values is derived from Hall (1989) which in turn is largely derived from a Farkas 1981 publication of facial norms. Interestingly, even with this more conservative cut off point, more of our sample is classified as having abnormally short palpebral fissure in the Astley method because the Hall/Farkas means are notably larger than the Thomas means. PFL measurements had strong reliability with all but 2 of 135 comparisons (Table 2) scoring within +/- 2mm of each other.

Small differences in measurements of small magnitude can have a large effect (Ward and Jamison 1991). Similarly, small differences in PFL can have a large impact on whether or not an individual is classified as FAS. This can be seen in the relatively low concordance across methods for this trait. There was concordance in defining PFL as normal or abnormal in only 64% of the cases (across all three methods). More specifically the measurements of the dysmorphologists tended to be smaller than those obtained indirectly from either the 2D or 3D image. As noted previously, Astley's method uses a more conservative cut off of the lower 5^{th} percentile. Not surprisingly, 33% of the subjects were discordant when comparing the 3D image with the 2D Astley method and over a quarter of (27%) of the individuals were discordant for the palpebral fissure criteria when comparing the assessment by dysmorphologists with those obtained using the 2D Astley method. However, there was good concordance between the dysmorphologist and the 3dMD assessment (89%). Clearly, the use of different standards and different cutoff points leads to different classification of individuals and argues for some agreement on common guidelines and standards.

Table 3: PFL results among the 3 methods [ruler (dysmorphologist), 2D and 3D].

Difference in PFL (mm)	Ruler minus 2D (N = 45)	Ruler minus 3D (N = 45)	2D minus 3D (N = 45)
-3 mm	2	0	0
-2.5 mm	0	0	0
-2 mm	0	4	0
-1.5 mm	4	7	1
-1 mm	4	5	4
-0.5 mm	13	12	8
0 mm	18	11	25
0.5 mm	3	4	7
1 mm	1	1	0
1.5 mm	0	1	0
2 mm	0	0	0
2.5 mm	0	0	0
3 mm	0	0	0

* *The chart used in the CIFASD classification program has different cutoff points and mean values than does the chart in this publication. Dr. Ken Lyon Jones had provided the chart from his personal files and believed it to be the same as that in the citation (Jones - personal communication)*

A more relevant question when comparing methods is the degree to which they concur in identifying individuals who have the FAS facial features. This is typically defined as those individuals who have at least two of the three facial features, although the Astley method allows for mild, moderate and severe categories depending on the combined feature scores. Interestingly, concordance between methods improves if we combine features to assess the face as a whole. Using a simple dichotomous approach (must have at least two FAS facial features as determined by scoring 4 or 5 on Likert scale features and/or having PFL in the abnormally small range as defined by the method), 40/45 individuals (89%) are concordant for the face.

It can be reasoned that at least some individuals in the study sample of "heavily exposed" subjects should have some features of intrauterine alcohol damage. Only Astley's method allows for a continuum of facial affects (absent, mild, moderate, severe). Table 3 indicates that nearly half (7/15 or 47%) of the HE group have some (mild) facial features associated with FAS. However, nearly as many controls (6/15 or 40%) also have mild features.

Table 4: Comparison of FAS and Control individuals with individuals without FAS but with Heavy Intrauterine Exposure* to Alcohol

ASTLEY METHOD	FAS	CONTROL	HEAVY EXPOSURE
No Facial Traits	0 (0%)	8 (53%)	8 (53%)
Mild Facial Traits	4 (27%)	6 (40%)	7 (47%)
Moderate Facial Traits	6 (40%)	1 (7%)	0 (0%)
Severe Facial Traits	5 (33%)	0 (0%)	0 (0%)
TOTAL	15	15	15

* The mothers of the children in the HE group drank a minimum of 1 oz AA/day or at least 4 binges (at least 4 standard drinks or 2 oz AA/occasion) during pregnancy. The median was 6.7 drinks/occasion and median frequency of drinking was 53.2 days during pregnancy.

Discussion

Comparison of two photogrammetric methods of identifying individuals with FAS facial features with "gold standard" of clinical diagnosis by trained dysmorphologists reveals strong correspondence with a nearly 90% agreement between the three approaches. This suggests that indirect assessment of the face using standardized criteria on carefully collected photographs or 3D images provides a reliable alternative diagnosis by a trained clinician. However, the comparison also suggests that the least reliable diagnostic feature is that collected with the greatest precision, palpebral fissure length. In the case of this single variable there was a relatively low concordance between Astley's method and either of the other methods. A portion of the difficulty can be traced to the tendency of photogrammetric approaches to generate consistently larger measurements for this variable but a more important source of variation is the use of different normative standards and disagreement on critical values.

In addition, no current method seems to offer a reliable means of identifying individuals who have the broader range of fetal alcohol spectrum disorders. It might be argued that this problem stems in part from the fact that all three methods rely on the diagnostic criteria assigned to FAS. Thus, circularity is introduced wherein only those individuals with features identified with FAS can be considered for inclusion in an FASD category. Even when allowing for mild expression of the philtrum flatness, lip thinness and/or palpebral fissure shortness, it is unclear that we can separate individuals who have intrauterine alcohol damage from normal variation of these features in a control population.

Approaches that consider a larger number of metric features (Moore et al, 2007) or facial shape (Hammond et al, 2008) and other "higher order" features (Fang et al. 2008, Klingenberg et al 2010) and utilize multivariate statistics to categorize individuals may offer a more reasonable approach to identifying individuals from the broader spectrum FASD. This study has shown that both 3D and 2D photogrammetric images offer a reliable alternative to data obtained directly from a subject by trained dysmorphologists.

Goal 3: Establish whether there is a relationship between FAS dysmorphic features and the specific underlying impairments in brain function.

We currently do not have a sufficient number of subjects with facial and brain imaging to perform these analyses. However, we have targeted collection of data so that we can augment these numbers. As described below, we will be obtaining facial images from ~30 subjects in South Africa who will also have brain imaging. We have also obtained facial images from subjects in Atlanta who will also be undergoing brain imaging.

VI. Discussion:

We have continued to employ a multifaceted approach to tackle the primary goals of this project. We have multiple researchers applying different approaches to these data to identify key features that distinguish those with FAS from controls but perhaps more importantly differ in those with and without prenatal alcohol exposure. These later analyses have grown tremendously with several of the researchers focusing initially on the data collected in South Africa. As the number of images grow in the United States, our goal is to replicate our results obtained in the South Africa sample in the US samples.

It is clear from all of our analyses that the face of FAS differs across race and age and perhaps sex, making the diagnosis challenging. Compounding these effects, there is also substantial variability in the FAS phenotype introduced potentially by variable prenatal alcohol exposure, but which may also simply reflect variability in human development.

A challenge for this core is to develop novel approaches that can help elucidate overall patterns of the effects of alcohol exposure and develop algorithms that could be used to improve our ability to diagnosis FAS. In concert with this goal, the studies of animal models provide a means to reduce the inherent variability among individuals by using genetically identical animals with known dose and frequency of exposure. Working together, the human and animal cores have begun to employ common methods such as dense surface modeling, pattern recognition and network analysis. This will allow us to more easily compare results across projects and discern the effects of prenatal alcohol exposure on a growing fetus.

VII. Interrelation with Aims of the Consortium and Other Projects:

Face-Brain Subgroup – Currently, there are 256 individuals with both 3D image and neurocognitive data. There are 85 individuals with data from three domains: 3D image, neurocognitive, and brain volume data. All subjects were seen by a member of the Dysmorphology Core and assigned a diagnosis of FAS, deferred, or no FAS. Hypotheses are being developed which will explore the impact of alcohol exposure on these various domains in this growing sample.

Coordination with the Bioinformatics Core – The Bioinformatics Core members have created a new interface for uploading the data so that xml and mdl files can be uploaded simultaneously. The x,y,z, coordinate data are now a part of the data base. Finally, the new interface accommodates longitudinal data. The Bioinformatics Core has also improved the rate at which images can be uploaded to the central repository.

Coordination with Dysmorphology Core - Members of this project conducted preliminary analyses with Dr. Ken Jones (PI of the Dysmorphology Core), comparing a diagnosis made employing the CIFASD algorithm on data obtained from the 3D images to the diagnosis made by the dysmorphologist. This comparison was then expanded to include Astley's methods (see summary in Goal 2 by Drs. Ward and Moore).

Coordination with mouse project – Members of this project are working with Dr. Zhou who is using a mouse model of alcohol exposure. Results based on anthropometric measurements on embryos at day 7 paralleled those of Moore et al (2007) in that two genetically identical substrains required different sets of measurements for prediction, and that width, depth, and height measurements were necessary for adequate sensitivity and specificity. Results based on mice exposed to a 4.8% alcohol diet from embryo days E7 to E16 compared to pair-fed and chows demonstrated that primarily width and depth measurements were necessary for classifying animals based on diet. Data based on a 3.6% alcohol diet are currently being analyzed and compared to the 4.8% alcohol exposure diet. Dr. Shen's group has also been studying effect of fetal alcohol on craniofacial bone development in C57BL/6J mouse models (Shen et al 2010). Special attention will be paid to relating mouse bone discovery to human facial findings.

Coordination with sheep project - Members of this project are working with Drs. Cudd and Goodlett who are using the sheep model. Results show that while there are no significant

differences between treatments in the mean anthropometric measurements in newborn sheep, width and depth measurements classify alcohol-exposed sheep from normal controls (sensitivity = 79%, specificity = 87%). Similarly, after 2 months of age, width and depth measurements discriminate between alcohol-exposed sheep and controls (sensitivity = 60%, specificity = 83%). The same measurements employed after 5 months accurately classify non-alcohol exposed sheep (88%) but not alcohol-exposed sheep (50%). These results have been submitted for a 2011 RSA symposium, in tangent with the mice results.

VIII. Plans for the Next Year:

Sites for data collection – We will be initiating data collection at 2 new sites.

In South Africa, members of this project will be traveling to South Africa in January to train PASS staff at Stellenbosch University to collect images in infants as well as children. Once trained, PASS subjects will be asked to complete a 3D facial imaging protocol at their 1 month and 12 month visits. We anticipate obtaining longitudinal images from approximately 1200 infants. Approximately 40% will have been exposed to alcohol prenatally and over 50% will not. This latter group will provide a powerful control group that will be needed for the analyses currently being undertaken by Dr. Peter Hammond, which require large numbers of controls.

In addition, a subset of the subjects participating in Dr. Phil May's study (~30) will also complete the 3D facial imaging protocol. These subjects will have their images collected at the time of their MRI visit – ensuring that we will have a set of subjects with both brain and facial imaging.

We will also complete a 3 day visit to Minnesota in early April, 2011 to collect 3D facial images as well as complete a dysmorphology evaluation in a set of ~20 individuals with heavy alcohol exposure. Given the new methods developed by Dr. Hammond, we will also collect a similar number of healthy controls who are not alcohol exposed.

Data collection – We will continue to collect both 3D facial images and saliva for DNA collection from all participating CIFASD sites. We do not anticipate any modifications in our data collection protocols.

Data analysis – Initial analyses of the imaging data collected with the new camera have focused on the South Africa cohort; however, in the coming year we will have sufficient numbers of subjects at the US sites to begin to explore analyses in both the Caucasian and African American subsets of the US sample. This will allow us to compare anthropometric features that differ between alcohol-exposed vs non-exposed children in two ethnically diverse subgroups of the same age (African American vs Caucasian). In addition it will allow us to compare the similar ethnic yet genetically diverse subgroups of American Caucasians vs Finnish Caucasians as well as African Americans and Cape Coloured children. We will continue to distribute images to all project collaborators and facilitate with the analysis of data.

IX. Publications:

1. Klingenberg CP, Wetherill L, Rogers J, Moore E, Ward R, Autti-Rämö I, Fagerlund Å, Jacobson SW, Robinson LK, Hoyme HE, Mattson SN, Li TK, Riley EP, Foroud T, and the CIFASD. Fetal alcohol syndrome alters the patterns of facial asymmetry. *Alcohol*. 2010 Nov-Dec; 44(7-8):649-57. PMID: 20060678

2. Anthony B, Vinci-Booher S, Wetherill L, Ward R, Goodlett C, Zhou F, and the CIFASD. Alcohol induced facial dysmorphology in C57BL/6 mouse models of fetal alcohol spectrum disorder. *Alcohol*. 2010 Nov-Dec; 44(7-8):659-71. PMID: 20570474.
3. Wan J, Shen L, Fang S, McLaughlin J, Autti-Ramo I, Fagerlund A, Riley E, Hoyme HE, Moore ES, Foroud T, CIFASD. A framework for 3D analysis of facial morphology in fetal alcohol syndrome. *Lecture Notes in Computer Science (LNCS)* 6326, pp. 118-127, Springer, Heidelberg, 2010.
4. McLaughlin J, Fang S, Huang J, Jacobson S, Hoyme HE, Robinson L, Foroud T. Interactive Feature Visualization and Detection for 3D Face Classification, Proc. The 9th IEEE International Conference on Cognitive Informatics. July, 2010. (An extended version of this paper has also been invited to the *International Journal of Cognitive Informatics and Natural Intelligence).*

X. Posters and Presentations:

1. Wan J. Shen L, Fang S, et al. A framework for 3D analysis of facial morphology in fetal alcohol syndrome. MIAR 2010: the 5th Int. Workshop on Medical Imaging and Augmented Reality, Sept. 2010, Beijing, China. A platform talk delivered by Wan J.
2. McLaughlin J, Fang S, Huang J, Jacobson SW, Hoyme HE, Robinson LK, Foroud T, and the CIFASD. Interactive feature visualization and detection for 3D face classification. Submitted to 3D Data Processing, Visualization and Transmission Conference 2010.

RSA abstracts (2010)

1. Shen L, Ai H, Liang Y, Anthony B, Zhou FC, CIFASD. Effect of fetal alcohol on craniofacial bone development in C57BL/6J mouse models. 33rd Annual RSA (Research Society on Alcoholism) Scientific Meeting, June 26-30, 2010, San Antonio, Texas.

2. Wetherill L, Hoyme HE, Robinson L, Rogers J, Moore ES, Ward R, Vinci-Booher S, Molteno CD, Jacobson JL, Jacobson SW, Foroud T, CIFASD Consortium. Longitudinal changes in facial measurements from 3D images in children with heavy prenatal alcohol exposure. 33rd Annual RSA (Research Society on Alcoholism) Scientific Meeting, June 26-30, 2010, San Antonio, Texas.
3. Goodlett CR, Wetherill L, Moore ES, Johnson TB, Lunde RE, Cudd TA. Evaluation of facial dysmorphology in an ovine model of fetal alcohol spectrum disorders. 33rd Annual RSA (Research Society on Alcoholism) Scientific Meeting, June 26-30, 2010, San Antonio, Texas.
4. Anthony B, Liang Y, Ai HS, Wetherill L, Ward R, Vinci-Booher S, Goodlett C, Zhou F, CIFASD Consortium. Facial measurement in mouse as a model of diagnosis for fetal alcohol spectrum disorders. 33rd Annual RSA (Research Society on Alcoholism) Scientific Meeting, June 26-30, 2010, San Antonio, Texas.

RSA (2011)

We submitted a symposium to the programming committee and are waiting to hear whether it will be accepted.

Fetal Alcohol Spectrum Disorder: What the face can tell us over time and across species
Organizers: Tatiana Foroud and Michael Charness

INTRODUCTION: Luther Robinson, M.D

PRESENTATION 1: Robert Lipinski, Ph.D.
MRM-based 3-D face/brain correlations in an FASD mouse model

PRESENTATION 2: Leah Wetherill, M.S.
Effect of prenatal alcohol exposure in sheep and mice on craniofacial measurements and their change with age

PRESENTATION 3: Peter Hammond, Ph.D.
Analysing facial dysmorphism in 3D

PRESENTATION 4: Tatiana Foroud, Ph.D.
Longitudinal changes in the face of individuals exposed to alcohol prenatally

DISCUSSANT: Michael Charness, M.D.

I. Principal Investigator: Sarah N. Mattson, Ph.D.

Key Personnel: Colleen Adnams, MD, Co-PI, subcontract
Claire D. Coles, Ph.D., PI, subcontract
Julie A. Kable, Ph.D., Co-I, subcontract
Wendy Kalberg, Ph.D., Co-PI, subcontract
Philip A. May, Ph.D., PI, subcontract
Edward P. Riley, Ph.D., Co-PI
Elizabeth R. Sowell, Ph.D., PI, subcontract

II. Title of Project: A Multisite Neurobehavioral Assessment of FASD, U01 AA014834

III. Objectives (unchanged):

The primary aim of this project is to determine whether a neurobehavioral phenotype exists in children with fetal alcohol syndrome, whether the same phenotype exists in children with FASD who lack facial dysmorphology, and whether the phenotype can be used for differential diagnosis. Secondary aims, involving collaboration with other CIFASD projects and cores, are to determine the relationship between brain dysmorphology, facial dysmorphology, and neurobehavioral function.

IV. Methods (unchanged):

A standard neurobehavioral protocol will be administered to four groups of children at six sites and will address the functional domains of executive function, working memory, verbal function, and psychological symptomology. In addition to children with FASD and non-exposed controls, children with low IQ scores or ADHD will be included as contrast samples. Using this heterogeneous sample and multivariate statistical methods, neurobehavioral profile specific to FASD will be sought. In addition, participants will be assessed using methodology prescribed by the Dysmorphology Core and the facial and brain imaging projects of the CIFASD. Data from three broad domains (neurobehavior, dysmorphology, and brain morphology and function) will be analyzed both separately and together to address the main aim of the CIFASD: improving the diagnostic criteria for FASD.

The current project assumed responsibility for the functions previously carried out by the Neurobehavioral Core. As such, the main site (San Diego) is now responsible for preparing all testing materials including purchasing tests and test materials, and creating and distributing administration manuals, summary forms, reliability protocols and forms, and scoring manuals. Training sessions are to be conducted at the San Diego site during the first year with follow-up sessions each year for reliability purposes, either in San Diego or in conjunction with one of the annual PI meetings. In addition, we are responsible for collaborating with the Informatics Core on creating the data dictionary for the input tool for the current data and on pilot testing of the input and upload tools created for this project.

Six sites are used for participant recruitment. Three of these sites were included in the last funding period for the CIFASD through 2 separate projects. The current sites are: (1) Center for Behavioral Teratology, San Diego State University, San Diego, CA; (2) Marcus Institute at Emory University, Atlanta, GA; (3) University of New Mexico, Albuquerque, NM; (4) Seven Northern Plains communities, including six Indian reservations; (5) University of Cape Town, South Africa, and a town in the Western Cape Province; and (5) the University of California, Los Angeles, Los Angeles, CA. The combination of these sites allows for a study population that is both large in number and heterogeneous in nature. Such a sample will ensure our results are both accurate and unbiased by specific site characteristics.

V. Accomplishments and Results:

Funding for Phase II began on September 30, 2007, significant progress has been achieved as follows:

A. General Progress (San Diego).

1. *IRB Approvals*. We have active IRB approval for the neuropsychological, dysmorphology, brain imaging, and 3D imaging portions of the study at each site (where relevant).
2. *Subcontracts*. All three subcontracts are active.
3. *Hiring*. We have in place staff necessary to conduct the study, including a psychometrist, recruiter, and research associates/assistants. The subcontract sites have personnel in place.
4. *Purchasing*. Necessary materials and equipment are in place.
5. *Material Development*. We have finalized versions of test administration and scoring materials.
6. *Training*. We conducted three training meetings in San Diego on December 3-5, 2007 (data collection staff from University of New Mexico and UCLA), January 14-15, 2008 (all sites present), and March 9-13, 2009 (UCLA, Atlanta, South Africa present). During the past year, we also conduced an onsite training at UNM on February 7-11, 2010.
7. *Database Development*. We continue to work with the informatics core to refine the input tool for the neurobehavioral test battery (CIFASD Neurobehavioral Phase II) and the neuro demographics database. The database is in place and has been used successfully by all sites but we have identified issues and improvements that require the informatics team. Data is present in both databases but there remains a continued need for sites to enter and upload both neuropsychological and demographics data.

B. Neurobehavioral Testing. Data collection for Phase II continued at all sites, and a total of 698 subjects have been tested, which is more than double what was reported last year. See below for details on CIFASD II data collection. In San Diego, we have tested 188 subjects using the Phase II test battery.

C. MRI Evaluations: The San Diego site is also involved in the brain imaging project (E. Sowell, PI). We are actively collecting data and thus far, have scanned 46 of our planned 40 subjects for scan 1. We scanned extra subjects to account for lost data due to movement and a few subjects that were outside the age range (older). We are scheduled to begin our second scans in January.

D. 3-D Facial Imaging: In San Diego, we received the original 3-D camera at the beginning of April 2005 and evaluated 107 children. We received the new camera in July of 2008 and have evaluated 192 children with this camera. Of these, 41 children have been evaluated with both cameras and two have been evaluated twice with the new camera. Including both cameras, 254 individual children have been imaged. All data have been transferred to IU.

E. Dysmorphology: Since the beginning of the CIFASD Phase I, 196 children have been examined by the dysmorphology core at the San Diego site.

F. Genetics: Since 2009, we (SDSU) have collecting saliva samples for genotyping under the direction of Dr. Tatiana Foroud. Standardized procedures are used according to IU specifications. Thus far, we have collected samples from 158 children.

G. Supplemental Funds: Two ARRA supplements were sought and received. One is described below and was used to supplement the San Diego ($100,000) and UNM/Plains ($100,000) sites. The second is for student research activity. Three students (1 full time, 2 part time) were supported for Summer 2009 and 4 (all part time) were supported for Summer 2010. The addition of these summer students to our research staff accelerated our ability to get data into the database and freed up time for the other staff to focus on data collection and larger database issues.

H. Site-Specific Progress: All sites have approved IRB approval and attended one or more training meetings. Site progress reports were requested from all sites. Reports from Dr. Coles (Atlanta) and Dr. Adnams (South Africa) are attached to this document (Appendix 1 and 2). Our budget allowed data collection to begin in month 7 (April 2008). Data collection is proceeding as follows:

	San Diego	Los Angeles	Atlanta	New Mexico	Northern Plains	South Africa
IRB Approval	Yes	Yes	Yes	Yes	Yes	Yes
Trained & Validated Tester	Yes	Yes	Yes	In progress (new tester)	Yes	Yes
Data Collection Begun/Active	Yes/Yes	Yes/Yes	Yes/No	Yes/Yes	Yes/Yes	Yes/Yes
Subjects Tested with Neurobehavioral Test Battery[1]	188	65	87	61	90	207
% of 12/2010 Goal	125.33	76.47	69.60	55.45	60.00	103.50
Phase II cases with partial data in CR (Neuro)[1]	177	62	91	56	87	204
Phase II cases with partial data in CR (Demo)[1]	249	36	97	54	82	195
Dysmorphology	Yes	Yes	Yes	Yes	Yes	Yes
3-D Facial Imaging	Yes	Yes	Yes	N/A	N/A	N/A
Genetics	Yes	Yes	Yes	N/A	N/A	N/A

[1] As of 12/30/2010

I. Scientific Progress. This year we have made considerable progress toward our specific aims. We published two papers (see list below) detailing (1) the general methodology of the CIFASD clinical projects and (2) a neurobehavioral profile determined using data collected from the CIFASD cohort. We are also currently conducting several sets of analyses using CIFASD data.

1. *Longitudinal study of executive function (EF).* A previous cross-sectional study indicated that EF deficits increase over time in alcohol-exposed youth. Using longitudinal data from CIFASD I and CIFASD II, we examined the development of EF in children from the CIFASD cohort. Our results supported previous reports of EF deficits in alcohol-exposed children but did not support the increase in EF disturbance over time. We presented a poster on these data at RSA 2010 and are hoping to increase the sample size.

2. *Analysis of sluggish cognitive tempo scale (SCT).* Children with FASD often meet criteria for attention-deficit/hyperactivity disorder (ADHD). Children with ADHD, particularly the predominantly inattentive subtype (ADHD-PI), have been noted to exhibit a symptom profile characteristic of a sluggish cognitive tempo. This set of symptoms is also related

to elevated internalizing behaviors in ADHD. We are interested in whether children with FASD demonstrate elevated SCT symptoms and if they do, is it related to internalizing symptoms. Using CIFASD data, we have examined SCT data from 161 children in the ALC, ADHD, and CON groups. Our preliminary analyses indicate that SCT scores are elevated in the ALC group but may not be specific to those children with ADHD-PI subtype. We are planning on submitting this as an abstract to the 2011 RSA meeting.

3. *Analysis of the relation between EF and behavior problems.* Previous research in ADHD has indicated that diminished EF is related to increased behavior problems in children. We were interested if the same relation existed in children with FASD but also hoped to expand it to include both parent ratings and direct measures of EF. Using data from the CIFASD cohort (N=216), we examined EF and behavior measures from 216 children in the ALC, ADHD, and CON groups. The measures included were the CBCL and the Behavior Rating Inventory of Executive Function (BRIEF) and portions of the Delis-Kaplan Executive Function System (D-KEFS). While our results are still preliminary, our hypothesis was supported in that performance on both parent ratings (BRIEF) and direct measures (D-KEFS) of EF predicted behavioral outcome on the CBCL. We are planning on submitting this as an abstract to the 2011 RSA meeting.

4. *Analysis of CANTAB data comparing children with FASD to those with ADHD.* 4 groups of children were compared on measures of delayed memory, working memory, and aspects of executive function using a computerized battery of neuropsychological measures: (1) children with heavy prenatal alcohol exposure, (2) typically developing controls, (3) non-exposed children diagnosed with ADHD, and (4) non-exposed children with low IQ scores. Tests administered were from the Cambridge Neuropsychological Test Automated Battery and included the Delayed Matching to Sample Test, the Spatial Working Memory Test, and the Intra/Extra Dimensional Shift Test. Primary scores derived from each of the tests were analyzed using repeated measures ANOVA with group as the between subjects factor and test score as the within subjects factor. Children with heavy prenatal alcohol exposure could be differentiated from controls on all three tests. Alcohol-exposed children performed similarly to children with ADHD on the DMS and IED measures, but performed more poorly on SWM outcomes. These findings do not appear to be attributable to IQ as children in the low IQ group performed similarly to the ADHD group on these measures. These findings are of significance because they demonstrate the utility of a computerized measure in differentiating children with FASD from similar clinical groups and determining the specificity of deficits related to heavy prenatal alcohol exposure. We are planning on submitting this as an abstract to the 2011 RSA meeting.

5. *Comparison of dysmorphology data from CIFASD to the IOM schema.* Under the assumption that it would be useful to compare th CIFASD dysmorphology criteria to the IOM/Hoyme scheme for diagnosing alcohol-related conditions, we examined the distribution of diagnostic labels using both schema. This question arose because of reviewer comments about the CIFASD diagnostic schema. Preliminary analysis of CIFASD dysmorphology data for data from the San Diego site (N = 193) indicates that the CIFASD criteria are more liberal than the IOM/Hoyme criteria. Of the 24 children labeled as FAS by CIFASD criteria, 12 (50%) would be labeled as FAS and 12 as partial FAS using IOM/Hoyme criteria. In addition, all of the children identified as Deferred (N=62) using CIFASD criteria would be labeled “Not FAS or partial FAS” using the IOM/Hoyme criteria. The remaining 107 children were labeled as “Not FAS” (CIFASD) and “Not FAS or partial FAS” (IOM/Hoyme). Thus, there was consistent classification in 129 cases and discrepant classification in 74 cases. These data indicate that CIFASD

criteria are more inclusive than the IOM/Hoyme criteria; the results are useful and could be followed up with much larger sample sizes from multiple sites. The analysis idea was briefly discussed on one of the monthly conference calls, and we are awaiting instruction on whether to pursue it further.

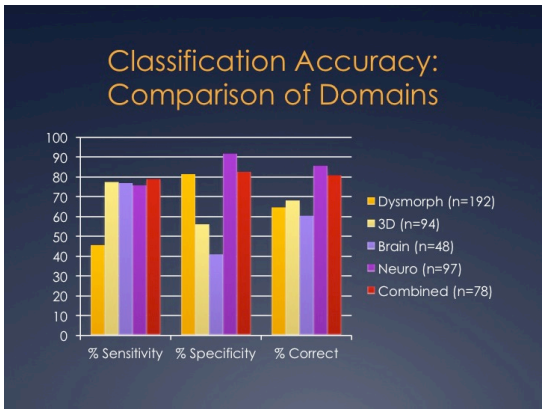
6. *5 Questions.* As a group, the clinical researchers of CIFASD were charged with 5 questions. Of these two are relevant here.

   a. *Determining if a more concise profile can be identified than the one submitted for publication from Phase 1 data.* This is a critical question and central to the aims of CIFASD. To this end, we are in the process of conducting several sets of analyses, which are described above.

   b. *Defining criteria for the FASD (excluding FAS) group.* The goal is to determine if children with heavy prenatal alcohol who do not have FAS can be distinguished from unexposed control children using all available data. A group of clinical reasearchers, Sarah Mattson, Ken Jones, Tatiana Foroud, and Elizabeth Sowell identified key variables from each domain. Initially, 28 variables were selected and this group was reduced to 11 key variables using logistic regression. Data from 3 sites were included (Emory, UCLA, SDSU). Brain variables were dropped from the analysis because of limited sample sizes. 78 children with data on all 11 variables were included; children with FAS were excluded. The following table lists the variables included.

Domain	Variables
Neurobehavior	FSIQ; CANTAB variables: BLC Mean Correct Latency, IED Total Errors, SWM Mean Time to First Response (6), CRT Max Latency, SRT Mean Latency, SRT SD of Latency
Dysmorphology	Camptodactyly, Heart Murmur
3D Facial Imaging	Left Upper Face (depth), Total Facial Height (length)

The sensitivity, specificity, and overall accuracy for this model were all around 80%. As follow-up, we examined how each domain did in the overall analysis. The following graph illustrates the results.



Sensitivity is about the same for all of the domains except dysmorphology, which is lower. Specificity is best for dysmorphology, neurobehaior, and the combination of all the domains. Overall accuracy is best for neurobehavior and the combination. As a point of comparison, in this sample (without FAS), using classic dysmorphology measures (growth, microcephaly, short PF, thin vermillion, smooth philtrum), classification accuracy is 65%, sensitivity is 46%, specificity is 82%. Our results suggest that we can identify children along the spectrum combining measures from neurobehavior, dysmorphology, and 3D facial imaging domains. These analyses were presented at the RSA 2010 conference.

J. <u>Obstacles and Plans for Overcoming Obstacles:</u>

1. *Data Collection.* Last year our biggest obstacle was in getting data collection to commence and continue in a reliable and valid fashion. All sites have begun data

collection and data collection was active at all sites. The Atlanta site recently lost their psychometrist and thus, has temporarily suspended data collection. Training of a new psychometrist is in progress and data collection should begin in early 2011. South Africa was the slowest to start but has been (amazingly) actively collecting data for the last year and completed the year at 103% of the year goal. At the UNM and Plains sites, data collection remains slower than expected for several reasons. Recruitment at the Plains site was dependent on a separate NIAAA award that ended April 30, 2009. We sought and received ARRA funding to supplement the existing CIFASD award in 2009 and these funds were used to provide employment at four established research sites in the Northern Plains region of the United States. These funds were requested to retain one employee at each of four established sites to focus exclusively on recruitment of subjects with FASD (those subjects who were already diagnosed in the previous grants who have not yet received testing with the CIFASD neuropsychological battery and a few newly diagnosed cases) and controls. In addition, the long-standing psychometrist at the UNM and Plains sites left the project in early September, 2009. Two psychometrists were hired and testing proceeded at both sites. In addition, we "loaned" the psychometrist from the SDSU site to UNM to travel to the Northern Plains site to increase their ability to test subjects at that site. Recruitment has improved but is still slow at the UNM and Plains sites. At UCLA, recruitment has slowed in the last year, although they remain at 76% of the year goal.

2. *Data Uploading.* An additional, pressing obstacle has been uploading of data in the neurobehavioral demographics central repositories (CR). A recent push to upload data has improved things dramatically, but work remains to be done. As of 12/30, sites had uploaded at least partial data for nearly all cases (96%) tested into the neurobehavioral CR. However, the demographics database has less data, only 56-100% (83% overall) of cases had at least partial data uploaded. This results in a large number of cases that cannot be used for analysis by any project since they have missing data. Importantly, this is actually an overestimate because these percentages count cases that have even partial data uploaded. We have been working with the informatics group to develop a missing data tracking tool and plan to implement in at the beginning of 2011. The following table lists the number of cases included in the two CRs by site (as of 12/30/2010).

Site	Cases Tested	Neuro CR	Demo CR	% Tested in Neuro CR	% Tested in Demo CR
San Diego	188	177	249[1]	94.15%	132.45%
Los Angeles	65	62	36	95.38%	55.38%
Atlanta	87	91	97	104.60%	111.49%
New Mexico	61	56	54	91.80%	88.52%
Northern Plains	90	87	82	96.67%	91.11%
South Africa	207	204	195	98.55%	94.20%
Total	698	677	580	96.99%	83.09%

[1] Includes CIFASD I cases

3. *Grouping Subjects.* A third obstacle we faced this year was related to the missing data and our previous strategy for grouping subjects. We have developed a detailed flow chart to be used to group subjects and have tested it on subjects from all sites. This chart allows us to assign a group in a consistent way across sites. A copy of the flow

chart is included in Appendix 3. In collaboration with the informatics group, we have implemented an automation of this flowchart. Using this automated system and the data that exist in the neurobehavioral and demographics databases, we have calculated a new variable (DemGroupClass) that indicates the correct grouping variable for each subject. As mentioned, an obstacle is that the variable is limited by missing data. It is calculated based on 8 variables from three databases (neurobehavior, demographics, and dysmorphology). Not all missing data in these 8 variables is fatal, but in a fair number of cases, DemGroupClass cannot be calculated. The following table summarizes the results of this calculation (as of 12/29/2010). The data presented in this table illustrate the problem. Of 698 children that have been tested, we cannot calculate a grouping variable (DemGroupClass) for 189 or 27%, which is unacceptable. Our hope is that with the introduction of the missing data tracking tool, this number will be reduced. Of note, about 11% of cases also are unclassified, but for a different reason (noted as "Unknown" in the table, below). These cases have adequate data but are classified in our Unknown group because they do not meet inclusion criteria for any group. Examples of this group include a child that was recruited as a control but who has more than minimal alcohol exposure or a child who was recruited as an ADHD subject but who doesn't meet criteria for ADHD. While we would like to use every subject and will work to reduce the number of children tested that cannot be used, 11% data loss is not unreasonable for this type of project.

Site	ALC	CON	ADHD	IQ	Missing	Unknown	Total	Tested	%Missing
San Diego	42	39	27	3	44	22	177	188	23.40%
Los Angeles	7	0	0	0	37	18	62	65	56.92%
Atlanta	25	14	12	10	23	6	90	87	26.44%
New Mexico	1	15	3	1	31	5	56	61	50.82%
Northern Plains	20	16	10	4	19	18	87	90	21.11%
South Africa	47	17	--	9	35	9	117	207	16.91%
Total	142	101	52	27	189	78	589	698	27.08%

4. *Fixing Errors in Dysmorphology Database.* A fourth obstacle we faced this year is the lack of a system for making changes in the dysmorphology database. We have several cases that need to be edited, but no way to do it. At this point, there is no apparent plan to remedy this problem, which will result in subjects being misclassified.
5. *Missing Dysmorphology Data.* Finally, an obstacle that is specific to the San Diego site is that we have a large number of cases for which dysmorphology data are missing. Of the 259 subjects that have been tested in either CIFASD I or CIFASD II, 112 (43.2%) subjects are missing a dysmorphology exam. A small number of these are either from outside of San Diego County (5, 4.5%) or California (4, 3.6%). The remaining are within San Diego County. In 2010 we planned to hold dysmorphology clinics monthly and did so 7 times (February, March, June, July, August, October, and December). We are aware of this weakness and have asked to schedule monthly clinics in 2011. We will

continue to work with Dr. Jones to schedule more clinics to remedy this problem. We are limited by his schedule, however. Already we know that he is not available in January and February.

VI. Discussion:

In the 40 months since funding began, we have made considerable progress at some sites. Challenges remain at all sites and include data collection and uploading. Ultimately, data collection will be conducted at multiple sites with varied subject characteristics, which will put us in the position to have access to a large, heterogeneous population on which to test our hypotheses.

VII. Interrelation with Aims of the Consortium and Other Projects:

This project relates to the overall aim of the consortium project in that our primary goal is to assess children with FASD and controls and to determine whether a profile of function exists in this population.

VIII. Plans for the Next Year:

During the next year, we plan to complete data collection at all sites and upload data for all subjects tested into the neurobehavioral and demographics database. As mentioned, we will initiate a missing data tracking system and attempt to increase the number of subjects with dysmorphology exams (San Diego). We will continue to provide administrative support as well as ongoing training and reliability assessment to the sites involved in neurobehavioral testing. We will continue to work with the informatics core as well as the 3D facial imaging, brain imaging, and dysmorphology projects. We will continue to analyze the data as described in the Scientific Progress section and submit manuscripts describing these studies for publication.

IX. Publications (2010):

Mattson, S. N., Roesch, S. C., Fagerlund, A., Autti-Ramo, I., Jones, K. L., May, P. A., Adnams, C. M., Konovalova, V., Riley, E. P. and the CIFASD (2010). Toward a neurobehavioral profile of fetal alcohol spectrum disorders. *Alcohol Clin Exp Res, 34*(9), 1640-1650. PMCID: 2946199.

Mattson, S. N., Foroud, T., Sowell, E. R., Jones, K. L., Coles, C. D., Fagerlund, A., Autti-Ramo, I., May, P. A., Adnams, C. M., Konovalova, V., Wetherill, L., Arenson, A. D., Barnett, W. K., Riley, E. P. and the CIFASD (2010). Collaborative initiative on fetal alcohol spectrum disorders: methodology of clinical projects. *Alcohol, 44*(7-8), 635-641. PMCID: 2888656

Klingenberg, C. P., Wetherill, L., Rogers, J., Moore, E., Ward, R., Autti-Ramo, I., Fagerlund, A., Jacobson, S. W., Robinson, L. K., Hoyme, H. E., Mattson, S. N., Li, T. K., Riley, E. P., & Foroud, T. (2010). Prenatal alcohol exposure alters the patterns of facial asymmetry. *Alcohol, 44*(7-8), 649-657. PMCID: 2891212.

Bjorkquist, O. A., Fryer, S. L., Reiss, A. L., Mattson, S. N., & Riley, E. P. (2010). Cingulate gyrus morphology in children and adolescents with fetal alcohol spectrum disorders. *Psychiatry Res, 181*(2), 101-107. PMCID: 2815126.

Papers under review citing this grant

Crocker, N., Nguyen, T.T., and Mattson, S.N. (Submitted 2010). Neuropsychological Review of fetal alcohol spectrum disorders. Submitted to *Neuropsychology Review*.

Mattson, S.N. and Riley, E.P. (Submitted 2010). The Quest for a Neurobehavioral Profile of Heavy Prenatal Alcohol Exposure. Submitted to *Alcohol Research & Health.*

O'Brien, J., and Mattson, S.N. (Submitted 2010). Neurobehavioural profiles of individuals with fetal alcohol spectrum disorders. Submitted to *Encyclopedia of Early Childhood Development*.

Vaurio, L., Riley, E.P., and Mattson, S.N. (Submitted 2010). Neuropsychological comparison of children with heavy prenatal alcohol exposure and an IQ-matched comparison group. Submitted to *Journal of the International Neuropsychological Society*. (In final stages of review, minor revisions requested)

Fagerlund, Å., Korkman, M., Autti- Rämö, I, Mattson, S.N., and Hoyme, H.E. (Submitted 2010). Risk and protective factors for mental and behavioral well-being in fetal alcohol spectrum disorders. Submitted to *Journal of Developmental and Behavioral Pediatrics*. (Originally submitted in 2009, revised and resubmitted 2010).

X. Posters and Presentations (2010):

Mattson, S.N., Roesch, S.C., Jones, K.L., Fagerlund, Å, Autti-Rämö, I, May, P.A., Adnams, C.M., Konovalova, V., Riley, E.P. and the CIFASD (2010). Neurobehavioral insights into fetal alcohol spectrum disorders: A multisite, multimethod approach. Presented at the Research Society on Alcoholism meeting, San Antonio, June 2010. Alcoholism: Clinical and Experimental Research, 34, Supplement S2, 245A. DOI: 10.1111/j.1530-0277.2010.01211.x, Published Online: 26 May 2010, http://www3.interscience.wiley.com/journal/122402770/abstract

Dudley, J.D., Wagner, A.E., Crocker, N., Riley, E.P., and Mattson, S.N. (2010). Development of executive functioning in children with heavy prenatal alcohol exposure: A longitudinal study. To be presented at the Research Society on Alcoholism meeting, San Antonio, June 2010. Alcoholism: Clinical and Experimental Research, 34, Supplement S2, 95A. DOI: 10.1111/j.1530-0277.2010.01210.x, Published Online: 26 May 2010, http://www3.interscience.wiley.com/journal/122402770/abstract

Planned RSA abstract submissions for RSA 2011(described in more detail above)

Graham, D.M., Mattson, S.N., et al. Are children with heavy prenatal alcohol exposure characterized by a sluggish cognitive tempo?

Ware, A., Mattson, S.N., et al. The relationship between executive function and behavior problems in children with heavy prenatal alcohol exposure.

Crocker, N., Mattson, S.N., et al. Spatial working memory distinguishes children with heavy prenatal alcohol exposure from children with ADHD.

Appendix 1: Progress Report from Dr. Claire Coles, PI of Atlanta Site

Submitted December 21, 2010

Marcus Subcontract Progress Report as of December 15, 2010

Study Goals remain unchanged. In the current Year, the following activities were carried out in pursuit of these goals.

Progress:

1. At the time of the previous report, it had been noted that Subject Classification was inexact. This issue was resolved by working with San Diego and other collaborators to develop a classification mechanism which has now been refined further and implemented throughout CIFASD.

2. Data Collection-Neuropsychology
    a. This has been an active year in terms of recruitment and testing particularly during the first half of the year. After the new fiscal year began, we had reduced funding and had to cut back on recruiting.
    b. We also collected CANTAB data from several participants (14) which had to be omitted earlier in the year when this system was malfunctioning. This was done during the May 14 and 15 Clinic.
    c. Please see below for the numbers of completed cases in each of the inclusion categories. Note that we have 29 potential participants identified or consented who can provide data .

Total Neuropsychology testing done as of December 21, 2010

Pilots	Unknown	IQ	ADHD	CONTROL	EXPOSED	TOTAL
2	3	14	18	23	29	89

3. Dysmorphology and 3-D Imaging
    a. We carried out Clinics for these protocols on May 14, and 15, and October 8 and 9, 2010, raising the number of completed cases for these outcomes to 99. Dr. For these visits, we held the Clinics on site at the Marcus Center and found that this change was very practical. Dr. Jones from the Dysmorphology Core and Dr. Wetherill from the 3-D imaging Core traveled to Atlanta for these Clinics.
    b. Dr. Wetherill and Dr. Coles are planning a submission to RSA based on adults seen and imaged during the October 2010 Clinic.

4. Data Processing
    a. During this year Dr. Kable identified and resolved a number of transmission problems with data entry forms. She has worked closely with colleagues in Indiana and San Diego on these on-going issues throughout 2010 the team has worked diligently to upload data on an on-going basis.

5. Staffing
    To support the higher rate of Neuropsychological testing, during 2009 and 2010, we trained 2 post doctoral fellows to supplement the activities of our psychometrist. Unfortunately, the psychometrist had a number of health problems related to pregnancy that interfered with her work; eventually she resigned to be placed on bed

rest. We are currently working with our current postdoctoral fellow to achieve reliability and, when that is achieved, will begin on the backlog of neuropsychological testings that remain.

6. Genetics Study
    a. With the cooperation of IU, we completed the IRB process for the Genetics Study and received approval for this protocol to begin in August 2009. Later, due to changes in the data sharing protocol, we submitted a supplement to the IRB approval which was completed September 2010 This modification was approved by the October Dysmorphology Clinic which allowed those participants to be included in the genetics study.

    b. As part of this protocol, we have collected 79 samples from children and their first degree relatives and sent these specimens to Indiana for processing.

Issues:

1. Funding Level. As noted previously, the project is underfunded for the scope of work requested. Although we were able to increase data collection in 2009-2010, due to supplementary support from CIFASD, this supplement was not available in the current grant year which required that we reduce recruitment. Unfunded projects like the Genetics project were continued when they do not require funding; that is, when samples can be collected at the time other data are being collected. However, we do not have staff available to "reconsent" participants who signed a previous version of the consent form (see above). We would happy to work with Indiana to find a way around this difficult. For instance, we will be happy to get in contact with participants if investigators from the genetics project would like to have staff come to Atlanta to make home visits for this purpose.
    a. Project Coordinator position. As there is no funding for this position in the current budget, the Recruiter has assumed a number of these duties with the support of the investigators. This arrangement has resulted in some delays in data collection and uploading and other activities.
    b. Investigator support. As anticipated, we reduced the salary support for Drs. Coles and Kable in order to support staff directly involved in data collection.

2. Data uploading to the Indiana database has been slower than testing. Specific issues:
    a. Checking and preparing data for upload is time intensive and requires professional expertise. As noted above, we do not have adequate professional time allocated. We have been training a post doctoral fellow to support Dr. Kable in this activity since this person is supported from other funds.
    b. We had adopted the strategy of waiting until a file was complete to upload to avoid confusion and overwriting of data. Currently, two specific sources of file incompleteness are (1) Teacher forms, whose format is inconsistent with High School structures in Georgia and (2) CANTAB computer malfunction(s).
    c. Many different uploading problems. Many of these have been resolved with the support of the Sand Diego and UI staff.

Plans

Subject Recruitment/Testing

    a. In the New Year, we will assess using the neuropsychological protocol, a number of individuals who have been "waiting" to complete this part of the protocol. We may also continue to recruit controls and children with other disabling conditions

to add to these contrast groups. We have found the activities of our recruitment specialist, Sharron Paige, to be essential for this aspect of the project.

b. Further Dysmorphology/3-D imaging “Clinics”. If appropriate we are willing to schedule further clinics at the convenience of the other participants.

c. Staffing. Based on our previous report and the current level of funding, we reduced the staff time and the paid involvement of the investigators. More specifically:
    i. The project coordinator position at .25FTE was eliminated.
    ii. Dr. Coles’ salary support was reduced. She will continue to be responsible for administrative activities related to grant management and IRB activities.
    iii. We are training a new Post Doctoral Fellow on the protocol. She has completed the pilot DVD. When she is reliable, she will be able to supplement data collection and checking at no cost until August 2011.

Appendix 2: Progress Report from Dr. Colleen Adnams, PI of South Africa Site

Submitted December 24, 2010

CIFASD II Neurobehavioral Study: Winter report 2010.

South African site (University of Cape Town).

Accomplishments and Results: 2010

In January 2010, the target numbers for this site were revised from 240 to 200. With continued accelerated rates of data collection throughout the year, we have caught up from 62 participants assessed (51.6% of target) at the end of 2009, to 207 participants assessed (103.5% of revised 2010 target). As of December 2010, the total numbers of participants assessed in study designated categories are: FASD = 102; Controls = 59; Low IQ Contrast = 40; Unknown = 6 (still to be categorized).

There are two age bands of participants in the CIFASD Phase 2 neurobehavioral study at the South African site. The first cohort recruited was from the May et. al. Wellington epidemiology study wave III (age range 15 – 16 years) and we more recently recruited participants from the May et. al. Wellington epidemiology study wave IV (age range 8 – 9 years).

Data has been regularly uploaded to the central repository with technical problems being addressed as and when they arose.

Discussion:

At the South African site, staffing and other resources and logistics including University IRB and Western Cape Department of Education approvals are currently in place. With respect to staff, the site team has worked with considerable commitment. Apart from our fieldworker, Loretta Hendricks, all staff employed on the study is part-time. We were fortunate to retain most staff members from the previous year, including Tania Pomario, our project research officer who co-ordinates assessments and data, and three of four post-graduate research assistants, Claire Corbett, Dominique Brand and Linda Santilli, who administer the neurobehavioral battery and questionnaires. Research assistant Karen van Eeden left at the end of 2009 and Malgorzata (Gosia) Lipinska joined the team in February 2010. Gosia underwent test administration and reliability training and commenced assessments in March. The three Cape Town based research assistants commute 75km to Wellington to administer the neurobehavioral battery to participants at our rented research premises in Wellington. Linda Santilli, is based in the Wellington area and because of low levels of literacy in our participants and their families, administers the self and parent questionnaires directly to all participants. The parent questionnaire data collection has proved time consuming and often challenging, requiring time flexibility to facilitate the administration of questionnaires to some of the working parents in the evenings. Linda also co-ordinates completion of the teacher questionnaires. Our fieldworker and two additional student assistants, who were hired to capture data and perform other general tasks, also contributed to our general progress in 2010.

Interrelation with Aims of the Consortium and Other Projects:

Selected participants in the neurobehavioral study were recruited for the Face and Brain Imaging study. Plans are being finalized to obtain 3-D imaging data for 30 of these participants (15 FASD and 15 Controls) between January and June 2011, through collaboration with the PASS study, University of Stellenbosch. The data obtained from these 30 participants will include dysmorphology, brain MRI, neurobehavioral and 3-D imaging.

Plans for the Next Year:

Data collection will continue and site neurobehavioral data will be analyzed, integrating imaging, dysmorphology and other demographic data.

Two abstracts of South African site data will be submitted for poster presentations at the 2010 RSA meeting.

Two of our staff are registered for further degrees at the University of Cape Town and are analyzing aspects of our CIFASD site data and additionally collected data. Tania Pomario is undertaking a PhD dissertation and Claire Corbett is completing an MSc in Research Psychology. Both candidates are co-supervised by Colleen Adnams.

Publications:

Mattson et al (2010) x 2

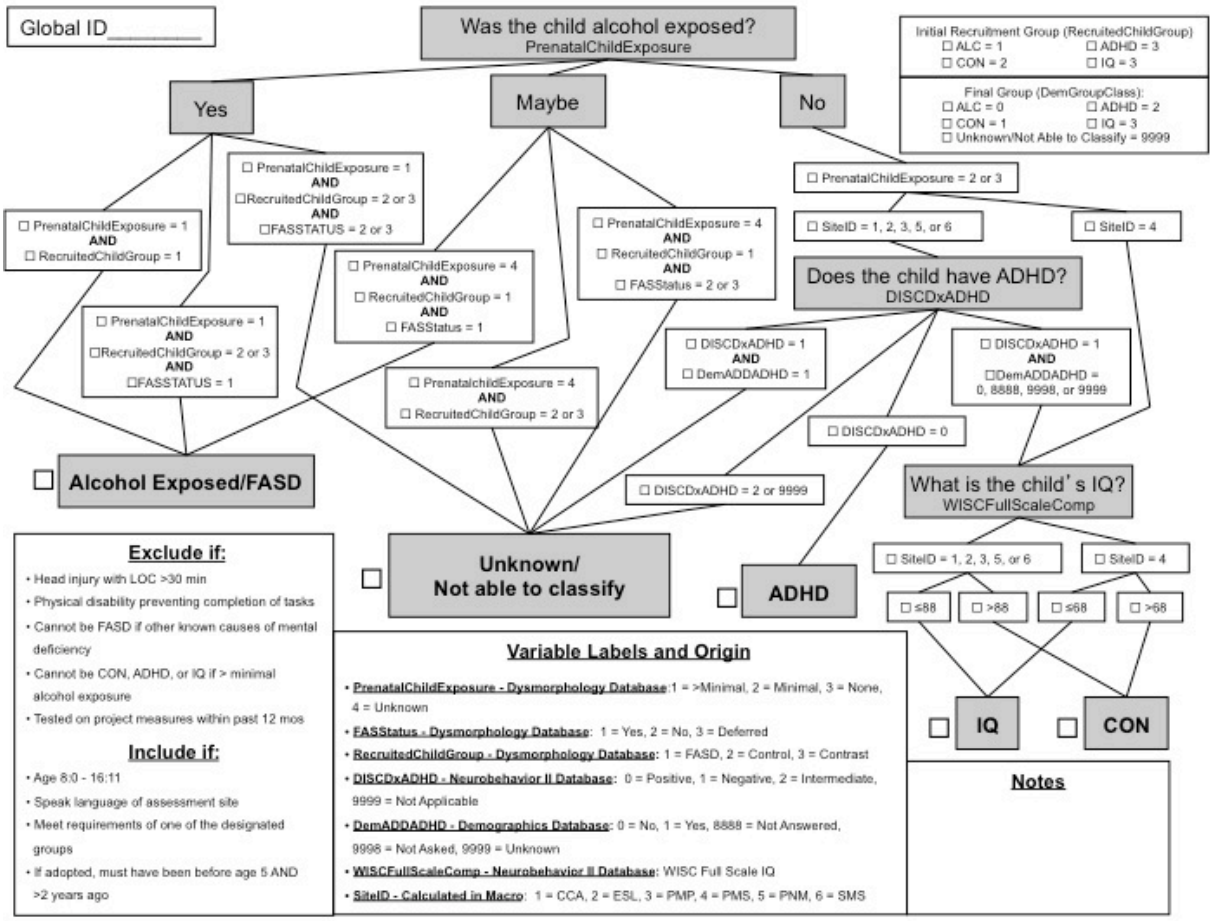
X. Posters and Presentations:

Colleen Adnams

University of Cape Town

23 December 2010.

Appendix 3: Flow Chart Developed for Grouping Subjects



I. Principal Investigator: Elizabeth R. Sowell, Ph.D.

II. Title of Project: Mapping the Brain, the Face and Neurocognitive Function in FASD, U01 AA017122

III. Objectives:

In our proposal, we outlined 4 specific aims:
Specific Aim 1: To evaluate cross-sectionally and longitudinally the effects of prenatal alcohol exposure on brain morphology and function. We will study differences in the patterns of results that occur across populations where drinking patterns may vary by making FASD/control comparisons within sites, and comparing the results across sites.

Specific Aim 2: To evaluate relationships between brain dysmorphology and facial dysmorphology both cross-sectionally and longitudinally to improve diagnostic criteria using facial morphology data from the dysmorphology core (cross-sectional data only) and the 3D camera project.

Specific Aim 3: To determine whether the anatomical "phenotype" relates to neurobehavioral profiles in children with fetal alcohol syndrome or FASDs.

Specific Aim 4: To investigate dysmorphology in the brains of human children based on findings in the mouse and sheep models conducted in the laboratories of Drs. Sulik, Zhou and Cudd.

IV. Methods:

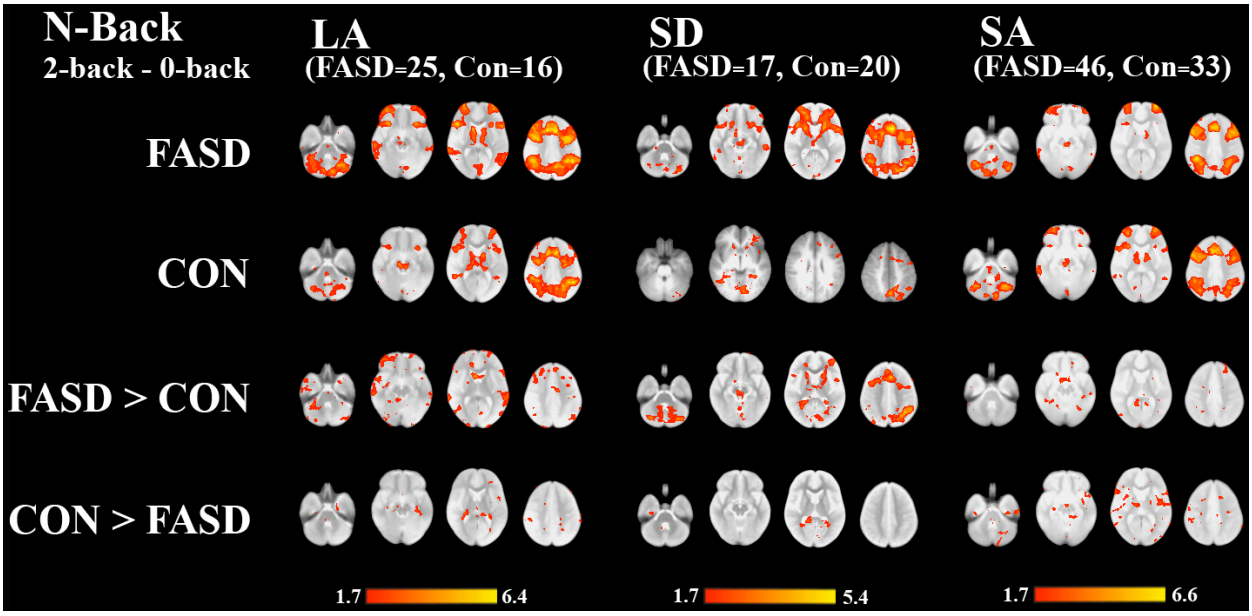
To conduct brain imaging, neuropsychological evaluations, and 3D facial imaging.

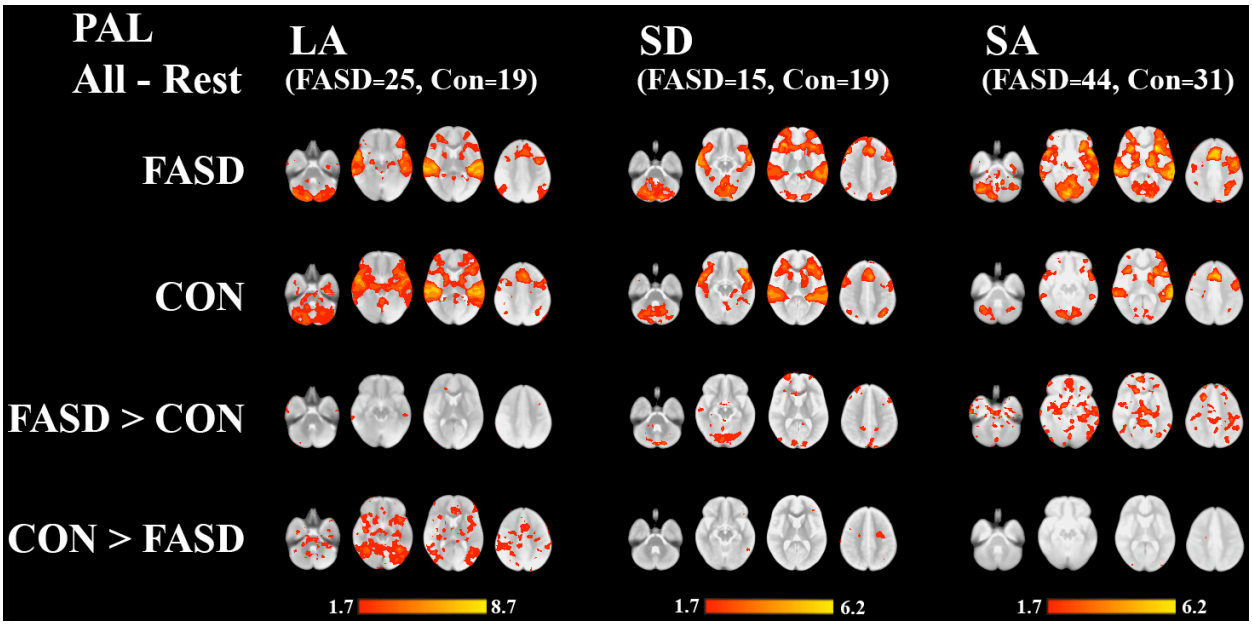
V. Accomplishments and Results:

Brain Image Acquisition:
UCLA: Structural MRI data has been collected for 56 CIFASD subjects (29 exposed and 27 controls). Three to four hi-resolution MPRAGE scans were acquired per subject. Longitudinal brain data has been collected and analyzed on 10 FASD and 16 control subjects. Functional MRI for visuospatial working memory and verbal learning have been collected on all of the same subjects. Time one data collection is complete, and longitudinal imaging will continue in 2011.
SDSU: Data from 46 CIFASD subjects (23 exposed, 21 control, 2 unknown) has been collected. Hi-resolution structural MRI, along with visuospatial working memory and verbal learning fMRI data sets have been collected. Time one data collection is complete, and longitudinal imaging will commense in 2011.
South Africa: Data from 81 CIFASD subjects (47 exposed, 34 control) has been collected. Hi-resolution structural MRI, along with visuospatial working memory and verbal learning fMRI data sets have been collected. Time one data collection is complete, and longitudinal imaging will commence in early 2011.
Emory: IRB approval has been approved to collect brain image data at the Emory site. UCLA has sent the Siemen's Trio protocols, and is preparing to send a mac laptop computer with the fMRI paradigms and software. Our goal with ARRA funding is to study 7 to 10 children with the full fetal alcohol syndrome. Imaging will commence in February 2011.

Neurobehavioral, Facial Imaging and Genetic (saliva) Data Collection:
Subjects began participating in May 2008, and since then 32 exposed, 21 control and 2 ADHD subjects have participated in the study, including completion of the neuropsychological battery and parent interviews/behavioral measures. Dr. Ken Jones has visited UCLA three times to conduct dysmorphology exams on alcohol-exposed and control subjects (January 15-16, 2009, May 19-20, 2009 and April 8-9, 2010) and to date, he has seen 52 children). Leah Flury Weatherhill was also present during these visits and 3-D photographs and saliva samples were taken from the same subjects.

Brain Image Analyses:
fMRI: Figures 1 and 2 show results from fMRI analyses for N-back and paired associates learning respectively.

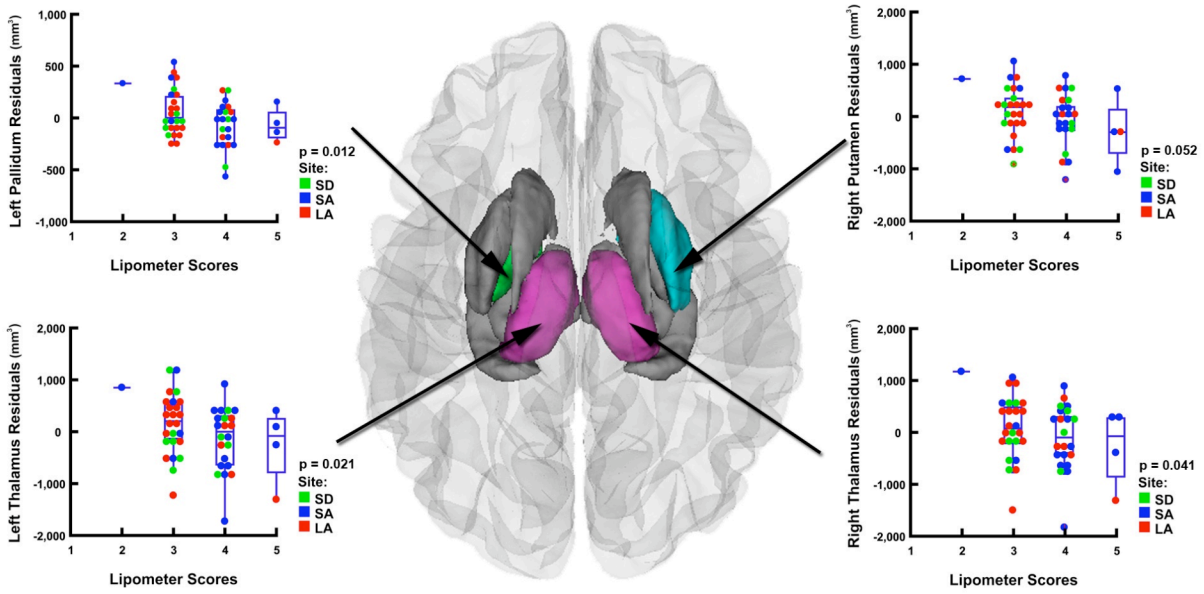


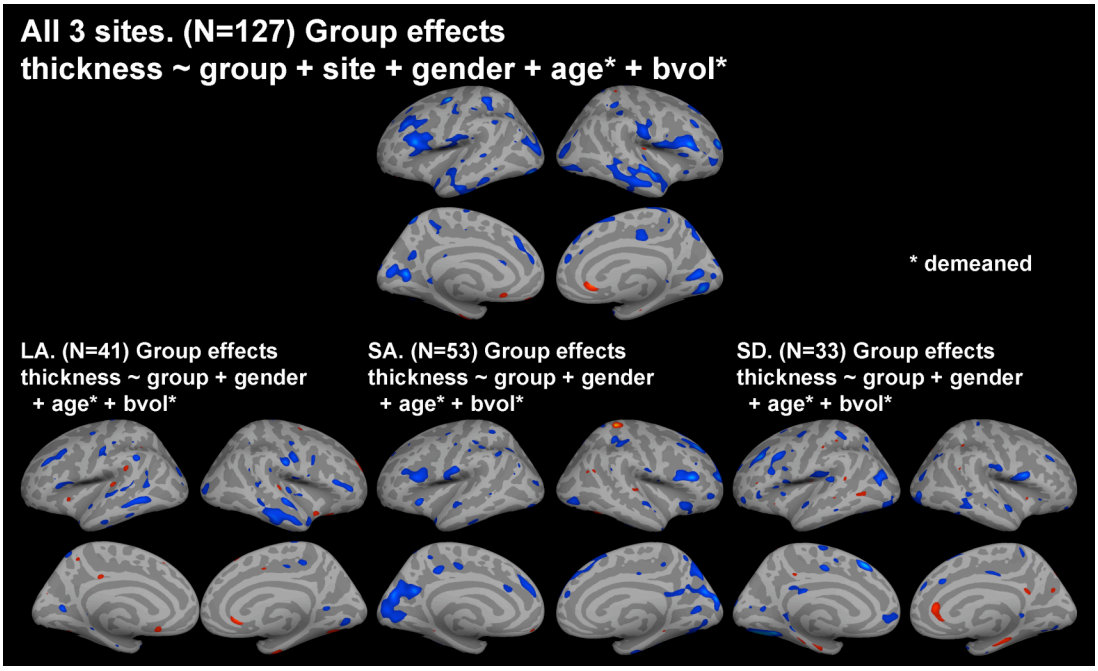


We will be moving forward with combined-site fMRI analysis in the next few months. Results thus far show some consistency across sites in activation patterns for task vs. rest conditions. Dr. Catherine Lebel will be leading these analyses.

Structural MRI:

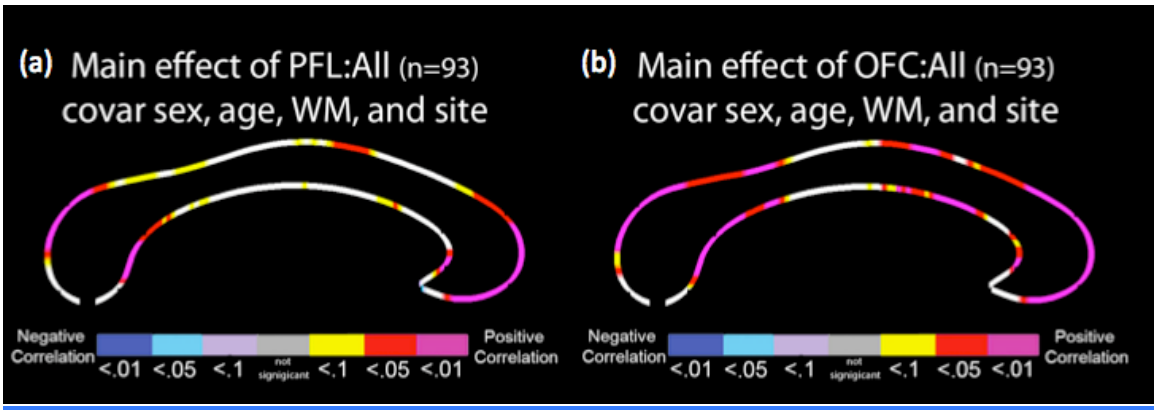
Subcortical: We have made considerable progress in analyzing the multisite structural MRI data. We have one manuscript in press in the journal Human Brain Mapping. In this report, we extend previous findings by illustrating relationships between specific measures of facial dysmorphology and the volumes of particular subcortical structures, and for the first time show that continuous measures of maternal alcohol consumption during the first trimester relates to overall brain volume reduction (see Figure 3).





Cortical thickness: Cortical thickness analyses (see image above) reveal increased cortical thickness in FASD relative to control subjects, as expected based on our earlier work in independent samples. Consistent at all 3 data collection sites were observations of increased cortical thickness in inferior frontal cortex bilaterally, and increased thickness in this region was associated with more severe facial dysmorphology. This work is currently being prepared for submission (Yang et al., In Preparation).

Corpus callosum thickness: We have investigated callosal thickness (see image below) consistent with previous findings, this study reported reduced CC area and additionally showed regional reductions in callosal thickness in adolescents with FASD compared to non-exposed controls. Results further indicated the presence of associations between features of facial dysmorphology and callosal morphology (Yang et al., In Preparation).



VI. Discussion:

The studies and results described above illustrate our continued dedication to apply the most advanced brain image analysis tools to the brain imaging data collected by the CIFASD. We have collected and analyzed brain imaging data for over 120 subjects collected at all 3 data sites. Consistency in imaging findings across sites provides confidence in the validity of results, and for the first time in the literature, we are reporting statistically significant relationships between the brain and facial morphology.

VII. Integration with Aims of the Consortium and Other Projects:

Thus far, our integration with other projects has included evaluating relationships between the dysmorphology core, and the neurobehavioral project. We worked closely with Dr. Kathy Sulik on our In Press publication to investigate how our findings relate to what is known from her animal work.

VIII. Plans for the Next Year:

We will continue or begin data collection for the longitudinal component. Data analyses and manuscript preparation will continue.

IX. Publications (2010):

Roussotte, FF, Sulik, KK, Mattson, SN, Riley, EP, Jones, KL, Adnams, CM, May, PA, O'Connor, MJ, Narr, KL, **Sowell, ER** (2010) Regional brain volume reductions relate to facial dysmorphology and neurocognitive function in fetal alcohol spectrum disorders (In Press,

Human Brain Mapping, 2010).

Sowell E.R., Leow A.D., Bookheimer S.Y., Smith L.M., O'Connor M.J., Kan E., Rosso C., Houston S., Dinov I.D., & Thompson P.M. (2010). Differentiating prenatal methamphetamine from alcohol exposure using tensor based brain morphometry and discriminant analysis. *Journal of Neuroscience*. (30)11:3876-3885.

Lebel C, Roussotte, FF, **Sowell, ER** (2011) Imaging the impact of prenatal alcohol exposure on the structure of the developing human brain: The face as a window into the brain (Submitted, Neuropsychology Review, 2011).

Nuñez, SC, Roussotte, FF, **Sowell, ER** (2009) Structural and functional brain abnormalities in fetal alcohol spectrum disorders, Alcohol Research & Health (In Press, Alcohol Research and Health).

Roussotte F. F., Bramen J.E., Nunez S.C., Quandt L.C., Smith L., O'Connor M.J., Bookheimer S.Y., and **Sowell E.R.** (In Press). Abnormal brain activation during working memory in children with prenatal exposure to drugs of abuse: the effects of methamphetamine, alcohol, and polydrug exposure. NeuroImage.

In Preparation:

Yang Y, Roussotte F, Sulik KK, Kan E, Narr KL, Sowell ER. and CIFASD. In Preparation. Abnormal cortical thickness alterations in fetal alcohol spectrum disorders.

Yang Y, Phillips O, Kan E, Narr KL, Sowell ER and CIFASD. In Preparation. Abnormal callosal morphology in adolescents with prenatal alcohol exposure.

X. Posters and Presentations (2010):

Roussotte, FF, Nunez, SC, Mattson, SN, Riley, EP, Jones, KL, Adnams, C, O'Connor, MJ, May, PA, Narr, K, **Sowell, ER** (2010) Regional Brain Volume Reductions are Related to Measures of Facial Dysmorphology and Neurocognitive Function in FASD: the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD). **Among Top-ranked abstract selected for an electronic poster presentation at the Organization for Human Brain Mapping (OHBM) 2010 Meeting in Barcelona, Spain.**

Yang Y., Philips OR, Kan E, Narr KL. Sulik KK, Mattson SN, Riley EP, Jones KL, Adnams CM, May PA, O'Connor MJ and **Sowell ER.** (submitted). Abnormal Callosal Morphology of Adolescents with Prenatal Alcohol Exposure. Abstract for the 17th Human Brain Mapping Annual Meeting June 26-30, 2011, in Quebec City, Canada