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## TABLE OF CONTENTS

### **Letter to the Editor**

1. Pregnancy in Takayasu Arteritis Patients Exposed to Anti-Tumour Necrosis Factor (Anti-TNF)- $\alpha$  Therapy 96-98  
– Valentina Canti, Elena Baldissera, Susanna Rosa, Giuseppe A. Ramirez, Isadora Vaglio Tessitore, Maria Grazia Sabbadini, Maria Teresa Castiglioni and Patrizia Rovere-Querini

### **Case Report**

2. Ovarian Cystectomy for Huge Mature Cystic Teratoma Developed in Less than Five Years: A Case Report 99-101  
– Evanthia G. Tsalkidou\*, Ilker H. Memet, Giasar M. Chasan, Apostolos-Kyriakos Zolotas, Achmet T. Achmet, Paris Poursanidis and Dimitrios Babalis

### **Research**

3. Use of the Partogram in the Bamenda Health District, North-West Region, Cameroon: A Cross-Sectional Study 102-111  
– Thomas Obinchemti Egbe\*, Evaristus Ngong Ncham, William Takang, Eta-Nkongho Egbe and Gregory Edie Halle-Ekane

### **Research**

4. Potential Co-Factor Role of Tobacco Specific Nitrosamine Exposures in the Pathogenesis of Fetal Alcohol Spectrum Disorder 112-125  
– Valerie Zabala, Elizabeth Silbermann, Edward Re, Tomas Andreani, Ming Tong, Teresa Ramirez, Fusun Gundogan and Suzanne M. de la Monte\*

### **Short Communication**

5. Changing the Algorithm in the Evaluation of Pelvic Anatomy in the Infertile Patient: Is Hysterosalpingo Contrast Sonography With Saline-Air Device the Appropriate Test for Everyone? 126-128  
– Kathryn Coyne, Thomas C. Winter III, Rose Maxwell and Steven R. Lindheim\*

Letter to the Editor

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# Pregnancy in Takayasu Arteritis Patients Exposed to Anti-Tumour Necrosis Factor (Anti-TNF)- $\alpha$ Therapy

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Dear Editor,

In our clinic with 30/83 patients with Takayasu Arteritis (TA) receiving anti-Tumour Necrosis Factor (anti-TNF)- $\alpha$  agents in the years 2013-2014, two women successfully delivered while under therapy with Infliximab (IFX).<sup>1</sup> (Table 1).

	First Patient	Second Patient
Age at delivery (years)	35	27
Duration of TA (years)	5	10
Concomitant diseases	Ulcerative Colitis	\
Arterial involvement	Subclavian artery (left); common carotid artery (right and left); aortic arch; celiac tripod (Type V)	Carotid artery (right and left); anoma artery; subclavian artery (right and left); aortic arch (Type IIa)
Main previous manifestations	Upper and lower limbs claudication; carotidynia; <i>angina abdominis</i>	Low-grade fever; carotidynia; upper and lower limbs claudication
Infliximab	7 mg/kg from 05/2013	7 mg/kg from 02/2009 → 10 mg/kg from 10/2009
Therapy at conception	Mesalazine 1600 mg, prednisone 5 mg and AZA 100 mg daily	Prednisone 5 mg and AZA 100 mg daily
TADS before pregnancy	7/40	6/40
TADS after delivery	7/40	6/40
MRA post pregnancy	Reducing of the thickening of involved arteries	Unmodified (disease stability)
Delivery	elective CS for previous CS (37.5 wg, 2610 g)	elective CS for previous CS (38 wg, 3165 g)

TA: Takayasu Arteritis; TADS: TA Damage Score; CS: Caesarean Section; wg: weeks gestation

Table 1: Patients characteristics.

First patient, a 35 year old woman with a diagnosis of ulcerative colitis since childhood, was diagnosed with TA in 2009. She had an uncomplicated pregnancy in 2004. When she planned a second pregnancy, she was receiving IFX 7 mg/kg every-six-weeks plus chronic therapy (Table 1). At conception she had type-V-disease (generalized involvement of all aortic segments, thoracic and abdominal) based on angiography.<sup>2</sup> Magnetic Resonance Angiography (MRA) revealed thickening of the common carotid arteries, aortic-arch, sovraortic branches and a 30-40% stenosis of the celiac tripod. Positron Emission Tomography (PET) scan revealed increase vascular uptake in the thoracic aorta and the aortic-arch.<sup>3</sup> Azathioprine (AZA) was discontinued in the first 12 weeks gestation (wg) and IFX was discontinued at 28 wg, respectively. Aspirin was administered 100 mg daily until 35 wg. Echocardiography at 12 and 28 wg was normal and carotid ultrasonography at 24 wg was stable. Fetal growth, umbilical and placental flow was repeatedly normal at ultrasonography examination. Arterial blood pressure was consistent and well-controlled. At 37.5 wg she delivered a healthy child. IFX was started four weeks postpartum. MRA two months after delivery revealed significantly reduced thickening of involved arteries. TA Damage Score (TADS), a clinical score of TA damage, remained unchanged.<sup>4</sup>

Second patient, a 27 year old woman and a mother of a 8 year old son, was diagnosed with TA in 2004 and had received corticosteroids, cyclophosphamide followed by Methotrexate (MTX) and AZA with addition of IFX (7 and then 10 mg/kg) in 2009. MRA performed in 2013 was consistent with extensive and active arteritis: thickening of the common, right and left carotid arteries, occlusion of the right carotid artery, bilateral occlusion of the subclavian artery and thickening of the aortic-arch. PET-scan confirmed increased uptake of contrast media in the aortic-arch and brachiocephalic, left subclavian and left axillary arteries. At conception she had type IIa disease (involvement of the ascending portion of aorta and the aortic arch).<sup>2</sup> AZA was discontinued in the first 12 wg and IFX was stopped at 28 wg; aspirin 100 mg daily was administered until 35 wg. Blood pressure remained consistently normal. At 30 wg Erythrocyte Sedimentation Rate (ESR) and C-Reactive Protein (CRP) elevation in association with anemia were observed and the patient complained of carotidodynia. Prednisone was increased (15 mg) and then tapered. Echocardiography at 12 and 28 wg and carotid ultrasonography at 24 wg were consistent with TA stability. Ultrasonography repeatedly showed normal fetal growth, umbilical and placental flow. At 38 wg she delivered a healthy child. TADS score was stable during pregnancy. Histopathological analysis of the placenta revealed no arteritic feature. Breastfeeding was allowed. One month after delivery the patient discontinued the therapy and was lost to clinical follow-up for two months. She developed low-grade fever, carotidodynia, upper and lower limbs claudication in addition to high ESR and CRP. IFX was re-introduced with immediate benefit. MRA three months after delivery was consistent with disease stability.

TA mainly affects women during child-bearing years.<sup>2</sup>

Preventing fetal toxicity due to treatments and disease progression during pregnancy is challenging. Furthermore, maternal complications, including arterial hypertension, pre-eclampsia and cardiovascular involvement can impact on maternal and fetal outcomes.<sup>5</sup> Relatively few TA pregnancies have been described without consensus on the optimal management of disease during pregnancy.

Anti-TNF agents, IFX in particular,<sup>1</sup> appear safe and effective in rheumatic patients during pregnancy. Here, we described two successful pregnancies of TA patients treated with IFX before conception and until 28 wg. Blood pressure remained optimal and the vascular inflammation/remodelling did not worsen.<sup>6</sup> Newborns were healthy. We followed the indication to discontinue IFX during the last trimester<sup>6</sup>: This approach seems to be safe for the mother and minimizes fetal exposition to the drug. The risk of infections is low. In our reported cases infections were not observed in postpartum period till one years follow-up of the babies. In infants exposed to infliximab in utero a normal antibody response to standard vaccinations is ensuring in the early childhood.<sup>7</sup> Live vaccines are recommended after 6 months of age in infants exposed to anti-TNF in utero.<sup>8</sup> To the best of our knowledge, this is the first report describing the use of anti-TNF agents in pregnant TA patients.

#### CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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#### CONSENT

The patients have signed a generic informed consent.

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## Case Report

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# Ovarian Cystectomy for Huge Mature Cystic Teratoma Developed in Less than Five Years: A Case Report

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## BACKGROUND

Cystic ovarian teratomas, also known as dermoid cysts, are the most common benign ovarian germ cell tumors. They typically occur during reproductive age. Due to the readable accessibility to ultrasonography, the finding of large cystic teratomas over 10 cm is an unusual event.

We report a huge mature ovarian cystic teratoma developed in less than five years and was successfully treated by cystectomy.

## CASE REPORT

A 26-year-old woman was admitted due to progressive abdominal distension and discomfort, five years after delivery. Symptoms, such as severe abdominal pain, nevertheless other symptoms, such as vomiting, change in bowel habits, urinary symptoms or change in menstrual pattern were denied. Physical examination on admission demonstrated a distended abdomen more obvious at the right side, soft with no area of tenderness. In laboratory testing haemoglobin was 12.6 g/dL, white cell count was 7.170  $\mu$ /L, and urinalysis was normal. Serum tumor markers, CEA, CA 125, CA 19-9,  $\alpha$ -fetoprotein (AFP) and  $\beta$ -subunit of Human chorionic gonadotropin ( $\beta$ -hCG) levels included, were also normal. Abdominal ultrasonography revealed a large unilocular cystic mass 14 $\times$ 20 $\times$ 18 cm rather than 12.58 $\times$ 21.26 $\times$ 19.38 cm with fat attenuation within the cyst and calcificated areas in the wall, found in abdominal contrast computed tomography (Figures 1 and 2). This cystic mass not existed five years ago on transvaginal ultrasound performed forty days after delivery.



**Figure 1:** Computed tomography.



**Figure 2:** Computed tomography.

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The patient underwent a median incisional exploratory laparotomy. Pelvic and abdominal organs were closely inspected. The cystic mass originated from the left ovary, was predominantly located in the right abdomen and no organ was infiltrated. The right ovary was not affected grossly and the uterus was normal in size. An ovarian cystectomy was performed. Histopathological examination of the excised cyst showed that it was a benign cystic teratoma. Her postoperative recovery was uneventful and she was discharged on the 5<sup>th</sup> postoperative day. Three months after operation there was functioning left ovarian tissue on follow-up transvaginal ultrasound.

## DISCUSSION

Cystic ovarian teratomas, especially mature cases (dermoid cysts), constitute 10-13% of all ovarian tumors and are the most common benign ovarian germ cell tumors.<sup>1</sup> They are composed of well-differentiated tissues derived from three germ cell layers: endoderm, mesoderm and ectoderm. They may contain skin, hair follicle, and sweat gland, bones, nail and teeth. Dermoid cysts may be asymptomatic or present with abdominal swelling, discomfort, and pain<sup>2,3</sup> and typically occur during reproductive age (mean age, 27 years).<sup>4</sup>

Ovarian cysts are labelled as large when they are over 5 cm in diameter and giant voluminous when they exceed 15 cm. According to bibliography mature cystic teratomas grow slowly at a mean average rate of 1.77±3.86 each year for the premenopausal women.<sup>5</sup> A significant point in this case is the fact that although the cyst was large, it had not developed over a long period. No cystic mass existed five years ago, on transvaginal ultrasound performed forty days after the patient's delivery.

Ovarian teratomas can be associated with various complications, including torsion (16%), rupture (1-4%), infection (1%), and autoimmune hemolytic anemia (<1%). Malignancy reported in teratomas ranges between 1-2%.<sup>6-8</sup>

The typical imaging finding of an ovarian teratoma is a cystic mass with intratumoral fat. At ultrasound, the most common finding of an ovarian teratoma is the Rokitansky nodule, i.e. a cystic mass with a densely echogenic tubercle projecting into the cystic lumen. The Rokitansky nodule or dermoid plug is described as a protrusion arising from the inner surface of a cyst, which may contain hair, teeth and fat causing acoustic shadowing.<sup>7</sup> Negative attenuation of this intratumoral fat at Computed Tomography (CT) and the combination of T1-weighted imaging and fat-saturated T1-weighted imaging for fat diagnosis at Mental Retardation (MR), rend these two diagnostic modalities fairly straightforward.<sup>7,10-12</sup>

Advanced surgical techniques have established the use of ovarian cystectomy as a safe method for tumor removal in the adult and pediatric population and as the first line of treatment in lesions that are preoperatively consistent with teratoma, i.e. without malignant characteristics in radiological and laboratory

evaluation.<sup>13,14</sup>

Taking under consideration the patient's age and cyst's characteristics, cystectomy *versus* oophorectomy was preferred. Contrary to appearances, the thinned cortical tissue that remains after cystectomy contains numerous viable follicles which can serve for future hormonal production and oocytes for reproduction. After laparoscopic ovarian biopsy in women 11-34 years old each was found that in 1 mm<sup>2</sup> of surface ovarian tissue contained approximately 35 primordial follicle.<sup>14</sup> Functioning ovarian tissue on follow-up supported our surgical approach.

## CONCLUSION

A mature cystic teratoma could grow at an average rate of up to 4 cm per year. Despite dimension, cystectomy should be the recommended surgical approach in women of reproductive age with negative pre-operative indications of malignancy.

## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

## CONSENT

The patient has provided written permission for publication of the case details.

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## Research

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# Use of the Partogram in the Bamenda Health District, North-West Region, Cameroon: A Cross-Sectional Study

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### ABSTRACT

**Background:** The partogram is an effective instrument in the follow-up of labor. It enables timely diagnoses of abnormalities and helps in decision-making.

**Objectives:** This study was carried out to 1) establish and compare the proportion of labor cases followed up with the partogram in primary and secondary healthcare facilities in the Bamenda Health District and 2) appraise the attitudes of the health workers towards the partogram and how those attitudes impact outcomes.

**Methods:** A cross-sectional study was carried out in which 383 files were reviewed and 42 questionnaires were administered to health workers. The information extracted from the files focused mainly on the number of partograms used and the standard criteria under study were: cervical dilatation, station, the state of the amniotic fluid and maternal temperature monitored every four hours. Maternal blood pressure, pulse rate and Fetal Heart Rate (FHR) were monitored every hour. Each of the parameters was judged as correctly filled if they met the above criteria; as not correctly filled if the criteria were not met; and as not filled if no information was recorded. Statistical analysis was with Epi-Info™.

**Results:** The results showed that 223(58.2%) deliveries were followed up with the partogram, only 4(1.0%) of which had all the parameters filled to standard. Two hundred and six (86.2%) deliveries in the Bamenda Regional Hospital and 17(11.8%) in the primary healthcare facilities were followed up with the partogram. Forty (95.2%) health workers agreed that the partogram was useful in following up labor.

**Conclusions:** The health workers had a positive attitude towards the partogram, but on the whole it was incorrectly used. The instrument was for the most part unavailable, and even where it was, poor supervision and absence of guidelines on its use led to poor diagnoses. We recommend that supportive supervision and regular in-service training be encouraged; and that partograms and guidelines be made available.

**KEYWORDS:** Partogram; Maternal mortality; Labor; Perinatal mortality.

### BACKGROUND

The partogram is a graphic representation of the progress of labor. It enables clinicians (midwives and doctors) to plot cervical dilatation, frequency, intensity and duration of uterine contractions, maternal condition (pulse rate, blood pressure and temperature), fetal condition (fetal heart rate and the state of amniotic fluid), descent of the fetal head and other features that aid the progress of labor.<sup>1,2</sup>

The first person to use a graph to follow up labor was Friedman in 1954.<sup>1</sup> Following studies in Zimbabwe to fully utilize midwives where there was a shortage of doctors, the

alert line<sup>3</sup> and then the action line<sup>4</sup> were added to the original cervicograph developed by Friedman. Since then, studies have shown that the partogram is a useful tool in the management of labor, especially in developing countries.<sup>5</sup>

Following results of a large multicentre randomized trial in South East Asia, WHO recommended the use of the partogram in all labor wards.<sup>5</sup> In the procedure, the action line is drawn 4 hours to the right of the alert line to denote the time of intervention in cases of obstructed labor. Where there are inadequate facilities, early intervention is recommended to allow for referral.<sup>6</sup>

The partogram is widely used in African countries to monitor the progress of labor and the fetal and maternal condition during labor. Drouin et al in 1979 in Yaoundé, Cameroon, showed that the use of the partogram reduced perinatal deaths by 10/1000 births.<sup>7</sup> The use of the alert and action lines offered accurate and reliable guidelines for the detection of abnormal labor.<sup>3,4</sup> Doh et al, 1989 reported a maternal mortality rate of 33 per 100,000 births in the University Teaching Hospital Yaounde, which at the time was considered one of the lowest in Africa. They concluded that, among other factors, the use of the partogram contributed significantly to this low rate.<sup>8</sup>

Appropriate use of the partogram requires skilled health workers, especially midwives,<sup>6,9</sup> who have a positive attitude towards its use. The partogram tools must also be available at all times and at all levels of healthcare facilities, especially given that over 95% of reported stillbirths and neonatal deaths occur in less developed countries.<sup>10,11</sup>

In 2005, it was estimated that in sub-Saharan Africa women have one chance in 22 of dying during childbirth. In the developed world, that chance is 1 in 8000.<sup>12</sup> According to the WHO 2012 statistics for Cameroon, the maternal mortality ratio was 690/100,000 births, a far cry from the 170/100,000 births target set by the Millennium Development Goals.<sup>13</sup>

The use of the partogram has been shown to be important in reducing maternal and neonatal deaths. Dohbit et al in 2010, working in hospitals around Yaoundé, found that the staff caring for women in labor had satisfactory knowledge of the partogram, and more than 83% of them desired additional training in its use.<sup>14</sup>

Since the introduction of the partogram in Bamenda in 2003, no study has been carried out to assess the extent of its use. It was also observed that the use of the partogram was infrequent and sometimes in-appropriate.

The aim of this study was to 1) establish and compare the proportion of labor cases followed up with the partogram in primary and secondary healthcare facilities in the Bamenda Health District and 2) appraise the attitudes of the health workers towards the partogram and how those attitudes impact outcomes.

## MATERIALS AND METHODS

### Study Design and Study Area

This was a health-facility-based observational cross-sectional study in the Bamenda Health District (BHD), North-West Region (NWR), Cameroon during the period 1<sup>st</sup> to 30<sup>th</sup> April 2013.

The BHD is an urban and semi-urban area with one main hospital (Bamenda Regional Hospital (BRH)) that functions as the referral hospital, and many public, lay private and mission health facilities. With its roughly 337,036 inhabitants, it has 17 health areas and covers a total surface area of 560 square kilometers.

The maternity service of the BRH has a capacity of 90 beds: 10 beds in the delivery room and the post-natal ward, and 40 beds in the General Obstetrics and Gynecology Section and a Neonatal Intensive Care Unit (NICU) attached to the labor room under the charge of a pediatrician; and an antenatal care unit and a family planning unit.

The staffing levels are low compared to the staff ratio recommended by WHO. The labor room numbers 12 nurses who work in two shifts, day and night, with an average of 3 nurses per shift. The maternity ward has one general practitioner and two obstetricians, and handles about 3360 deliveries per year. The obstetrician does emergencies and cesarean sections. All the other health facilities are primary care centers.

### Study Population and Sampling

**Study population:** All files filled in all the health facilities of the BHD in the 12 months preceding the study were exploited, as were all the personnel (doctors, nurses and midwives) of the maternity wards of the different health facilities in the Bamenda Health District who accepted to participate in the study. A total of 3 obstetricians, 4 general practitioners, 7 midwives and 28 nurses participated in the study.

Were excluded from the study files of women who came to the hospital already in the second stage of labor and those who came for elective cesarean section or with an obvious indication for cesarean section.

**Sample Size Estimation:** The proportion of files with partograms filled to standard was about 40%, with a 5% chance of error and a 95% confidence interval.<sup>15</sup> A minimum of 369 files was required for study.

The month, April 2013, was selected by simple random sampling from the period May 2012 and June 2013 preceding the study. All the files filled during the month of April in the selected health facilities were analyzed. A total of 383 files were reviewed out of 446.

**Study procedures:** After obtaining administrative and ethical approvals, the doctors, midwives and nurses were contacted in their places of work and made to sign an informed consent form. The questionnaires were then administered to the participants who were given two weeks to fill and deposit in a box left in the office of the head-nurse. We paid regular follow-up visits to the healthcare facilities in order to ensure a high response rate.

Access to the files was obtained by a written authorization from the Director of the Hospital and Heads of the different Health Institutions involved. Information from the files was collected using a pre-designed data collection form.

The relevant information obtained from the files focused on the number of partograms used and labor variables (frequency, duration and intensity of uterine contractions, cervical dilatation, station, fetal heart rate, state of the amniotic fluid, maternal blood pressure, pulse rate and temperature) were recorded. Cervical dilatation, station, state of the amniotic fluid and maternal temperature were monitored every four hours. Maternal blood pressure, pulse rate and FHR were monitored every hour. Each of the parameters was judged as correct if they met the above criteria; not correct if the criteria were not met, and inexistent if no information was recorded.

#### Data Management and Analysis

The questionnaires were crosschecked for completeness and coded to ensure participant's confidentiality. We entered and analyzed the collected data into Epi Info™ version 7.1.1.14. Continuous variables were described using means, medians, standard deviations and interquartile ranges. Absolute and relative frequencies were used to describe categorical variables.

The predictor variable was the number of deliveries carried out in the selected healthcare facilities during the month of April 2013 while the outcome was the number of partograms filled and the number filled to standard. The standards that were adopted in this study included cervical dilatation, station, state of the amniotic fluid and maternal temperature monitored at least once every 4 hours, fetal heart rate, maternal blood pressure and pulse rate monitored hourly. The different sections of the partogram were judged to be standard if the parameters met the above criteria, substandard if the above criteria were not met, and not monitored if they were not filled.

We also studied as predictor variable: level of the health facility while the outcome variables were the number of partograms filled.

Furthermore, we studied as predictor variable the number of health workers who took part in the study, while the outcome variable was the number of health workers who considered the partogram to be a useful tool in the monitoring of women in labor and the number of health workers who desired to have more training in the use of the partogram. Finally, we also studied as predictor variables the availability of partograms

in the health facilities, availability of guidelines on the use of the partogram, in-service training in the use of the partogram, supervision by the service heads, and the attitude of the health workers towards the use of the partogram. The outcome variable, for its part, was the use of the partogram to monitor women in labor. Chi squared, Risk ratios and *p*-values were computed with Epi Info™. *P*-values <0.05 were considered to be statistically significant.

#### Ethical considerations

Before carrying out the study, ethical approval was obtained from the Institutional Review Board of the Faculty of Health Sciences (IRB/FHS), University of Buea, Cameroon. Administrative approval was obtained from the North West Regional Delegation of Public Health and from the Heads of the different Institutions included in the study. Participants signed an informed consent form before taking part in the study and information obtained was confidential.

#### RESULTS

##### The Use of the Partogram

The partogram was used in only 3 of the 7 selected healthcare facilities. Three hundred and eighty-three files were reviewed, representing the number of deliveries in the selected healthcare facilities that met our criteria. One hundred and forty-four (37.6%) were from primary healthcare facilities while 239(62.4%) were from the secondary healthcare facility. Two hundred and twenty-three (58.2%) deliveries were followed up with the partogram. Only 4(1.0%) had all the parameters in the different sections of the partogram recorded to standard.

##### Parameters of the Progress of Labor

**Table 1:** Of the 383 files reviewed, parameters of the progress of labor were recorded in 220(57.4%). Cervical dilatation 155(40.5%), station 144(37.6%) and uterine contractions 138(36.0%) were monitored to standard. Only 113(29.5%) deliveries had parameters of the progress of labor monitored up to standard.

One hundred and sixty-three (42.6%) cervical dilatations, 165(43.1%) stations and 175(45.7%) uterine contractions were not recorded on the partogram.

##### Parameters of fetal monitoring

In 167(43.6%) deliveries, the fetal heart rate was not recorded on the partogram. One hundred and twenty-nine (33.7%) deliveries had fetal heart rate monitored to recommended standard while in 87 (22.7%) deliveries the monitoring of the fetal heart rate was substandard.

In 356 (93.0%) deliveries, the state of the liquor was not recorded on the partogram. Only 16 (4.2%) deliveries had

	Not Monitored N (%)	Substandard Monitoring N (%)	Standard Monitoring N (%)	Total=383
Variables				
Progress of Labor				
• Cervical dilatation	163(42.6%)	65 (17.0%)	155(40.5%)	383(100%)
• Station	165(43.1%)	74 (19.3%)	144(37.6%)	383(100%)
• Uterine contractions	175(45.7%)	70 (18.3%)	138(36.0%)	383(100%)
• Overall monitoring	163(42.6%)	107(27.9%)	113(29.5%)	383(100%)
Fetal Monitoring				
• Fetal heart rate	167(43.6%)	87(22.7%)	129(33.7%)	383(100%)
• State of liquor	356(93.0%)	11(2.9%)	16 (4.2%)	383(100%)
• Overall	167(43.6%)	201(52.5%)	15 (3.9%)	383(100%)
Maternal Monitoring				
• Blood pressure	201(52.5%)	135(35.3%)	47(12.3%)	383(100%)
• Pulse	261(68.2%)	88(23.0%)	34(8.9%)	383(100%)
• Temperature	357(93.2%)	07(1.8%)	19(5.0%)	383(100%)
• Overall	201(52.5%)	174(45.4%)	08(2.1%)	383(100%)

Table 1: Recording of the Parameters of Labor.

that state recorded to recommended standard.

Overall, only 15 (3.9%) deliveries had fetal monitoring recorded to standard.

**Parameters of Maternal Monitoring**

**Table 1:** Maternal blood pressure, pulse and temperature were not recorded in 52.5% (201/383), 68.2%(261/383) and 93.2%(357/383) deliveries respectively. Blood pressure, pulse and temperature were recorded to standard in 12.3%(47/383), 8.9%(34/383) and 5.0%(19/383) deliveries respectively. Furthermore, only 2.1%(8/383) deliveries had all the parameters of maternal monitoring recorded to recommended standard.

**Figure 1:** The progress of labor was monitored according to the recommended standard in 29.5% of the files as compared to 3.9% for the fetal monitoring and 2.1% for the maternal condition.

**Figure 2:** Only about 1% of the files had partograms that were filled to standard. 57.2% were substandard. The partogram was not used at all in 41.8% of the files.

**Comparing The Use of the Partogram in Primary and Secondary Healthcare Facilities**

**Table 2:** Of the 383 files reviewed, 144 were from primary healthcare facilities and 239 from secondary healthcare facilities.

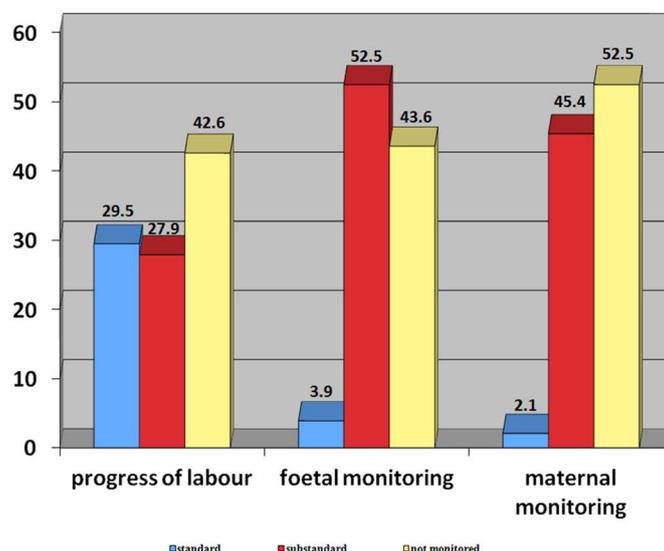


Figure 1: Distribution of the filling of the different sections of the partograph.

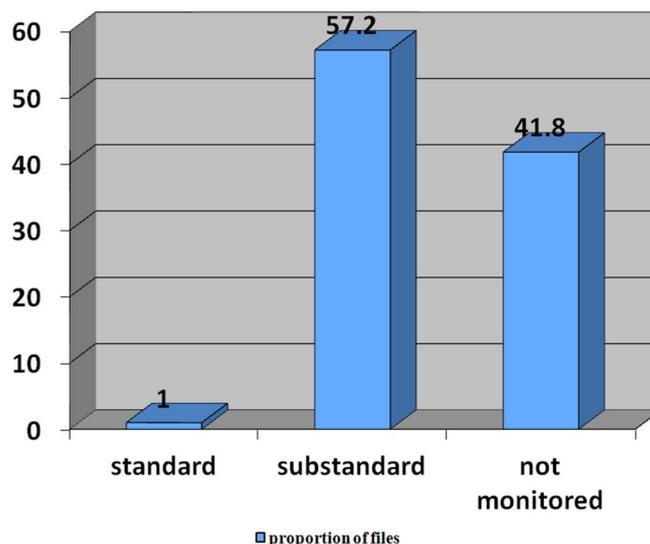


Figure 2: Distribution of evaluation of deliveries followed up with the partograph.

	Primary N (%)	Secondary N (%)	Total N (%)	X <sup>2</sup>	P value
<b>Partogram used</b>					
Facility				204.42	<0.01
Yes	17 (11.8%)	206 (86.2%)	223(58.2%)		
No	127 (88.2%)	33 (13.8%)	160(41.8%)		
Total	144 (100%)	239 (100%)	383 (100%)		
<b>Progress of labor</b>					
	N=144	N=239	N=383	202.73	<0.01
Standard	7(4.9%)	106(44.4%)	113(29.5%)		
Substandard	9(6.3%)	98(41.0%)	107(27.9%)		
Not monitored	128(88.9%)	35(14.6%)	163(42.6%)		
Total	144(100%)	239(100%)	383(100%)		
<b>Fetal monitoring</b>					
				195.0	<0.01
Standard	4(2.8%)	11(4.6%)	15(3.9%)		
Substandard	12(8.3%)	189(79.1%)	201(52.5%)		
Not monitored	128(88.9%)	39(16.3%)	167(43.6%)		
Total	144(100%)	239(100%)	383(100%)		
<b>Maternal monitoring</b>					
				125.60	<0.01
Standard	3(2.1%)	5(2.1%)	8(2.1%)		
Substandard	13(9.0%)	161(67.4%)	174(45.4%)		
Not monitored	128(88.9%)	73(30.5%)	201(52.5%)		
Total	144(100%)	239(100%)	383(100%)		

Table 2: Distribution of partographs filled and comparison of quality of completed partographs in the primary and secondary healthcare facilities.

Only 11,8%(17/144) of women in primary healthcare facilities were followed up with the partogram during labor as opposed to 86.2% (206/239) in secondary healthcare facilities.

There was a significant difference in the proportion of deliveries for which parameters of the progress of labor were monitored to standard in the primary 4.9%(7/144) and secondary 44.4%(106/239) healthcare facilities ( $P<0.01$ ). Only 2.8%(4/144) and 4.6%(11/239) of deliveries in the primary and secondary healthcare facilities respectively had parameters of fetal condition monitored to standard ( $P<0.01$ ). Standard monitoring of parameters of maternal condition was 2.1% for both primary and secondary healthcare facilities ( $P<0.01$ ).

Up to 79.1%(189/239) and 67.4%(161/239) of

deliveries in the secondary healthcare facilities had substandard monitoring of parameters of fetal and maternal conditions as opposed to 8.3%(12/144) and 9.0%(13/144) in the primary healthcare facilities, respectively.

### Characteristics of the Health Workers

**Table 3:** Of the 42 health workers who responded to the questionnaire, 15(35.7%) were from the secondary healthcare facilities while 27(64.3%) were from primary healthcare facilities. Fifteen (35.7%) of them were in the age range 20-30, 15(35.7%) between 30 to 40 years, 8(19.1%) between 40 to 50 years and 4(9.4%) were above 50 years. Majority of the participants, 29(69.1%), were female. Most of the participants, 28(66.7%), were nurses; 7(16.7%) were midwives, 4(9.4%)

	Nurses	Midwives	General practitioners	Obstetricians	Total
BRH	9	3	1	2	15
CMA Nkwen	5	1	1	1	8
God's will maternity	0	1	0	0	1
Mendankwe IHC	5	0	1	0	6
Ntakah IHC	3	0	1	0	4
PHC Ntamulung	4	1	0	0	5
St Blaise clinic	2	1	0	0	3
Total	28	7	4	3	42

Table 3: Qualification of the health workers.

were GPs while 3(7.1%) were obstetricians.

The BRH, the only secondary healthcare facility, had more qualified staff than any of the primary healthcare facilities.

#### Attitudes of the health workers towards the use of the partograms

**Table 4:** 40/42(95.2%) of the health workers attending to women in labor thought that the partogram was necessary in the follow up of women in labor. 2/42(4.8%) of the health workers did not see the partogram as a useful tool in the follow up of women in labor. They thought that the partogram was cumbersome and entailed filling in too many time-consuming details. Thirty-four (81.0%) desired more training in the use of the partogram.

Overall, the health workers had a positive attitude towards the use of the partogram.

#### Factors Affecting the Use of the Partogram

**Figure 3:** Of the 42 health workers who responded to the questionnaire, 30(71.4%) said they knew how to use the partogram; 10(23.8%) said they had difficulties using it, and 2(4.8%) did not know how to use it at all. Of the 40 who could use it though with difficulty, 37(92.5%) said they learnt to use the tool while still in school; the others (7.5%) learnt to use it in seminars and workshops.

The Figure shows that of the 40 health workers who knew how to use the partogram, only 25% of them used it always. 22.5% used it most of the time while 12.5% rarely used it. Up to 45% of these health workers did not use the partogram to follow up any woman in labor.

**Table 4:** Seventeen (56.7%) health workers, all from primary health facilities, blamed the non-utilization of the partogram on its unavailability. Two (6.7%) said the partogram took too much time while 3/30(10%) said they had difficulties using it. Eight (26.7%) gave other reasons for not using the partogram, among which were shortage of staff, especially during night duty shifts when sometimes one person had to attend to more than one woman at a time.

**Table 5:** Only 19/42(45.2%) health workers admitted that they had regular supervision from the service head. Supervision was prompted by the 4.6 fold increase in the relative risk (RR=4.6, 95% CI=2.1-10.0) that the partogram will be used in the follow-up of women in labor. All the 19 health workers who admitted to being supervised by their service heads were using the partogram. Of the remaining 23, only 5 (21.7%) were using the partogram.

Nine (21.4%) health workers said they had had in-service training on the use of the partogram. In-service training was found to be associated with a 2.2 increase in the relative risk (RR=2.2, 95% CI=1.5-3.2) of using the partogram. All those

Level of Health Facility			
Variables	Primary N =27	Secondary N=15	Total
Partogram necessary in monitoring labor?			
Yes			
No	25(92.6%) 2(7.4%)	15(100%) 0.0(0%)	40(95.2%) 2(4.8%)
Do you Desire more Training on partogram?			
Yes	23(85.2%)	11(73.3%)	34(81.0%)
No	4(14.8%)	4(26.7%)	8(19.1%)
Reason why partogram is not used (n=30)			
Partogram not available	17(56.7%)	0(0.0%)	17(56.7%)
It takes too much time	2(6.7%)	0(0.0%)	2(6.7%)
Difficulties using it	3(10.0%)	0(0.0%)	3(10.0%)
Others	3(10.0%)	5(16.7%)	8(26.7%)
Total	25(83.3%)	5(16.7%)	30(100%)

Table 4: Attitude of health workers toward the partogram and reasons why partogram are not used.

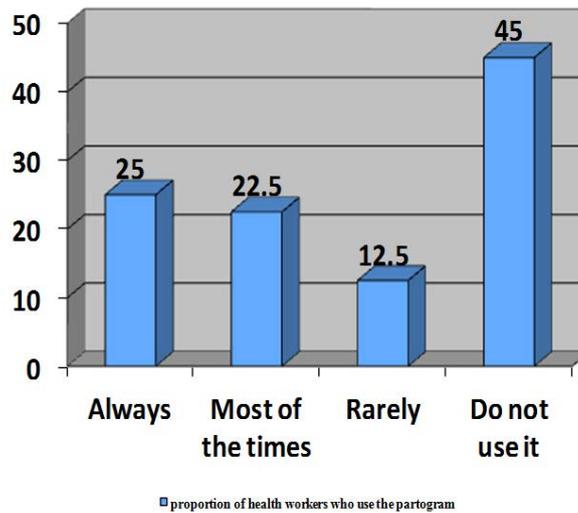


Figure 3: Proportion of Healthcare workers who use the partogram.

	Do you use the partogram?		Total	RR	95% CI
	Yes	No			
Supervision?				4.6	(2.12-10)
Yes	19(100%)	0(0.00%)	19(100%)		
No	5(21.7%)	18(78.3%)	23(100%)		
Total	24(57.1%)	18(42.9%)	42(100%)		
In-service training?				2.2	(1.5- 3.2)
Yes	9(100%)	0(0.00%)	9(0.00%)		
No	15(45.5%)	18(54.5%)	33(100%)		
Total	24(57.1%)	18(42.9%)	42(100%)		

Table 5: Relationship between supervision, in-service training and the use of the partogram.

who had had some form of in-service training were using the partogram. About half (45.5%) of those who had never received any in-service training were using the partogram.

We also noticed that there were no guidelines on the use of the partogram in any of the healthcare facilities.

**DISCUSSION**

This study was carried out to see the proportion of deliveries followed up with the partogram in the primary and secondary healthcare facilities, and how the attitudes of the health workers and other factors impact the use of the partogram.

**The Use of the Partogram**

The partogram was used in 3 out of the 7 healthcare facilities selected for this study. This tool was completely absent in the other 4 healthcare facilities. This was comparable to results by Ogwang et al in Uganda where they found that 4 out of the 8 facilities considered in their study were using the partogram.<sup>16</sup> On the other hand, our results contrasted with those reached by Vikumitsi in Buea in 2012 where only 1 out of 7 health facilities was using the partogram (A. G. Vikumitsi, MD, unpublished

date, 2012).

Many, i.e. 58.2% of the deliveries were followed up using the partogram. The study in Buea found that 70% of the deliveries in the Buea Regional Hospital were followed up with the Partogram, though the utilization rate for the Buea Health District as a whole was not calculated. This was similar to results by Ogwang et al in Uganda in 2008 in which they reported an overall utilization rate of 69%.<sup>16</sup> Azandegbe et al in a survey in Benin reported a high utilization rate of 98%.<sup>17</sup>

There was a high proportion of unrecorded parameters on the partogram. Only 1.0% of the files had all the parameters of labor recorded up to standard. In a study to assess the knowledge and utilization of the partogram in the Niger Delta Region of Nigeria, Opiah et al found that standard filling of the partogram was done in 32.6% and 37.5% of the files in the two healthcare facilities studied.<sup>18</sup> This difference could be explained by the fact that their study was done in tertiary hospitals, unlike ours, which was done in primary and secondary healthcare facilities. A similar study carried out in secondary and tertiary hospitals in Malawi reported standard utilization of the partogram in 3.9% cases.<sup>19</sup> Parameters of the progress of labor were relatively well monitored compared to the other sections of the partogram.

Cervical dilatation, station and uterine contractions were well monitored in 40.5%, 37.6% and 36.0% of the files respectively. Ogwang et al<sup>16</sup> and Yisma et al<sup>20</sup>, working in Uganda and Ethiopia respectively, also found that cervical dilatation was relatively well monitored compared to the other parameters of labor; 42% and 30.7% of partographs, respectively. Another study carried out in Tanzania reported that cervical dilatation was monitored in 97% of the cases.<sup>21</sup> The preferential recording of parameters of the progress of labor may suggest that health workers prioritize documentation of the parameters of the progress of labor over parameters of the maternal and fetal conditions, which is not correct.

The fetal heart rate was monitored based on recommended standard in 33.7% of files as opposed to the state of the amniotic fluid, which was well monitored in only 4.2% of the files. The overall monitoring of the fetal condition was up to standard in only 3.9% of the files. The study in Ethiopia found a similar result with 32.9% of fetal heart rate monitored to recommended standard although they did not assess recording of the state of the liquor.<sup>20</sup> This was contrary to the study in Uganda where only 2% of the partograms had fetal heart rate recorded according to recommended standard.<sup>22</sup> Nyamtema et al in Tanzania found that the FHR was monitored in 94% of the cases but up to 91% of these were substandard.<sup>21</sup> This difference could be due to a difference in the health system policy or simply due to the time gap between this study and the study in Uganda that was carried out from May-June, 2008.

Maternal blood pressure, pulse rate and temperature were monitored to standard in 12.3%, 8.9% and 5.0% respectively. This was similar to the results from the study in Uganda where maternal blood pressure was recorded to standard in 10% of the partograms. The study in Ethiopia found that maternal BP was well monitored in 18.6% of the files.<sup>20</sup>

Two hundred and nineteen (57.2%) deliveries had an overall substandard monitoring of the parameters of labor and 41.8% of the deliveries were not followed up with a partogram at all. As shown by Drouin et al in 1979, the use of the partogram reduces perinatal deaths and allows early detection of abnormal labor.<sup>7</sup> Several other studies have shown the effectiveness of the partogram in reducing unnecessary interventions during labor and in improving maternal and neonatal outcomes (A. G. Vikumitsi, MD, unpublished date, 2012).<sup>9,16,21,23</sup> Doh et al, working in the University Teaching Hospital in Yaoundé, found a maternal mortality rate of 33 per 100,000 births over a period of five years, which was considered one of the lowest in Africa, and concluded that the use of the partogram contributed greatly to this low rate.<sup>8</sup> For the partogram to be used as a tool for the timely diagnoses of abnormal labor, all the parameters should be recorded and to the recommended standard. Substandard recording of the parameters of labor means that there is a discontinuity in the follow-up of patients in labor and many abnormalities can be missed or not diagnosed on time. Accurate and timely diagnoses will lead to accurate timely intervention

and to better outcomes.

This study also found that there was better documentation on the parameters of labor in the secondary than in the primary healthcare facilities; 86.2% and 11.8% respectively. This was however expected because secondary healthcare facilities have more resources and better supervision than primary healthcare facilities. Furthermore, in secondary healthcare facilities, there is a good mix of skills where nurses and midwives work with doctors and obstetricians who can promote knowledge transfer and so improve the performance of the staff. Similar results were found by Fawole et al in South West Nigeria in 2009.<sup>24</sup>

#### Attitude of the Health Workers Towards the Partogram

The participants in this study included 28(66.7%) nurses, 7(16.7%) midwives, 4(9.4%) general practitioners and 3(7.1%) obstetricians. This was similar to ratios in the study carried out in Buea in 2012 where 64.1% were nurses and 5.1% general practitioners (A. G. Vikumitsi, MD, unpublished date, 2012). However, the study in Buea had more midwives (28.2%) and fewer obstetricians (2.6%). Dohbit et al in Yaoundé had similar proportion of midwives and nurses (42.4% and 45.5% respectively) and more obstetricians (7.6%) than general practitioners (4.5%).<sup>14</sup>

Forty (95.2%) health workers admitted that the partogram was necessary for the proper monitoring of women in labor. Vikumitsi in the Buea Health District in 2012 and Dohbit et al in Yaoundé in 2010 found similar results where 92% and 94.8% of the health workers respectively said the partogram was useful in following up labor.<sup>14</sup> This is in line with the findings that the partogram has been shown to be an effective tool for monitoring labor and identifying women in need of an intervention.<sup>5</sup> Thirty-four (81.0%) of workers expressed the desire to have more training on how to use the partogram. A similar result was obtained by Dohbit et al in Yaoundé in 2010 where 96.55% of nurses and 62.5% of doctors desired to have more training on the use of the partogram.<sup>14</sup> The study in Buea reported that 92% of the health workers were willing to have more training on the use of the partogram (A. G. Vikumitsi, MD, unpublished date, 2012). This can be taken to mean that the health workers admit to the fact that their knowledge of the partogram may not be enough to enable them to use it appropriately. This likelihood is reflected in the number and use of the partograms in the monitoring of labour: most of the parameters are either not monitored to standard or not even monitored at all.

#### Factors Affecting the Use of the Partogram

In this study, it was realized that partograms were available in only 42.9% of the health facilities. Most (56.7%) of the participants said unavailability of the partogram meant that they could not use it to monitor women in labour. This result was higher than the results reached in the study in Buea where only 17.9% of the health workers gave unavailability of the

partogram as a reason for not using it (A. G. Vikumitsi, MD, unpublished date, 2012). Dohbit et al found that 25% of doctors and 39.7% of nurses were not using the partogram because of its unavailability.<sup>14</sup> Similar results were found by Opiyah et al in Nigeria where more than 50% of the health workers acknowledged the unavailability of the partogram in their labour room.<sup>18</sup> The study in Uganda also found unavailability of the partogram as one of the factors affecting its use.<sup>16</sup>

Inadequate training of the staff was found to be another reason why the partogram was not used. Although 92.5% of the participants said they had formal training in the use of the partogram, 81.0% of them expressed the wish to have more training. Similar results were found by Dohbit et al<sup>14</sup> Although the health workers had good theoretical knowledge, their practical knowledge was inadequate as most of the parameters were not recorded to standard. Most of them had never had any form of in-service training since they started working.

There was a significant relationship between supervision by the service heads and the use of the partogram (RR=4.6; 95% CI=2.1-10.0). About 45.2% of the health workers admitted to having regular supervision from their service heads. Bosse et al in Tanzania and Ogwang et al in Uganda also reported that health workers lacked follow-up and supervision and this impacted on the number and quality of the partograms used.<sup>16,22</sup> In this study, the BRH had the highest number of deliveries followed up with the partogram (86.2%), and more supervision and qualified staff than any of the other healthcare facilities.

Just as in results found by the studies in Uganda and Tanzania,<sup>16,22</sup> none of the health facilities had guidelines on the use of the partogram.

#### Limitations of the Study

The study did not analyze biochemical data such as urinalysis and medications and fluids administered during labor but involved review of records already filled and as such may not give a real picture of what is practiced since filling the partogram does not necessarily mean actually using it to monitor the progress of labor. Furthermore, there might also be recall bias in collecting the information and the cross-sectional nature of the study done in one part of Cameroon may not reflect what happens in other regions of the country.

#### CONCLUSIONS

Our results showed that 58.2% of deliveries were followed up with the partogram. Most (57.2%) of the deliveries had substandard recording of the parameters of labor and only 1.0% was monitored to the recommended standards. More deliveries were monitored with the partogram in the secondary healthcare facility (86.2%) than in the primary healthcare facilities (11.8%). Majority of the health workers (95.2%) had a positive attitude towards the use of the partogram. The factors

affecting the use of the partogram in the monitoring of labor include: Unavailability of the partograms, inadequate training of the health workers in the use of the partogram, lack of supervision and lack of guidelines on the use of the partogram.

#### RECOMMENDATIONS

Health facilities should have a constant supply of partograms together with guidelines on how to use them. Equally, supportive supervision by the service heads and the head nurses of the labor room should be a standard procedure in maternities. There is need for constant in-service training and seminars on the use of the partogram to improve the practical skills of the health workers. Finally, there is need to strengthen the health system policy to make the use of the partogram compulsory in all health facilities offering delivery services.

#### COMPETING INTERESTS

We declare that we have no competing interests.

#### AUTHORS CONTRIBUTION

TOE contributed in the project conception, analysis and interpretation of data, and writing of the manuscript. He is the corresponding author. ENN and ENE contributed in data collection, analysis and interpretation, and proofread the manuscript. WT and GEHE proofread the manuscript. All authors proofread and agreed on the final version of the manuscript.

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# Potential Co-Factor Role of Tobacco Specific Nitrosamine Exposures in the Pathogenesis of Fetal Alcohol Spectrum Disorder

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### ABSTRACT

**Background:** Cerebellar developmental abnormalities in Fetal Alcohol Spectrum Disorder (FASD) are linked to impairments in insulin signaling. However, co-morbid alcohol and tobacco abuses during pregnancy are common. Since smoking leads to tobacco specific Nitrosamine (NNK) exposures which have been shown to cause brain insulin resistance, we hypothesized that neurodevelopmental abnormalities in FASD could be mediated by ethanol and/or NNK.

**Methods:** Long Evans rat pups were intraperitoneal (IP) administered ethanol (2 g/kg) on postnatal days (P) 2, 4, 6 and/or NNK (2 mg/kg) on P3, P5, and P7 to simulate third trimester human exposures. The Cerebellar function, histology, insulin and Insulin-like Growth Factor (IGF) signaling, and neuroglial protein expression were assessed.

**Results:** Ethanol, NNK and ethanol+NNK groups had significant impairments in motor function (rotarod tests), abnormalities in cerebellar structure (Purkinje cell loss, simplification and irregularity of folia, and altered white matter), signaling through the insulin and IGF-1 receptors, IRS-1, Akt and GSK-3 $\beta$ , and reduced expression of several important neuroglial proteins. Despite similar functional effects, the mechanisms and severity of NNK and ethanol+NNK induced alterations in cerebellar protein expression differed from those of ethanol.

**Conclusions:** Ethanol and NNK exert independent but overlapping adverse effects on cerebellar development, function, insulin signaling through cell survival, plasticity, metabolic pathways, and neuroglial protein expression. The results support the hypothesis that tobacco smoke exposure can serve as a co-factor mediating long-term effects on brain structure and function in FASD.

**KEYWORDS:** Insulin resistance; Fetal alcohol spectrum disorder; Tobacco; Cerebellum; NNK; Nitrosamine.

### INTRODUCTION

Alcohol abuse during pregnancy causes Fetal Alcohol Spectrum Disorder (FASD)

which leads to long-term neurodevelopmental deficits.<sup>1,2</sup> Ethanol mediates its adverse effects on the immature brain by inhibiting insulin and Insulin-like Growth Factor (IGF) signaling at multiple points within the cascade, beginning with ligand-receptor binding.<sup>3</sup> Consequently, ethanol inhibits: 1) insulin and IGF-1 Receptor Tyrosine Kinases (RTKs); 2) Insulin Receptor Substrate (IRS) signaling<sup>4-6</sup>; 3) Phosphatidylinositol-3-Kinase (PI3K) and Akt activation; 4) Akt suppression of Glycogen Synthase Kinase-3 $\beta$  (GSK-3 $\beta$ )<sup>3,5-8</sup>; and 5) inactivation of phosphatases that negatively regulate RTKs, i.e. PTP-1b or PI3K, i.e. PTEN.<sup>6,8,9</sup> These effects compromise growth, survival, metabolism, neuronal migration, and plasticity during development.<sup>10-15</sup> In addition, acetaldehyde build-up from incomplete alcohol metabolism causes neurotoxic injury due to oxidative stress, DNA damage, mitochondrial dysfunction, and probably adduct formation in brain.<sup>16-21</sup> Therefore, ethanol inhibition of insulin/IGF signaling combined with acetaldehyde-mediated oxidative injury could be responsible for many of the structural and functional Central Nervous System (CNS) abnormalities in FASD, including motor impairments, cerebellar hypoplasia, and neuronal migration disorders.

Although ethanol exposure is sufficient to cause FASD, in reality, heavy alcohol abusers are often smokers<sup>22</sup> and both heavy drinking and cigarette smoking adversely affect neurocognitive function.<sup>23</sup> Review of an outpatient substance abuse treatment center database for pregnant women revealed that from 2010 to 2013, 74% of the pregnant alcohol users (N=57) smoked cigarettes compared with 42% of controls (N=31) (P=0.0053).<sup>24</sup> Despite strong evidence linking cigarette smoking during pregnancy to impaired fetal growth and development, and neurocognitive function,<sup>25</sup> the mechanisms are not well understood. Given the frequency of overlapping exposures and their known independent adverse effects on development, further research is needed to determine the degree to which alcohol and tobacco exposures produce additive or synergistic adverse effects on the immature brain.

Nicotine is the main stimulant and addictive substance in tobacco. Nicotine's half-life is short (1-2 hours), but its chief stable metabolites, cotinine, can cause significant harm. Moreover, tobacco-specific nitrosamines, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosomnicotine (NNN), nitrosaminoaldehyde (NNAL), N-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB), iso-NNAL, iso-N-nitrosamino acids (iso-NNAC)<sup>26,27</sup> are problematic because they are present in tobacco smoke and cause DNA damage and form adducts with proteins, lipids, and nucleic acids.<sup>28-30</sup> In previous studies, we found that low-dose exposures to N-nitrosodiethylamine, a nitrosamine found in processed and preserved foods, cause sustained deficits in neurobehavioral function with impairments in brain insulin/IGF signaling, increased oxidative stress, and reduced expression of neuroglial genes.<sup>31</sup> Subsequently, we demonstrated similar independent or additive adverse effects of ethanol and low-dose NNK exposures in adolescent rats,<sup>32,33</sup> and that low-dose NDEA exposures exacerbate the injurious effects of ethanol on the developing

brain.<sup>34</sup> These findings suggest that further studies concerning additive and interactive effects of ethanol and nitrosamine exposures during development are needed to understand the nature and mechanisms of their long-term effects.

The prevalent co-morbid exposures to alcohol and tobacco during pregnancy prompted us to evaluate neurotoxic and teratogenic effects of tobacco-specific nitrosamine exposures in relation to the effects of alcohol. This study utilized an *in vivo* model of ethanol and/or NNK exposures during the early postnatal period to examine effects on cerebellar function, structure, insulin and IGF-1 signaling through survival, metabolic, and plasticity pathways, and neuroglial protein expression. The findings demonstrate that early postnatal exposures to ethanol, NNK, or ethanol+NNK produce molecular, biochemical, and histopathological effects that correspond with abnormalities in FASD. Therefore, the pathogenesis of sustained neurodevelopmental abnormalities are typically linked to prenatal alcohol exposures may be mediated in part by co-exposures to tobacco smoke.

## METHODS AND MATERIALS

### *In vivo* Model

The models generated were intended to simulate 3<sup>rd</sup> trimester pregnancy human exposures to alcohol and/or tobacco toxins. Long Evans rat pups were divided into 4 groups and administered 50  $\mu$ l i.p. injections of: saline vehicle as control; pharmaceutical grade ethanol (2 g/kg in saline); NNK (2 mg/kg in saline); and ethanol+NNK. Ethanol treatments (binge) were administered on postnatal days (P) 2, 4, 6, and 8,<sup>35-37</sup> and NNK was administered on P3, P5, P7, and P9. All rats survived the treatments and growth trajectories were similar across the groups. All experiments were performed in accordance with protocols approved by Institutional Animal Care and Use Committee at the Lifespan-Rhode Island Hospital, and they conformed to guidelines established by the National Institutes of Health.

### Rotarod Testing

We used rotarod tests to assess cerebellar motor function.<sup>38</sup> On P16, rats (N=6-8 per group) were administered 10 rotarod trials at incremental speeds up to 10 rpm using a Rotamex-5 (Columbus Instruments, Columbus, OH, USA), with 10 minutes rest between trials. The latencies to fall were automatically recorded with photocells placed over the rod; however, trials were stopped after 30 seconds to avoid exercise fatigue. Data from trials 1-3 (2-5 rpm), 4-7 (5-7 rpm), and 8-10 (8-10 rpm) were culled and analyzed using the Mann-Whitney test.

### Cerebellar Protein Extraction

On P30, the rats were sacrificed to harvest cerebella for histological and molecular studies. Fresh cerebella were divided in the mid-sagittal plane. One hemisphere was fixed in 10% neutral buffered formalin and embedded in paraffin. His-

tological sections (5  $\mu\text{m}$ -thick) were stained with Luxol fast blue-Hematoxylin and Eosin (LHE). The other hemisphere was snap frozen and stored at  $-80\text{ }^{\circ}\text{C}$ . For multiplex and duplex Enzyme-Linked Immunosorbant Assays (ELISAs), fresh frozen cerebellar tissue was homogenized in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 50 mM NaF, 0.1% Triton X-100, and protease and phosphatase inhibitors. Supernatants obtained by centrifuging the samples at  $14,000 \times g$  for 15 minutes at  $4\text{ }^{\circ}\text{C}$  were used in ELISAs. Protein concentrations were measured using the bicinchoninic assay.

### Multiplex ELISAs

Bead-based Total and Phospho Akt Panels were used to examine effects of ethanol, NNK and ethanol+NNK exposures on the expression and phosphorylation of proteins integrally involved in Insulin (IN) and IGF-1 signaling through Akt and Glycogen Synthase Kinase-3 $\beta$  (GSK-3 $\beta$ ). The Total Akt multiplex panel measured immunoreactivity to the insulin and IGF-1 receptors, Insulin Receptor Substrate, type 1 (IRS-1), Akt, and GSK-3 $\beta$ . The Phospho-Akt panel measured immunoreactivity to: pYpY1162/1163-IN-R, pYpY1135/1136-IGF-1R, pS312-IRS-1, pS473-Akt, pT246- and pS9-GSK3 $\beta$ . Protein samples (100  $\mu\text{g}$  each) were incubated with antibody-coated beads to capture specific antigens following the manufacturer's protocol. Biotinylated second epitope antibodies and phycoerythrin-conjugated Streptavidin were reacted with antigen-bound beads, and immunoreactivity was detected and quantified in a MAGPIX (Bio-Rad, Hercules, CA, USA). Data are expressed as Fluorescence Light Units (FLU) corrected for protein concentration.<sup>30,39</sup>

### Duplex ELISAs

Duplex direct-binding ELISAs were used to measure cerebellar protein expression of Hu (neuronal), Myelin-associated glycoprotein-1 (MAG-1; oligodendroglia), Glial fibrillary acidic protein (GFAP; astrocytes), Choline acetyl transferase (ChAT), Acetylcholinesterase (AChE), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tau, phospho-tau, ubiquitin, 4-hydroxy-2-nonenal (HNE), and Aspartyl-asparaginyl- $\beta$ -hydroxylase (ASPH). Quadruplicate 50  $\mu\text{l}$  aliquots containing 2  $\mu\text{g}/\text{ml}$  of protein were adsorbed to the bottoms of 96-well MaxiSorp plates by overnight incubation at  $4\text{ }^{\circ}\text{C}$ . Non-specific sites were adsorbed with Superblock-TBS. Proteins were reacted with primary antibody (0.1-0.4  $\mu\text{g}/\text{ml}$ ) overnight at  $4\text{ }^{\circ}\text{C}$ . Immunoreactivity was detected with HRP-conjugated secondary antibody and Amplex UltraRed soluble fluorophore. Protein homogenates were subsequently incubated with biotin-conjugated antibodies to large ribosomal protein (RPLPO), and immunoreactivity was detected with streptavidin-conjugated alkaline phosphatase (1:1000) and 4-Methylumbelliferyl phosphate (4-MUP) fluorophore. Fluorescence intensity was measured in a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at Ex565nm/Em595nm for Amplex UltraRed, and Ex360nm/Em450nm for 4-MUP. Binding specificity was assessed with control incubations in which the primary or secondary antibody was omitted. The calculated ratios of immu-

noreactivity corresponding to the specific proteins/RPLPO were used for inter-group comparisons.<sup>39</sup>

### STATISTICS

All assays were performed with 6 or 8 samples per group. Inter-group comparisons were made using two-way ANOVA and the Tukey multiple comparisons post-hoc tests (GraphPad Prism 6, San Diego, CA, USA).

### MATERIALS

Pharmaceutical grade ethanol was used in the *in vivo* experiments. The A85G6 and A85E6 monoclonal antibodies to ASPH were generated to human recombinant protein<sup>40</sup> and purified over Protein G columns (Healthcare, Piscataway, NJ, USA). Otherwise, antibodies used for duplex ELISAs were purchased from Abcam (Cambridge, MA, USA). RPLPO antibody was from the Proteintech Group Inc (Chicago, IL, USA). ELISA MaxiSorp 96-well plates were purchased from Nunc (Rochester, NY, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody, Amplex Red soluble fluorophore, and the Akt Pathway Total and Phospho panels were purchased from Invitrogen (Carlsbad, CA, USA). HRP-labeled polymer conjugated secondary antibody was purchased from Dako Corp (Carpinteria, CA, USA). The SpectraMax M5 microplate reader was purchased from Molecular Devices Corp. (Sunnyvale, CA, USA). BCA reagents were from Pierce Chemical Corp. (Rockford, IL, USA). All other fine chemicals, including NNK were purchased from CalBiochem (Carlsbad, CA, USA), Pierce (Rockford, IL, USA), or Sigma (St. Louis, MO, USA).

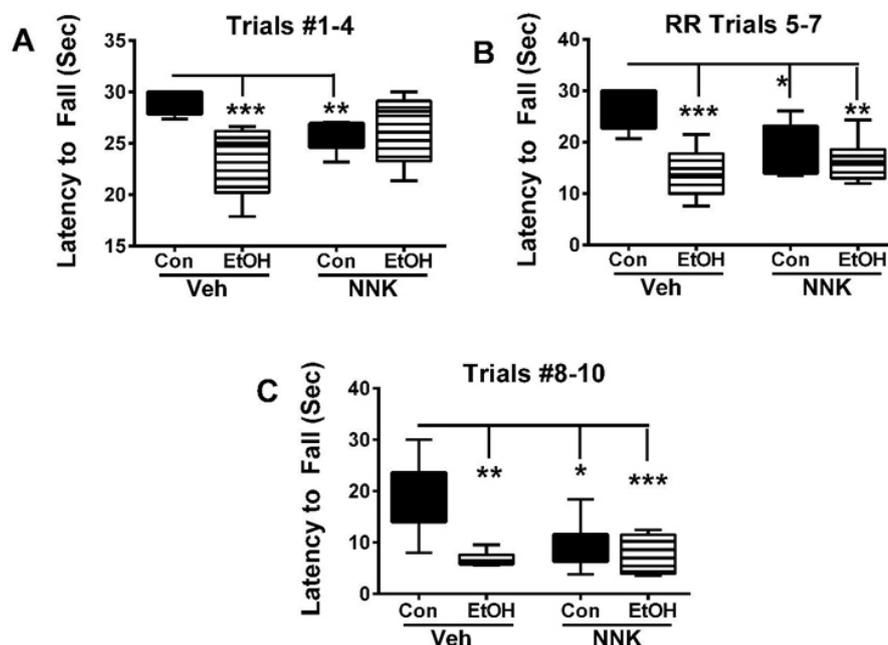
### RESULTS

#### Effects of Ethanol and NNK on Motor Function

Growth curves were virtually identical for all groups (Supplementary Figure 1). Rotarod latency to fall data were culled for Trials 1-4, 5-7, and 8-10 and analyzed using the Kruskal-Wallis ANOVA and Dunn's multiple comparison test. Significant inter-group differences were detected for Trials 1-3 ( $p=0.0003$ ), 5-7 ( $p<0.0001$ ), and 8-10 ( $p<0.0001$ ). In Trials 1-4 (least challenging), significant differences from control occurred in the ethanol ( $p<0.001$ ) and NNK ( $p<0.01$ ), but not in the ethanol+NNK group (Figure 1A), but in subsequent more demanding trials, the mean latencies to fall were significantly reduced in all three experimental groups relative to control. Furthermore, the effect sizes increased with trial difficulty (Figures 1B,C). Although ethanol and NNK independently impaired motor performance to similar degrees, dual exposure effects were not additive or synergistic.

#### Histopathology

LHE stained histological sections of the cerebellar vermis (mid-sagittal section, anterior superior region-lobules IV-VIII) revealed the characteristic 3-layered cortical architecture



**Figure 1:** Effects of early postnatal exposures to ethanol, NNK, or ethanol+NNK on motor performance as early adolescence. Long Evans rat pups were treated with i.p. saline (control-vehicle), ethanol (2 g/kg), NNK (2 mg/kg), or ethanol+NNK from postnatal day 2 (P2) through P12. Ethanol was administered on Mondays, Wednesdays, and Fridays, and NNK was administered on Tuesdays, Thursdays, and Saturdays. On postnatal day 16, the rats were subjected to 10 trials of rotarod testing with incremental speeds. Data from Trials (A) 1-4, (B) 5-7, and (C) 8-10 were culled and analyzed using the Mann-Whitney test. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .

with distinct white matter cores in all groups (Figure 2). Control cerebella had relatively uniform thicknesses of the molecular and granule cell layers, a well-populated Purkinje cell layer, and delicately arborized, well-myelinated white matter cores (Figures 2A,E,I). Ethanol exposure altered the patterns of foliation, causing mainly shallow grooving, irregular thickness of the molecular layer, loss of Purkinje cells, and prominent broadening with reduced myelin staining and increased vacuolation of white matter cores (Figures 2B,F,J). NNK treatment resulted in thin simplified (straighter) foliation with sulcal widening, prominent irregularity in granule cell layer thickness within the depths of sulci, atrophy and loss of Purkinje cells, and moderate broadening and tapering of white matter cores compared with the effects of ethanol (Figures 2C,G,K). Ethanol+NNK exposures had similar effects on the cortex and white matter as observed with NNK (Figures 2D,H,L).

### Insulin/IGF-1/IRS-1 Signaling

The multiplex ELISA data corresponding to effects of ethanol, NNK, and ethanol+NNK on signaling proteins, phospho-proteins and relative degrees of phosphorylation within the insulin/IGF-1 pathways were analyzed by two-way ANOVA (Table 1).

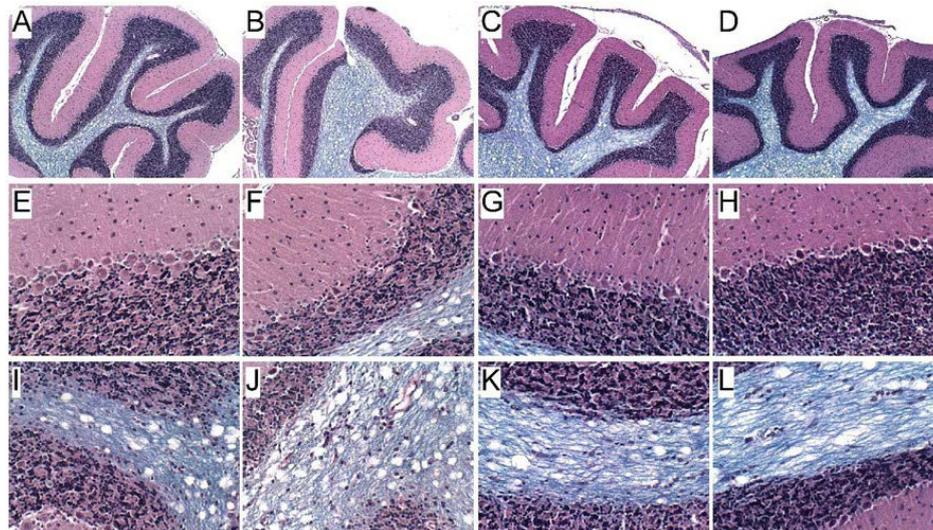
### Signaling protein expression

Ethanol had significant effects on IGF-1R, IRS-1, and Akt protein levels, and trend effects ( $0.05 < p < 0.10$ ) on insulin

receptor and GSK-3 $\beta$ . NNK exposures had significant effects on insulin receptor, IRS-1, and GSK-3 $\beta$  expression. Significant ethanol x NNK interactive effects occurred with respect to IGF-1R, IRS-1, and Akt. Post-hoc tests demonstrated that ethanol significantly increased expression of IGF-1R relative to all other groups (Figure 3B), and increased IRS-1 (Figure 3C), but decreased Akt (Figure 4A) relative to control. Effects of NNK and ethanol+NNK were similar in that both types of exposures significantly increased insulin receptor (Figure 3A), IRS-1 (Figure 3C), and GSK-3 $\beta$  (Figure 4B) relative to control and ethanol, and decreased Akt expression relative to control (similar to the effects of ethanol only) (Figure 4A).

### Phospho-protein expression

Ethanol had significant effects on pYpY1135/1135-IGF-1R and pS473-Akt expression. NNK had significant effects on pYpY1162/1163-InsulinR, pYpY1135/1135-IGF-1R, pS473-Akt, and pS9-GSK-3 $\beta$ , and a trend effect on S312-IRS-1 expression. Significant ethanol x NNK interactive effects occurred with respect to pYpY1135/1135-IGF-1R, pS473-Akt, and pS9-GSK-3 $\beta$  overlapping with the effects of NNK- and/or ethanol-only exposures. Graphs and post-hoc tests demonstrated that ethanol significantly increased pYpY1135/1135-IGF-1R expression relative to all other groups (Figure 3E), and decreased pS473-Akt relative to control (Figure 4C), paralleling its effects on IGF-1R (Figure 3C) and Akt (Figure 4A). The effects of NNK and ethanol+NNK were similar in that both inhibited pYpY1162/1163-InsulinR relative to control (Figure 3D) and pS473-Akt relative to control and ethanol (Figure 4C), and increased pS9-GSK-3 $\beta$  relative to control and ethanol-

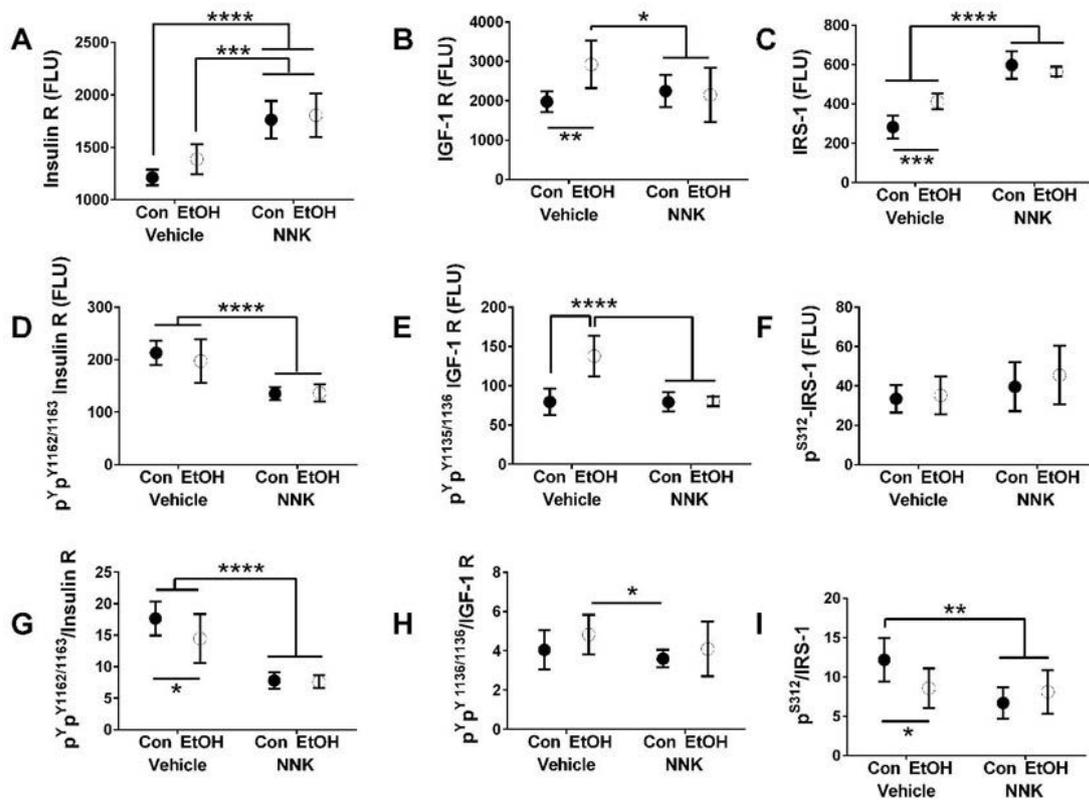


**Figure 2:** Ethanol and NNK effects of cerebellar structure. Fresh cerebella were hemi-sectioned in the mid-sagittal plane (along the vermis). One half was immersion fixed in formalin and other was snap frozen in a dry ice-methanol bath for later molecular studies. Paraffin-embedded histological sections (8 μm thick) were stained with Luxol fast blue, Hematoxylin and Eosin (LHE). (A,E,I) Vehicle treated control cerebellar cortex showing (A) complex foliation (folding), (E) uniform molecular layer and well-populated granule and Purkinje cell layers, and (I) well-delineated and densely stained (blue) white matter cores. (B,F,J) NNK-, (C,G,L) ethanol-, and (D,H,M) ethanol+NNK-exposed cerebella showing relative simplification of folia with reduced secondary sulcation (arrows in A), thinner molecular (m) layers (compare B-D to A), reduced densities of granule cells (gc) and Purkinje cells (P) in Panels F-H versus E, and broader white matter (wm) cores with reduced intensity of myelin staining (compare wm in J-M to I). Original magnifications, A-D, 50x; E-H, 200x; I-M, 100x.

Protein	Ethanol Effect		NNK Effect		Ethanol x NNK Effect	
	F-Ratio	P-Value	F-Ratio	P-Value	F-Ratio	P-Value
Insulin R	2.944	0.099	59.25	<0.0001	1.092	N.S.
IGF-1 R	4.436	0.046	1.588	N.S.	6.729	0.016
IRS-1	6.107	0.021	140.9	<0.0001	17.05	0.0004
Akt	55.26	<0.0001	0.057	N.S.	68.55	<0.0001
GSK-3β	3.056	0.093	68.91	<0.0001	0.346	N.S.
p-Insulin-R	0.565	N.S.	55.36	<0.0001	0.852	N.S.
p-IGF-1R	23.64	<0.0001	22.43	<0.0001	22.38	<0.0001
p-IRS-1	0.702	N.S.	3.317	0.081	0.202	N.S.
p-Akt	42.57	<0.0001	488.5	<0.0001	51.05	<0.0001
p-GSK-3β	0.075	N.S.	40.47	<0.0001	4.968	0.035
p/T-Insulin R	3.620	0.069	87.61	<0.0001	2.817	0.101
p/T-IGF-1R	2.668	N.S.	2.287	N.S.	0.122	N.S.
p/T-IRS-1	1.320	N.S.	9.585	0.005	6.799	0.015
p/T-Akt	0.137	N.S.	272.9	<0.0001	0.526	N.S.
p/T-GSK-3β	0.062	N.S.	4.723	0.039	4.419	0.046

Cerebellar homogenates were used to measure total and phosphorylated (p) proteins by multiplex bead-based ELISAs. The ratios of phosphorylated/total (p/T) protein were calculated. Data were analyzed by two-way ANOVA. F-ratios reflect variances of the ethanol effects, NNK effects, and ethanol x NNK interactive effects. Italicized P-values represent trend effects. Corresponding graphs with results of post-tests are shown in Figures 3 and 4.

**Table 1:** Two-Way ANOVA summary of ethanol and NNK effects on Insulin/IGF-1/Akt signaling networks in the cerebellum-multiplex ELISA results



**Figure 3:** Ethanol and NNK effects on insulin/IGF-1/IRS-1 pathway activation. Cerebellar protein homogenates were used in bead-based multiplex ELISAs to measure immunoreactivity to: (A) insulin receptor (R), (B) IGF-1R, (C) IRS-1, (D) p<sup>Y1162/1163</sup>-Insulin-R, (E) p<sup>Y1135/1136</sup>-IGF-1R, and (F) p<sup>S312</sup>-IRS-1. Relative levels of protein phosphorylation are represented by the calculated ratios of (G) p<sup>Y1162/1163</sup>/total Insulin-R, (H) p<sup>Y1135/1136</sup>/total IGF-1R, and (I) p<sup>S312</sup>/total IRS-1. Data were analyzed by 2-way ANOVA (Table 1). Graphs depict mean±S.E.M. (N=6 rats/group). 1). Post-hoc Tukey test results are depicted in the graphs (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001).

only exposures (Figure 4D). S<sup>312</sup>-IRS-1 levels were similar across the groups (Figure 3F).

**Relative Protein Phosphorylation**

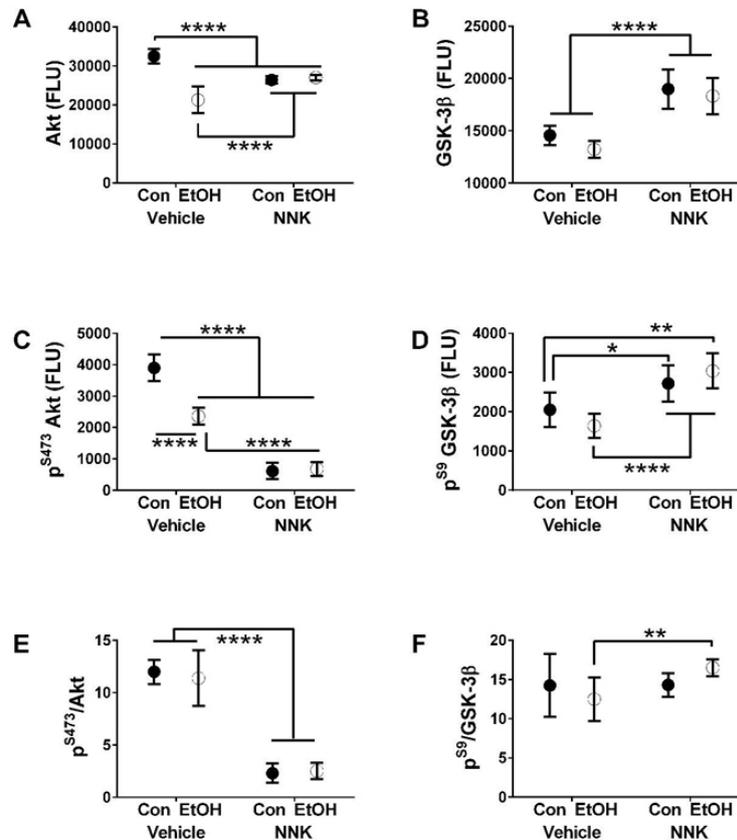
Relative levels of protein phosphorylation were calculated from the ratios of phosphorylated to total protein. An ethanol trend effect occurred with respect to p<sup>Y1162/1163</sup>-InsulinR/InsulinR. Significant NNK effects occurred with respect to p<sup>Y1162/1163</sup>-InsulinR/InsulinR, S<sup>312</sup>-IRS-1/IRS-1, p<sup>S473</sup>-Akt/Akt, and p<sup>S9</sup>-GSK-3β/GSK-3β. Significant ethanol x NNK interactive effects were observed for S<sup>312</sup>-IRS-1/IRS-1 and p<sup>S9</sup>-GSK-3β/GSK-3β, and a trend effect for p<sup>Y1162/1163</sup>-InsulinR/InsulinR. Ethanol reduced expression of p<sup>Y1162/1163</sup>-InsulinR/InsulinR (Figure 3G) and S<sup>312</sup>-IRS-1/IRS-1 (Figure 3I) relative to control. NNK significantly reduced p<sup>Y1162/1163</sup>-InsulinR/InsulinR (Figure 3G), S<sup>312</sup>-IRS-1/IRS-1 (Figure 3I), and p<sup>S473</sup>-Akt/Akt (Figure 4E) relative to control. Ethanol+NNK exposures significantly reduced p<sup>Y1162/1163</sup>-InsulinR/InsulinR (Figure 3G), S<sup>312</sup>-IRS-1/IRS-1 (Figure 3I), and p<sup>S473</sup>-Akt/Akt (Figure 4E) relative to control, and increased p<sup>S9</sup>-GSK-3β/GSK-3β (Figure 4F) relative to ethanol-only treatment.

**Neuronal and Glial Protein Expression**

Duplex ELISAs were used to measure Hu (neuronal marker), Myelin-associated glycoprotein-1 (MAG-1; mature

oligodendrocyte marker), Glial fibrillary acidic protein (GFAP; astrocyte marker), Choline acetyltransferase (ChAT), Acetylcholinesterase (AChE), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tau (major neuronal cytoskeletal protein), phospho-tau (phosphorylated on S396 and T205), ubiquitin, 4-hydroxy-nonenal (HNE; lipid peroxidation marker), and Aspartyl-β-hydroxylase (ASPH; protein regulating neuronal migration). Immunoreactivity was normalized to large ribosomal protein (RPLPO). Two-way ANOVAs demonstrated significant ethanol effects on ASPH-A86G6, and a trend effect for HNE (Table 2). Significant NNK effects occurred with respect to MAG-1, GFAP, ChAT, AChE, GAPDH, Tau, pTau, ubiquitin, HNE, ASPH-A85G6, and ASPH-A85E6, but not Hu. Significant ethanol x NNK effects occurred with respect to MAG-1, ChAT, AChE, GAPDH, ubiquitin, HNE, ASPH-A85G6 and ASPH-A85E6.

Post-hoc tests demonstrated that ethanol significantly reduced expression of MAG-1 (Figure 5B), ubiquitin (Figure 6C), ASPH-A85G6 (Figure 6E), and ASPH-A85E6 (Figure 6F) relative to control. NNK and ethanol+NNK inhibited expression of MAG-1 (Figure 5B), GFAP (Figure 5C), ChAT (Figure 5D), AChE (Figure 5E), Tau (Figure 6A), pTau (Figure 6B), ubiquitin (Figure 6C), ASPH-A85G6 (Figure 6E), and ASPH-A85E6 (Figure 6F) relative to control. In addition, the expression levels of MAG-1, GFAP, ChAT, AChE, Tau, pTau, HNE, ASPH-A85G6 and ASPH-A85E6 were significantly re-

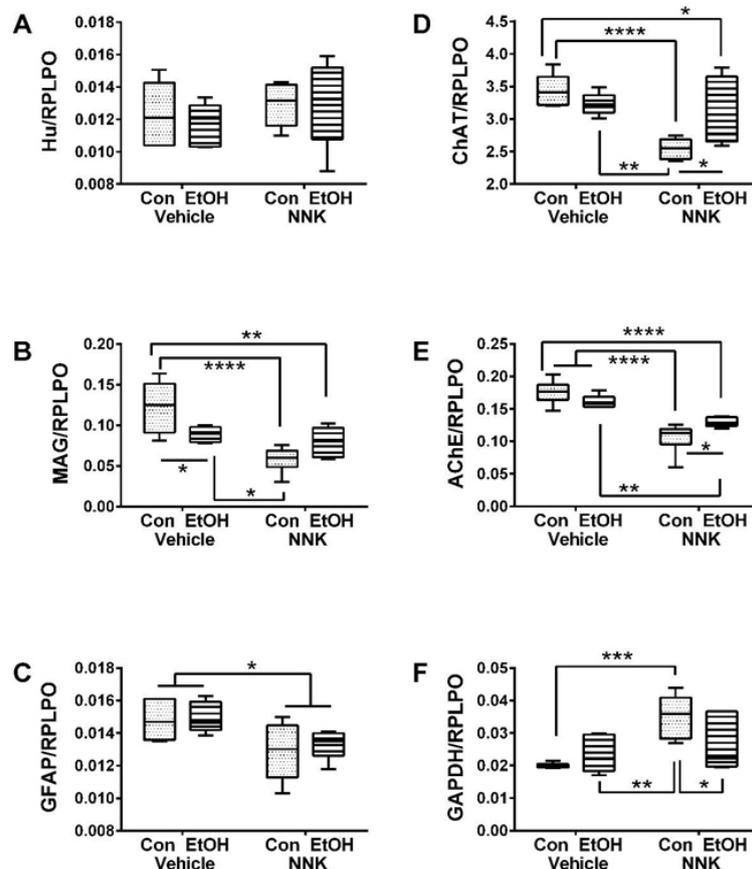


**Figure 4:** Ethanol and NNK effects on Akt and GSK-3 $\beta$  signaling proteins. Cerebellar protein homogenates were used in bead-based multiplex ELISAs to measure immunoreactivity to: (A) Akt, (B) GSK-3 $\beta$ , (C) p<sup>S473</sup> AKT, and (D) p<sup>S9</sup>-GSK-3 $\beta$ . Relative levels of phosphorylation are represented by the calculated ratios of (E) p<sup>S473</sup>/total AKT and (F) p<sup>S9</sup>/total GSK-3 $\beta$ . Graphs depict mean  $\pm$  S.E.M. (N=6 rats/group). Data were analyzed by 2-way ANOVA (Table 1). Post-hoc Tukey test results are depicted in the graphs ( $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\*\* $p < 0.0001$ ).

Protein	Ethanol Effect		NNK Effect		Ethanol x NNK Effect	
	F-Ratio	P-value	F-Ratio	P-Value	F-Ratio	P-Value
Hu	0.118	N.S.	1.195	N.S.	0.126	N.S.
MAG-1	0.508	N.S.	19.36	0.0003	10.87	0.0036
GFAP	0.307	N.S.	12.63	0.002	0.081	N.S.
ChAT	1.185	N.S.	18.89	0.0003	7.573	0.012
AChE	0.393	N.S.	60.49	<0.0001	8.455	0.009
GAPDH	1.405	N.S.	13.88	0.0013	6.458	0.019
Tau	0.013	N.S.	100.2	<0.0001	0.889	N.S.
pTau	0.016	N.S.	65.19	<0.0001	0.163	N.S.
Ubiquitin	0.525	N.S.	40.38	<0.0001	6.807	0.017
HNE	3.520	0.075	5.982	0.024	8.874	0.007
ASPH-A85G6	14.29	0.001	161.4	<0.0001	20.04	0.0002
ASPH-A85E6	0.949	N.S.	110.8	<0.0001	14.93	0.001

Immunoreactivity was measured by duplex ELISAs with results normalized to the RPLPO control. Data were analyzed by 2-way ANOVA. F-ratios reflect variances for ethanol effects, NNK effects, and ethanol x NNK interactive effects. Italicized p-values represent trend effects. Corresponding graphs with results of post-tests are shown in Figures 5 and 6.

**Table 2:** Two-way ANOVA summary of ethanol and NNK effects on neuronal and glial protein expression in the cerebellum-duplex ELISA results.



**Figure 5:** Ethanol and NNK effects on cerebellar neuronal and glial protein expression. Duplex ELISAs were used to measure Immunoreactivity to (A) Hu, (B) myelin-associated glycoprotein 1 (MAG-1), (C) glial fibrillary acidic protein (GFAP), (D) choline acetyltransferase (ChAT), (E) acetylcholinesterase (AChE), and (F) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with results normalized to RPLPO (control). Data were analyzed by two-way ANOVA (Table 2). Post hoc significance tests determined the specific inter-group differences as shown in the panels. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .

duced in NNK relative to ethanol-only cerebella. Similarly, the mean expression levels of GFAP, AChE, Tau, pTau, ubiquitin, ASPH-A85G6 and ASPH-A85E6 were significantly reduced in the ethanol+NNK relative to the ethanol-only group. Finally, significant differences between NNK and ethanol+NNK were detected for ChAT (Figure 5D), AChE (Figure 5E), GAPDH (Figure 5F), and HNE (Figure 6D), although those responses tended to be normalizing rather than additive or synergistic. Hu was the only protein found to be similarly expressed in all four groups (Figure 5A), and GAPDH was the only protein that was selectively increased by NNK relative to the other groups (Figure 5F).

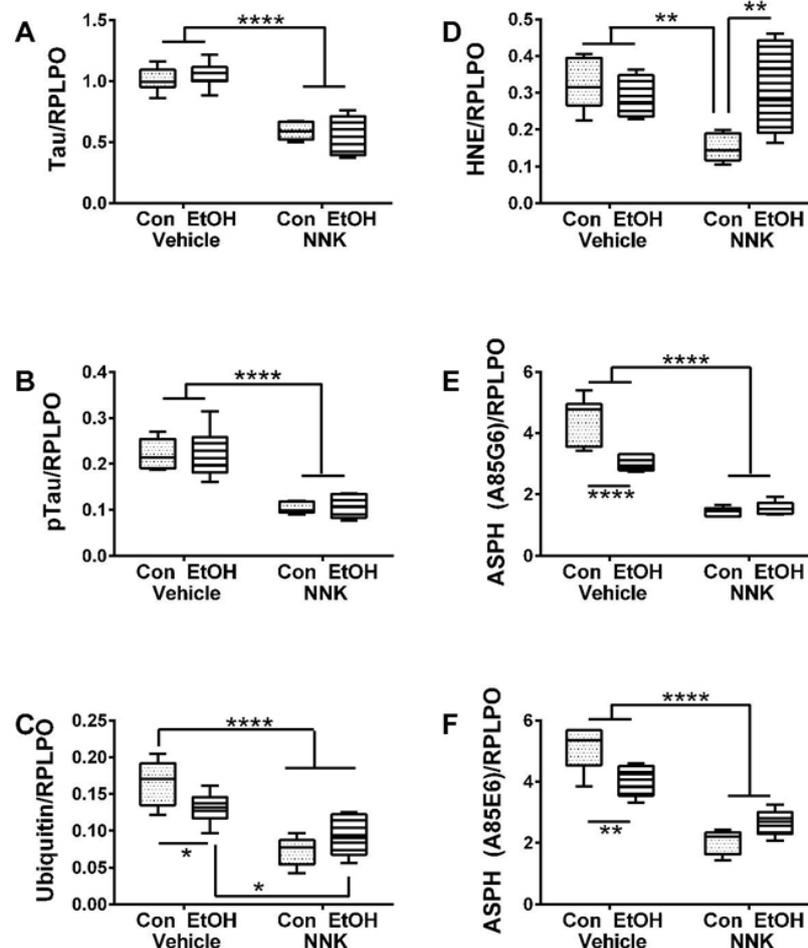
**DISCUSSION**

FASD is caused by prenatal alcohol exposures, either chronic or binge, and associated with a range of neurocognitive and motor deficits. Impairments in motor function are associated with hypoplasia and disordered migration of cerebellar neurons. Mechanistically, alcohol disrupts neuronal insulin and IGF signaling networks needed for neuronal growth, survival, metabolism, migration, plasticity and neurotransmitter function.<sup>1,2</sup> These adverse effects of ethanol are mediated at all levels of

the cascade, from ligand-receptor binding and activation of receptor tyrosine kinases, through downstream Akt and GSK-3 $\beta$  pathways.<sup>3,6,7</sup>

Despite evidence that developmental exposures to ethanol are sufficient to cause FASD, variability in the phenotypic features, including dose-effects, prompted us to consider potential co-factor exposure contributions. The finding of high rates of smoking in women who drank during pregnancy<sup>24</sup> turned our attention to the concept that tobacco smoke and toxin exposures may contribute to neurodevelopmental abnormalities in FASD. Although tobacco smoke contains hundreds of toxins, tobacco-specific nitrosamines draw interest because, like other nitrosamines, their chronic low-level exposures can cause insulin resistance and neurodegeneration.<sup>32,33</sup> The present study extends those gains by testing the hypothesis that developmental exposures to low levels of NNK cause cerebellar abnormalities that overlap with or mimic effects of FASD. NNK exposures occur with direct and sidestream (second-hand) tobacco smoke exposures.<sup>41,42</sup>

The third-trimester equivalent exposures were used to target the most vulnerable period of cerebellar development in



**Figure 6:** Ethanol and NNK modulation of neuronal and stress protein expression. Cerebellar protein homogenates were used to measure (A) Tau, (B) pTau, (C) ubiquitin, (D) 4-hydroxy-2-nonenal (HNE), (E) aspartate- $\beta$ -hydroxylase (ASPH<sub>A85G6</sub>), and (F) ASPH-Humbug (A85E6) immunoreactivity by duplex ELISAs with results was normalized to RPLPO. Inter-group comparisons were made by two-way ANOVA (Table 2) with the post-hoc Tukey multiple comparison test.

rats.<sup>43</sup> Ethanol, NNK, and ethanol+NNK exposures significantly impaired motor performance on rotarod tests and altered cerebellar architecture, including foliation (folding), Purkinje cell density, granule cell layer thickness, white matter structure, and intensity of myelin staining. Therefore, this study demonstrates that low-level NNK exposures during development can impair cerebellar function, but the corresponding effects on cerebellar structure differ. Ethanol-associated alterations in cerebellar foliation (folding and grooving) were associated with neuronal loss in the Purkinje cell layer, whereas NNK and ethanol+NNK caused simplification of cerebellar folia, irregular granule cell layer organization, some neuronal loss in the Purkinje layer, and broadening with pallor of myelin staining in the white matter cores. Therefore, these findings link NNK exposures to sustained functional and structural abnormalities in the cerebellum as occur in FASD. However, the findings also suggest that the mediators of these responses differ for ethanol and NNK.

Multiplex ELISA studies demonstrated that ethanol and NNK impaired signaling through the insulin receptor. However, the ethanol-treated group had increased expression levels of

total and p<sup>Y1135/1135</sup>-IGF-1R, and all 3 experimental groups had increased levels of IRS-1 protein but decreased levels of S<sup>312</sup>-IRS-1. Increased expression of IRS-1 enhances downstream signaling through growth and metabolic pathways.<sup>44</sup> Since <sup>312</sup>S-phosphorylation of IRS-1 is inhibitory,<sup>45</sup> its reduced levels *vis-à-vis* increased IRS-1 protein expression could represent positive compensatory responses to impaired signaling through the insulin receptor.

The findings that Akt and S<sup>473</sup>-Akt were inhibited by ethanol, NNK, and ethanol+NNK exposures indicates that downstream signaling through growth, metabolic, plasticity, and cell survival pathways were impaired, correlating with cell loss observed in the cerebellar cortex. While these effects of ethanol on the developing brain has been reported,<sup>6,7,46,47</sup> the new information derived from these studies is that NNK's neurotoxic effects mechanistically overlap with those of ethanol. Furthermore, the results suggest that the adverse effects of NNK (and smoking) can be worse than ethanol since its inhibitory effects on insulin receptor and Akt signaling were more striking than observed with ethanol. The finding that the dual ethanol

and NNK exposures did not produce additive adverse effects on these pathways suggests that corresponds with the broad NNK effects demonstrated by two-way ANOVAs and suggests that maximum pathway dysregulation was produced by the NNK exposures.

Ethanol, NNK and ethanol+NNK significantly altered the expression of several neuroglial proteins in the cerebellum. However, one of the consistent themes was that NNK's and ethanol+NNK's inhibitory effects were broader and more striking in severity than those of ethanol. Reductions in MAG-1 expression correlate with the reductions in myelin staining with Luxol fast blue dye and the less sharply delimited architecture of the white matter cores. White matter atrophy, degeneration, and developmental hypotrophy are well-recognized consequences of prenatal ethanol exposure. Recently, we demonstrated similar experimental effects of NNK and tobacco smoke exposures in adolescent or adult brains.<sup>32,48</sup> The present work provides further evidence that NNK (and probably smoking) impairs white matter myelin development in part by inhibiting expression of mature myelin-associated proteins, including MAG-1. NNK-associated inhibition of GFAP could also indicate that NNK disrupts the structural integrity/scaffolding of the brain, including components needed for blood-brain barrier maintenance and axonal function.

Tau is a key neuronal cytoskeletal protein and tau phosphorylation is critical for translocating the neuronal cytoskeleton from perikarya into neurites, in order to establish and maintain synaptic connections and help axons course through white matter. Since tau expression and phosphorylation are regulated by insulin and IGF-1 signaling and resistance,<sup>49-51</sup> it is not surprising that both were significantly reduced in NNK- and NNK+ethanol exposed cerebella, given the prominent inhibition of insulin receptor expression and tyrosine phosphorylation in those groups. Similarly, NNK-associated inhibition of ChAT could be attributed to the sustained impairments in insulin signaling because ChAT is regulated by insulin/IGF-1.<sup>7</sup> Decreased ChAT expression can impair motor function due to reduced cholinergic actions. AChE was also reduced by NNK exposures. Although the mechanism is not well understood, AChE inhibition can be consequential to persistent oxidative stress,<sup>52-55</sup> as occurs with dysregulation of energy metabolism due to inhibition of Akt signaling. The ethanol and NNK associated reductions in ubiquitin immunoreactivity could reflect deficits in the ubiquitin-proteasome system, as this phenomenon has been reported following chronic ethanol exposure,<sup>56,57</sup> and in association with various degenerative diseases.<sup>58,59</sup> Deficits in the ubiquitin-proteasome pathway could lead to increased oxidative and endoplasmic reticulum stress due to activation of the unfolded protein response.<sup>60,61</sup>

For the ASPH measurements, we used two monoclonal antibodies. A85G6 binds to the C-terminal region of ASPH which contains a catalytic domain<sup>40</sup> required to promote cell motility<sup>62-66</sup> and neuronal plasticity.<sup>62,63,65,67-70</sup> A85E6 binds to the

N-terminal Humbug-homologous region of ASPH; Humbug has a role in regulating calcium sequestration in the ER.<sup>71</sup> ASPH is regulated by insulin/IGF-1 signaling through IRS-1 and Akt.<sup>62,67,68</sup> Inhibition of ASPH perturbs cell motility and adhesion,<sup>63,70,72</sup> and in the case of FASD, ethanol's inhibitory effects on ASPH expression correlate with impairments in cerebellar neuronal migration and motor dysfunction.<sup>40,47</sup> The findings herein demonstrate that developmental exposures to ethanol or NNK significantly inhibit expression of cerebellar ASPH and Humbug proteins, correlating with the persistent alterations in cerebellar architecture and impairments in rotarod motor performance.

In conclusion, ethanol and NNK exposures during development cause structural and functional abnormalities in the cerebellum with associated impairments in signaling through the insulin receptor and Akt. In addition, ethanol and NNK reduced expression of several neuroglial proteins that help maintain the structural and functional integrity of the cerebellum. The effect sizes were generally greater with NNK than ethanol, yet the absence of additive responses suggests that the observed adverse effects of NNK were maximum. Differential responses to ethanol and NNK were evident, indicating that the manners in which they interfere with development also differ. Finally, these studies demonstrate that the CNS abnormalities typically regarded as consequences of prenatal alcohol exposure can be caused by NNK *via* tobacco smoke exposures. Moreover, the findings suggest that the heterogeneity in occurrence and severity of FASD could be due to independent or co-factor effects of tobacco smoke exposures.

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**CONFLICTS OF INTEREST:** None.

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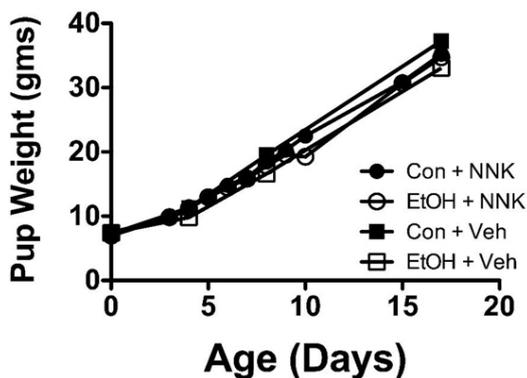
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#### Supplementary Data



**Supplementary Figure 1:** Effects of early postnatal ethanol, NNK, and ethanol+NNK exposures on body growth. Long Evans rat pups were divided into 4 groups and administered 50  $\mu$ l i.p. injections of: saline vehicle as control; pharmaceutical grade ethanol (2g/kg in saline); NNK (2 mg/kg in saline); and ethanol+NNK. Ethanol treatments (binge) were administered on postnatal days (P) 2, 4, 6, and 8, and NNK was administered on P3, P5, P7, and P9. Body weights were obtained at weekly intervals to monitor growth.

## Short Communication

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# Changing the Algorithm in the Evaluation of Pelvic Anatomy in the Infertile Patient: Is Hysterosalpingo Contrast Sonography With Saline-Air Device the Appropriate Test for Everyone?

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The evaluation of the fallopian tubes is an essential part of the infertility workup, with abnormalities related to the fallopian tubes accounting for up to 40% of female subfertility.<sup>1</sup> Laparoscopy is still considered the gold standard in the diagnostic evaluation of fallopian tubes, though the hysterosalpingogram (HSG) has long been recognized as complementary to laparoscopy, since tubal anatomy can be distinctly be seen.<sup>2</sup> That said, previous investigations using laparoscopy as the gold standard demonstrate HSG has a sensitivity and specificity of 53% and 87%, respectively, for any tubal pathology and 46% and 95%, respectively, for bilateral tubal pathology.<sup>3</sup>

In last few years, there has been a move away from these methods and towards the use of Hysterosalpingo Contrast Sonography (HyCoSy). Laparoscopy mandates regional or general anesthesia and incurs significant operative costs and risks.<sup>4</sup> In contrast, while HSG obviates the need for hysteroscopy and/or laparoscopy, it is associated with exposure to ionizing radiation and the need for iodinated contrast material.<sup>5-8</sup> While HyCoSy has been advocated as an alternative to the HSG since the 1980s, its use in evaluating tubal patency has been limited as the normal fallopian tube is a poor sonic reflector, devoid of the defined interfaces that produce clear organ outlines.<sup>9-11</sup> Various agents to enhance transvaginal ultrasound visualization of the fallopian tubes have been described; however, given storage issues, expense, and lack of FDA approval, it has obviated their routine use in the office setting. Others have substituted a mixture of saline and/or air for more elaborate distending media: some vigorously shake a syringe of saline and air creating air bubbles immediately before infusion, while others have described filling a syringe with both air and saline and tilting the syringe with the intermittent infusion of air followed by saline in increments of 1-3 mL.<sup>12-14</sup> Recently, the FDA approved saline-air devices, which create and deliver a constant alternating pattern of saline and air as a continuous stream in a controlled fashion allowing for fallopian tube evaluation under ultrasound guidance.<sup>15</sup>

The allure of the HyCoSy-saline-air device (HyCoSy-SAD) is that: 1) it is a relatively quick and non-invasive procedure that can be done in the office setting and does not require exposure to ionizing radiation; 2) a number of studies, including a meta-analysis, have demonstrated concordance rates of 83% to 100% and a diagnostic accuracy of 65% to 85% between HyCoSy-SAD, laparoscopy, and HSG in establishing tubal patency when detecting tubal pathology<sup>2,8-19</sup>; and 3) the low cost of air and saline solutions makes this particular HyCoSy procedure attractive to determine tubal patency (price points range from USD 10-40 for the SIS catheter and USD 90-150 for the saline-air device).

However, caution needs to be heeded when embracing new technology and discarding gold standards. Work by our research team concurs with reports from other investigators that

Algorithm for Initial Infertility Assessment

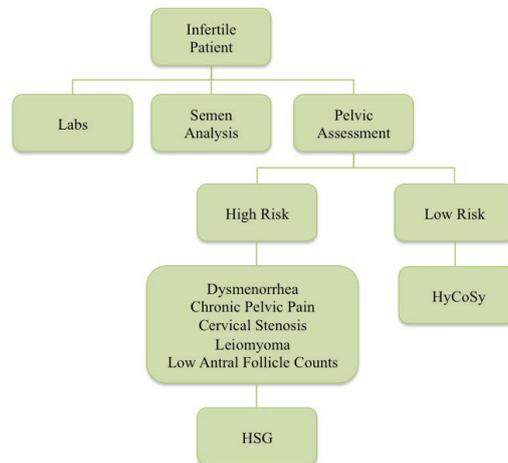


Figure 1: Algorithm for Initial Infertility Assessment. The initial workup for an infertile patient should include labs, semen analysis, and a pelvic assessment. If the patient presents with risk factors for poor visualization under HyCoSy, pelvic assessment should be executed firstly with hysterosalpingogram (HSG). If the patient carries low risk, pelvic assessment may proceed directly with HyCoSy.

Risk factors Associated with Indeterminate-Inconclusive HyCoSy-SAD

- Dysmenorrhea
- Cervical Stenosis
- Uterine Fibroids
- Low Antral Follicle Counts

Figure 2: Risk Factors associated with Indeterminate-Inconclusive HyCoSy-SAD. The risk factors for poor visualization resulting in indeterminate-inconclusive HyCoSy-SAD include dysmenorrhea, cervical stenosis, uterine fibroids (leiomyoma), and low antral follicle counts.

indicate a high degree of concordance with HSG when using a HyCoSy-SAD to detect tubal patency; however, the converse is not true with respect to tubal occlusion.<sup>20</sup> Indeterminate or inconclusive studies using the HyCoSy-SAD tend to be associated with patients with cervical stenosis, as air bubbles are less likely to traverse the cervix and ascend upwards towards the fallopian tubes, and with diminished ovarian reserve, where smaller ovaries may make it harder to track the air-bubbles. In our experience, there is nearly a two times greater likelihood of incorrectly reading a fallopian tube as occluded on HyCoSy-SAD, and between 5-10% of seemingly normal HyCoSy-SAD studies are identified as phimotic or having loculated spill on HSG. Unlike the distinct tubal anatomy seen with the HSG, distinct tubal architecture cannot be delineated with HyCoSy-SAD unless a hydrosalpinx is seen on ultrasound.

Perhaps before we leap to the HyCoSy-SAD as a first-step procedure of choice in the assessment of tubal patency, we should consider creating an algorithm and triaging patients based upon risk factors (Figure 1). The HSG should be considered in patients with cervical stenosis and without easily apparent ovaries. Other risk factors for an unsuccessful HyCoSy-SAD study to be considered should be severe dysmenorrhea (indicating possible endometriosis and/or pelvic adhesions) and uterine

fibroids, which make identification of the uterine cornua and ovaries challenging (Figure 2). While the HyCoSy-SAD allows for the simple delivery of saline and air as a continuous stream in a controlled fashion, further refinement with proper patient selection may advance this procedure as a useful first-line modality in the evaluation of tubal patency and reduce the false positives and negative studies.

CONFLICTS OF INTEREST: None.

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