

## Preface

The remarkable evolution of research on alcoholism in the last years, focusing on the importance of gender in alcohol biological effects, is highlighted in this special issue of *Annali dell'Istituto Superiore di Sanità*. The paper is aimed to give a scientific, reliable update on the most recent studies about female alcohol problems covering a broad range of topics related to female drinking, including animal studies, socio-cultural issues, and considerations about the health risk due to concomitant exposure to alcohol and environmental pollutants.

Alcohol drinking is one of the most ancient traditions in western countries and it has been part of people life from many generations, thus leading to a widespread underestimation of excess-drinking-related risks for health. The new models of life, modifying cultural tradition and drinking patterns, lead to an increase of the spread of alcohol damage. Women are more affected by these lifestyle modifications, with increased risk for their well-being. Social acceptance of female drinking is low in some cultural contexts, and female drinking affects more than male drinking the life and health of the family. Recent studies evidenced that alcohol adverse effects are heavier in female than in male (the so-called "telescopic effect", reported for drug-addicted women), as well as the risk for children prenatally exposed to alcohol.

Prenatal alcohol damage, globally defined fetal alcohol spectrum disorders (FASD), is not reversible and the only way to avoid it is the prevention of maternal alcohol ingestion during the gestational period by a widespread information.

Female drinking is often underestimated because of the female tendency to hide alcohol problems. This makes difficult early diagnosis and intervention, in spite of the improved assessment of the spectrum of alcohol effects devoting more attention to gender differences.

In 2004, a Research Protocol was signed by the Dipartimento di Ambiente e Connessa Prevenzione Primaria (Department of Environment and Primary Prevention) of the Istituto Superiore di Sanità (ISS, Italian Health Institute) and the Centro di Riferimento Alcolologico Regione Lazio (CRARL, Regional Reference Centre for Alcoholism), University "La Sapienza", aimed to the implementation of biological and clinical alcoholism research. The researchers involved in the research protocol for alcohol studies set up informally the "Italian Group for Alcoholism Research" (IGAR)\*. The collaborative Project ISS/NIH (NIAAA Depart-

ment) "Woman, health, alcohol. Risks and damages from alcohol in different woman ages: the role of abuse markers" is one of the first results of this collaboration. Several studies are being performed by teams of the different groups: clinical aspects are investigated by the research teams of the University "La Sapienza" coordinated by the CRARL; laboratory research about genetics, immunology, biochemistry, environmental toxicology is performed in the ISS.

Moreover, free phone services for information about alcohol problems are operative in ISS (Telefono Verde Alcol: 800 63 2000), and in CRARL (Alcoltel: 800 04 6655). Well trained operators are available to give information about alcohol drinking and about Italian and European organizations devoted to alcohol problems. Phone services are aimed to support structures dealing with alcohol prevention and health promotion and to help young people and their families suggesting possible solutions and informing about the most suitable tools against alcohol problems.

The project of the present issue was born from the need to give an update on research about women problems in consideration of the increasing female drinking, the lowering of their first-use age and the risks of severe damage for children exposed to alcohol in utero. The aim of this issue is also to promote safer life habits and more respect for the environment and the individual health. Furthermore, this issue was aimed to increase the awareness of alcohol diseases in social and health operators, including family physicians, gynaecologists and paediatrics, obtaining an early detection and an effective treatment of women with alcohol-related problems and FASD children.

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# National Institute on Alcohol Abuse and Alcoholism and the study of fetal alcohol spectrum disorders. The International Consortium

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**Summary.** Fetal alcohol syndrome (FAS) is a large and rapidly increasing public health problem worldwide. Aside the full-blown FAS, multiple terms are used to describe the continuum of effects that result from prenatal exposure to alcohol, including the whole fetal alcohol spectrum disorders (FASD). The revised Institute of Medicine (IOM) Diagnostic Classification System and the diagnostic criteria for FAS and FASD are reported, as well as the formation of the four-state FAS International Consortium and its aims, as the development of an information base that systematizes data collection that helps to determine at-high-risk populations, and to implement and test a scientific-based prevention/intervention model for at risk women. The Consortium was further enlarged, with the inclusion of some more states (including Italy), leading to the formation of the International Consortium for the Investigation of FASD. The objectives of the Consortium are reported, as well as its previous activities, the South Africa and Italy Projects (active case ascertainment initiatives), and its future activities.

*Key words:* fetal alcohol syndrome, diagnosis, intervention strategies.

**Riassunto** (*NIAAA e lo studio della FASD. Il Consorzio Internazionale*). La sindrome fetoalcolica (FAS) è un problema di salute pubblica, sempre più grave, in tutto il mondo. Oltre al termine FAS, che descrive la sindrome completa, sono stati proposti diversi termini per descrivere la gamma di quadri clinici dovuti all'esposizione prenatale all'alcol. Lo spettro completo dei problemi fetali da alcol è definito FASD. Il sistema di classificazione (r-IOM diagnostic Classification System) ed i criteri diagnostici per FAS e FASD sono descritti. Viene riassunta la costituzione del Consorzio Internazionale dei Quattro Stati per la FAS ed i suoi scopi, lo sviluppo di un sistema di informazione che sistematizza la raccolta di dati su popolazioni a rischio, e lo sviluppo e la verifica di modelli di intervento e prevenzione, su base scientifica, per donne a rischio. Il Consorzio è stato successivamente ingrandito, con l'inclusione di altri stati (compresa l'Italia), formando il Consorzio Internazionale di studio sulla FASD. Vengono esposti gli obiettivi del Consorzio, le sue attività, i progetti Sud Africa e Italia (iniziative di casistica ad accertamento attivo) e le sue future attività.

*Parole chiave:* sindrome fetoalcolica, diagnosi, strategie di intervento.

## INTRODUCTION

Fetal alcohol syndrome (FAS) is a large and rapidly increasing public health problem. The prevalence of FAS in the United States has been reported as 1-3 per 1000 live births and the rate of fetal alcohol spectrum disorders (FASD) as 9.1 per 1000 live births [1]. Prevalence studies completed in the United States have suggested estimates of FAS among chronically alcoholic woman of 25 per 1000. This is about twenty times higher than the estimates of prevalence in the general population [2]. By 1998, the cost of medical care for treatment of FAS-related growth retardation, surgical repair of organic anomalies, and treatment of vision defects and mental retardation had exceeded 4 billion dollars per years. The

consequences of fetal exposure to maternal alcohol consumption, therefore, is a serious problem for the individual and for society, in terms of human suffering, lost productivity and medical and social monetary costs [3].

## FETAL ALCOHOL SPECTRUM DISORDER

Multiple terms are used to describe the continuum of effects that result from prenatal exposure to alcohol, including fetal alcohol effects, alcohol-related birth defects (ARBD), alcohol-related neurodevelopment disorder (ARND), and, more recently, fetal alcohol spectrum disorders (FASD) [4, 5]. In April 2004, the National Organization on Fetal Alcohol Syndrome (NOFAS)

convened a meeting of representatives from three federal agencies (the National Institutes of Health - NIH -, CDC, and the Substance Abuse and Mental Health Services Administration - SAMHSA -) and persons with expertise in the field to develop a consensus definition of FASD. The resulting definition, which is used in this report, defined FASD as the range of effects that can occur in a person whose mother drank alcohol during pregnancy, including physical, mental, behavior, and learning disabilities, with possible lifelong implications. As this definition indicates, multiple diagnostic categories (*e.g.*, FAS, ARND, and ARBD) are subsumed under the term FASD. However, FASD is not a diagnostic category and should be used only when referring to the collection of diagnostic terms resulting from prenatal exposure to alcohol.

### DIAGNOSTIC CRITERIA

Despite the known adverse effects of prenatal exposure to alcohol [6], children who experience these effects often do not receive a correct diagnosis or referral for diagnostic evaluation because of the absence of uniformly accepted diagnostic criteria and guidelines for referral. Early identification and diagnosis of FAS in affected persons are essential components to providing health, education, and social services that promote optimal well-being. In 1996, a committee appointed by the Institute of Medicine (IOM) recommended adopting a new classification of fetal alcohol spectrum disorders. This included: FAS with and without a confirmed history of alcohol exposure, partial FAS, alcohol-related birth defects (ARBD), and alcohol-related neurodevelopmental disorders (ARND) [7]. In the late 1990s, another diagnostic system was developed by Astley and Clarren, which came to be known as the 4-Digit Diagnostic Code [8]. To increase the reliability of diagnosis, Astley and Clarren developed scales to assess clinically significant characteristics of the face such as philtrum and the vermilion border [9].

Although the IOM criteria and the 4-Digit Code have recognized the wide spectrum of outcomes produced by prenatal alcohol exposure, these two diagnostic systems have limitations. For example, two diagnostic categories introduced by the IOM report to replace the label FAE were found to be ambiguous. The question of the utility of multiple categories created by the 4-Digit Code has been questioned [10]. Accordingly, a few new diagnostic systems have been introduced recently.

The Canadian diagnostic guidelines are one new classification system, developed by harmonizing the IOM criteria and the 4-Digit Code. The Canadian Guidelines employ the objective measures from the 4-Digit Code and use the IOM diagnostic categories. The authors have described the diagnostic process in detail (which consists of screening and referral, physical examination and differential diagnosis, and neurobehavioral assessment), and underscore the necessity of a multidisciplinary team for accurate and comprehensive diagnosis [11]. The sensitivity and specificity of the Canadian guidelines have not been established yet.

The Revised IOM Diagnostic Classification System is very similar in its approach to that proposed by the Canadian Working Group. Like the Canadian Guidelines, it recommends a multidisciplinary approach (including input from experienced physicians, psychologists, educational diagnosticians and skilled maternal interviewers), sets forth an objective method of morphological assessment and stresses differential diagnosis prior to assigning a diagnosis in the FASD continuum. However, as opposed to the Canadian system, the Revised IOM Diagnostic Criteria have been field tested in a large multiracial international cohort of children prenatally exposed to alcohol and have been found to accurately define the range of FASD [10].

Recently, the National Task Force on Fetal Alcohol Syndrome and Fetal Alcohol Effects have also issued guidelines for Referral and Diagnosis (Centers for Disease Control and Prevention, 2004) [12]. The focus of this task force was to provide diagnostic criteria for fetal alcohol syndrome. The task force underscored, however, the necessity of developing science-based guidelines for identifying other alcohol-related conditions such as alcohol-related neurodevelopmental disorders.

To increase understanding and reduce the risk factors that affect the prevalence of this preventable condition in 2000, under the guide of National Institute on Alcohol Abuse and Alcoholism, the states of Minnesota, Montana, North Dakota and South Dakota, formed the Four-State International Consortium.

### HOW THE FOUR-STATE FAS CONSORTIUM STARTED

The Four-State FAS Consortium was funded for 5 years with a start date of September 29, 2000 and initially operated on a largely rural, sparsely populated area covered 380 000 square miles. In Montana, North Dakota, and South Dakota there are vast distances between population centers. Minnesota is the urban exception in the Consortium, however it shares the rural, reservation characteristics of the other three states. All four states are economically dependent on agriculture and related industry.

Together the four states have a combined population of 7.2 million people and a combined total of approximately 95 000 births per years. Thirty-three Indian reservations are located within the four states. The rural nature of the states and the large numbers of Native American reservations are cited in several national studies as two relevant risk factors for alcohol use. In fact, in small rural communities alcohol is the drug of choice because it is more readily available than other drugs and, according with the data of Indian Health Service, the alcohol related death rates is as much as 14.4 times higher in Native Americans than in other population.

Therefore, the four states show a significant high-risk population for alcohol abuse, and similarities in landmass, economy and territorial characteristics: in this way, activities that could be effective in one state can be modified and replicated in the other Consortium states [13].

## ADMINISTRATIVE STRUCTURE OF CONSORTIUM

The Four-State Consortium operates through a cooperative agreement with funding from the Center for Substance Abuse Prevention (CSAP).

The Four-State Consortium is administered by the Center for Disabilities at the University of South Dakota School of Medicine with subcontracts to the other three states. The Minnesota Organization on Fetal Alcohol Syndrome (MOFAS) is the lead agency for the Consortium activities within Minnesota. The Department of Public Health and Human Services is the lead agency for Montana. The FAS Center at the University of North Dakota School of Medicine is the lead agency in North Dakota [13].

## OBJECTIVES OF THE CONSORTIUM

Three primary objectives are addressed through the Consortium structure. These include: to develop and evaluate the formation of the Consortium itself (objective 1); to develop an information base that systematizes data collection that helps determine populations and areas considered high risk (objective 2); and to implement and test a scientific-based prevention/intervention model for woman considered high risk of abusing alcohol during their childbearing years (objective 3).

*Objective 1:* The first objective was realized through significant event tracking forms to collect important information on the success and barriers encountered by the Consortium. In addition, advisory groups and consortium participants and staff are asked to complete interviews and surveys on a periodic basis. The analysis of this information provided a logical and comprehensive look at the effectiveness of supporting multi-state efforts in the area of the prevention of FAS/FASD [13].

*Objective 2:* To realize the second objective was activated a project to examine risk factors using a Prenatal Questionnaire (PNQ) that included substance-screening questions with a large sample of pregnant woman. The participants (no. = 4676) for the study were sampled from four states. Clinic sites for the administration of the prenatal screening instrument were selected in each state. Univariate and multivariate procedures were used to determine predictive factors of alcohol use. The study results indicate that there are a number of maternal risk factor present: women at high risk for alcohol use when pregnant tended to be younger, less educated, single, and unemployed. Other variable associated are past sexual abuse, current or past physical abuse, using tobacco, using other drugs, living with substance users, and believing that drinking any amount of alcohol while pregnant is acceptable [14].

To systematize those data was used the FAS Data Entry Form. This instrument is helpful in determining what risk factors have been used to refer the individual for a possible diagnosis as well as what criteria was used in making the diagnosis. This instrument is also useful in collecting information on the biological mothers of the individual referred.

*Objective 3:* The third objective was realized through a multifaceted intervention procedure to reduce the substance use of high-risk women of child-bearing age. The intervention arm featured an intensive home visit/case management system delivered by support specialists through direct programming or referrals. Six domains (mental health, social support, family functioning, self-efficacy/general well being, alcohol use, and tobacco and other drug use) were the focus of the program. The intervention model is based on the Health Belief Model (HBM) [15, 16].

The application of the HBM are: define population at risk and personalize risks based on person's characteristics; specify consequences of the risk and the condition; identify and reduce barriers and risk factors, through reassurance, incentives and assistance; define specific action and clarify the positive effects to be expected; provide how-to information and promote awareness; use goal setting and verbal reinforcement; reduce stress; improve mental health.

Within the construct of the HBM, if a pregnant mother thinks or feels that her baby is susceptible to harm as a result of her actions, then the mother will perceive that she and her baby are susceptible to the consequences of her actions.

Through those projects, the Consortium has produced excellent data on prenatal care risk markers in 9360 women from the four states. Hundreds of woman at increased risk to have a child with FASD have received education and 604 women at the highest risk have been enrolled in the Consortium's prevention trial to reduce risk factors for FASD [13].

## FURTHER STEPS

In the last five years other countries decided to agree to the Consortium projects to investigate the national prevalence and the specific risk factors for FASD. Those countries includes: South Africa, Italy, Ukraine, Finland, Russia. Therefore, the International Consortium for the Investigation of FASD was founded.

The active projects are focused on different objectives, according to the resources and infrastructures available, the economy and territorial characteristics, the population.

The South Africa Project was an active case ascertainment initiative funded by US and Africa sources to establish the prevalence of Fetal Alcohol Syndrome in a community in the Western Cape Province of the Republic of South Africa. This area is densely populated, constituted by small towns and rural settlements and involved in growing grapes and producing wine.

The study was based on a school-based screening program: dysmorphology, growth, developmental and maternal risk data were collected and utilized to identify 626 children to evaluate with a full dysmorphology examinations. Full examinations for so many children also provided intensive training for South African physician trainees.

The results showed a high risk rate of fetal alcohol syndrome in the schools: 40.5 to 46.4 per 1000 children aged 5 to 9 years. These rates are 18 to 141 times greater than in the United States [17].

The aims of the Finland Project were to examine the spectrum of dysmorphic features in a genetically homogeneous subgroup of children with known heavy alcohol exposure; to determine whether a weighted dysmorphology score could be used as an adjunct clinical diagnostic tool when an FASD diagnosis is suspected; to evaluate determinants of cognitive development in Finnish children with FASD; to compare the newly revised IOM criteria for FAS diagnosis with the original alcohol-related diagnoses in Finnish population.

Seventy-seven children born between 1984 and 1996 and diagnosed as having FAS or FASD in one of the major hospitals in Helsinki were evaluated using six subtests from the Wechsler Intelligence Scale for Children III and were undergone to a systematic dysmorphic examination.

Using the Revised IOM criteria, was identified 23 children with full FAS, 7 children with partial FAS, and 2 children with ARND. In addition, 14/77 children evaluated had congenital heart disease related to FAS [18].

Finally, the Italian Project utilized active case ascertainment to determine prevalence and risk factors in Italy. A school-based project was realized in the partial rural and wine-producing area around Rome (Lazio).

Children were ascertained: by referral from teachers and/or parents, occipito-frontal circumference (OFC) < 10%, height and weight < 10%. All subjects and controls underwent: blinded dysmorphology examination, neuro-psychological testing, maternal interview. Preliminary data analysis following the first wave of screening indicates FAS as a significant problem in Italy, too [19].

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## CONCLUSIONS

Data from the projects realized by the International Consortium for the Investigation of FASD indicate that drinking during pregnancy is a universal problem which requires a comprehensive international approach to be successful in diagnosing, treating and preventing this tragic spectrum of disabilities.

The identification of affected people was been crucial for early entry into intervention programs and for the development of prevalence estimates and those projects have demonstrated effectiveness and efficiency of a community-based screening strategy to obtain this result. Besides, the prevention programs activated in several countries have obtained excellent outcomes among women considered at high risk for alcohol use during their childbearing years.

*Next steps:* Those results indicate that each state needs to have a FAS task force to implement appropriate identification, treatment, and prevention strategies for FAS and related disorders. This would include screening to identify high-risk women and a system to link mothers with diagnosed children in order to identify them for immediate substance abuse treatment. This system also needs to emphasize early identification of affected children, early entry into treatment, prevention of secondary disabilities, and development of a specialized service delivery system for affected adults.

The cost of inaction will be higher than the cost of science-based action against alcohol related damage.

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# Individual susceptibility and alcohol effects: biochemical and genetic aspects

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**Summary.** The large interethnic and interindividual variability in alcohol-induced toxic effects comes from a combination of genetic and environmental factors, influencing ethanol toxicokinetics. The hepatic enzymatic systems involved in ethanol metabolism are alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and microsomal P4502E1 (CYP2E1). ADH oxidizes ethanol to acetaldehyde, which is very efficiently oxidized to acetate by ALDH. About 10% of moderate quantities of ethanol is metabolised by CYP2E1; the percentage increases when ADH is saturated. During ethanol metabolism reactive oxygen species and hydroxyethyl radicals are generated, causing oxidative stress, responsible for most ethanol-induced liver damage. For their critical role in detoxifying radicals, glutathione S-transferase are gaining attention in the etiology of alcoholism. All these enzymes have been shown to be polymorphic, giving rise to altered phenotypes. For this reason recent studies have looked for a correlation between metabolic variability and differences in alcohol abuse-related effects.

**Key words:** ethanol, interindividual variability, polymorphism, alcohol dehydrogenase, aldehyde dehydrogenase, CYP2E1, glutathione S-transferase.

**Riassunto** (*Variabilità individuale ed effetti dell'alcol: aspetti biochimici e genetici*). L'ampia variabilità interetnica ed interindividuale degli effetti tossici dovuti all'alcol deriva da una combinazione di fattori genetici ed ambientali che influenzano la tossicocinetica dell'etanolo. I sistemi enzimatici epatici coinvolti nel metabolismo dell'etanolo sono l'alcol deidrogenasi (ADH), l'aldeide deidrogenasi (ALDH) e il citocromo P450 2E1 (CYP2E1). L'ADH ossida l'etanolo ad acetaldeide che è efficientemente biotrasformata ad acetato dalla ALDH. Circa il 10% di moderate quantità di etanolo viene metabolizzato dal CYP2E1; la percentuale aumenta quando l'ADH è saturata. Durante il metabolismo dell'etanolo si generano specie reattive dell'ossigeno e radicali idrossietilici che causano stress ossidativo, principale responsabile del danno epatico indotto da etanolo. A causa del ruolo critico delle glutathione S-transferasi nella detossificazione dei radicali, il loro coinvolgimento nell'eziologia dell'alcolismo sta ricevendo crescente attenzione. Tutti questi enzimi sono presenti come forme polimorfiche che possono originare fenotipi alterati. Per questa ragione studi recenti hanno cercato una correlazione fra variabilità metabolica e differenze negli effetti dell'abuso di alcol.

**Parole chiave:** etanolo, variabilità interindividuale, polimorfismo, alcol deidrogenasi, aldeide deidrogenasi, CYP2E1, glutathione S-transferasi.

## INTRODUCTION

Alcoholism is a common disorder with a complex origin and outcome, since individuals react differently when exposed to comparable amounts of alcohol. Many epidemiological, biomedical and psychosocial studies support the hypothesis that some individuals suffer more severe adverse effects following alcohol use. Physiological features (such as age and gender) and socio-cultural/psychological factors may play a relevant role in determining the huge interindividual variability in the thresholds and lifetime prevalence of this disease. Indeed, social restrictions have been shown to have a huge influence on the risk for alcohol dependence, particularly in societies with a high prevalence of alcoholism [1]. Excessive and prolonged use of alcoholic beverages is the cause of serious social

and medical disorders in a significant number of individuals associated with socioeconomic consequences for the rest of the population. Alcohol related pathologies are very often related to the deficient nutritional status of chronic drinkers, due to unbalanced diet and ethanol interference in the uptake and utilization of carbohydrates, lipids and vitamins, particularly vitamin A. Indeed, ethanol has been shown to inhibit the oxidation of retinol to retinoic acid (the active form of vitamin A) by competing for alcohol dehydrogenases [2]. As a consequence, the levels of retinoic acid, which is essential for growth and maintenance of normal epithelial function, are decreased in alcoholics. Ethanol has also been demonstrated to have teratogenic potential: alcohol consumption during pregnancy can potentially result in effects in the foetus ranging

from transient outcome to a quite severe neurologic disorder known as foetal alcohol syndrome (FAS). The syndrome has been associated to both ethanol metabolism and related oxidative stress and to reduction in retinoic acid production during gestation.

Family studies on twins and adoptee estimated that individual risk for alcoholism can be equally addressed to environmental and genetic factors which show a high degree of interaction [3].

Ethnicity seems also to confer different susceptibility to ethanol toxicity, as suggested by studies showing that, when compared with African Americans or native Americans, Caucasians have higher and lower rates of ethanol elimination, respectively. Differences in liver mass may only partially explain ethnic and gender differences measured in alcohol clearance [4]. Indeed, recent molecular genetic research has assigned to functional polymorphisms at those genes encoding enzymes involved in ethanol toxicokinetics, the pivotal role in determining the differential susceptibility in alcohol-induced toxic effects. This genetic features may act in combination with environmental factors, such as nutrition, life-style and exposure to other xenobiotics, responsible for the acquired modulation (induction/inhibition) of the same enzymatic activities [5].

In the paper the main features of ethanol metabolism and the polymorphisms of the most relevant enzymes involved in determining the eventual different susceptibility to alcohol-induced effect will be briefly presented.

### **Ethanol toxicokinetics and metabolism**

Ethanol-induced effects are due to both ethanol *per se* and to the products of its metabolism, including redox changes related to the production of acetaldehyde and acetate.

The time course of ethanol blood concentration after ingestion of alcoholic drinks is strictly dependent on its toxicokinetics, which determines the dose to the target organs and the toxicodynamic responses to ethanol [6].

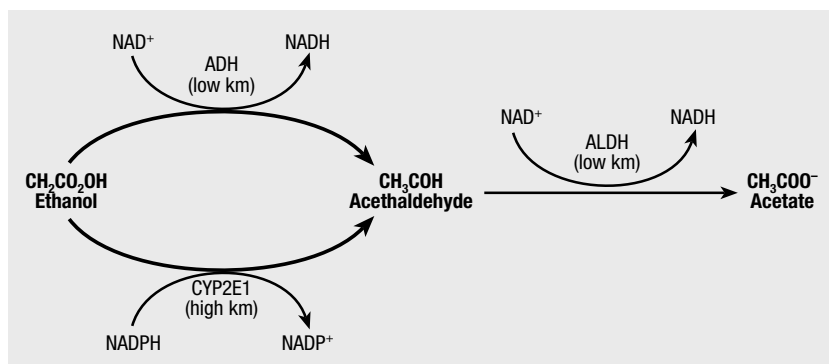
After oral administration, ethanol is readily absorbed by the gastrointestinal tract; absorption takes place by passive diffusion through the stomach wall (about 20%), being the remaining 80% absorbed through the duodenum and small intestine wall [5]. The rate of

absorption varies with the time of the day, the dosage form, the concentration of ethanol and the drinking pattern, mainly related to the gastric emptying status. After oral absorption of ingested doses  $<0.3$  g/kg, the removal of ethanol by the liver, before it reaches systemically other organs (hepatic first-pass effect), is pronounced. At higher ethanol doses, this effect is not easy to be quantitatively defined, also due to the great interindividual variation in the percentage of absorbed dose. Once in the bloodstream, ethanol is uniformly distributed in the body water space; indeed, being highly water soluble, it does not need to bind to plasma proteins and therefore there is a strong correlation between ethanol volume of distribution and total body water [5].

Elimination of absorbed ethanol occurs primarily through metabolism (95-98%), with small fractions of the administered dose being excreted unchanged in the breath (0.7%), sweat (0.1%), and urine (0.3%) [5, 7].

In adult nonalcoholic individuals, most ethanol biotransformation occurs in the liver (*Fig. 1*) mainly *via* oxidation catalyzed by alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and by a cytochrome P450 isoform (CYP2E1) [8, 9].

In the cytosol of hepatocytes, ethanol is oxidized to acetaldehyde, in a reversible reaction catalyzed by class I ADH, a high affinity ( $K_m = 0.05-0.1$  g/l) and low capacity enzyme, becoming saturated after only few drinks. Acetaldehyde is then oxidized in a non-reversible reaction to acetate, by the mitochondrial form of ALDH. Since the enzyme has a very low  $K_m$ , the elimination of acetaldehyde is very efficient, so that the product of ethanol oxidation, which is highly toxic, is eliminated soon after its formation. It has been estimated that during ethanol intoxication only 1-2% of the acetaldehyde formed in the liver enter the bloodstream, giving rise to negligible peripheral venous levels ( $\approx 1$   $\mu\text{mol/l}$ ) [10]. The activated form of acetate, acetyl CoA, may be further metabolized leading to ketone bodies, amino acids, fatty acids and steroids [8]; when it is oxidized in the Krebs cycle,  $\text{CO}_2$  and water are formed as the end-products of ethanol oxidation. Both ADH and ALDH utilize as the cofactor  $\text{NAD}^+$ , which is reduced to NADH (*Fig. 1*): as a consequence, during ethanol oxidation the ratio  $\text{NADH}/\text{NAD}^+$  is significantly increased, altering the cellular



**Fig. 1** | Pathways of ethanol metabolism in the liver. ALDH=acetaldehyde dehydrogenase;  $\text{NAD}^+$ =nicotinamide adenine dinucleotide;  $\text{NADH}$ =reduced  $\text{NAD}^+$ ;  $\text{NADP}^+$ =nicotinamide adenine dinucleotide phosphate;  $\text{NADPH}$ =reduced  $\text{NADP}^+$ ; ADH = alcohol dehydrogenase; CYP2E1 = cytochrome P-450 isoform 2E1.

**Table 1** | Polymorphisms of alcohol dehydrogenase (ADH) genes

Class (Protein)	Gene	Subunit	Nucleotide change	Effect	Chromosomal location
<b>Class I ADH</b>					
ADH1	ADH1*1	$\alpha$		wild-type	4q22
ADH2	ADH2*1	$\beta$ 1		wild-type	4q22
	ADH*2	$\beta$ 2	47G > A	His <sup>47</sup> ; increased $V_{max}$	
	ADH2*3	$\beta$ 3	369C > T	Cys <sup>369</sup> ; increased $V_{max}$	
ADH3	ADH3*1	$\gamma$ 1		wild-type	4q22
	ADH3*2	$\gamma$ 2	271C > T; 349G > A	Gln <sup>271</sup> ; Val <sup>349</sup>	
<b>Class II ADH</b>					
ADH4	ADH4	$\pi$	192T > A; 159G > A; 75A > C	altered expression	4q21-25
<b>Class III ADH</b>					
ADH5	ADH5	$\chi$		wild-type	4q21-25
<b>Class IV ADH</b>					
ADH7	ADH7	$\sigma$		wild-type	4q23-24
<b>Class V ADH</b>					
ADH6	ADH6	? <sup>§</sup>		? <sup>§</sup>	4q21-25

<sup>§</sup>Subunit composition not known. \*Adapted from [7].

redox state and triggering a number of adverse effects, related to alcohol consumption [5]. The hepatic NADH re-oxidation seems to be the rate limiting step of the process and together with the functional ADH and ALDH activities regulate the steady-state of ethanol oxidation rate.

When a moderate dose of ethanol is ingested, a small but significant amount ( $\approx 10\%$ ) is metabolized by the microsomal NADPH dependent-oxidation catalyzed by CYP2E1 [8, 9]. This enzyme is characterized by a lower affinity ( $K_m = 0.5-0.8$  g/l) with respect to ADH: its role in ethanol oxidation becomes relevant when large amounts of alcohol able to saturate ADH are ingested ( $>100$  g per day). The capacity-limited elimination, due to CYP2E1 saturation, is counteracted by enzyme induction by ethanol itself, which is thus able to increase its own clearance in heavy drinkers and alcoholics.

#### **Role of polymorphisms in ethanol metabolism and ethanol-induced effects**

The important contribution of genetic factors to alcoholism is not explained by Mendelian inheritance of single genes, but there are strong evidences suggesting that this pathology is a genetically-influenced complex multifactorial disease. The increasing utilization of molecular technologies in the genetic research on alcoholism has focused the attention on the possible role of functional polymorphisms in genes encoding ethanol metabolizing enzymes. Those genetic variants produce enzymes with altered activity, changing the rate of toxic metabolites production or of their detoxication.

Thus, elucidation of the molecular mechanisms that control and influence elimination and metabolism of ethanol is important in understanding the biochemical basis of ethanol toxicity and alcohol abuse-related

pharmacological and addictive consequences in humans. In addition, the identification of genetic polymorphisms and the characterization of their putative role in alcoholism vulnerability may help in improving prevention and treatment approach.

In the following the major genetic variants, giving rise to functional polymorphisms of enzymes involved both in ethanol metabolism (ADH, ALDH, CYP2E1) or in the onset of effects due to the alteration of the redox status (glutathione S-transferase enzymes), will be presented.

#### **Alcohol dehydrogenase**

Alcohol dehydrogenase (ADH) is a cytosolic enzyme able to metabolize ethanol and a wide variety of substrates, including other aliphatic alcohols, hydroxysteroids and lipid peroxidation products. ADH exists as a polygenic family of seven genes located on chromosome 4, translated in various human ADH forms (*Table 1*). They can be divided into five major classes or distinct groups (I-V), according to their subunit composition as well as their physicochemical properties [11].

Human ADH is a dimeric protein, resulting from the association of different subunits with a molecular weight of 40 kD each. Class I ADH (ADH1, ADH2 and ADH3) isoenzymes are formed by different combinations of subunits ( $\alpha$ ,  $\beta$ ,  $\gamma$ ), coded by genes from either the same locus or different loci. Homodimeric proteins are formed by identical subunits coded by the same locus, while heterodimeric proteins are formed by alleles coded from different loci (*e.g.*,  $\alpha\beta$ ,  $\alpha\gamma$ ) or by different alleles at the same locus (*e.g.*,  $\beta1\beta2$ ,  $\gamma1\gamma2$ ).

Although 20 ADH isoenzymes are known, which vary in their catalytic properties, relevant functional polymorphism has been found only for the beta and gamma subunits forming ADH2 and ADH3 [12]. Up to now no allelic polymorphism has been reported in



**Table 2** | Polymorphism of aldehyde dehydrogenase (ALDH) genes

Locus	Gene	Nucleotide change	Effect	Chromosomal location
ALDH1B1	ALDH1B1*1		wild-type	9p13
	ALDH1B1*2	257C > T	Ala <sup>69</sup>	
	ALDH1B1*3	320T > G	Leu <sup>90</sup>	
	ALDH1B1*4	183C > T	silent	
ALDH2	ALDH2*1		wild-type	12q24.2
	ALDH2*2	1510G > A	Lys <sup>487</sup>	
	ALDH2*3	1486G > A	Lys <sup>479</sup>	
	ALDH2*4	1464G > A	silent	
ALDH3A1	ALDH3A1*1		wild-type	17p11.2
	ALDH3A1*2	985C > G	Ala <sup>329</sup>	
ALDH3A2	ALDH3A2*1		wild-type	17p11.2
	ALDH3A2*2	521T del	frameshit	
	ALDH3A2*3	808G del	frameshit	
	ALDH3A2*4	941 del 3bp, 941 ins 21bp	Gly <sup>314</sup> , Ala <sup>315</sup> , 6 aa ins	
	ALDH3A2*5	641G > A	Tyr <sup>214</sup>	
	ALDH3A2*6	1297GA del	frameshit	
	ALDH3A2*7	1311 ins 5bp	frameshit	
	ALDH3A2*8	1297GA del	frameshit	
ALDH4A1	ALDH4A1*1		wild-type	1
	ALDH4A1*2	21G del	frameshit	
	ALDH4A1*3	1055C > T	Leu <sup>352</sup>	
	ALDH4A1*4	47C > T	Leu <sup>16</sup>	
	ALDH4A1*5	1563T ins	frameshit	
ALDH9A1	ALDH9A1*1		wild-type	1q22-q23
	ALDH9A1*2	344G > C	Ser <sup>115</sup>	
	ALDH9A1*3	327C > T	silent	

ins: insertion; del: deletion; bp: base pair; aa: amino acids. \*Adapted from [7].

human populations for the  $\alpha$ -,  $\pi$ - and  $\chi$ -subunits of ADH [8]. It has been suggested that the ADH variants may be involved in different attitude to alcoholism, since allele frequencies differ between alcoholics and controls [13].

The *ADH2* gene may be present as *ADH2\*1*, *ADH2\*2* and *ADH2\*3* encoding for  $\beta$ 1,  $\beta$ 2, and  $\beta$ 3 subunit, respectively, which differ by single nucleotide exchanges; however, the difference of a single amino acid determines in the protein quite different catalytic properties. The enzyme containing the  $\beta$ 1 subunit has high affinity and low capacity for ethanol, whereas the  $\beta$ 2 and the  $\beta$ 3 forms show lower affinity and higher capacity: the  $V_{\max}$  of  $\beta$ 2 homodimers is around 40-fold higher than that of  $\beta$ 1 homodimers. As a consequence, the activities related to  $\beta$ 2 and  $\beta$ 3 subunits are not highly capacity-limited by large amount of ethanol ingestion.

*ADH3* (encoding the  $\gamma$  subunits) is also polymorphic; however, the functional meaning of these variants is limited, since the  $\gamma$ 1 homodimers (encoded by *ADH3\*1*) have an only two-fold higher  $V_{\max}$  than the one measured for  $\gamma$ 2 homodimers (encoded by *ADH3\*2*) [14].

Different tissues show differentially measurable human *ADH* gene expression; liver contains a large amount of ADH (representing about 3% of total soluble proteins in the hepatocyte) and expresses the widest number of isozymes, mainly class I. ADH5 ( $\chi$ -ADH) is ubiquitously expressed in all human tissues tested so far; ADH4 ( $\pi$ -ADH) is solely expressed in liver, while ADH7 ( $\sigma$ -ADH) is the only isoform expressed at low level in the liver [6], but present at significant amounts in gastrointestinal tissue, mainly in the gastric mucosa of Caucasian, but nearly absent in Asians [15]. Similarly, a low ADH activity has been demonstrated in the gastric mucosa of females of Caucasian origin [16]. This feature has been associated with the lower gastric first pass effect in the toxicokinetics of ethanol observed both in Asian populations and females, as well as with ethanol decreased clearance and consequently with increased alcohol blood levels which may contribute to the higher susceptibility of females to ethanol-induced effects.

The actual role of ADH in alcohol related pathologies has not been elucidated yet. It is not clear the mechanism according to which heavy drinkers with liver damage and other gastrointestinal disorders show

a higher serum ADH activity [17], in spite of an equal rate of ethanol metabolism with respect to controls. Although some correlation between ADH polymorphisms and ethanol susceptibility has been observed, the effect of ADH variants on the risk of alcoholic liver disease could be complex: high activity-ADH variants decrease alcoholism risk in carrying individuals, but if they persist in drinking, the risk for hepatic injury might increase, resulting from high intrahepatic concentrations of acetaldehyde [18].

The prevalence of the variant forms of ADH vary in different ethnic populations: 95% of Caucasians have the  $\beta$ 1 enzyme form (*ADH2\*1*); the 90% Orientals have the  $\beta$ 2 form (*ADH2\*2*) which is present in Europe with frequency varying from 5–10% of the English up to 20% of the Swiss population; the  $\beta$ 3 form (*ADH2\*3*) is present in the 24% Africans and African-Americans [14]. Analogously for *ADH3\*1* and *ADH3\*2*: 90% of Asians and 50% of Caucasians have the  $\gamma$ 1 and  $\gamma$ 2 form, respectively [19]. In *Table 1*, the molecular basis of polymorphic changes in various ADH alleles and the effect on enzyme activity are summarized.

### ALDEHYDE DEHYDROGENASES

Acetaldehyde, the toxic metabolite produced by enzymatic ethanol oxidation in the human liver, is further metabolized by ALDH in a  $\text{NAD}^+$ -dependent reaction. These enzymes have broad substrate specificity for aliphatic and aromatic aldehydes, which are irreversibly oxidized to their corresponding carboxylic acids. The ALDH are cytosolic enzymes, expressed in a wide range of tissues [8]. A number of isoenzymes of ALDH coded by different gene loci have been detected in humans, which differ in their electrophoretic mobility, kinetic properties, as well as in their cellular and tissue distribution and show a certain degree of overlapping substrate specificity.

Genes coding for ALDH enzymes are divided into nine major families (*Table 2*); the major ones are family 1 corresponding to cytosolic ALDHs (ALDH1), family 2 to mitochondrial ALDHs (ALDH2), and family 3 which groups the major constitutive and inducible ALDH forms (ALDH3) found in human stomach, saliva, and hepatocarcinoma [8]. On the basis of kinetic properties and sequence similarities, the nomenclature for ALDH proteins has been recently revised; they have been tentatively classified as class 1 (low  $K_m$ , cytosolic), class 2 (low  $K_m$ , mitochondrial) and class 3 (high- $K_m$  ALDH, such as those expressed in tumors, stomach and cornea) [18].

There are multiple molecular forms of ALDH in human liver, but only class I and class II isozymes, encoded by *ALDH1* and *ALDH2* genes, respectively, are thought to be involved in acetaldehyde oxidation [6].

Both ALDH1 and ALDH2 are homotetrameric enzymes, characterized by isoenzyme specific subunits (MW  $\approx$ 54 kD each) and by different catalytic properties. The lowest  $K_m$  values for acetaldehyde has been measured for the mitochondrial ALDH2 (0.2–1  $\mu\text{M}$ ), although also ALDH1 has a relatively high affinity for

the substrate ( $K_m$  value around 30  $\mu\text{M}$ ), consistently with their relevant involvement in ethanol oxidation process. ALDH3 and ALDH4 show a lower affinity towards both acetaldehyde and propionaldehyde as substrates: indeed their  $K_m$  values are in the millimolar range ( $\approx$ 11 mM).  $\text{NAD}^+$  is the preferred coenzyme for the low  $K_m$  isoenzymes (ALDH1 and ALDH2), whereas the high  $K_m$  isoenzymes (ALDH3 and ALDH4) can use either  $\text{NAD}^+$  or  $\text{NADP}^+$ .

In addition to the above mentioned genes, a number of additional genes have been cloned and characterized in humans [20]; the major ones will be briefly listed in the following.

*ALDH1B* (ALDH5): it is expressed in various tissues including liver, brain, adrenal gland, testis, stomach, and parotid gland. *ALDH1A6* (ALDH6): this isoenzyme is primarily expressed in the salivary gland, stomach, and kidney. The cDNA encodes 512 amino acid residues and shows a 70% sequence homology with ALDH1. *ALDH3B1* (ALDH7): the isoenzyme is expressed mainly in the kidneys and lungs; the cDNA encodes 468 residues with a 52% positional identity with ALDH3. *ALDH3B2* (ALDH8): the gene product shows 85% homology with of ALDH7. *ALDH9A1* (ALDH9): the isoenzyme has a high activity for oxidation of gamma-aminobutyraldehyde and other amino aldehydes [8].

Genetic polymorphisms have been reported in a number of *ALDH* genes: nucleotide changes and effect on encoded proteins are listed in *table 2*. Due to the major role of mitochondrial ALDH2 in acetaldehyde oxidation [21], the genetic factor that most strongly correlates with reduced ethanol consumption and incidence of alcoholism is human *ALDH2* functional gene polymorphism. The enzyme is encoded by two distinct alleles in chromosome 6: *ALDH2\*1* (wild type allele) and *ALDH2\*2*, differing for the substitution glutamate-to-lysine at position 487 (E487K) due to a single point mutation (transition G $\Rightarrow$ A). Although the difference between the two alleles appears to be minimal, the proximity in the primary structure between the mutation site and the region containing cysteine residues, very likely involved in the catalytic cycle, is compatible with the phenotypic decrease in ALDH2 activity, associated with the variant genotype. Indeed, individuals homozygous for the mutated *ALDH2\*2* allele are completely lacking ALDH2 activity, whereas heterozygous individuals showing the *ALDH2\*1,2* genotype maintain about 30–50% of the ALDH activity, shown by individuals carrying wild type gene. Blood acetaldehyde levels of *ALDH2\*2* homozygous individuals are 6-to-20 fold higher than in *ALDH2\*1* gene carriers, in which acetaldehyde is hardly detectable after ethanol consumption. The acetaldehyde blood concentrations reached in individuals homozygous for *ALDH2\*2* cause unpleasant side-effects (flush syndrome) which protects them from alcoholism. However, heterozygous individuals may become heavy drinkers or even alcoholics, thus experiencing the toxic effects due to acetaldehyde production [22].

Oriental show the presence of the inactive ALDH2 isoenzyme phenotype in approximately 50% of the individuals [17, 23], whereas no ALDH2-deficient Caucasian or Negroid have been identified so far [24]. This is the reason why Orientals exhibit intense facial flushing after a mild dose of alcohol as compared to Caucasians, thus affecting their drinking habits. The percentage of heavy and moderate drinkers is higher among Caucasians, while abstainers and infrequent drinkers are more frequent among the Chinese and Japanese [8].

About 40% of the South American Native Indians (Mapuche, Atacamen's, Shuara tribes) showed the presence of *ALDH2\*2*, which on the contrary has been detected only in a very small percentage of the North American Indians (Sioux, Navajo and Mestizo) [25].

### Microsomal CYP2E1

After ingestion of low amount of alcohol, about 10% of ethanol is metabolized in the liver by the microsomal cytochrome P450 CYP2E1, which catalyzes its oxidation to acetaldehyde and then to acetate [26]. During the reaction, CYP2E1 generates reactive oxygen species (ROS) such as H<sub>2</sub>O<sub>2</sub>, superoxide anion (O<sub>2</sub><sup>-</sup>), hydroxyl (•OH) and substrate-derived radicals (1-hydroxyethyl radical), which can cause oxidative stress, triggering lipid peroxidation, protein inactivation, increased cytokine

production, mitochondria and DNA damage leading to cell death [27].

Liver damage associated to ethanol consumption is hypothesized to be due at least partially to oxidative stress associated to its metabolism. Indeed alcohol-induced liver disease (ALD) has been related to the increased production of free radicals as well as to the decreased availability of antioxidants and/or impaired activity of a number of enzymatic systems able to detoxify ROS and their by-products, including Glutathione S-Transferases (GST), superoxide dismutase, glutathione peroxidase and catalase.

The potential sources of ROS in ALD are compartmentalized to (i) microsomes, *via* CYP2E1 and cytochrome P450 reductase; (ii) mitochondria, *via* the electron transport chain; (iii) peroxisomes, *via* fatty acid oxidases; and (iv) cytosol, *via* xanthine oxidase and aldehyde oxidase. However, among all the potential hepatic sources of ROS, CYP2E1 has been a center of attention for its pathogenic role in ALD [28].

In addition to ROS, the 1-hydroxyethyl radicals produced by CYP2E1 during ethanol oxidation bind covalently to proteins forming adducts able to induce autoantibodies which have been found in human alcoholics [29, 30]. These antibodies may represent markers of the production of ethanol-derived free radical ad-

**Table 3** | Polymorphisms of CYP2E1, glutathione S-transferase M1, P1 and T1 genes

Protein	Gene	Nucleotide change	Effect	RFLP	Chromosomal location
CYP2E1.1	CYP2E1*1A	None (Wild Type)		PstI-/RsaI + (c1 allele)	10q24.3-qter
	CYP2E1*1B	9893C >G		TaqI -	
	CYP2E1*1C	6 repeats in the 5' flanking region			
	CYP2E1*1D	8 repeats in the 5' flanking region		DraI- + XbaI	
CYP2E1.2	CYP2E1*2	1132G > A	His76		
CYP2E1.3	CYP2E1*3	10023G > A	Ile389		
CYP2E1.4	CYP2E1*4	4768G > A	Ile179		
CYP2E1.1	CYP2E1*5A	-1293G > C; -1053C > T; 7632T >A		PstI+/RsaI-/DraI-	
	CYP2E1*5B	-1293G > C; -1053C > T		PstI+/RsaI-(c2 allele)	
	CYP2E1*6	7632T >A		DraI-	
	CYP2E1*7A	261T > A			
	CYP2E1*7B	-71G > T; 261T > A			
	CYP2E1*7C	-261T > A; 280G > A			
GSTM1	GSTM1*A	G519	Lys173		1p13.3
	GSTM1*B	C519	Asn173		
	GSTM1*0	deleted	no expression		
	GSTM1*A/B x2	duplicated	overexpression		
GSTP1	GSTP1*A	A313; C341	Ile105; Ala114		11q13.3
	GSTP1*B	313 A > G	Val105; Ala114		
	GSTP1*C	313 A > G; 341C > T	Val105; Val114		
	GSTP1*D	341C > T	Ile105; Val114		
GSTT1	GSTT1*A	wild-type	wild-type		22q11.23
	GSTT1*0	deleted	no expression		

*CYP2E1: cytochrome P450 Isoform CYP2E1; GSTM1: glutathione S-Transferase M1; GSTP1: glutathione S-transferase P1; GSTT1: glutathione S-transferase T1.*  
\*Adapted from [7] and [36].

ducts and contribute to the hepatotoxicity of ethanol in promoting immune mechanisms of liver injury [30].

The CYP2E1 protein is regulated both transcriptionally and post-transcriptionally through a substrate-induced protein stabilization; chronic ethanol consumption leads to an increase in CYP2E1 protein, by decreasing its degradation, without affecting its mRNA. Beside ethanol consumption other xenobiotics (acetone), a fatty diet, diabetes, obesity or starvation may lead to CYP2E1 induction, contributing to modulate ethanol metabolism.

The induction of CYP2E1 hepatic content, beside increasing ethanol clearance, has been demonstrated to positively correlate with the generation of hydroxethyl radicals and lipid peroxidation. Consistently, induction of CYP2E1 has been shown to result in enhanced hepatic injury, whereas inhibition of CYP2E1 was associated with an improvement of these lesions [22]. In addition, increased ethanol metabolism may contribute to the development of alcohol dependence: faster ethanol inactivation during long-term alcohol drinking may increase motivation to consume more alcohol in order to maintain a desired level of ethanol at target sites [31].

The *CYP2E1* gene has been localized to chromosome 10 and consists of 9 exons and 8 introns, encoding a 493-amino acid protein. Ten polymorphic loci on human *CYP2E1* gene have been reported so far, most of them in the promoter and intron regions. In addition, a tandem repeat was identified in *CYP2E1* regulatory region [32]. *CYP2E1* gene polymorphisms are listed in Table 3.

A *RsaI* restriction fragment length polymorphism (RFLP) has been reported in the 5'-flanking region of the *CYP2E1* gene. The rare mutant allele (*c2* allele) lacking the *RsaI* restriction site has been found to be associated with higher transcriptional activity, protein levels and enzyme activity than the wild-type *c1* allele. Moreover, the frequency of *RsaI c2* allele varies in different populations: the highest frequency has been observed in the Taiwanese (28%) and Japanese populations (19-27%), while the frequency is much lower (1-5%) in Africans [8]. The enhanced transcriptional activity of *CYP2E1 c2/c2* might play a role in the development of severe ALD [33]. In individuals carrying the *ADH3\*2* allele, the presence of the *CYP2E1 c2* allele increases the risk of ALD, presumably reflecting increased metabolism of ethanol by CYP2E1. The relevance of combination of different genotypes in modulating the risk is suggested by the fact that in the absence of the *CYP2E1*, *ADH3* genotype itself does not influence the risk of ALD [34].

The polymorphic *CYP2E1\*ID* has been associated with greater CYP2E1 inducibility and it has been suggested to contribute to the development of alcohol and nicotine dependence. *CYP2E1\*ID* allele contains a repeat sequence in the 5' flanking region of the gene that may disrupt negative regulatory elements. Homo- and heterozygous individuals for *CYP2E1\*ID* gene were found to have greater CYP2E1 activity after ethanol consumption [32].

CYP2E1 is also involved in the metabolism of various other xenobiotics, including procarcinogens, industrial and environmentally relevant small molecular weight volatile organic chemicals. Therefore, chronic ethanol consumption, leading to CYP2E1 induction, may result in the increased conversion of known hepatotoxic agents to their toxic metabolites [22], possibly explaining the enhanced susceptibility of alcoholics to the adverse effects of industrial solvents [33].

The finding of CYP2E1-mediated bioactivation of xenobiotic in prenatal human brain tissue seems of extreme interest. Significant levels of activity and specific mRNA were detectable between gestational days 45 and 53, a period during which embryogenesis overlaps with organogenesis, taking place at 50-60<sup>th</sup> days of gestation. The mRNA levels increase up to days 80-84, then remain almost constant throughout the early foetal period [35] and may be increased by ethanol itself or by its strong effect on maternal nutritional status (*i.e.* altered fat or vitamin A and B intake). The presence of CYP2E1 during organogenesis in the brain, the target organ of alcohol teratogenesis, has been associated with the appearance of foetal alcohol syndrome (FAS), as a result of alcohol consumption during pregnancy. FAS is characterised by a particular pattern of facial anomalies, growth retardation and developmental abnormalities in the central nervous system that could result in mental retardation. Even if FAS is not evident, some evidences indicate that adults, who had been prenatally exposed to alcohol, frequently suffer from mental disorders and maladaptive behaviours and are prone to become alcoholics themselves. Damages in the foetal brain due to alcohol consumption by the mother during gestation has been associated to the presence of many polyunsaturated fatty acid side chains in the membranes of embryonic and foetal brain, making the tissue a highly susceptible target for ROS and radicals arising from CYP2E1-mediated ethanol metabolism *in situ*. The damages in the brain caused by lipid peroxidative processes triggered by ROS might be manifested as the central nervous system dysfunction after birth, described as FAS, although other factors such as decreased levels of retinoic acid may act concurrently.

#### **Glutathione S-transferases**

Glutathione S-transferases (GST) are phase II xenobiotic metabolizing enzymes, acting as a highly efficient detoxification system. They catalyze the conjugation of harmful electrophilic compounds with reduced glutathione (a tripeptide present at relatively high concentrations in the cytosol), to produce less toxic or readily excreted metabolites. Moreover, these enzymes have a strong antioxidant function and protect cells from the natural by-products of lipid peroxidation and oxidative stress [36].

Since the implication of ROS, generated during ethanol metabolism and by ethanol-induced cell damage, has been postulated in the etiology of alcohol-induced pathologies, GST activity may play a central role by detoxifying both ROS and other ethanol-derived free radicals, as suggested by the alteration of GST expression in the liver of patients with ALD [37].

The cytosolic GSTs are dimeric proteins with each subunit having 22–28 kD MW. On the basis of their amino acid sequence, in humans 8 families of the cytosolic forms have been identified and named with greek symbols, each class consisting of various isoenzymes; GSTs mainly involved in ROS detoxication belong to Alpha, Mu, Theta and Pi families [38].

The  $\alpha$ -GST are very abundant hepatic homo- or heterodimers, in humans accounting for about 85% of the total GST protein. The dimerization of two distinct subunits (A1 and A2) gives rise to GSTA1-1, GSTA1-2 and GSTA2-2. Other  $\alpha$ -GST have been localized in extrahepatic tissues, such as GSTA3 and GSTA5, mainly expressed in the skin.

Alpha, Mu and Pi GST can detoxify harmful  $\alpha,\beta$ -unsaturated carbonyl, such as 4-hydroxynonenal generated by lipid peroxidation and the products of oxidative DNA damage mediated by free radicals. The alpha-GST, as well as the microsomal membrane bound GST, exhibits glutathione peroxidase activity, suggesting an additional defense mechanism against lipid peroxidation associated with ethanol consumption.

In humans, genetic polymorphisms have been described in *GSTM1*, *GSTT1* and *GSTP1* genes. Among the described polymorphisms at the *GSTM1* locus on chromosome 1p13.3, the most studied encodes for a gene deletion (*GSTM1* null genotype), resulting in a complete absence of *GSTM1* enzyme activity. The frequency of the *GSTM1* null genotype ranges from 23 to 62% in different populations and is approximately 50% in Caucasians [38].

For *GSTT1* locus, located on chromosome 12q11.2, one polymorphism has been described. The *GSTT1* null genotype represents a gene deletion and is associated with the absence of functional enzyme activity. The frequency of these null genotypes ranges from 16 to 64% in different populations being approximately 20% in Caucasians [39].

Although the absence of an active GST isoform may be of relevance for the total detoxifying capacity of the cell, compensation mechanisms due to the overlapping substrate specificities exhibited by different GST can limit the consequent functional impairment. However, individuals with the homozygous *GSTM1* or *GSTT1* null genotypes express no protein of two major human GST isoforms, highly express in the gastric and intestinal mucosa, are expected to have a reduced ability to detoxify reactive metabolites resulting from ethanol metabolism.

At least four different polymorphisms have been described at the *GSTP1* locus on chromosome 11, the most important of which encoding for an enzyme with altered activity. Polymorphisms of the *GSTP1* gene

consists of an A-to-G transition of nucleotide 313 in exon 5 (*GSTP1\*B*) and a C-to-T transition of nucleotide 341 in exon 6 (*GSTP1\*C*), involving the substitution of two amino acids in the enzyme active site, Ile  $\Rightarrow$  Val and Ala  $\Rightarrow$  Val. These allelic variants appear to influence *GSTP1* activity, therefore modulating the risks of damage [36]. The amino acid 105 is proximal to the enzymatic active site, therefore it is not surprising that this residue can modulate the catalytic activity. The same transition may occur also in position 114, but the functional consequences of this mutation have not been clarified yet.

The *GSTP1* Val<sup>105</sup>/Val<sup>105</sup> polymorphism is very common and may result either in reduction or increase of the enzyme activity of the compared to the wild type form (Ile<sup>105</sup>), dependent on the electrophilic structure of the substrate [38]. As an example *GSTP1* Val<sup>105</sup>/Val<sup>105</sup> genotype has been shown to be protective against asthma symptoms, since the mutated gene is more efficient in scavenging ROS formed during the chronic inflammation process associated with the pathology, thus protecting lung cells from damages produced by oxidative stress [38].

## CONCLUSIONS

Ethanol-induced adverse effects result from a broad range of complex interactions between environmental, behavioral, genetic and social factors. There is a high ethnic and interindividual variability in the occurrence and gravity of alcohol related pathologies, often not correlated to the amount of ethanol intake.

In the last years the gender-related different susceptibility has focused the attention on women vulnerability to ethanol and particularly to the possible teratogenic effects, as a consequence of alcohol consumption during gestation, resulting in neurobehavioral disorders in the adult. These differences have been mainly ascribed to ethanol toxicokinetics and polymorphisms of metabolic enzymes, in combination to socio-cultural factors, whose contribution cannot be disregarded.

However, in order to identify the determinants of multifactorial diseases such as ALD and other alcohol-related disorders, evaluation of functional polymorphism at multiple genes is necessary. The identification of possible biomarkers of susceptibility will represent the main goal of the near future and will contribute to the implementation of adequate prevention strategies, to the development of effective diagnostic test strategies, to detect higher risk drinking behavior and early indicators of tissue damage.

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# Alcohol intake during prenatal life affects neuroimmune mediators and brain neurogenesis

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**Summary.** Several lines of evidence suggest that alcohol exposure during prenatal gestation, or during early postnatal life may be a risk factor for the manifestation of neurological and for immune-related disorders in later life. The cellular, biochemical and molecular mechanisms of ethanol toxicity, however, have not been yet clearly established. Recent studies indicated that neurotrophin signaling pathways may be involved in ethanol mediated cell death. The present investigation addressed the question of whether nerve growth factor (NGF), which is the first and best characterized member of the neurotrophin family, and NGF-target cells are affected by prenatal exposure to alcohol. The result of our study indicates that NGF synthesis and the functional activity of NGF-target cells localized in the brain are markedly influenced by ethanol intake. The possible link between such changes and the hypothesis that these alterations may contribute to certain of the neuropathology observed following alcohol exposure would be discussed.

*Key words:* ethanol, neurotrophins, NGF-receptors, brain, ChAT, brain development, brain damages.

**Riassunto** (*Effetto dell'esposizione prenatale all'alcol sui mediatori neuroimmunologici e sulla neurogenesi del cervello*). Diverse osservazioni sperimentali suggeriscono che l'esposizione all'etanolo durante il periodo gestazionale o in una fase precoce della vita post-natale costituisce un fattore di rischio per la manifestazione di disordini neurologici e di natura immunitaria nell'età adulta. I meccanismi cellulari, biochimici e molecolari alla base della tossicità da etanolo, comunque, non sono stati ancora completamente chiariti. Studi recenti indicano che il segnale cellulare trasdotto dalle neurotrofine potrebbe essere coinvolto nella morte cellulare indotta da etanolo. In questa ricerca abbiamo cercato di capire se il fattore di crescita nervoso (NGF), che è il primo e meglio caratterizzato membro della famiglia delle neurotrofine, e le cellule target dell'NGF sono sensibili all'esposizione di etanolo in età prenatale. I risultati del nostro studio indicano che la sintesi di NGF e l'attività funzionale delle cellule target dell'NGF nel cervello sono fortemente compromesse a seguito di assunzione di etanolo. Il possibile legame tra questi fenomeni osservati e l'ipotesi che questi eventi possano contribuire ad alcune alterazioni neurologiche osservate a seguito di esposizione all'etanolo saranno oggetto di discussione.

*Parole chiave:* etanolo, neurotrofine, recettori dell'NGF, cervello, ChAT, sviluppo cerebrale, danni cerebrali.

## INTRODUCTION

Several lines of evidence indicate that alcohol exposure during prenatal gestation can influence cell proliferation and differentiation in the central nervous system (CNS) causing brain growth retardation and deficits in the limbic areas involved in cognitive functions [1-4]. The cellular, biochemical and molecular mechanism implicated in the deleterious action of alcohol intake are not fully known. Studies reported in recent years led to the hypothesis that alcohol can act on biological mediators, including growth factors synthesis and release by cells of the CNS. Nerve growth factor (NGF) is a member of the family of proteins known as neurotrophins, including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5 and NGF is one of the most thoroughly studied neurotrophic factors, playing a crucial role in the survival and development of specific peripheral and brain neurons [5-10]. NGF is

produced and released by a variety of cells localized in the central and peripheral nervous system and by cells of the immune and endocrine systems [11]. Under normal conditions NGF is present in the bloodstream of rodents and humans and increases during stressful events, neuroendocrine alterations, and following neurological insults [12, 13]. Within the CNS, NGF is produced in frontal cortex, the hippocampus (HI) and the hypothalamus and exerts a trophic action on the cholinergic neurons of the basal forebrain, particularly at the level of the medial septum and the nucleus basalis of Meynert and Broca's diagonal band, both during development and in adulthood [8, 10]. Studies published in recent years have shown that alcohol abuse can also lead to immune neuroimmune-related pathologies suggesting that NGF plays a crucial role of survival, differentiation and function, not only of nerve cells, but also of immuno-competent cells [11, 12].

Aim of the present study was to address further the question of brain NGF distribution in the CNS of rodents that have experienced ethanol abuse during prenatal life and to assess whether ethanol intake affected the distribution of brain progenitor cells in postnatal life.

## MATERIALS AND METHODS

### *Animals and housing*

Adult pregnant Sprague-Dawley rats were purchased from Charles River, Italy. The animal was kept under standardized conditions with pellet food and tap water available *ad libitum*. Animal care procedures were implemented according to the intramural committee and institutional guidelines that are in compliance with the national and international laws and policies (EEC Council Directive 86/609, OJL 358, I December 12, 1987). At day 15 of pregnancy one group of rats (no. = 6) received a single administration of intragastric ethanol (20%, v/v), 4 g/kg body weight (bw), under anesthesia. A second group of pregnant rats (no. = 8) were similarly treated, but the ethanol was replaced by an isocaloric equivalent of sucrose (4% v/v, 2g/kg body weight) as control. This stage of pregnancy was chosen because previous studies have shown that brain cells are particularly affected during this particular prenatal developmental age.

At birth each litter was reduced to 8 pups and divided in two different groups: (no. = 4)-sucrose group, 0.15 ml/10 g body weight (bw) of a 5% w: v sucrose solution, equivalent caloric for ethanol intake. At 4 weeks of postnatal life, sucrose and ethanol-treated rats were deeply anaesthetized with sodium pentobarbital and animals sacrificed for biochemical and structural studies.

### *NGF determination*

Brain tissues were dissected and processed for quantification of endogenous NGF by a highly sensitive and specific two-site enzyme immunoassay, described in detail previously [14]. Briefly, 96-well immunoplates (NUNC) were coated with 50  $\mu$ l per well of 0.4  $\mu$ g/ml monoclonal anti-mouse-betaNGF antibody 27/21 (Boheringer, Mannheim, Germany). Parallel wells were coated with mouse IgG for evaluation of non-specific signals. After an overnight incubation at room temperature (20 °C) the plates were washed three times with washing buffer and the samples were incubated in the coated wells (50  $\mu$ l each) overnight at room temperature. After an additional three washes the immobilized antigen was incubated with 0.5 mU per well monoclonal antibody 27/21 conjugated with b-D-galactosidase (Boheringer, Mannheim, Germany) for 2 h at 37 °C. The plates were again washed with washing buffer, and then finally incubated with chlorophenol-red-b-D-galactopyranoside (Boheringer, Mannheim, Germany) in substrate buffer for another 2 h at 37 °C. The colorimetric reaction product was measured at 570 nm using a microplate reader (Dynatech MR 5000, PBI International). NGF concentrations were determined

from the regression line for the NGF standard (ranging from 1.56 to 50. pg/well purified mouse NGF) incubated under similar conditions in each assay. Recombinant BDNF is not recognized in the ELISA at concentrations up to 20 ng/ml [14].

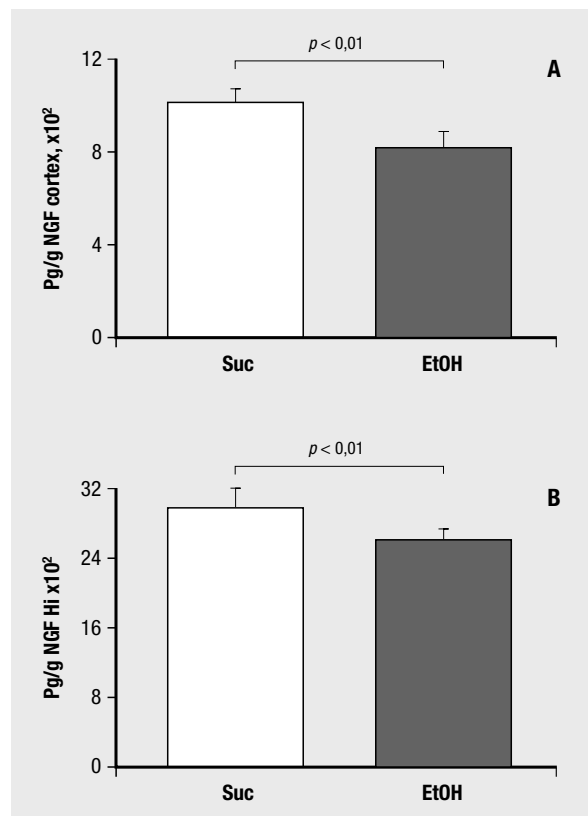
Data are presented as means  $\pm$  SEM for each tissue and animal group investigated. Means of alcoholics and controls were compared by analysis of variance (ANOVA) and statistical significance was accepted at a level of  $p < 0.05$ .

### *ChAT and low-affinity*

#### *NGF-receptor immunohistochemistry*

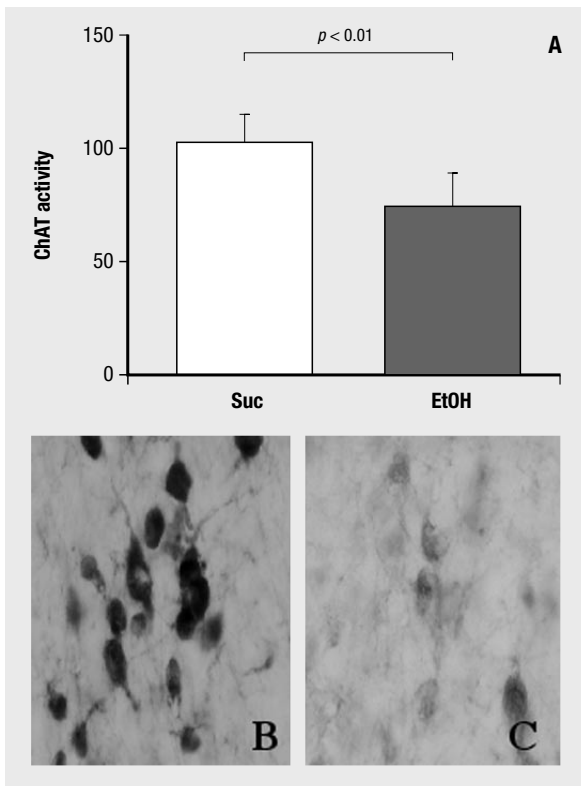
For these studies rats were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS), pH 7.4, the brains removed and placed in a cryoprotectant solution of 20% sucrose in PBS, serial coronal sections (30  $\mu$ m) were cut on a cryostat and used for routine histological analysis and stained with toluidine blue, or for immunohistochemistry to identify NGF-target cells.

For immunohistochemical localization in brain NGF-responsive cells we used ChAT monoclonal antibody (Mab17 kindly provided by Dr. Costantino Cozzari, Institute of Cellular Biology, CNR Italy) and low-af-



**Fig. 1** | Levels of NGF in the cortex (A) and HI (B) of rats exposed at 15 days of prenatal life to sucrose (Suc) or ethanol (EtOH). Note the decrease of NGF concentration in these two brain regions after prenatal ethanol intake. These differences are statistically significant,  $p < 0.01$ .





**Fig. 2** | ChAT activity in the HI (A) of rats exposed at 15 days of prenatal life to sucrose (Suc) or ethanol (EtOH). Note the decrease, compared to control, of this cholinergic enzyme after ethanol intake (A). Figures B,C show ChAT-immunoreactive neurons in the septum of rats exposed to sucrose (B) or ethanol (C). Magnification X-260.

finity (p-75) monoclonal antibody, (kindly provided by Dr. E.J. Johnson from Washington University, St. Louis, MO, USA). Brain sections, including the septum, were incubated overnight with the mentioned antibodies and processed for immunoperoxidase with the ABC Vectastain Kit (Vector Lab. Inc. Burlingame, USA) following the manufacturer's instructions. Staining specificity was assessed by omission of the primary antibody and by isotypic IgG.

Quantitative analysis of ChAT- and p75-positive neurons in the basal forebrain cholinergic neurons was carried out using a Zeiss Axiophot microscope and an image analysis program (IAS 2000, Delta System, and Rome, Italy) connected to a PC computer. The number of positive neurons present in 10-matched sections/animal containing the medial septum were evaluated in experimental and control groups (no. = 4 rats/group). The average values of the pooled cell counts from each group were compared.

## RESULTS

### General observations

The amount of alcohol administered to pregnant rats does not cause lethal effect on the mother and no abor-

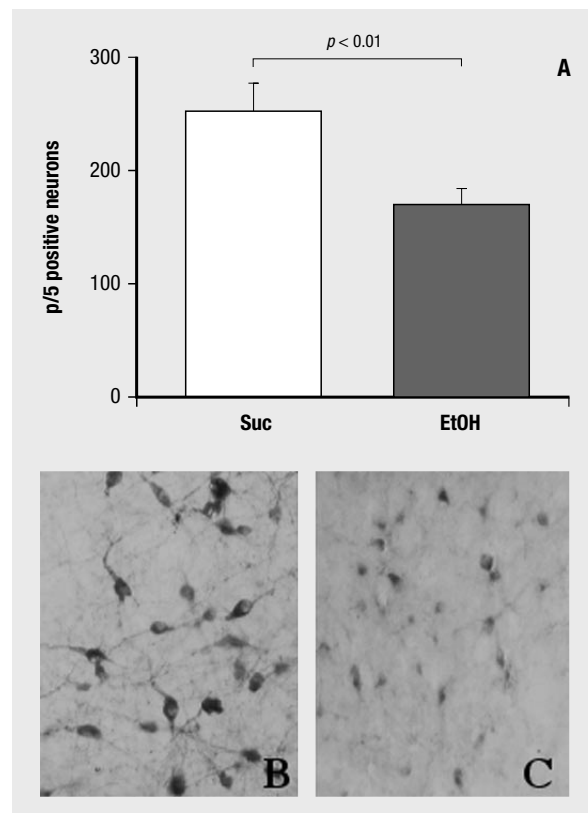
tion. At birth all pups exposed to alcohol appear normal, they weighted less compared to the pups exposed to sucrose. Two weeks after birth no differences in bw and other somatic features were observed between the two groups of rats.

### Effect of ethanol on NGF level in the cortex and HI

The cortex and HI produce and store the largest amount of NGF in the brain. As shown in Fig. 1A and Fig. 1B, respectively, the concentration of NGF in these two brain structures of rats exposed to ethanol during prenatal life are significantly lower compared to the concentration of NGF in the same brain structure of non-ethanol exposed rats.

### Effect of ethanol on NGF-target cells

Because one prominent action of the NGF produced in the HI is to provide trophic support of NGF-target neurons and to regulate the biosynthesis of the cholinergic enzyme ChAT, we measured the activity of this enzyme in the HI. As reported in Fig. 2A, ethanol exposure during prenatal life reduces significantly the presence of ChAT activity in this brain region. Rats exposed to ethanol also showed a marked decrease in ChAT immunoreactivity in cholinergic neurons located in the septum (Fig. 2B-C).



**Fig. 3** | P75-immunoreactive neurons in the septum of rats exposed at 15 days of prenatal life to sucrose (Suc) or ethanol (EtOH). Note the reduction in the number of these NGF-receptive neurons after ethanol intake (C) compared to control (B). Magnification X-120.

### ***Effect of ethanol on NGF-receptor expression***

To further characterize the role of ethanol on NGF-target cells, we analyzed the presence of NGF receptor-immunoreactivity in neurons of the septum, which receive trophic support from the NGF produced in the HI. As shown in *Fig. 3A-C*, the number of p75-positive, NGF-receptive neurons located in this brain region of rats exposed to alcohol are markedly reduced compared to sucrose-exposed rat brains.

### ***Effect of ethanol on HI progenitor cells***

The dentate gyrus of the HI generates new granule neurons also during the postnatal life. To further explore the effect of prenatal ethanol intake in brain cell plasticity, we investigated whether prenatal alcohol exposure affects brain progenitor cells. Histological and immunohistochemical analyses revealed that cells located in the dentate gyrus of ethanol-exposed rats display characteristics of cells death, not evident in the dentate gyrus of rats exposed to sucrose. The effect of ethanol on cell survival is more clearly evident when rats were received BrdU, a marker for the identification of cell proliferation. As reported in *Fig. 4A*, the dentate gyrus of rats exposed to ethanol displays a reduced number of BrdU-positive cells compared to the dentate gyrus of sucrose-exposed rats *Fig. 4B*, indicating alcohol intake can interfere with the proliferating and/or survival activity of this brain progenitor cells.

## **DISCUSSION**

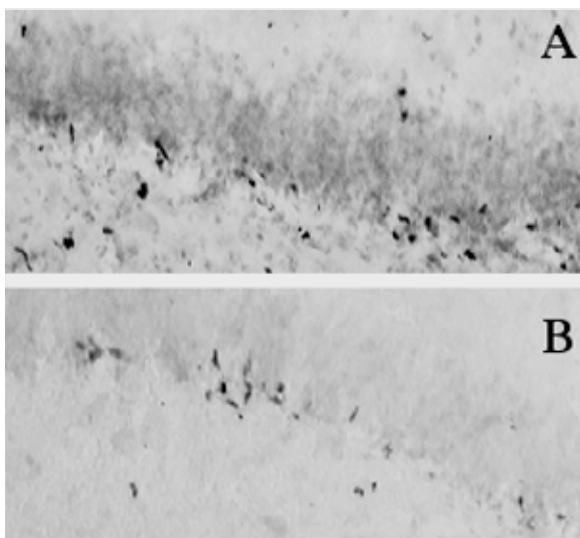
The aim of this study was to investigate the effect of prenatal alcohol exposure on NGF expression in the HI and on the cholinergic enzyme, ChAT activity in septal neurons, since survival and functional activity of these neu-

rons are NGF-dependent [10, 13]. The result of this study showed that alcohol intake during prenatal life reduces the basal level of NGF in the HI, NGF receptor immunoreactivity in septal cholinergic neurons and the presence of ChAT activity in the septum. This observation suggests that alcohol assumption during prenatal life might impair irreversibly the NGF signaling pathways. This hypothesis is consistent with our previous studies showing that prenatal ethanol exposure reduces NGF synthesis in the HI and expression of p-75 NGF-receptor in the offspring [6, 15]. Since NGF is known to be implicated in preventing neuronal damages and in protecting cell death, the reduced synthesis and release of NGF represent negative event for neuronal growth, survival, and differentiation. Our observation therefore suggests that the reduced presence of NGF concurs to the development manifestation of neurological deficits induced by ethanol intake.

Moreover, because brain cholinergic neurons are critically involved in learning and associative processes, the reduced activity of this cholinergic enzyme indicates the existence of a link between brain NGF synthesis and release and functional activity of brain cholinergic neurons. This hypothesis is consistent with findings obtained with aged animals showing that impairments of the brain cholinergic pathway can be attributed at an insufficient production and/or uptake of NGF. Thus, the deficit in learning and cognitive abilities resulting after ethanol intake might be also associated to a reduced synthesis of NGF, a molecule which plays a crucial role in the regulation of basal forebrain cholinergic pathways and learning processes [8-10].

Several lines of evidences suggest that deficits in cell survival and/or cell migration during brain development is a prominent risk factor for the manifestation of neurological disorders in later life [16, 17]. Given the role of NGF in neurogenesis in shaping hippocampal plasticity, it is possible that NGF-hippocampal interaction underlies the decrease of cell proliferation.

We have recently reported that NGF is involved in the survival and differentiation of brain progenitor cells [18, 19]. Given that alcohol intake induces down-regulation of NGF availability, it is possible that a low presence of NGF can cause attenuation in neurogenesis. This interpretation predicts that animals treated with alcohol prior to BrdU injection should show similar changes. Our results indeed indicate that rats prenatally exposed to alcohol also display a decrease in cell proliferation in the dentate gyrus. The observation that reduced NGF presence in the HI of alcohol-exposed rats prompted us to investigate whether low presence of NGF might reduce the properties of progenitor cells present in the dentate gyrus. The observation that the number of these cells are reduced in the dentate gyrus of rats exposed during fetal life to alcohol suggests that the neurotoxic action of alcohol include also impairment of functional activity of brain progenitor cells, occurring, most probably through a mechanism involving down-regulation of NGF and NGF-receptors expression. The report that ethanol exposure of neonatal rats reduced the expression of both p75 and trkA NGF receptors on the Purkinje cell dendrites further supports the hypothesis that ethanol in-



**Fig. 4** | Immunolocalization of proliferating cells in the hippocampal formation showing a reduced number of BrdU-positive cells in the dentate gyrus of ethanol exposed rats (B) compared to the presence of BrdU-positive cells in the dentate gyrus of sucrose exposed rats (A). Magnification X-180.

terferes with neurotrophic support of these brain cells by reducing the levels of available NGF receptor. A number of studies have reported that injured brain induced by neurotoxic compounds or surgery intervention can lead in HI-based learning, it is reasonable to hypothesize that one mechanism by which alcohol affects neurogenesis is to provoke changes in NGF synthesis.

Alcohol consumption is known to be associated with decreased cellular immune response and increased susceptibility to infections [20, 21]. It is also known that a number of immuno-competent cells are able to produce NGF and/or are receptive to the action of this growth factor [22, 23] and that ethanol intake, both during prenatal, or postnatal life can severely affect the functional activity of the immune system. We have recently reported that human macrophages exposed *in vitro* to ethanol are characterized by altered ability to produce and to respond to the action of NGF [22]. This finding along with the observations that the exposure to etha-

nol induced in M/M cell population a sharp decrease of phagocytosis and bactericidal activity [24] suggests that the lack of NGF synthesis might lead to an impairment of innate immunity in alcohol addiction.

In conclusion, the evidence that alcohol-induced decrease of hippocampal NGF down-regulates ChAT activity and reduces cell neurogenesis in the dentate gyrus, suggests that the altered brain basal level of NGF need to be considered as an important mechanism implicated in neurological and neuro-immune based deficits during development and most probably also in adult life.

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# Exposure to low and moderate doses of alcohol on late gestation modifies infantile response to and preference for alcohol in rats

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**Summary.** Several studies in rats have found that maternal administration of low or moderate doses of ethanol result in fetal perception of the chemosensory and toxic effects of ethanol. This prenatal experience with the drug enhances the palatability of ethanol's flavor and increases ethanol consumption during infancy and adolescence. The acquired preference for ethanol seems to be a conditioned response established prenatally, by the association of ethanol's sensory and reinforcing aspects, the latter mediated by the opioid system. These results are in accordance with data of studies in humans, and should be taken into account for clinical studies analyzing the relationship between prenatal ethanol exposure and later ethanol abuse problems.

*Key words:* ethanol, odor, taste, preference, consumption, rat, fetus.

**Riassunto** (*Esposizione a basse e moderate dosi di etanolo in gravidanza. Modificazioni nel comportamento verso l'alcol nel ratto*). Numerosi studi sugli animali hanno dimostrato che la somministrazione di basse o moderate dosi di alcol alla madre costituisce una esperienza prenatale che aumenta la gradevolezza dell'etanolo nella prole e favorisce il consumo di etanolo durante infanzia e adolescenza. La preferenza per l'etanolo sembra essere una risposta condizionata che si instaura in fase prenatale sia per gli effetti sensoriali associati all'etanolo che per gli aspetti di rinforzo mediati dal sistema oppioide. Questi studi sono in accordo con i dati ottenuti nell'uomo e possono essere presi in considerazione per gli studi clinici che riguardano la relazione tra esposizione prenatale e problemi di abuso alcolico nel corso della vita.

*Parole chiave:* etanolo, odore, gusto, preferenza, consumo, ratto, feto.

## INTRODUCTION

Studies in both humans and animals have extensively demonstrated the deleterious effects on the fetus of maternal alcohol ingestion. Although deficits produced by prenatal exposure to high ethanol levels are most severe and have been documented most significantly in children with fetal alcohol syndrome (FAS), there are data showing that children prenatally exposed to lower levels of alcohol frequently exhibit similar problems, especially those related to neurobehavioral impairment [1-3].

In recent years a growing amount of research has been published in relation to the effects of moderate drinking in humans. But, how is defined moderate drinking? Some authors have described moderate ethanol consumption as the one with a low risk for generating ethanol related problems [4]. Although the amount of factors that influence the effect of ethanol intake makes very difficult to establish a quantitative limit, in general for humans a moderate ethanol intake has been set between 1 and 1.99 drink/day for men and no more than 1 drink/day for women [5, 6]. Considering that one drink corresponds approximately to 0.5 fl oz ethanol, this consumption is equivalent to 24-28 g/day

for men and 12-14 g/day for women [4]. The inferior limit for the moderate consumers' category has been established on 4 drink/week [7]. In clinical studies on the effects of ethanol consumption during gestation a moderate drinking pattern is considered as an intake of 7 to 14 drink/week. In two longitudinal clinical studies [8, 9] in which the effects of prenatal ethanol on neurobehavioral development were analyzed, concluded that there is no clear threshold for these effects. For some behaviors, such as mental development, even the smallest dose of ethanol (0.02 to 3.49 drink/week) seems to have effects on the fetus, although most neurobehavioral outcomes have higher thresholds [5]. So far, it is not clear whether exists a limit of ethanol intake during gestation that does not produce any effect on the development of the fetus. The difficulty for conclusions on this respect comes from the impossibility to control all the factors intervening in clinical studies. Some authors indicate that for concluding whether or not prenatal ethanol exposure affects a given cognitive function, certain factors should be considered such as the test used and the age of the tested subject [5]. Other factors such as acute stress that may help the expression of deficits produced by ethanol prenatal exposure

that in normal conditions are not evidenced should be also taken into consideration [5]. Results of longitudinal studies also indicate a clear relationship between moderate ethanol intake during pregnancy and ethanol abuse problems when measured at age 14 [10] and 21 [11]. They have found that binge ethanol drinking during pregnancy is better predictor for young alcohol involvement and alcohol-related problems than other important factors such as family history for alcoholism, nicotine exposure, and other ambient variables like parent's consumption of drugs. These studies conclude that even modest levels of fetal alcohol exposure have to be considered in studies of the etiology of alcoholism and family history. Within the complex relationship between prenatal ethanol exposure and subsequent alcohol abuse several factors may influence the initiation of ethanol consumption during early stages of development. Besides the already known teratological effects of prenatal ethanol, there are other ways by which ethanol exposure *in utero* may promote subsequent ethanol directed behaviors. The use of animal models provides useful means for analyzing each of these ways in isolation as well as their interactions in controlled conditions.

#### ANIMAL STUDIES

Experimental studies in animals have indicated that prenatal ethanol may alter neurochemical systems that are involved in the motivational and reinforcing aspects of the drug's consumption. Other way by which ethanol exposure could be modifying subsequent patterns of response to the drug is related to fetal perception of its chemosensory characteristics (odor and probably taste) *in utero*. Respect to this, studies in humans have shown that human fetuses are capable of detecting chemosensory stimuli in the amniotic fluid [12]. Additional support for this has been recently presented in a study showing that flavors contained in maternal diet during pregnancy are later preferred by the infant when compared to other novel flavors [13]. In another study in which newborn babies (24-48 hours postpartum) were tested in their behavioral response to ethanol odor, it was reported that neonatal responses to ethanol odor were affected by maternal ethanol ingestion during gestation. It was observed that babies born from mothers who reported moderate ethanol intake during pregnancy displayed higher reactivity levels to ethanol odor than babies from mothers classified as infrequent drinkers [14]. Newborns from both groups of mothers were also tested in their reaction to lemon odor and, in this case, no differences in behavioral reaction were observed between subjects. In all these studies it seems clear that the fetus recognizes chemosensory aspects of substances presented in their amniotic environment, including ethanol, and that this prenatal experience may change its response to them.

#### *Acute prenatal ethanol exposure*

Similarly to what has been described in humans, in studies with animals a direct relationship between prenatal ethanol exposure and changes in postnatal

response to ethanol's odor and taste has been reported. This was clearly observed in a series of studies in which ethanol was directly administered into the amniotic fluid of each rat fetus, during only 10 min just before birth. This acute and brief alcohol exposure was enough to produce in the newborn rat an increase in motor activity in response to ethanol odor as well as an increase in ethanol intake and preference for ethanol odor in infant rats [15]. In addition, it was found an interaction between this short prenatal experience with only the chemosensory aspects of alcohol and postnatal conditioned learning involving ethanol odor or taste. Conditioned preferences for alcohol odor were obtained in infant rats after receiving paired presentations of alcohol and intraoral infusions of a sucrose solution. This conditioned preference for alcohol was enhanced in pups prenatally exposed to the drug [16]. In this same study it was also observed that the prenatal alcohol experience attenuated a conditioned aversion towards alcohol odor. Further studies showed that these effects were more clearly observed when alcohol was presented 10 min prior to cesarean delivery than with a longer delay, 30 min, or a shorter one, 3 min [16-18]. These last studies indicated that the effect observed was the result of an association between the prenatal ethanol chemosensory cues and tactile stimulation occurring during cesarean delivery. Due to the fact that in all these studies alcohol intoxication of the fetuses was explicitly avoided it can be concluded that those results are mainly related to processing of alcohol's chemosensory characteristics. Moreover, similar results were found when a non-alcohol stimulus, such as lemon, was presented to the rat fetus [16, 19].

These results are not unexpected when taking into account that, similarly to what has been described for human fetuses [12], in the rat fetus, during the last days of gestation certain olfactory subsystems are already functional [20]. Clear evidence of fetal perception and recognition of substances present in the amniotic fluid has been reported [21-23]. This fetal perception of the chemosensory properties of the amniotic environment before birth seems to be directly related to postnatal responses towards those substances. For example, it has been demonstrated that olfactory cues guiding rat neonates in their first nipple attachment are substances contained in the amniotic fluid [24]. Contamination of the amniotic fluid with a particular flavor has been found to modify that early behavior [25], and also may result in an increased consumption of that substance later in life [26]. It has been also shown that from gestational day 17 (GD 17) the rat fetus has the capacity for acquiring and displaying basic forms of non-associative and associative learning, such as stimulus sensitization and habituation or conditioned responses to tactile and chemosensory stimuli [27-33].

#### *Toxic effects and behavioral changes*

When ethanol is administered to the pregnant rat, the drug is rapidly distributed to fetal tissues reaching levels in fetal blood equal to those in maternal circulation

[34]. Alcohol also accumulates in the amniotic fluid, and previous data have demonstrated that 60 min after the administration of a relatively low ethanol dose to the pregnant dam, the concentration of the drug in the amniotic fluid is sufficient to be perceived by the rat fetus [15, 34]. Thus, maternal administration of alcohol during the last days of gestation results in fetal exposure to both, the ethanol toxic and chemosensory properties.

In studies in which low (1 g/kg) or moderate (2 g/kg) dose of alcohol were administered to the pregnant rat during the last days of gestation, *i.e.* gestational days (GD) 17 to 20, no significant teratological effects were detected. In those studies ethanol exposure failed to affect several maternal-fetal and perinatal physical parameters such as placenta weight, umbilical cord length, offspring's body weight, weight and size of olfactory bulbs, cerebral hemispheres, and cerebellum [35]. However, in some cases these same alcohol doses were found to induce an increase in baseline motor activity [17, 36]. This hyperactivity effect was significantly reduced in the presence of alcohol odor, but not when other novel odors, such as lemon, were presented [35]. Nevertheless, this seems to be a very weak effect and not very consistent since no differences in baseline motor activity were found in other studies with neonates prenatally exposed to 1 g/kg alcohol [37] or in 14 day old pups treated *in utero* with 1 or 2 g/kg alcohol [38].

What has been consistently found in those studies is that ethanol exposure promotes subsequent changes in responsiveness to the drug. For instance, repeated administrations of low to moderate ethanol doses (1-2 g/kg) to the pregnant rat during GD 17-20 promoted changes in behavioral and autonomic responses to ethanol odor interacting with postnatal re-exposure to the drug [39]. In that study, pups exposed to alcohol pre and postnatally showed a stronger orienting response towards alcohol odor, which is a marked bradycardia, when compared to pups not exposed to alcohol prenatally. This same prenatal experience with alcohol has been also found to change fetuses responsiveness to the toxic as well as to the sensory aspects of alcohol. In a study in which fetuses' behavior was evaluated during GD 20, it was found that maternal ethanol intoxication with 1 or 2 g/kg induced a drastic reduction in fetal movements [36]. In general, alcohol administration during pregnancy results in a decrease in fetal motor activity and breathing movements. This has been documented in humans [40], in sheep [41], as well as in rats [42]. What was more surprising from that study was that, besides this acute effect of alcohol intoxication, it has been also observed that rat fetuses, whose mothers were repeatedly administered with low doses of alcohol during the previous three days of gestation showed a more marked decrease in motor activity while intoxicated when compared to fetuses never exposed to the drug before [36]. This indicated that the fetus became sensitized to the sedative effects of ethanol, what results surprising since repeated exposure to ethanol usually results in the opposite process, *i.e.* tol-

erance. Another interesting result from this study was that those same fetuses, when tested sober, displayed more mouthing to the taste of alcohol and also it was observed a trend to display less facial wiping to this taste, when compared to fetuses with no previous experience with ethanol. No differences between ethanol exposed and non-exposed fetuses were observed when the response to saline or lemon was tested, confirming that ethanol experience was affecting specifically the response to the flavor of the drug.

#### *Changes in ethanol preference*

Repeated prenatal alcohol experience has been also found to produce an increase in ethanol consumption when tested during postnatal stages. This enhanced alcohol intake effect has been consistently found in several studies in which pregnant females were administered either 1 or 2 g/kg alcohol during the last days of gestation [35, 43-45]. Rat neonates exposed prenatally to those alcohol doses subsequently recognized alcohol odor when presented alone or in compound with amniotic fluid, and reduced their general activity in the presence of the drug [35]. Those same subjects were tested as infants on postnatal day 15, in terms of ethanol intake, and it was reported that only pups prenatally exposed to the lower ethanol dose, *i.e.* 1 g/kg, showed an increased ethanol intake when compared to saline pre-exposed pups. These pups showed also a higher consumption of a compound of quinine and sucrose which has been previously proved to be perceived by the rat as very similar to the taste of alcohol, measured at behavioral and physiological levels [46, 47]. On the other hand, subjects who had been exposed prenatally to the moderate alcohol dose, 2 g/kg, showed intermediate ethanol intake scores, not differing from pups whose mothers were administered with water during pregnancy or pups experiencing ethanol but in a lower concentration. In addition, no differences were observed between both prenatal alcohol treatments when intake of other substances were tested, such as water, a sucrose solution, or a quinine solution [44]. With similar prenatal treatments, however, in a recent study slightly different results were observed [43]. It was found that preweanlings exposed *in utero* to the 2 g/kg alcohol dose were the ones showing a strong increase of alcohol intake when compared to controls or to pups treated with the lower alcohol dose. In that study it was also observed that the effect of enhanced consumption of alcohol could be observed also during postweaning periods, but in that case the expression of the effect was affected by gender of the subjects.

#### *Gender effect*

Males treated prenatally with the higher alcohol dose increased significantly their alcohol intake on postnatal day 28, while the increase in alcohol consumption was observed more clearly in those female subjects exposed to the lower dose. The effect observed in adolescent rats indicates a long-term retention of the prenatal experience with ethanol, what corroborates

the importance of the near-term fetus' knowledge of the chemosensory aspects of its amniotic milieu [24]. Also may indicate that this experience is strong enough to be retained after the numerous changes in the internal and external context that overcomes the developing subject. For instance, the drastic changes from prenatal to postnatal environment, during infancy all those changes related to the development of the visual and auditive perception, such as eye and ear opening, and later from preweaning to postweaning stages. The gender-specific effect found at that age could be related to a different sensibility to ethanol by females in comparison to male subjects. Although this effect related to gender has not been observed in preweaning rats, in adolescent rats there has been reported differential ethanol intake as a function of sex [48].

### ***Increase in ethanol consumption***

The effect mainly observed after prenatal exposure to low or moderate alcohol doses is an increase in ethanol consumption. This increased alcohol intake could be indicating a preference for it, and the main hypothesis for explaining this preference is an appetitive conditioning occurring during fetal stages. The conditioned stimulus (CS) in this case could be the chemosensory characteristics of the drug and the unconditioned stimulus (US), the reinforcing aspects of alcohol probably mediated by the opioid system.

As mentioned before, in infant rats the taste and/or smell of ethanol can act as a CS, which paired with appetitive or aversive reinforcers may result in conditioned preferences or aversions, respectively [49-51]. It has been also shown that ethanol can work as a US promoting aversions when paired with an unfamiliar flavored substance, either pre- or postnatally [37, 52]. In studies of conditioned place preference, low ethanol doses have been found to act as positive reinforcers, especially at younger ages [53, 54]. Furthermore, in some cases ethanol can act as a CS and a US at the same time. For example, 10 or 11-day old infant rats administered intragastrically with a 3 g/kg dose of ethanol, but not with a 1.5 g/kg dose, showed a strong aversion to ethanol odor or taste [49-51]. As has been mentioned before, fetuses have also the capacity for perceiving alcohol chemosensory aspects *in utero* and also of associative learning about stimuli present in their environment. As stated before, conditioned responses to alcohol odor have been observed after the association of alcohol chemosensory aspects and stimulation occurring during cesarean delivery [16, 18, 55].

### ***Reinforcing aspects***

Respect to the reinforcing aspects of alcohol consumption, considerable evidence has been accumulated supporting the role of the endogenous opioid system in the mediation of them. Indeed, low blood ethanol levels have been found to stimulate the activity of the opioid system [56] and also the administration of  $\mu$ -receptor agonists has been found to increase ethanol intake [57]. On the other hand, the administration of non-selective opioid antagonists naloxone or naltrex-

one has been shown to reduce ethanol intake in rats [58, 59]. In human subjects treatment with naltrexone has been successfully used for reducing ethanol craving and clinical relapse in recovering alcoholics [60]. Several investigations indicate that the reinforcing properties of ethanol are regulated by the activity of the  $\mu$ - and  $\delta$ -opioid receptors, although more recent studies using selective antagonists conclude that voluntary consumption of ethanol is primarily modulated by  $\mu$ -opioid receptors [57, 61].

In the rat fetus, two opioid receptor subtypes,  $\mu$  (mu) and  $\kappa$  (kappa), are functional and are capable of modulating fetal behavior during the last days of gestation [62, 63]. It has been also demonstrated that the activity of the opioid system (specifically  $\mu$ -opioid receptors) can be conditioned prenatally after pairing a chemosensory CS with a US that promotes the release of endogenous opioids, and that subsequently the rat fetus is capable of exhibiting a conditioned opioid response when the CS is again presented [64]. The administration of opioid antagonists, either nonselective or selective for each receptor subtype, has been found to be effective modifying those fetal responses known to be regulated by the opioid system [28, 62].

The hypothesis of a conditioned preference established *in utero* as a consequence of the association between ethanol chemosensory and reinforcing aspects, the latter mediated by the opioid system, was supported by data showing that the effect of augmented ethanol intake was not observed in pups whose mothers were administered naloxone together with ethanol. Similarly, postnatal re-exposure to ethanol flavor and naloxone decreased subsequent ethanol intake in pups prenatally exposed to the drug [43]. The administration of the non-selective opioid antagonist to the pregnant rat seemed to alter the enhanced ethanol intake effect obtained after maternal ethanol intoxication. Therefore, it can be assumed that the activity of the opioid system was closely involved in the establishment of that effect. The increased ethanol intake cannot be merely explained in terms of habituation of neophobia or increased familiarity with the stimulus, since subjects with the same prenatal experience with ethanol flavor but differing in terms of naloxone treatment, displayed significantly different levels of ethanol consumption. It could be argued that naloxone treatment may have changed the palatability of ethanol when administered together. Indeed, opioid antagonists have been shown to alter the palatability or hedonic value of ethanol and other substances, when measured with a taste reactivity test [65-67]. To our knowledge, however, there are no evidences that this change in the taste value of ethanol is related to a change in the perception of the chemosensory aspects of this drug. Therefore, in that study, subjects (fetuses or infants) receiving ethanol together with naloxone could have experienced a qualitatively different cue, but different in terms of affective properties and not in terms of chemosensory properties of ethanol. By administering naloxone, the intention was to "block" the reinforcing properties of ethanol. But, it is possible that instead of becoming a neutral cue etha-

nol has become a cue with negative value. If that were the case, an alternative explanation should be considered for the reduced consumption observed in pups treated with ethanol and naloxone. When fetuses were exposed to both substances, if naloxone were turning ethanol in an aversive stimulus, then the reduced ethanol intake on postnatal stages could be the result of an aversive conditioning acquired *in utero*. While, when naloxone was administered postnatally together with the taste of ethanol, if ethanol would acquire a negative value, instead of an extinction effect we would have a counter-conditioning situation. In any case, results of that study seem to support the idea that the increased ethanol consumption observed in pups exposed prenatally to this drug is a conditioned appetitive response established *in utero* as a consequence of the association between the chemosensory properties of ethanol and its reinforcing aspects mediated by the endogenous opioid system. Additional support for these conclusions is provided by numerous evidences of fetal capacities for acquiring conditioned responses to stimuli presented in its environment and the implication of the opioid system in the establishment and expression of conditioned responses [28, 29, 64]. Also in agreement with those results are studies in which prenatal ethanol exposure resulted in increased mouthing in response to ethanol taste in rat fetuses and neonates [35, 36], an index that has been related to enhanced palatability of substances [68, 69]. In addition, the  $\mu$ -opioid system has been found to be responsible for increasing mouthing and licking responses in rat fetuses and, in general, to regulate fetal oral appetitive responses [28, 29].

#### **PRENATAL EXPERIENCE: PREFERENCE, AVERSION, PALATABILITY**

There are studies providing data that call into question the interpretation of a preference for ethanol as a consequence of the prenatal experience with the drug. For example, in one study rat fetuses on GD 17-20 received paired presentations (through maternal intragastric administration) of ethanol and cineole, a substance that has been proved to reach the amniotic fluid and to be perceived by the near term fetus [70]. This paired condition was compared to another one in which fetuses were exposed to these same two substances but separated by a 4-hour interval, and also to a control group in which dams received only water. On postnatal day 16 all pups received repeated intraoral infusions of milk and once their mouthing responses were habituated they were tested in terms of their dishabituation when milk was presented contaminated with alcohol or cineole. It was observed that pups which have received prenatally cineole paired with ethanol responded to cineole with less mouthing than pups from the unpaired condition or than the water group. It was also reported that the prenatal experience did not affect the response to milk contaminated with ethanol [70]. These results were interpreted as an aversive response to cineole after its prenatal pairing with ethanol intoxication. An interpretation that was confirmed by the results of an-

other study, with similar prenatal treatments, in which it was found that 15 day old pups prenatally exposed to cineole paired with ethanol consumed less cineole than pups receiving those same two substances, but 4 hours apart [37]. Although in that study pups from the paired condition did not consume significantly less cineole than the water control pups, these authors conclude that the difference in consumption between unpaired and paired groups is due to a conditioned aversion to cineole acquired by the pups which received cineole paired with ethanol intoxication *in utero*. Those same pups received after the intake test (cineole or water) an intragastric (i.g.) administration of either ethanol (1g/kg) or water in order to induce a conditioned aversion to cineole using ethanol as a US. The following day (PD 16) they were tested in a habituation-dishabituation test. As was the case of the previous described study, they were first habituated to milk and then presented with a milk-cineole mixture. The results showed that all pups receiving cineole and alcohol showed less mouthing to cineole than the remaining groups, however pups from the paired group mouth less than the unpaired group especially during the first testing trials. In summary, according to the results of these last described studies, low doses of alcohol may act as aversive US when administered prenatally or postnatally together with a olfactory CS [37, 70].

Nevertheless, in another recent study, it has been reported that neonate rats prenatally exposed to cineole paired with ethanol attached more time to surrogate nipples scented with cineole than controls, a response that could be considered evidence of a learned preference [71]. Yet, the authors of that study do not discard the hypothesis that rat pups could be attaching to the nipples in order to obtain calming effects and counteract anxiety promoted by the aversive memory generated prenatally. Although, in view of these results, they conclude that the affective valence of the experience occurring *in utero* remains unclear.

Another possible way of understanding this apparent contradiction in the pup's response to ethanol and/or in the unconditioned effects of prenatal ethanol exposure, is considering studies about drugs that are rewarding when tested in some paradigms but can generate a conditioned taste aversion (CTA) when associated with other flavors [72-74]. So, for instance, *in utero* ethanol could be generating a CTA when presented together with the taste of cineole, what will be reflected in reduced cineole consumption, but this may not affect the rat's auto-administration of the drug. Furthermore, in the case of ethanol, that has in addition to its pharmacological effects a particular taste and odor, its palatability can remain unchanged or even be enhanced after several exposure trials [75].

Increased intake of a flavored solution does not imply necessarily a preference for it [76, 77], on the other hand, the palatability of a substance can be increased without evident changes in a consumption test [65, 66]. Taking this into account as well as the apparent contradictory results of the above mentioned studies, in order to further investigate the hedonic nature of the prenatal



ethanol experience, it was analyzed whether the increased ethanol intake effect observed in pups prenatally exposed to the drug is accompanied by a change in palatability of the substance. This was measured using a taste reactivity test adapted for infant rats by Hall and Bryan [68] who found that rat pups could express differential behavioral responses - preference or aversion - to several tastes [78]. It was found that pups exposed to both ethanol doses (1 and 2 g/kg) *in utero* not only consumed more ethanol when tested on PD 15 but also displayed more appetitive responses (mouthing and paw licking) and less aversive behaviors (general motor activity and wall climbing) in reaction to the taste of ethanol than controls [38]. In that same study, pups were tested in their reaction to a sucrose-quinine compound, a mixture that is perceived by the rat as very similar to ethanol taste. If increased mouthing and paw-licks on one hand, and decreased general activity and wall climbing, on the other hand can be considered as behavioral appetitive manifestations for a substance intraorally infused [68, 74, 78], these last results suggest that in the infant rat the palatability of the taste of ethanol was enhanced after exposure to the drug during the last days of gestation. This enhanced palatability effect has been also shown to be blocked or reduced when naloxone was administered to the pregnant dam together with ethanol [79]. In this last study, it was found that naloxone administered together with ethanol to the pregnant rat not only reduced ethanol intake in the infant offspring but also decreased the appetitive behaviors and increased the aversive reactions to the taste of ethanol observed in those pups whose mothers were administered only alcohol during gestation.

In sum, the administration of a low or moderate dose of ethanol to the pregnant rat during the last days of gestation clearly modifies the offspring's response to the flavor of ethanol. In most studies, this response seems to be a conditioned preference for ethanol, mediated by the opioid system, what results in an increased palatability of the drug and a high ethanol intake during the rat's infancy.

Several studies have demonstrated that the opioid system is implicated in learning processes modulating the acquisition of taste or odor preferences during early infancy [80]. Some researchers suggest that this neurochemical system has a distinctive role in neonatal rat learning, temporally limited to a sensitive period that ends on postnatal day 9 and coincides with the emergence of walking [81]. One characteristic of this sensitive period is that pups tend to learn easily odor preferences [81-83]. Sullivan and collaborators have demonstrated that an odor paired with foot-shock on PD 7-8 generates a conditioned preference for that odor, while after the sensitive period, on PD 11-12, that same association generates a conditioned odor aversion [82-84]. It has been also reported that the administration of the opioid antagonist naltrexone disrupted the shock-induced odor preference in the younger pups but not the odor aversion in the older group [81]. Although it is not specified by those authors, it is conceivable that this sensitive period may also include the last prenatal period. In fact during the last days of gestation it has been observed that, as

previously mentioned, chemosensory preferences are readily acquired [26, 33, 37, 38, 43] and also that the opioid system is involved in prenatal learning processes [43, 64, 85]. Within this framework it results easy to explain the apparent paradox that has been raised in light of the results of recent studies: why doses of ethanol that promote conditioned aversions during postnatal stages result in clear preferences when administered during the gestational period? Indeed, conditioned aversions in adult rats have been observed using ethanol doses equivalent to those employed in all the above described studies, *i.e.* 1 and 2 g/kg, [53, 86, 87], but also with doses as low as 0.8 g/kg [86]. In preweanling rats, learned aversions were also reported after intoxication with ethanol doses ranging between 0.4 and 3 g/kg, but in all cases this was observed after PD 10 [49, 52, 88, 89]. Intriguingly, there are no studies showing that ethanol can act as an aversive stimulus for infant rats before PD 10. In studies in which the unconditioned effects of ethanol were analyzed in newborn rats, it was found that ethanol when administered *i.p.* in doses between 0.125 and 0.5 g/kg was reinforcing, as well as when using 0.75 g/kg dose, but the latter only in neonates prenatally exposed to ethanol [90, 91]. In a recent study, a preference for ethanol was found when rat pups were intoxicated with a relatively high ethanol dose (3 g/kg) before PD 9, while an aversion was observed when intoxication occurred after PD 9 [92]. So, the preference for ethanol observed in pups that were prenatally exposed to ethanol toxic and chemosensory aspects could be explained, if not completely at least in part, by those processes that have been described to occur during the sensitive period. In other words, in view of the unique role of the opioid system during this developmental period and that ethanol reinforcing properties are mediated by this neurochemical system [56], it seems probable that ethanol intoxication, particularly during this developmental stage, will be perceived by the fetus as a positive reinforcer. Additional support for this hypothesis is derived from the fact that, similarly to what has been observed in neonate rats, the prenatal administration of an opioid antagonist disrupts the acquisition of a preference for ethanol.

## CONCLUSIONS

To sum up, animal studies about the effects of moderate or low doses of ethanol during the last gestational days indicate that, although no apparent teratological effects are evidenced with this treatment, the prenatal experience with ethanol may alter normal patterns of response to the drug. In this case, prenatal exposure to ethanol results in fetal learning about its sensory and toxic properties, which in turn is expressed during infancy, and even in adolescent periods as an increase in ethanol intake and a preference for its flavor. In general, the outcome of this animal research is congruent with data from human studies showing infantile recognition of and preference for substances previously experienced [14, 93, 94]. One potential implication of the data presented here involves the influence of early prenatal learning about ethanol on alcohol consump-

tion in humans. In our opinion, these results should be taken into account in studies in which the relation between prenatal exposure to the drug and later ethanol abuse problems is analyzed. Nevertheless, more research is necessary for a complete understanding of the consequences of ethanol exposure during gestation,

as well as for the identification of the mechanisms by which this prenatal experience may lead to increase ethanol consumption.

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# Female drinking, environment and biological markers

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**Summary.** The rate of women involved in alcohol abuse is rapidly increasing and the age of first use tends dramatically to decrease. The health and social costs are high both for the adverse effects on physical and psychological woman health, and for the teratogenic effect of alcohol on fetal development. The review takes in account physiological aspects of alcohol effects according to age and gender differences. Interaction between alcohol habit and environment are discussed together with the risk of co-exposure to alcohol and pollutants. The role of biomarkers may be invaluable for clinical utility, prevention and early intervention above all to avoid prenatal, not reversible damages. The update of alcohol studies shows the greater severity of alcohol damage in female and the need of gender-targeted intervention.

*Key words:* alcohol, woman, gender, biomarker, environment, prenatal exposure.

**Riassunto** (*Bere femminile, ambiente e indicatori biologici*). L'aumento delle donne coinvolte in problemi alcolici e il drammatico abbassamento dell'età di primo uso, costituiscono un problema di sanità pubblica sia per gli effetti sulla salute fisica e psichica della donna, sia per gli effetti teratogeni dell'alcol assunto in gravidanza sullo sviluppo del feto. Il lavoro presenta gli effetti fisiologici dell'alcol in considerazione delle differenze di genere e di età, e i fattori di rischio per la salute dovuti all'interazione tra assunzione alcolica e contaminanti ambientali. Il ruolo degli indicatori biologici può essere essenziale nella pratica clinica, nella prevenzione e nell'intervento precoce soprattutto per evitare danni prenatali non reversibili. Gli studi sull'alcol concordano sulla maggiore severità del danno alcolico nella donna e sulla necessità di interventi specifici per il genere.

*Parole chiave:* alcol, donna, indicatori biologici, ambiente, esposizione prenatale.

## INTRODUCTION

Excessive drinking is a major health problem worldwide and could be considered a social "emergency". Alcohol problems rise both from alcoholic beverages diffusion and from changes in drinking habits that are involving young people and women more and more. Alcohol related problems are generated by a lot of factors with personal, social, health aspects. The approach to alcohol problems tended to be socio-ethical rather than scientific since the availability of alcoholic beverages, social acceptance, traditional use into the family makes the perception of risk of alcohol misuse very difficult. The concept itself of excessive drinking is debated and even some health operators have difficulties in the correct evaluation and approach to alcohol problems [1].

Ethanol exposure, together with other environmental risky factors due to lifestyle (tobacco, drugs use) and to exposure to pesticides, heavy metals, pollutants etc., has an amplifying action on health damage in addiction to ethanol "reinforcing" properties on brain functions. Information about side effects due to simultaneous exposure to ethanol and environmental toxics are not well known, but a synergic action is likely.

The effects on public health may be heavy especially for women and children. Several studies support the hypothesis that women are more vulnerable to ethanol effects for their physiological, metabolic, hormonal peculiarities [2, 3], and the increase of female drinking is an emerging alert in our days. USA epidemiological studies in the early '80s revealed a situation of alcohol abuse and dependence for the 23% of men and 4% of women (ratio = 5.75); ten years after the same problems involved the 20% of men and 8% of women (ratio = 2.50). At the end of 90's the ratio men/women among at-risk drinking adolescents (teen agers 12-17 yrs), was close to 1:1 [4, 5].

Epidemiological studies across Europe confirm that, in 2004, the rate of at risk drinking female was increasing more quickly than men and that the rate among female teenagers was quite similar (or outstripping) to male [6].

Children may be exposed to alcohol effects from conception, as maternal alcohol drinking during pregnancy may have dramatic effects [7]. They cannot be considered "little adults" in environmental medicine, since there are differences in exposure, pathways of

absorption, tissue distribution, ability to biotransform or eliminate chemicals, responses to chemical and radiation. The differences vary with the developmental stage of child and little is known about the impact of environmental factors on children [8]. The epidemiological data, pointing out the increase of female drinking, support the alert for the health of woman and child.

Self-report questionnaires are used to evaluate drinking habits and the type of alcoholic beverage taken, but their reliability is questionable, since most alcoholics are quite reticent about their drinking patterns and their real alcohol consumption. The role of excess drinking biomarkers, together with clinical examination, instrumental data and psychological tests as MAST (Michigan Alcoholism Screening Test), MALT (Munich Alcoholism Test), SADQ (Severity of Alcohol Dependence Questionnaire), AUDIT (Alcohol Use Disorders Identification Test) may be essential to ascertain and evaluate at risk drinking patterns and to identify health hazard for women, pregnant women and children [9].

Early identification of risky behavior could prevent prenatal damage and could avoid or minimize disabilities, mental health problems or trouble with the law in the future adults. Furthermore advances in the field of biomarkers of exposure, effect and susceptibility, may have important implications for the detection, prevention and treatment of environmentally induced disabilities.

This paper focuses on some aspects of alcohol-related problem in woman during adolescence, reproductive years and older age. Peculiarities of female drinking and possible interactions between alcohol and environmental toxics will be considered here, as well as the role of biomarkers in evaluating alcohol-related health hazard.

### FEMALE DRINKING AND METABOLISM

Blood alcohol concentration (BAC) depends on alcohol ingested and adsorbed by gastrointestinal tract, by volume of distribution in the body and by elimination rate. The higher will be BAC, the greater will be ethanol effects. Ethanol is adsorbed in different way depending on concentration and amount of alcohol ingested, on amount and type of food (if any) in the stomach, on drinking pattern.

Most tissues of the body contain enzymes capable of ethanol oxidation or non-oxidative metabolism, but significant activity occurs only in the liver and, to a lesser extent, in the stomach. Hence, medical consequences are predominant in these organs. In the liver, ethanol oxidation generates an excess of reducing equivalents, mainly NADH, causing hepato-toxicity. An additional system, containing cytochromes P-4502E1 (CYP2E1) inducible by chronic alcohol feeding, was demonstrated in liver microsomes and is a major cause of hepatotoxicity [10]. CYP2E1 is also induced in Kupffer cells, promoting their activation and the release of inflammatory cytokines, including tumor necrosis factor (TNF)- $\alpha$  [11].

Before metabolism by the liver, during the first pass metabolism (FPM) in the stomach ethanol is metabolized by the gastric isoenzyme alcohol dehydrogenase (ADH). Finally, ethanol is metabolized by hepatic ADH.

The enhanced vulnerability of women to alcohol-related damage may be due to their higher blood alcohol levels after drinking, but the mechanisms are debated. BAC is strongly dependent on body mass index (BMI) and body water. Female BMI and total body water are lower than male, leading to lowered ethanol diffusion in the body and resulting in higher BAC. If BAC value was normalized according to content of total body water, gender differences were flattened [12,13]. Several years ago it was demonstrated that activity of gastric ADH responsible for FPM is significantly lower in females than in males and is close to zero in heavy drinking females [14].

Thus, in woman a larger amount of alcohol ingested will reach the liver directly, promoting a more rapid progression of liver damage. A recent experimental study demonstrated that the gender differences in alcohol levels is due mainly to a significantly lesser activity of female  $\chi$ -ADH, rather than to differences in gastric emptying or in hepatic oxidation of ethanol. These effects were demonstrated to be concentration-dependent, since women had a gastric first pass metabolism lower than men, when given 10% or 40% but not 5% alcohol solutions [15].

Furthermore, ADH activity was demonstrated dependent not only on gender but also on age [16]. In social drinking male, gastric ADH activity is at the top level at 20-40 yrs and decreases with ageing until, at 61-80 yrs, it becomes approximately the half of 20-40 yrs activity. In women, gastric ADH activity is at the lowest level at 20-40 yrs, reaches the top at 41-60 yrs and decreases at 61-80 yrs. Thus, significant gender-related differences in gastric ADH activity appear during lifetime and with a trend to flatten in old age. The critical point is just at young age (20-40 yrs), when the gender difference is at the top level and females are more exposed to alcohol effects. The age 20-40 yrs is the age of fertility, and the increased risk just during the reproductive years results in an improved risk of fetal exposure and damage. BAC levels are related to an impairment of cognitive and psychomotor performance that increases together with BAC levels [17]; thus, the higher BAC levels result in a noticeable risk for female performance.

Drinking females may outstrip BAC legal limits for car driving easily (Italy: 50 mg/100 ml of blood) and undergo legal consequences, as well as may be victims of accident and violence. Recent investigations indicate that alcoholic brain damage is much more common than previously suspected and the potential sex-related difference in the susceptibility to the detrimental effects of chronic alcohol exposure on subsequent behaviour (cognitive aspects are mainly impaired) are investigated.

Even physiologic hormonal pattern have a role in vulnerability of female organism to alcohol intake.

Different reactions to alcohol intake may be observed in different phases of the menstrual cycle [18] and the mechanisms involving the estrogens in inflammatory processes are suggested to explain the more rapid progress of liver damage in female [19]. Furthermore, in the reproductive years, the use of oral contraceptives may worsen alcoholic damage by hormonal activation of endotoxin-induced liver injury [20]. Epidemiological and experimental data demonstrate that alcohol drinking may lead to female fertility impairment, [21] and increase of the risk of breast cancer [22-24].

Moreover, the teratogenic effect of alcohol is to be considered. Alcohol passes through placenta and reaches the fetus that is no tolerant to ethanol. Ethanol may interfere with fetal development causing abortion, fetal death, premature birth, low birth weight, abnormalities in mental and physical development, somatic alterations. The teratogenic effects of alcohol are globally defined as fetal alcohol spectrum disorders (FASD) and the fetal alcohol syndrome (FAS) is the worst occurrence. Fetal damage is not dose-related and may occur even at low levels of maternal alcohol intake, above all if ingested in the early pregnancy. Fetal alcohol damage is not reversible, and may be preventable avoiding any maternal alcohol intake during pregnancy.

#### **FEMALE DRINKING AT DIFFERENT AGES**

Adolescent age is a period of great physical changes and brain modifications, leading to a wide range of effects on the psychological functions and behaviour of adolescent [25]. Risk-taking behaviour, as experimenting with alcohol and other drugs (novelty seeking), is more common at this age. The volume of the hippocampus, a brain region important for learning and memory, in adolescents with alcohol-related problems is significantly smaller than in controls [26]. Women may be more susceptible than men to modifications of brain regions [27]. During adolescence dramatic changes in hormone levels and patterns occur and gender differences in the body's hormonal response to stress emerge. Girls may be especially vulnerable to stress [28, 29] and the levels of perceived stress may be a predictor of alcohol and other drug use [30]. Severe depression too plays a role amplifying the impact of substance use and abuse. Because females of all ages appear to be at greater risk for affective disorders, alcohol use/abuse among adolescent females is particularly worrisome. Animal studies suggest that alcohol may affect the adolescents differently than adults and the adolescents appear to be more sensitive to alcohol-induced damage in certain types of memory [31, 32]. Gender differences regarding the alcohol effects on developing adolescent brain and other body systems need further research. However, several evidences suggest that the younger a person begins to drink, the more likely he or she will develop alcohol problems later in life [33].

In the reproductive years, heavy drinking has been shown to disrupt normal menstrual cycling and reproductive function, leading to infertility and increased risk for spontaneous abortion [34, 35]. Maternal drinking during pregnancy may generate impaired fetal growth and development with a wide range of effects on exposed offspring (hyperactivity, attention problems, learning and memory deficits, and problems with social and emotional development).

These problems usually emerge at school age, and are globally defined as fetal alcohol spectrum disorders (FASD). The most serious consequence of maternal drinking during pregnancy is the fetal alcohol syndrome (FAS), that shows a distinctive set of facial anomalies, growth retardation, and significant learning and/or behavioural problems. A generalized deficit in complex information processing constitutes the central feature of the cognitive-behavioral phenotype of FASD [36].

As no safe threshold of alcohol use during pregnancy has been established, women who are pregnant, planning a pregnancy, or at risk for pregnancy should not drink alcohol at all: even children exposed to low levels of alcohol in their prenatal life may exhibit learning and behavioral problems [37].

Some evidences suggest that alcohol consumption may increase the risk of breast cancer. Moreover, even low levels of drinking may be a risk factor for women with family history of breast cancer [38, 39]. In middle-aged women, the use of hormone replacement therapy (HRT) during menopausal stage is a known risk factor for breast cancer and even moderate amounts of alcohol may increase significantly this risk [40].

In the last years, many topics about alcohol effects on different organs are under discussion. In postmenopausal women, alcohol consumption may affect several organs, like liver, brain, and gastrointestinal tract, directly; indirectly, altering the blood levels of sex steroids, may increase the risk for some diseases [41]. A debate matter is: may moderate alcohol drinking modulate a possible beneficial effect on health at this age? Many epidemiological evidences suggest that light-to-moderate alcohol consumption significantly reduces the risk of atherosclerosis in both genders by lowering the low-density lipoprotein (LDL), or "bad" cholesterol, increasing the high-density lipoprotein (HDL), or "good" cholesterol, and reducing blood clotting and the "stickiness" of platelets. On the contrary, heavy drinking can damage the heart. The problem is: what is the "safe" alcohol consumption for each individual?

A well-known problem of older age is osteoporosis, a skeletal disease characterized by low bone mass, increased bone fragility, and susceptibility to fracture [42, 43]. At menopause, the rate of bone loss increases significantly and some epidemiological studies suggest that light-to-moderate alcohol consumption may be associated with increased bone mineral density and decreased fracture risk in postmenopausal women [44-46]. However, heavy drinking may compromise bone health and increase the risk of osteoporosis, leading to

decreased bone density and impaired bone mechanical properties. These effects are mainly patent in young women, whose bones are still developing, but also chronic alcohol use in adulthood can harm bone health [47] in addition with other lifestyle factors, as tobacco smoking, that may increase the risk of osteoporosis and fractures [48].

Among elderly people, memory and brain function Alzheimer's disease (AD) is the most common form of dementia. It is characterized by progressive changes in cognitive ability, memory and mood. Women appear to be at greater risk than men for AD, also taking into account the women's longer life span [49]. Heavy alcohol drinking may increase the risk for AD in both genders, mainly in women, as they appear to be more vulnerable than men to alcohol-induced brain damage. However, moderate alcohol consumption doesn't seem to have negative effects on brain function, and moderate drinking could protect the blood vessels in the brain, as in the heart, against atherosclerosis. But the safe limits of alcohol intake are difficult to be defined because of individual variations in susceptibility to damage. The "optimum" amount of alcohol for each individual cannot reasonably be established.

#### ALCOHOL AND ENVIRONMENT

Heavy drinking is *per se* a risk factor for health, since it can trigger many pathological processes. Research on ethanol metabolism have established that alcohol is hepatotoxic, both because of secondary malnutrition and through metabolic disorders associated with ethanol oxidation. These effects are due to redox changes produced by the nicotinamide adenine dinucleotide NADH generated via the liver ADH pathway which in turn affects the metabolism of lipids, carbohydrates, proteins and purines. In addition to ADH, ethanol can be oxidized by liver microsomes by the ethanol-inducible cytochrome P450 (CYP2E1) which contributes to ethanol metabolism and tolerance and to the selective hepatic perivenular toxicity of various xenobiotics. This may explain the increased susceptibility of the heavy drinkers to the toxicity of industrial solvents, anesthetic agents, commonly prescribed drugs, chemical carcinogens and even nutritional factors such as vitamin A. The induction of the microsomal pathway contributes to increased acetaldehyde generation, with formation of protein adducts. This results in antibody production, enzyme inactivation, decreased DNA repair, and a striking impairment of the ability of the liver to utilize oxygen. Moreover, acetaldehyde promotes GSH depletion, free-radical-mediated toxicity and lipid peroxidation and increase hepatic collagen synthesis and accumulation [50].

Thus, alcohol drinking significantly enhances negative effects due to simultaneous exposure, professional or environmental, to other toxics. Probably never as in our days lifestyle and environment together play significant role for the health hazard of worldwide population. At present, alcohol drinking is usually considered in work places as a risk factor only for

its acute effects on performance, mainly for activities such as pilots, car, bus drivers, industrial workers. There is a lack of data about organic damages due to professional (or environmental) exposure to toxics together with heavy drinking. Indeed, also the assessment of ethanol intake in toxicological studies is quite questionable: ethanol intake is estimated by self reported consumption, which resulted poorly reliable in several studies. Real at risk drinking should be identified by specific biological markers, as blood alcohol concentration (BAC), transaminases (AST and ALT), mean corpuscular volume (MCV), gamma-glutamyl-transferase (GGT); alcoholism biomarkers may be a useful tool to evaluate the real alcohol consumption, but their values may be modified by the exposure of toxics. Thus, the classic biomarkers of alcoholism cannot fully discriminate between the effects of alcohol or some other toxics.

Dangerous lifestyle, such as smoking, drug abuse, alcoholism, may act synergically together with environmental toxics and promotes neurotoxicity, GABA system alteration, mitochondrial damage, impairment of immune system and teratogenic effects [51].

Chronic heavy drinking may be associated with an unbalance of some essential elements, such as iron, zinc, copper and selenium, because of an impairment of homeostatic mechanisms that in physiological conditions maintain these elements within the physiological limits. Levels of toxic metals may increase in chronic alcohol intoxication, because of a reduced availability or activity of regulatory essential nutrients and substances. Thus, concentrations of metals considered safe for general population can become unsafe for heavy drinkers. For example, lead is one of the most toxic metals and, in the last years, many limits for the use of this element were set to reduce the general exposure to this element. Nevertheless, the modern lifestyle produces many sources of exposition, continuously renewed, which cause a wide spread of lead; moreover, changes of population habit may trigger health risk situations, sometime ignored or underestimated. Most of ingested lead is rapidly excreted but, when the dose increases, relatively more is absorbed. Much of adsorbed lead is immobilized and incorporated in bone and hair, but some of it is concentrated in the liver with deleterious effects. Old and more recent studies have outlined that prenatal and postnatal development are compromised significantly by the presence of lead in the body: cognitive performance is affected, and even the risk of developing psychiatric diseases as schizophrenia is debated [52-54]. Since ethanol too have teratogenic effects, female alcoholics are a population heavily at risk for lead exposure, and their children will have a very high risk of neurobehavioral damage from lead and ethanol, since prenatal exposure to alcohol and lead intoxication seem affect the same brain functions. The developing brain is mainly vulnerable to the toxicants that may be responsible for learning disabilities and behavioural problems in children. It was demonstrated that lead exposure, like prenatal exposure to alcohol, affects cognitive performances



severely in children and may trigger antisocial behaviour in adolescence [55, 56].

Furthermore, other factors may concur to synergic effect of lead and ethanol. It is well known that thiamine (vitamin B1) is an effective antidote against lead intoxication, but this vitamin results dramatically reduced in alcoholics, because of both malnutrition and impaired phosphorylation. Thiamin deficit is more severe in alcoholic women, resulting more exposed than men to side effects of lead [57].

Experimental data from animal models [58] demonstrated that co-exposure to lead and ethanol produced more elevation of blood zinc protoporphyrin and hepatic lipid peroxidation. Compared with the group treated with lead alone, lead-ethanol exposure lowered the concentration of blood and liver magnesium and calcium and increased the amount of lead in the blood, liver and brain. Chronic alcohol intake results in calcium and magnesium loss but co-exposure to lead and ethanol could result in more serious depletion of calcium and magnesium suggesting a synergism between alcohol consumption and lead poisoning. Results from another study [59] suggest that lead metabolism is modified by alcohol, and that heavy drinkers may be a risk population for saturnism.

The association of alcohol drinking with an increasing risk of certain types of cancer is a well known problem. Excess drinking can cause DNA damage by several mechanisms including increased cellular proliferation, oxidative stress, formation of lipid peroxidation products and related DNA adducts, inhibition of DNA repair.

The research focuses on the role of acetaldehyde, the metabolite formed from ethanol by the action of ADH and subsequently converted to acetate by aldehyde dehydrogenase (ALDH) [60].

The most recent studies have individuated the role of polyamines, natural compounds essential for cell growth, that reacts with acetaldehyde to trigger a series of reactions that damage DNA, an event that can lead to the development of cancer [61].

The polyamines facilitate the conversion of acetaldehyde into crotonaldehyde (CrA) that generates the DNA adduct, 1,N(2)-propano-2'-deoxyguanosine (PdG) called Cr-PdG. It was found that, under physiologically relevant conditions, polyamines stimulate the formation of Cr-PdG from acetaldehyde and dG or from acetaldehyde and DNA by directly reacting with acetaldehyde to generate CrA. The Cr-PdG adducts are also endogenous lesions apparently derived from lipid peroxidation. The adduct Cr-PdG is believed to be responsible for mutagenic and genotoxic effects [62]. But crotonaldehyde is not only an endogenous product but also a chemical present in the environment that is a powerful irritant, mainly for eyes and lungs, that may impair immune function and may cause cancers in animals [63, 64]. Crotonaldehyde is naturally present in food and is formed by the burning of fossil fuels (including waste gas from motors) and wood, cigarette smoke and cooking oils. Professional exposure may occur in chemical and war industry. Crotonaldehyde is produced mainly

as intermediate for sorbic acid production, and for the production of flavourings, solvents, substances of industrial interest, disinfectants for human, veterinary, domestic, and civil use, adhesives, inks and paints. The finding that polyamines may lead to the formation of endogenous crotonaldehyde and its DNA adducts, may be of great interest for understanding the mechanisms by which alcoholic beverage consumption increases the risk of cancer development. Furthermore, the possible role of both exogenous and endogenous crotonaldehyde in increasing cancer risk due to alcohol drinking and pollutants exposure, may stimulates further investigation about genetic factors, mainly those that influence DNA repair pathways, in relation to alcohol related risk of cancer and to individual susceptibility to alcohol drinking and environmental toxics.

### FEMALE DRINKING AND BIOMARKERS

At present, no laboratory test alone can detect and quantify alcohol use lasting over a protracted period and distinguish between a single drinking episode and chronic alcohol use. Because biological markers currently in use may not be effective in screening for at risk alcohol use in a longer period, such as during pregnancy, clinicians most commonly use brief screening measures that rely on maternal self-reports to assess drinking pattern. Such screening measures have major disadvantages. One is that often is difficult for people to correctly recall their actual amount and frequency of alcohol intake. Furthermore, above all for women and pregnant women, the fear of punishment and disapproval for drinking alcohol can make them reluctant to reveal alcohol use and prenatal alcohol use, especially if heavy use. Supplementing self-reports with a carefully selected panel of biological markers as AST, ALT, MCV, GGT, would allow the earlier identification of women who are at risk for heavy drinking and the earlier monitoring of their drinking behaviour during pregnancy. A large number of patients seen in clinical practice have an underlying alcohol problem and there is a pressing need for accurate methods to objectively diagnose alcohol over-consumption. The problem is: how best to use biological markers to support the diagnosis of alcoholism [65]? Until few years ago, many longitudinal studies on alcohol dependence examined only male population. Women are poorly represented also in treatment studies even because they enter to alcohol facilities less than men. Thus, data from alcohol studies come essentially from males but results are often generalized to both sexes. This problem involves also alcohol biomarkers studies and only recently there is a greater attention to differences between sexes and gender studies are promoted. In fact it was demonstrated that gender has to be taken into account when the results of such a test are evaluated since significant interaction between gender and alcohol biomarkers was found [66]. In our previous studies about alcohol biomarkers, we verified the relevance of assessing actual drinking not only by a self-report questionnaire,

but also by the direct determination of BAC. This was demonstrated a good strategy for a correct evaluation of the diagnostic power of the biomarker on study. In the study about the mitochondrial isoenzyme of AST (mAST), we monitored a series of controls and on-treatment alcoholics, assessing simultaneously mAST and BAC. The results demonstrated mAST suitable to discriminate between controls and alcoholics; but the most relevant result was that mAST was suitable to discriminate between “BAC positive” actual drinkers (including self-claimed abstinent) and “BAC negative” actual abstinent. Furthermore mAST value increased or decreased in a short time (two days), in response to BAC level. Thus, acute alcohol consumption was demonstrated a significant, suggestive and until now inadequately examined factor in evaluating the diagnostic suitability of alcohol biomarkers [67].

In diagnostic evaluation, a debated problem is the usefulness of the evaluation of the area under the curve (AUC). Some authors show it as a good tool to create an algorithm and to increase the diagnostic accuracy of combined biomarkers above all when gender studies are considered [66]. In recent researches on thiamine (vitamin B1) and its esters, we found highly significant differences between alcoholics and control for thiamine (T) and thiamine difosphate (TDP or cocarboxylase) values, and no gender differences among the values in alcoholics. But, when the AUC of the ROC curves were evaluated, differences between alcoholic men and women were found and female AUC were significantly closer to 1, both for T and TDP [57, 68].

Experience clearly shows that particular attention may be due to gender differences when biological data are to be evaluated. In pregnant women, alcohol abuse biomarkers have to be carefully evaluated, because of the risk of fetal damage. FASD is a preventable cause of mental retardation and birth defects, and an early identification of at risk alcohol use may adverse fetal outcomes. The clinical laboratory can help to assess prenatal alcohol use [69] and can give a valuable contribution to prevent fetal damage. The clinical utility of biomarkers as blood/breath alcohol concentration, GGT, MCV, hemoglobin associated acetaldehyde (HAA) would be a mainstay in alcohol use detection, mainly in pregnancy where the presence of several positive markers should be correlated with at risk pregnancies [70]. Alcohol use during pregnancy is a significant public health problem. The health and social costs of prenatal alcohol use are very high. Some evidence indicates that even low dose drinking during pregnancy can cause adverse fetal effects but how this damage occurs is not fully understood. Because of alcohol adverse effects on the fetus, all women should be advised to abstain from drinking during pregnancy. Bio-tests that could identify women who continue to drink while pregnant would be invaluable to facilitate intervention for helping to stop alcohol drinking during pregnancy, to identify children at risk for alcohol associated birth effects and to monitor them from the birth for potential problems and, if needed, to facilitate their early approaches to special facilities.

Above all, the diagnosis of FASD should be established in at risk children before school age, to reduce mental health problems, school failure, antisocial behaviours and future problems with alcohol and other drugs. The intervention for mothers may help them to reduce their problem drinking, to enhance their ability to care for their children and to reduce the risk of alcohol exposure in their subsequent pregnancies. Biological samples for detecting drinking during pregnancy are traditionally neonatal or maternal urine and blood. More recently, other samples could be used after delivery to assess biomarkers of prenatal alcohol consumption, as amniotic fluid, cord blood, neonatal hair, placenta, breast milk, meconium and vernix (*i.e.* the cheese-like material that covers the skin of fetus). No single marker is sensitive and specific enough to be considered a gold-standard biomarker for prenatal alcohol use and panels of two or more markers may yield greater sensitivity and specificity [71, 72]. Current biochemical markers are not as diagnostically effective in women as in men. Studies evaluating biomarkers in female are very limited and validated biomarkers with greater diagnostic sensitivity and specificity are needed, mainly to monitor pregnant women. In the last years, there is great interest in the study of fatty acid ethyl esters (FAEE) the metabolic products that result from the interaction between alcohol and fatty acids. FAEE can be detected in the cord blood, meconium and hair of children, and in other organs in adults [73, 74]. In the studies about thiamine, alcohol damage resulted higher in women than in men, confirming the so-called “telescoping effect” *i.e.* a more rapid progression of alcohol damage. Indeed, it was demonstrated that thiamine deficit has to be corrected in alcoholics, and above all in woman, since may be responsible of a worrisome worsening of the clinical situation of the patient. In drinking pregnant women, T deficit may be further increased by hyperemesis gravidarum with heavy consequences for woman health and with strong risk of damage for the central nervous system of the fetus [75]. So, the assessment of thiamine during pregnancy may be relevant, both as a marker of alcohol abuse and as an alert for the need of vitamin supplementation. More recent advances in the field of alcohol studies offer proteomics as new promises for developing biomarkers able to detect biological changes due to alcohol use and to distinguish between subjects currently drinking and true abstainers [76]. It was demonstrated that clusters of proteins can collectively distinguish between children with and without FAS and the aim is to monitor women at drinking risk. Development in proteomics would be a significant step in primary prevention of alcohol related prenatal damage.

Taken together, the results of many different studies outline the greater severity of alcohol-related damage in female, as well as the need of alcohol prevention programs especially targeted at women.

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# Diagnosis of fetal alcohol spectrum disorder (FASD): fatty acid ethyl esters and neonatal hair analysis

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**Summary.** Measuring levels of fatty acid ethyl esters (FAEE) in hair has been recently shown to discriminate between adult heavy and non-drinkers. Here, we review the potential of neonatal FAEE measurement in detecting infants exposed to alcohol *in utero* by outlining current progress in the development of a neonatal hair test for the diagnosis of fetal alcohol spectrum disorder (FASD). Developing a reproducible, accurate and predictable hair test for FAEE measurements in neonatal hair may prove to be a powerful tool in the detection of *in utero* alcohol exposure which is needed for the diagnosis of FASD. Such a neonatal hair test can revolutionize current FASD diagnostic methodology by providing early diagnosis, allowing intervention and treatment at stages where the adverse effects of alcohol can still be mitigated.

*Key words:* fetal alcohol spectrum disorder, *in utero* alcohol exposure, neonatal hair testing, alcohol biomarkers, fatty acid ethyl esters.

**Riassunto** (*Diagnosi di fetal alcohol spectrum disorders: etilesteri degli acidi grassi e analisi nei capelli di neonato*). Di recente è stato dimostrato che la determinazione quantitative di etil esteri degli acidi grassi (FAEE, fatty acid ethyl esters) nei capelli può discriminare tra adulti forti bevitori e non bevitori. In questo lavoro viene presa in considerazione la determinazione di FAEE nei capelli di neonati per riconoscere i bambini esposti ad alcol durante la gravidanza e vengono presentati i più recenti progressi in campo analitico. Lo sviluppo di un metodo affidabile per la determinazione di FAEE nei capelli del neonato potrebbe costituire un mezzo estremamente efficace per la rilevazione obiettiva di esposizione *in utero* necessaria per la diagnosi di fetal alcohol spectrum disorders (FASD). Ciò può innovare l'attuale metodologia diagnostica e rendere possibile una diagnosi precoce permettendo intervento e trattamenti adeguati in uno stadio in cui gli effetti negativi dell'alcol possono ancora essere limitati

*Parole chiave:* fetal alcohol spectrum disorder, gravidanza, alcol, capelli, analisi, biomarcatori.

## INTRODUCTION

Prenatal alcohol exposure is associated with a wide spectrum of adverse effects known as fetal alcohol spectrum disorder (FASD). Diagnosing an infant who has been exposed to alcohol *in utero* can be an extremely difficult task since often, the effects of gestational drinking on the fetus may not be clinically evident at birth or shortly thereafter. In less apparent cases of FASD where no physical signs have manifested, (*e.g.*, the alcohol related neurodevelopmental disorder, ARND), children exposed to ethanol during pregnancy may go undetected until the adverse effects of impaired brain growth become evident [1].

Diagnosis of FASD is difficult since this requires in most cases positive confirmation of heavy maternal drinking. According to current diagnostic guidelines [2], without the distinctive pathognomonic facial features seen in fetal alcohol syndrome (FAS), confirmation of *in utero* alcohol exposure is required. Admission to gestational drinking, especially of addictive patterns, may not be the most accurate information source for *in utero* ethanol exposure [3]. Maternal self-reporting is often

unreliable because of the countless stigmas associated with a pregnant mother's admission to risky behaviours [3]. Questionnaires, such as the TWEAK and T-ACE, have been developed to facilitate a physician's ability to screen their pregnant patients for problem drinking. Unfortunately, the effectiveness of these tests is dependent on frank maternal reports [3].

In attempting to find an accurate method to detect problem drinking in pregnancy, laboratory biochemical blood markers have often attempted alone or in combination to identify alcohol consumption. Immediately following ingestion, ethanol can be measured in blood, breath or urine. However, using ethanol itself or its aldehyde can only indicate recent exposure due to their relatively rapid metabolism and lack of appreciable accumulation for long periods of time. The association between gestational alcohol consumption and maternal biochemical markers such as gamma-glutamyl transferase (GGT), mean corpuscular volume (MCV), haemoglobin-associated acetylaldehyde (HAA) and carbohydrate deficient transferrin (CDT) have some potential, but many of these tests are unavailable in

most settings [4]. Studies evaluating the effectiveness of these biochemical markers in pregnancy are still very limited and to date, no single laboratory test exists that is sufficiently reliable for the identification of heavy gestational drinking.

Unlike biochemical blood markers, hair analysis is often used as a tool for the retrospective detection of illicit and/or therapeutic drug exposure over a prolonged time period. It has been successfully described for detection of cocaine, marijuana, nicotine, opiates and amphetamine use [5]. The parent compounds and their metabolites are deposited in the cortex of the hair shaft through the blood stream [6]. Hydrophobic drugs tend to accumulate significantly more into the hair shaft and remain for the life of the hair or until cut [7].

In typical hair analysis, substances are extracted from the hair shaft and the hair extract is screened using an immunoassay. Results are then confirmed by gas chromatography/mass spectrometry (GC/MS). Hair may also be dissolved and compounds detected by radioimmunoassays (RIA) [6].

Since many drugs of abuse are retained in hair for prolonged periods of time, maternal hair analysis is a useful method for monitoring drug use in pregnancy [8]. This technique has also been performed successfully in the neonate to confirm suspected *in utero* exposures to such drugs as nicotine and cocaine [7, 9]. Neonatal hair begins to grow at approximately six to seven months of fetal life [6]. Thus, any exposures within the last trimester of pregnancy may be theoretically found in neonatal hair after birth.

Ethanol is a highly volatile compound and hence, it is not retained within the hair matrix. When looking for a possible hair biomarker for *in utero* alcohol exposure, candidates must have the capability of accumulating sufficiently within the hair shaft; ethyl glucuronide, phosphatidylethanol, cocaethylene, acetylaldehyde adducts to protein and fatty acid ethyl esters (FAEE) are the current main contenders.

Until recently, lack of neonatal biological markers for *in utero* exposure to ethanol has severely limited the ability of physicians to appropriately diagnose FASD. FAEE are products of the non-oxidative metabolism of

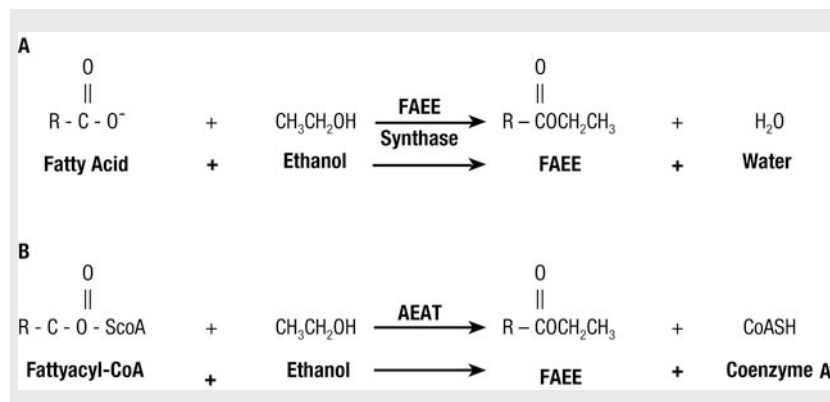
ethanol and have been proposed as biological markers of acute and chronic exposures to alcohol in adults due to their long half-life in blood and their ability to accumulate within various biological matrices [10, 11].

The following is a review of the usefulness of FAEE in detecting infants exposed to alcohol *in utero*, outlining current progress in the development of a neonatal hair test for the diagnosis of FASD. Developing a reproducible, accurate and predictable hair test for FAEE measurements in neonatal hair may prove to be a powerful tool in the detection of *in utero* alcohol exposure leading to the subsequent diagnosis of FASD in many infants. A neonatal hair test to identify infants exposed to alcohol *in utero* can revolutionize current FASD diagnostic methodology and provide early diagnosis, allowing intervention and treatment at stages where the adverse effects of alcohol can still be mitigated.

### FATTY ACID ETHYL ESTERS

FAEE are non-oxidative metabolites of ethanol. They are formed via the esterification of ethanol with endogenous fatty acids or fatty acyl-CoA (Figure 1). Two main enzymes are involved in FAEE formation: FAEE synthase and acyl-coA:ethanol O-acetyltransferase (AEAT). FAEE synthase is present in almost all human tissue with the highest levels reported in the pancreas [12, 13]. Studies have shown that AEAT activity can be several-fold higher than FAEE synthase activity in many human organs and tissues, including the heart, liver, duodenal mucosa, lung, adipose tissue and gall bladder [10]. Only pancreatic activities of AEAT and FAEE synthases have been found to be comparable [14]. Fatty acids most recently incorporated into the cell are preferred as substrates for FAEE synthesis [15].

In plasma, albumin transports the majority of FAEE [16]. Fatty acids have a higher affinity for albumin and effortlessly displace FAEE. Once displaced, free FAEE are readily and extensively broken down by a variety of cellular structures in the blood, such as white blood cells. Substantial degradation also takes place in the liver and pancreas [14].



**Fig. 1** | Formation of FAEE.  
**A.** FAEE synthase pathway using fatty acids as a substrate for FAEE production.  
**B.** AEAT pathway using acyl-CoA as a substrate for FAEE production.

Unlike ethanol, FAEE persist in blood for more than twenty-four hours after significant alcohol consumption and have been found to concentrate in adipose tissue quite readily (half-life = 16.5 hrs) [17, 18]. FAEE are highly hydrophobic and have been found to concentrate in organs commonly damaged by chronic alcohol abuse. Consequently, they have been implicated as mediators of alcohol-induced organ damage [19]. *In vitro*, FAEE have the ability to uncouple oxidative phosphorylation by binding to cell mitochondrial structures. Furthermore, they are able to disrupt the membrane bilayer, increase lysosomal fragility and decrease protein synthesis, showing a significant capacity to induce cell death [20].

There are some theories that FAEE may also play an important role in the development of FAS. Studies have indicated that human and mouse placentae have substantial amounts of FAEE synthase activity [21]. These same investigators also noted that mouse heart, liver, placenta and fetal tissues accumulate ample amounts of FAEE following maternal ethanol exposure. Additionally, FAEE have the ability to persist in placental tissue for up to 7 days in mouse placenta models [21]. Thus, the potentially toxic effects of FAEE may also extend into the neonate.

#### ANALYSIS OF FAEE IN MECONIUM

FAEE have the ability to concentrate in meconium, a matrix unique to the developing fetus which has been widely used in neonatal screening for *in utero* substance exposures [8]. The deposition of drugs in meconium begins at the 13th week of human gestation. Hence, unlike blood and urine samples, this cumulative matrix can yield a detailed history of fetal exposure in the second and third trimesters. Recent studies have been able to document significantly higher levels of FAEE in the meconium of neonates of self-reporting heavily drinking mothers when compared to non-drinking controls [22]. Ethyl palmitate (E16), ethyl oleate (E18:1), ethyl stearate (E18), and ethyl linoleate (E18:2) were the predominant FAEE detectable in the meconium of neonates exposed to excessive amounts of alcohol *in utero* [23].

These findings have documented the promise of using FAEE levels as a powerful biological marker of *in utero* ethanol exposure. One of the issues concerning meconium analysis, however, is that meconium exists only during the first three post-natal days. Consequently, diagnosis of maternal drinking may be missed thereafter. Unlike meconium, neonatal hair collection can occur up to 3 months after birth, at which point neonatal hair typically sheds. Thus, measuring FAEE in neonatal hair rather than meconium may increase the window of opportunity to confirm *in utero* alcohol exposure.

#### FAEE ACCUMULATION IN HAIR

Although much potential may exist for other biological hair markers to identify *in utero* alcohol exposure, presently none show as much promise and reliability as FAEE. As a result of their hydrophobic nature, FAEE have the potential to accumulate significantly into the hair and remain for the life of the hair or until it is cut

[7]. Pragst and colleagues have documented increased FAEE concentrations in the hair of adult alcoholics [24, 25]. Using headspace solid-phase micro extraction (HS/SPME) and gas chromatography-mass spectrometry (GC/MS), they succeeded in developing a reliable and sensitive method for the routine analysis of myristic (E14), E16, E18, and E18:1 in adult hair. Detection limits (LOD) ranged between 0.03 and 0.13 pmol/mg of hair with an assay reproducibility between 3.5 and 16% [2, 26]. E16 and E18:1 were found in the highest concentrations, with means of 5.94 and 7.08 pmol/mg of hair, respectively. In contrast, hair taken from children and teetotalers failed to yield detectable levels of FAEE. For social drinkers, defined as an alcohol consumption of approximately 2 to 4 standard drinks per week, levels of FAEE were much lower than what was seen in alcoholic samples, with maximum E16 and E18:1 levels of 1.40 and 1.03 pmol/mg. These results have provided solid evidence that the accumulation of FAEE in hair may be dose-dependant and that the measurement of FAEE concentrations in hair can be used as biological markers for excessive alcohol consumption in adults.

#### USING FAEE AS BIOLOGICAL MARKERS IN NEONATAL HAIR TO DETECT *IN UTERO* ALCOHOL EXPOSURE

A case study using the method described by Pragst *et al.* has revealed that FAEE are present in the hair of neonates exposed to alcohol prenatally. Klein *et al.* have documented the presence of measurable levels of FAEE in the hair of an admitted gestational drinker and her neonate [22]. Both maternal and newborn hair were positive for FAEE, at 2.6 and 0.4 pmol/mg respectively. This case study was the first to suggest that neonatal hair analysis of FAEE may hold much promise as a potential biomarker for *in utero* alcohol exposure.

Following these preliminary evidence, our laboratory has been extensively involved in establishing a valid method for the measurement of FAEE in neonatal hair. In investigating the possibility of using FAEE levels in neonatal hair as a means to identify gestational drinking, studies involved assay development, analysis of FAEE stability within the hair matrix, animal studies and baseline establishment of FAEE in neonates born to non-drinking mothers.

#### Assay development

In developing an assay for the measurement of FAEE in neonatal hair, one of the main challenges was to ensure the assay was sufficiently sensitive to detect the low levels of FAEE that were expected in this type of sample. To date, we have successfully created such an assay using solid-phase extraction (SPE) with GC/MS in chemical ionization (CI) mode. This method was modified from our meconium assay and a GC/MS assay currently implemented by Pragst *et al.* [25, 27]. Briefly, 10 to 20 mg of washed and dried, hair samples were cut into pieces of about 1 mm length and extracted overnight with a mixture of 0.5 ml of dimethylsulfoxide, 4.0 ml hexane and 100  $\mu$ l of heptadecanoic as an internal

**Table 1** | LOD and LOQ for each individual FAEE included in neonatal hair assay

FAEE	LOD (pmol/mg)	LOQ (pmol/mg)
Lauric (E12)	0.022	0.055 – 0.219
Myristic (E14)	< 0.010	0.010 – 0.049
Palmitoleic (E16:1)	0.018	0.044 – 0.177
Palmitic (E16)	< 0.009	0.009 – 0.044
Oleic (E18:1)	< 0.008	0.008 – 0.040
Stearic (E18)	< 0.008	0.008 – 0.040

standard for the quantification of FAEE. The hexane layer was then collected and SPE performed. Following a final reconstitution in 50 µl of hexane, samples were analysed for six FAEE using GC/MS/CI with isobutene as our ionizing gas. Extraction efficiencies range from 40% to 73% while the LOD ranged from 0.008 to 0.084 pmol/mg for individual esters (Table 1).

SPE has been extensively used in the isolation of FAEE from matrices such as blood, tissue and meconium [19, 27, 28]. This is the first time it has been used in isolating FAEE from hair. In addition, CI is a “softer” ionization mode which causes less fragmentation of FAEE upon impact. In choosing CI rather than EI for our analysis, we have been able to develop a method with analytical limits 4-fold lower than those found in current methods [24, 25]. We have also expanded the FAEE analysis profile to six rather than four esters, choosing to include lauric (E12), E14, E16, palmitoleic (E16:1), E18, E18:1 in our FAEE analysis (Table 2). There is a growing body of evidence to suggest that the cumulative analysis of all commonly occurring FAEE in biological matrices rather than the analysis of one specific species of FAEE is a more accurate way to identify neonates with suspected prenatal exposure to ethanol [29]. Thus including more FAEE in the analysis of hair may have increased the predictive value of this test to identify individuals, adults and neonates, who have been exposed to heavy levels of alcohol.

Using this assay, hair samples from three heavy drinkers (defined by a weekly alcohol consumption of more than 9 standard drinks for females and 14 standard drinks for males) and three non-drinkers were analysed. The average amount of FAEE quantified in our drinking cohort was  $6.33 \pm 1.03$  pmol/mg while only trace amounts of

FAEE were measured in the hair of our no drinking cohort, indicating that the current assay is able to discriminate between adult heavy drinkers and non-drinkers.

### Stability of FAEE in the hair matrix

Recent studies in our laboratory have found traces of FAEE in hair samples taken from adult Peruvian and Chilean mummies dating back to 1000-1250 AD [30]. Quantifiable FAEE included E16, E16:1, E18, and E18:1 while the mean cumulative FAEE concentration was  $0.773 \pm 1.136$  pmol/mg. Their presence indicates that FAEE are highly stable within the hair matrix and may be useful as long-term biological markers for alcohol exposure. Similar stability has been documented in mummy hair for cocaine and its metabolite, benzoylecgonine [31, 32]. Studies have indicated that FAEE have no general tendencies to deteriorate over several days in refrigerated meconium samples [33]. Given their stability in an enzymatically active matrix such as meconium, it is not surprising that FAEE exhibit prolonged stability in an enzymatically innate matrix like hair.

### Animal studies

In our attempts to establish whether FAEE accumulate in neonatal hair in a reproducible and predictable manner after chronic prenatal ethanol exposure, we used guinea pigs as an experimental animal to investigate the potential of FAEE in neonatal hair (Caprara *et al.*, *Pediatr Res*). Guinea pigs were chosen as an ideal animal model since, similar to humans and unlike rats and mice, pups are born with hair. The pharmacokinetics of ethanol in pregnant guinea pigs are well established with measured maternal ethanol blood concentrations corresponding to those shown in alcoholic mothers [34-36]. Furthermore, this model has been successfully used in studying ethanol teratogenesis [37-39]. Thus, this animal model of gestational alcohol exposure can provide needed insight into the feasibility of using FAEE in neonatal hair as potential biomarkers for *in utero* alcohol exposure.

Pregnant guinea pigs were dosed daily throughout their pregnancy with chronic maternal ethanol regimens mimicking a binge-type drinking pattern with an apparent blood-alcohol concentration (BAC) of 260 mg/dl, more than twice the legal BAC limit in Canada. It is a dosing regimen shown to cause fetal neurotoxicity in the guinea pig [40]. Dosing began on gestational day (GD) 2 through

**Table 2** | Individual FAEE molecular weights, retention times and quantification/qualifier ions

FAEE	Molecular weight (g/mol)	Approx. retention time (min)	Ions
Lauric (E12)	228.4	7.55	<b>229</b> , 227
Myristic (E14)	256.4	8.71	<b>257</b> , 255
Palmitoleic (E16:1)	282.5	9.66	<b>283</b> , 284
Palmitic (E16)	284.5	9.75	<b>285</b> , 286
Heptadecanoic IS	298.5	10.25	<b>299</b> , 300, 297, 298
Oleic (E18:1)	310.5	10.60	<b>311</b> , 312
Stearic (E18)	312.5	10.70	<b>313</b> , 314

Quantification ions are expressed in bold italic.



GD 67 and hair samples were taken from pregnant mothers at GD 57 and 65, as well as from offspring at postnatal day (PD) 1 and 10. Isocaloric sucrose and water control animals were used as a means for comparison.

Elevated levels of FAEE were repeatedly measured in the hair of chronically dosed pregnant guinea pigs and their pups in comparison to their respective controls. Overall, ethanol treated mothers had 10-fold higher levels of FAEE compared to their controls while ethanol exposed pups had a 15-fold higher cumulative FAEE level than their sucrose and water counterparts (Caprara *et al.*, *Pediatr Res*). Our results verify for the first time that chronic exposure to alcohol leads to increased levels of FAEE in both maternal and neonatal hair in guinea pigs. The documented presence of FAEE in the hair of neonatal guinea pigs provides evidence that FAEE do have the ability to accumulate to significant concentrations in the hair matrix of neonates exposed to heavy amounts of alcohol *in utero*. These data suggest that using FAEE in neonatal hair as biomarkers for gestational alcohol exposure in humans is feasible and may be useful in identifying children exposed to alcohol during pregnancy.

The use of an experimental animal model overcomes the most critical challenge of human studies extending the use of FAEE into neonatal hair; the reliance on maternal reports of alcohol consumption and drinking schedules. By using an animal model, one can administer a highly controlled dose of ethanol and have accurate account of prenatal alcohol exposure. Such a controlled environment has allowed us to evaluate the detailed relationship between FAEE levels in hair and ethanol exposure. We have been able to shed light on the full potential of FAEE as biological hair markers for *in utero* alcohol exposure. Future studies will employ this novel model to establish the full dose-response curve between steady-state maternal alcohol blood concentrations and neonatal outcomes (*e.g.*, neurobehavioral).

#### ***Human studies: baseline establishment of FAEE in neonatal hair***

Preliminary baseline studies indicate that certain FAEE can be found in the meconium and hair of neonates with-

out daily or prenatal alcohol exposure [27]. The reason for this phenomenon is unclear but may be due to physiologic and pathologic conditions. Ethanol is a common by-product of routine physiologic metabolism in the human gut [41]. Small quantities of alcohol may also be present in medications and food additives. Furthermore, fatty acid alkyl esters, mostly ethyl and methyl esters, are naturally present in different types of olive oil [42]. Thus, it is not unreasonable to expect endogenous levels of FAEE to exist that may originate from sources other than alcohol consumption.

To account for endogenous production and accumulation of FAEE in neonatal hair, a baseline study was conducted using samples taken from non-drinking and socially drinking mothers and their neonates (Caprara *et al.*, *Ther Drug Monit*). All drinking patterns were social. Fifty-six neonates up to 2 months of age were enrolled in the study and their hair was analysed for FAEE. Our results demonstrated that a baseline level of FAEE exists in the hair of neonates born to non-drinking mothers. No significant differences in total FAEE concentrations were found between neonates born to gestational drinkers and non-drinkers. As a group, the mean ( $\pm$  SEM) level of FAEE measured in neonatal hair was  $0.321 \pm 0.088$  pmol/mg with levels ranging from 0.000 to 2.953 pmol/mg. Within the ranges of alcohol consumption seen in this study population, quantity, time and duration of gestational alcohol exposure did not have a significant effect on total FAEE concentrations found in neonatal hair. Similar baseline results were found in meconium samples taken from infants born to non-drinking mothers [27]. As shown above, data from guinea pig model also demonstrated baseline concentrations of FAEE in hair taken from sucrose and water control pups that were not exposed to ethanol *in utero*. It is evident that measurable levels of FAEE are present in humans not actively consuming alcohol. Consequently, it is critical to define such a baseline level before FAEE levels in neonatal hair can be used to accurately identify infants exposed to alcohol *in utero*.

Data collected from our baseline cohort indicated that 39% of the women in our population consumed any amount of alcohol at some point throughout gestation (Table 3).

**Table 3** | Drinking patterns among women in baseline study

Number of women with a history of alcohol use	40 (65%)
Total number of gestational drinkers	24 (39%)
Total number of drinks in pregnancy among gestational drinkers (mean $\pm$ SD)	8.7 $\pm$ 12.9
Range	0.5-56
Number of women drinking in T <sub>1</sub>	16 (66%)*
Number of women drinking throughout pregnancy (T <sub>1</sub> - T <sub>3</sub> )	5 (21%)*
Drinking by trimester:	
T <sub>1</sub>	8 (33%)*
T <sub>1</sub> + T <sub>2</sub>	1 (4%)*
T <sub>1</sub> + T <sub>3</sub>	2 (8%)*
T <sub>2</sub> + T <sub>3</sub>	3 (15%)*
T <sub>3</sub>	5 (21%)*
* % of gestational drinkers	

No difference in FAEE concentrations in neonatal hair was seen between these gestational drinkers and those women who abstained completely from alcohol throughout their pregnancy. All of the gestational drinkers were categorized as mild, social drinkers. Our assay may not be able to discriminate between neonates unexposed to alcohol *in utero* from neonates exposed to mild levels of alcohol throughout gestation. Nonetheless, if a neonatal hair sample is analyzed and FAEE levels are found to be significantly higher than our baseline cohort (drinker and non-drinkers), this may be an indication that maternal drinking during pregnancy was at a heavier, problematic level; a consumption level whereby the toxic effects of *in utero* alcohol exposure may be much more prevalent.

In our sample population, 26% of women admitted to drinking small amounts of alcohol in the second and/or third trimester, a period of gestation when hair grows *in utero*. This constitutes the first evidence that mild, rare drinking in the second and third trimester will not lead to a significant accumulation of FAEE in neonatal hair above baseline levels.

#### **Future challenges**

The collection of drinking history using a standardized questionnaire is a major limitation to any study, as maternal admission to drinking in pregnancy is an unreliable and inaccurate method to identify *in utero* alcohol exposure [3]. We are assuming the women in our study cohort were truthful about their ethanol ingestion throughout pregnancy. However, recall bias, guilt and embarrassment may affect our data on gestational alcohol consumption.

Although a preliminary baseline for FAEE levels in neonatal hair has been established, much work is still needed to validate the use of FAEE in neonatal hair as potential biomarkers for *in utero* alcohol exposure. Using a guinea pig animal model where the chronic maternal ethanol regimen mimics the binge-type drinking pattern found in humans, we have shown that the use of a neonatal hair test to identify prenatal ethanol exposure is feasible. To properly validate such a test in humans and to determine clinical sensitivity and specificity of the assay, hair samples from confirmed heavy gestational drinkers and their neonates are critical to define a positive FAEE level screening cut-off in hair. Until such studies are conducted, expected FAEE levels in hair samples taken from this heavy drinking population will remain unknown.

When using hair analysis for any type of drug testing, there are other issues that must also be addressed. Studies have shown that for certain compounds like nicotine and cotinine, hair properties, such as colour and texture, may influence drug incorporation and distribution into hair [43]. Chemical treatments, such as hair dyes and perms can damage the structural integrity of the hair shaft and

may further affect drug accumulation and detection [44]. As the use of FAEE levels in hair increases, it will be necessary to investigate such issues that may influence the incorporation of FAEE into both adult and neonatal hair.

#### **CONCLUSIONS**

The development of a reliable biomarker of alcohol consumption during pregnancy is a critical step in allowing early diagnosis of FASD. Diagnosing FASD is a difficult task, especially in cases where characteristic physical facial abnormalities have not manifested.

FAEE have shown much promise as the first useful biomarkers for *in utero* alcohol exposure and have been used successfully in meconium to identify alcohol-exposed infants. Meconium, however, is a short-lived species. If collection is not performed days after birth, meconium is lost and so is the opportunity to confirm alcohol consumption during pregnancy in cases where maternal admission is unreliable or unavailable. By extending the use of FAEE into neonatal hair, we may extend the window of opportunity to collect samples up to 3 months postpartum, thus increasing our ability to identify children exposed to alcohol *in utero*.

We have successfully developed a sensitive, specific and reliable assay for the measurement of FAEE in hair able to discriminate adult non-drinkers from heavy drinkers. FAEE are highly stable within the hair matrix and that baseline, endogenous levels of FAEE exist, independent of active alcohol exposure in both guinea pig and human neonatal hair.

Using this novel assay, we have confirmed for the first time that chronic exposure to alcohol leads to increased levels of FAEE in both maternal and neonatal hair in guinea pigs. This provides solid evidence that using FAEE in neonatal hair as biomarkers for *in utero* alcohol exposure is feasible and may be useful in identifying children exposed to alcohol during pregnancy.

Our studies have served in extending the use of FAEE into neonatal hair to objectively identify children exposed to alcohol *in utero*. Such advancement will revolutionize a physician's ability to diagnose FASD and will facilitate the early diagnosis of FASD, providing intervention at stages where the effects of prenatal alcohol exposure can be minimized and/or prevented.

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# Brain imaging and fetal alcohol spectrum disorders

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**Summary.** Over thirty years of research has revealed that prenatal exposure to alcohol has a devastating impact on the structure and function of the developing central nervous system. Imaging studies over the past ten years have improved our understanding of the structural alterations related to prenatal alcohol exposure and provided researchers with potential hypotheses for brain-behavior relationships. Structural alterations associated with prenatal alcohol exposure have been found in overall brain size, shape, and symmetry, along with regional decreases in white and gray matter. In addition, abnormalities have been noted in specific structures such as the cerebellum, basal ganglia, and corpus callosum. This review demonstrates that specific areas of the brain may be more vulnerable to prenatal exposure to alcohol.

*Key words:* fetal alcohol syndrome, imaging, prenatal alcohol exposure, brain.

**Riassunto** (*Diagnostica per immagini e sindrome fetoalcolica*). Oltre trent'anni di ricerca hanno dimostrato che l'esposizione prenatale ad alcol ha un effetto devastante sulla struttura e funzione del sistema nervoso centrale in via di sviluppo. La diagnostica per immagini negli ultimi dieci anni ha molto migliorato la comprensione delle alterazioni strutturali legate all'esposizione prenatale all'alcol e ha fornito agli studiosi nuove ipotesi di relazione tra sviluppo cerebrale e comportamento. Le alterazioni strutturali alcol-relate riguardano dimensione, forma e simmetria del cervello, e diminuzioni della materia bianca e grigia. Inoltre, sono state trovate anomalie in strutture specifiche quali cervelletto e corpo calloso. Questo lavoro dimostra che specifiche aree del cervello possono essere più vulnerabili all'esposizione prenatale all'alcol.

*Parole chiave:* sindrome fetoalcolica, diagnostica per immagini, esposizione prenatale ad alcol, cervello.

## INTRODUCTION

The teratogenic effects of alcohol on the developing brain have been known for over thirty years [1-3]. During this period, a host of neuropsychological and behavioral deficits associated with prenatal alcohol exposure have been identified and have helped to increase awareness of this public health concern. Although a limited number of autopsy studies have been completed, it was not until the utilization of imaging techniques during the last ten years that the opportunity to view the range of structural effects resulting from prenatal alcohol exposure on the living brain has been available.

Perhaps the most widely recognized consequence of prenatal alcohol exposure is fetal alcohol syndrome (FAS). FAS is a major public health concern wherever women consume alcohol and the overall prevalence in the United States is estimated to be between 0.5 and 2.0 per 1000 births [4]. It is diagnosed based on a characteristic pattern of facial abnormalities, pre- and/or postnatal growth deficiency, and central nervous system (CNS) dysfunction. Facial alterations typically seen in individuals with FAS include short palpebral fissures, thin vermilion of the upper lip, and smooth philtrum. CNS dysfunction can be evidenced

by functional and/or structural alterations depending on the specific diagnostic criteria used [5, 6]. Common functional impairments include deficits in overall intellectual ability, problem solving, language, social skills, and motor functioning. Structural alterations, the focus of this article, can be determined by brain imaging or at autopsy, or a proxy such as head circumference or neurological signs may be used.

However, FAS is not the only consequence of prenatal alcohol exposure. Recently, the National Organization on Fetal Alcohol Syndrome coined the term fetal alcohol spectrum disorders (FASD) to describe the full range of effects resulting from prenatal exposure to alcohol. These effects range from FAS on the most severe end to subtle physical, cognitive, or behavioral effects. When considering the full range of effects, prevalence estimates of FASD are approximately 1 in 100 [7]. Various moderating factors have been proposed that may account for the variability of outcomes resulting from prenatal alcohol exposure. Some of these factors include genetic variation, timing and amount of alcohol consumed, nutrition, maternal age, socioeconomic status, and family and community resources [4]. FASD was not intended to be a diagnostic term, but serves to unify diagnostic labels such as

alcohol related neurodevelopmental disorder, alcohol related birth defects, and FAS as belonging to the same spectrum. FAS is sometimes referred to as dysmorphic FASD due to the presence of diagnostic facial features, whereas individuals on the remainder of the spectrum are nondysmorphic, or lack the constellation of characteristic facial features. Numerous studies have shown that individuals with prenatal exposure to alcohol, with or without facial dysmorphia, perform similarly on a number of neuropsychological and behavioral measures [8]. Thus, it is not unreasonable to expect similar patterns of structural brain alterations.

Autopsy studies have revealed heterogeneous findings in individuals with FASD including gross microcephaly, cellular disorganization, migration errors, microdysplasia, and anomalies to such structures as the corpus callosum and cerebellum. These findings have been reviewed elsewhere [9], and therefore the focus of this review will be on evidence of structural alterations found in imaging studies. Imaging technologies offer the ability to examine structural brain damage in the living brain, and thus are more representative and generalizable to the larger population of individuals prenatally exposed to alcohol. These imaging techniques utilize differences in biological tissue density (*i.e.*, bone, gray matter, white matter) and produce data that can be reconstructed into visual images. In a research setting, these images can be analyzed quantitatively using specialized software packages and group differences can be compared.

This review will begin by describing structural alterations to the cerebrum followed by more specialized structures such as the cerebellum, corpus callosum, basal ganglia, and hippocampus. Each section will progress from identifying more global findings to more detailed and specific structural alterations. When appropriate, brain-behavior relationships will be discussed, and comparisons to other developmental disorders will be explored.

### CEREBRUM AND WHOLE BRAIN ANALYSES

Imaging studies have consistently found size reductions in the cranial vault in individuals with prenatal alcohol exposure [10-20]. According to one study, adolescents with alcohol exposure had an average volume reduction of 12% compared to controls [19]. Findings of reduced brain size are not surprising given head circumference measurements indicating microcephaly below the 10<sup>th</sup> centile in many FAS cases. In addition to gross reductions in brain size, several studies with decent sample sizes have demonstrated significant overall gray and white matter reductions in individuals with prenatal alcohol exposure [10, 15]. When reductions in brain size were taken into account, white matter was disproportionately reduced and was more severe than gray matter hypoplasia [10]. In other words, relative to overall brain volume, individuals with histories of heavy prenatal exposure may have too much relative gray matter and not enough white matter [15].

More detailed analyses have been conducted to determine if brain reductions are diffuse or more specific in

nature. Volumetric reductions have been found in the parietal, frontal, and temporal lobes [16]. However, when overall brain size was considered, only the parietal lobe was disproportionately reduced [10, 16]. This disproportionate reduction indicates that brain tissue in individuals prenatally exposed to alcohol is not uniformly affected, but rather specific areas may be more vulnerable to insult. White and gray matter volumes have also been examined for each lobe, and both white and gray matter were disproportionately reduced in the parietal lobe [10]. When examined separately in comparison with controls, individuals in the alcohol exposed group without a FAS diagnosis tended to show a similar pattern of reductions, but comparisons did not reach significance [10]. Upon visual inspection of group means, the nondysmorphic group was generally intermediate to the FAS group and controls. These findings suggest that individuals without dysmorphic facial features show a similar pattern of alterations, but may be less severely affected than individuals with FAS.

Based on the disproportionate reductions found previously, voxel-based morphometric analyses have been conducted with individuals with prenatal alcohol exposure to examine regional alterations in brain tissue. Voxel-based morphometry allows the whole brain to be analyzed at once without the delineation of specific regions required in volumetric analyses. It also permits the examination of brain regions without clear gyral or structural boundaries and regional alterations that may be obscured with more global volumetric measurements can be identified with this approach. Analyses were conducted examining group differences for gray and white matter separately [15]. For the gray matter analysis, significant differences in tissue composition were found between groups for 17 clusters, with the largest occurring in the left posterior temporo-parietal region. The exposed group exhibited increases in gray matter for all clusters where the control group tended to have cerebral spinal fluid (CSF) or white matter. Similarly, the exposed group showed significant decreases in white matter for 25 clusters where voxels tended to segment as gray matter or CSF in controls. The largest region of white matter reduction corresponded with gray matter findings. In other words, alcohol exposed subjects tended to have increased gray matter and decreased white matter in the left posterior temporo-parietal region. In addition, separate analyses revealed similar patterns for both dysmorphic and nondysmorphic individuals with histories of prenatal exposure to alcohol. The number of significant clusters was greater and individual clusters tended to be larger for the group with FAS, whereas nondysmorphic individuals exhibited less robust clusters [15].

In concordance with the voxel-based findings, a separate study [16] found a 15% increase in gray matter density bilaterally in the inferior parietal and perisylvian cortex. These findings were robust when overall brain size was controlled, indicating a disproportionate increase in gray matter in this region. Evidence of increased gray matter likely reflects white matter

reductions in this region, although this was not directly measured in this study. In addition to volumetric and density measures, this study conducted regional shape analyses by calculating distances from the center of the brain for over 65 000 points on the brain surface. This measurement allows for the examination of regional differences in local brain growth and when taken together illustrates alterations to brain shape. Compared to controls, alcohol exposed subjects exhibited bilateral reductions in brain extent in inferior parietal and perisylvian cortex, independent of overall brain size. Brain growth and gray matter density have been shown to be inversely related [21]. In other words, decreases in local brain growth should be related to increases in gray matter density. Consistent with this relationship, increased gray matter density and reduced white matter density have been demonstrated with individuals with prenatal exposure to alcohol in the same region of reduced local brain growth [10, 15]. Thus, brains of individuals prenatally exposed to alcohol tend to be smaller and narrower in the inferior parietal and perisylvian areas than non-exposed individuals. This study also found reduced local brain growth in the anterior and orbitofrontal cortex, specifically in the left hemisphere. The orbitofrontal cortex is proposed to be involved in socially mediated behavior and emotion-related executive skills, and alterations in this region are consistent with reports of executive deficits and difficulties with social functioning seen in individuals with prenatal alcohol exposure [22].

Based on findings of disproportionate reductions in the left hemisphere [15], asymmetry patterns were examined in adolescents with prenatal exposure to alcohol [23]. From surface based analyses, both typically developing individuals and alcohol exposed subjects showed prominent asymmetry in the perisylvian region in which inferior parietal and superior temporal cortices were shifted backward in the left relative to the right hemisphere. However, when gray matter density asymmetry was examined, alcohol-exposed subjects lacked the prominent right greater than left asymmetry seen in the posterior inferior temporal lobe in controls. This region coincides with areas primarily involved in language and object/face recognition (Brodmann areas 21, 22, 37), which may be impaired in individuals with prenatal exposure to alcohol [24]. No other differences in asymmetry were found between alcohol exposed subjects and controls. This study provides additional evidence that specific areas of the brain are more vulnerable to teratogenic insult from alcohol.

Before concluding this section, one functional study has found specific alterations in metabolic activity that are consistent with structural findings discussed above [25]. Metabolic activity serves as a measure for brain functioning in that increased metabolic rate within a specific region indicates more neural activity. Compared to data from non-exposed children, children with FAS exhibited decreases in cerebral blood flow in the left parieto-occipital region. The authors speculated that altered functioning in this region is related to difficulties that children with

FAS have with arithmetic and speech. Additionally, children with FAS exhibited hyperperfusion, or increased uptake of the radiotracer, in the right frontal lobe compared to the left, a finding which could be related to the attention deficits commonly seen in this population. In comparison, similar studies with children with attention deficit hyperactivity disorders (ADHD) have found patterns of decreased uptake in portions of the left frontal lobe [26-28].

In summary, brains of individuals prenatally exposed to alcohol are reduced in size and show narrowing in the inferior parietal and perisylvian region. Increases in gray matter density and corresponding reductions in white matter likely contribute to narrowing in this region. In addition, reduced local brain growth has been demonstrated in the area of the frontal lobe believed to be responsible for socially mediated behavior. Finally, findings of altered functional activity have been found in areas corresponding to structural alterations. It has been proposed that white matter hypoplasia may be a result of abnormal myelination. Both frontal and parietal lobes, areas shown to be affected by prenatal alcohol exposure, continue to mature into early adulthood in association with late myelination [10]. Abnormal myelin deposition could prevent normal growth and thinning, which could result in reductions in synaptic density [16]. In addition, abnormal glial cell functioning, apoptotic regulation, and errors in proliferation and differentiation of new cells may have contributed to brain alterations seen in individuals with histories of prenatal alcohol exposure [15].

## CEREBELLUM

The cerebellum is a highly convoluted region of the brain that is largely involved in the coordination of movement and motor learning. Volumetric reductions of the cerebellum have been found in several studies with individuals prenatally exposed to alcohol [10-13, 25]. In comparison with controls, individuals with FAS exhibited significant gray and white matter reductions in the cerebellum. However, when total brain size was taken into account, neither gray or white matter was disproportionately reduced in volume.

The cerebellum consists of two hemispheres, divided by connecting tissue called the cerebellar vermis. Hypoplasia of the vermis has been found in a number of individuals with FAS [11]. In a quantitative study [29], alcohol-exposed subjects showed disproportionate reductions in the anterior portion of the vermis (lobules I-V), whereas mean area measurements of other regions were nearly identical to controls. In addition, the pattern of disproportionate vermal reduction is distinct from other developmental disorders. For example, reduction in lobules VI and VIII were found in individuals with autism [30], whereas increases were noted in lobules VI and VIII in individuals with Williams syndrome [31]. In addition, disproportionate reductions of vermal lobules VIII to X have been found in ADHD [32]. Thus it appears that some portions of the cerebellum are more vulnerable to prenatal

exposure to alcohol and these regions are distinct from alterations seen in other developmental disorders.

Cerebellar alterations may explain some of the neuropsychological impairments often seen in children prenatally exposed to alcohol. The cerebellum is most often linked to motor coordination and quantitative studies have demonstrated balance impairments in alcohol-exposed individuals [33, 34]. Although historically the cerebellum has been linked to motor coordination and learning, accumulating evidence has also supported its involvement in attentional processes [32, 35-38]. Thus, alterations to the cerebellum may also account for some of the attention problems often observed in children with histories of prenatal exposure to alcohol [39-42].

### CORPUS CALLOSUM

The corpus callosum is a tract of fibers that connects the two cerebral hemispheres and forms the roof of the lateral ventricles. Development of this structure begins relatively early, beginning between the sixth and eighth gestational weeks. A definite corpus callosum is formed by weeks 12-13 and continues to grow in the caudal direction for the next 5-7 weeks. Although the corpus callosum is fully formed, additional axons continue to pass through this structure to form connections with the other hemisphere up until the third decade of life.

Alterations of the corpus callosum are among the most frequently cited brain abnormalities in imaging studies with individuals with prenatal alcohol exposure. At the most extreme end, a number of studies have found agenesis, or complete absence of the corpus callosum in children and adolescents who were prenatally exposed to alcohol [12, 17, 18, 43, 44]. Riley *et al.* reported an incidence rate of 6.8% of agenesis of the corpus callosum in their larger sample of alcohol-exposed children [43], and FAS has been proposed to be one of the leading causes of this rare condition [45]. This rate is considerably higher than the general population rate of 0.3% and even the rate of 2.3% in developmentally disabled populations. In addition to complete agenesis, others have noted less severe alterations such as marked thinning, hypoplasia, or partial agenesis, which often occur in more posterior regions [11, 12, 17-20, 25, 44, 46]. As was noted above, the corpus callosum forms the roof of the lateral ventricles, and thus it is not surprising that ventricular and other midline abnormalities commonly coincide with such marked alterations of the corpus callosum.

However, the majority of individuals with prenatal alcohol exposure do not exhibit such severe alterations in callosal morphology. Several studies have conducted quantitative studies to determine if more subtle alterations are common in individuals with prenatal alcohol exposure. Overall, the corpus callosum of alcohol-exposed subjects has been found to be significantly smaller in area and length than controls [11, 19, 43]. However, once total brain volume was added as a predictor, the area of the corpus callosum in exposed subjects was

not significantly reduced compared to controls [19]. Although the overall structure is not disproportionately reduced in area, disproportionate reductions in regions of the corpus callosum have been found, most notably in the most posterior region corresponding to the splenium [19, 43]. In addition, similar alterations in callosal morphology have been reported in cases of attention deficit-hyperactivity disorder [47-49].

In addition to area reductions, alterations in shape and location of the corpus callosum have been found in individuals with prenatal alcohol exposure [19]. Specifically, the posterior region of the corpus callosum (*i.e.*, splenium) was shown to be displaced approximately 7 mm in the inferior and anterior direction. In contrast, no evidence of displacement was found in the anterior or posterior commissures, suggesting the specificity of damage to the corpus callosum. Similar to the whole-brain analyses described above, when dysmorphic and nondysmorphic groups were separated, the nondysmorphic group showed a similar but not statistically significant pattern of callosal alterations as the FAS and alcohol group as a whole. In addition to examining alterations to brain structure, this study assessed the relationship between callosal displacement and verbal learning. Anterior-posterior displacement was found to be a significant predictor of verbal learning for the exposed group. Specifically, individuals with more anterior displacement demonstrated more impaired verbal learning. Anterior displacement was general rather than region specific and was a better predictor of verbal learning than verbal intelligence. Thus, these findings suggest a somewhat specific relationship between callosal displacement and verbal learning rather than reflecting overall cognitive impairment. In addition, although not directly assessed, alterations to the corpus callosum are proposed to underlie the variable and inaccurate performance of children with prenatal alcohol exposure on a task of bimanual coordination [50].

An independent group of researchers studying a large prospectively identified sample has generally found no difference in the average size or shape of the corpus callosum when comparing alcohol-exposed individuals with controls [51, 52]. However, they found that individuals with prenatal exposure to alcohol have excess variability of callosal shape. Consistent with these findings, the authors [51] examined the data from Riley *et al.* and noted that the coefficients of variation for all sub-regions of the corpus callosum in exposed subjects were significantly larger than controls. Excess variation may be a result of the timing and levels of exposure during the development of the corpus callosum, which could have resulted in a number of patterns deviating from normal variation. Based on this excess variability, a classification algorithm was created that accurately classified subjects (alcohol-exposed *vs* not) with high sensitivity (100 out of 117) and specificity (49 out of 60) [52]. Bookstein *et al.* argue that by focusing on variances rather than mean differences more sensitive and clinically useful data can be obtained to discriminate alco-

hol-exposed individuals from non-exposed controls, which may result in a useful diagnostic tool.

In addition, this group assessed the relationship between callosal variation and neuropsychological performance [53]. In exposed individuals, excess shape variation was associated with two distinct profiles of cognitive impairment. A relatively thick callosum was associated with executive function deficits whereas a relatively thin callosum was related to motor deficits. The thicker corpus callosum was found not to extend as far into the frontal lobes as the thinner callosum. This may reflect abnormalities of the white matter fibers that connect the frontal lobes through this region of the corpus callosum and may result in the observed executive deficits. The authors propose that motor impairments seen in individuals with thinner callosa may be related to a loss of some white matter pathways projecting from the parietal sensory inputs, caudate nucleus, and cerebellum through the corpus callosum to motor and premotor centers. As identified in this review, abnormalities in the parietal lobe, caudate, and cerebellum have been described in individuals with prenatal alcohol exposure.

In summary, alterations of the corpus callosum have been frequently cited in imaging studies with individuals with prenatal alcohol exposure. In addition to gross abnormalities such as agenesis that are visible upon inspection, more subtle alterations have been found from quantitative studies. More subtle alterations of the corpus callosum include disproportionate area reductions especially in the most posterior region, excess variability in shape, and anterior and inferior displacement. Furthermore, alterations were related to impaired neuropsychological performance in several domains including verbal learning, executive functioning, and motor skill. In addition to the hypotheses described above, callosal dysmorphology may be due to arrested growth at the time of exposure or delayed continued development after the initial insult [19]. Several mechanisms may account for altered prenatal development including disruption of the glial facilitated migration of cells and errors in apoptosis [18]. As identified above, callosal morphological changes are consistent with alterations seen in other areas such as the caudate, cerebellum, and the perisylvian region as well as with observed neuropsychological impairments [15, 19, 53].

### BASAL GANGLIA

The basal ganglia are a group of subcortical nuclei including the caudate, nucleus accumbens, putamen, and globus pallidus. The putamen and globus pallidus are often grouped and referred to as the lenticular nucleus. Collectively, the basal ganglia are often considered to control voluntary motor function. However, as with many of the structures described in this review, the basal ganglia maintain many connections with other regions of the brain and may be involved in other critical functions such as executive functioning, motivation, and social behavior. Smaller case studies with

individuals with prenatal exposure to alcohol found disproportionate reductions in the volume of the basal ganglia [12-14]. However, when overall brain size was taken into account only disproportionate reductions were evident in the caudate [12, 14] with gray matter being disproportionately affected [12]. The volume of the lenticular nucleus was relatively spared [14]. A larger study found similar results with a disproportionate reduction of the caudate and relative sparing of the lenticular nucleus and nucleus accumbens [10]. In addition to structural alterations noted in the caudate, a functional study with adolescents with prenatal alcohol exposure found significant reductions in metabolic activity in the thalamus, the caudate heads, and the right portion of the caudate-putamen body [46]. Overall, research suggests that the caudate may be specifically affected both structurally and functionally by prenatal exposure to alcohol, which may be related to functional impairments observed in this population.

### HIPPOCAMPUS / AMYGDALA

The hippocampus and the amygdala are components of the limbic system and are generally considered to be involved in the formation of long term memories and emotion respectively. These structures maintain connections with each other as well as receive projections from other areas of the brain. Few imaging studies with individuals with prenatal alcohol exposure have noted alterations to the hippocampus, and those that have found abnormalities generally have reported on a few individuals with gross alterations [11, 44]. However, one study with a small sample of individuals with FAS noted significant differences in the asymmetry of the hippocampus with the right larger than the left [25]. In contrast, Archibald *et al.* found that the hippocampus was relatively spared when the whole brain volume was taken into account [10]. So far, studies have consistently found relative sparing of the amygdala [10, 13, 25].

### CONCLUSIONS

Prenatal alcohol exposure has been shown to have effects on brain development. This review has illustrated that specific portions of the brain are more vulnerable to the teratogenic insult of alcohol. While brains of individuals prenatally exposed to alcohol tend to be smaller in size, portions of the parietal and temporal lobes near the perisylvian region have been found to be disproportionately affected, exhibiting narrowing and decreases in white matter. In addition to these robust findings, some structural and functional abnormalities have been noted in the frontal lobe. Specific disproportionate alterations to the cerebellar vermis, corpus callosum, and caudate have been found in a number of studies. Such specific alterations were not expected based on the heterogeneity of autopsy findings. However, cases that reach autopsy may not be representative of the larger population affected by prenatal alcohol exposure. That is, these cases may represent the most severe end of the spectrum. Thus, imaging



studies are able to study a larger more representative portion of individuals with prenatal alcohol exposure and therefore have shown more consistent results.

Brain imaging is still a relatively young technique and advancements in acquiring and analyzing images are continually being made. Thirteen years have passed since the first report of brain imaging with two children with FAS [12]. Since that time, additional studies have provided a clearer understanding of the structural impact of prenatal alcohol exposure. However, much is still to be learned. Many of the findings have yet to be replicated with independent samples. Due to the extensive cost and time involved in imaging studies, sample sizes are typically small. Therefore there is a considerable chance for bias, which speaks to the need for replication. Future studies should continue applying newly developed analytic techniques such as the surface-based analyses described above. Additional imaging techniques such as diffusion tensor imaging may prove to be illustrative of structural damage resulting from prenatal alcohol exposure. Furthermore, few functional imaging studies have been conducted with individuals with prenatal alcohol exposure. Although we have evidence of specific structural brain alterations, knowledge of the location of functional anomalies will aid in interpreting structural and behavioral findings. A few studies reported above have directly examined brain-behavior relationships for specific brain alterations. Future studies should continue

to directly assess brain-behavior relationships rather than draw parallels from the literature base and neuropsychological theory. This is especially important for prenatal alcohol exposure and other developmental disorders because the developing brain may have a drastically different pattern of functional organization than the adult brain. Hopefully improvements in developmental neuropsychological theory will allow for more precise interpretations and provide additional hypotheses for brain-behavior relationships.

In conclusion, brain imaging has improved our understanding of alcohol's effects on brain structure during development and has demonstrated that not all areas of the brain are equally vulnerable. In addition to summarizing the existing literature on brain imaging and prenatal alcohol exposure, this review has attempted to draw some preliminary conclusions on brain-behavior relationships. Future studies will further clarify both the structural and functional effects of prenatal alcohol exposure on the developing brain. With this knowledge, recognition of brain alterations can be integrated with behavioral and neuropsychological findings to better understand the teratogenic effects of alcohol on the whole person and develop improved interventions.

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# Fetal alcohol syndrome disorders: experience on the field. The Lazio study preliminary report

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**Summary.** In Italy, little is known about the problems related to alcohol drinking during pregnancy. In this paper, the Italian literature about this subject is briefly reviewed. This first Italian experience of a field study, aimed to the assessment of the prevalence of fetal alcohol syndrome (FAS) and fetal alcohol spectrum disorders (FASD) in an area in the Rome province (Lazio region) is reported. This in-field study was performed in the school years 2003-2004 and 2004-2005 in cooperation with American researchers, most from University of New Mexico (Albuquerque), and Italian researchers from University "la Sapienza" of Rome. First grade children ( $n^{\circ} = 1086$ ) of primary school were contacted to enter in the in-school study for the detection of FAS and FASD and were examined by the experts team of clinicians, pediatrics, psychologists. Preliminary consideration and the implications of this study for FASD prevention are discussed.

*Key words:* fetal alcohol syndrome, epidemiological clinical study, Italy.

**Riassunto** (*Sindrome fetoalcolica: dati preliminari dello studio svolto nel Lazio*). In Italia, la conoscenza delle problematiche connesse al consumo di alcol in gravidanza è ancora scarsa. Dopo una breve rassegna della letteratura italiana sull'argomento, questo lavoro descrive l'esperienza effettuata sul campo durante uno screening (il primo studio italiano) volto ad individuare la prevalenza della sindrome fetoalcolica e, più globalmente, dei disordini legati ad esposizione fetale ad alcol in 15 comuni del Lazio nell'area della provincia di Roma. Lo studio è stato effettuato negli anni scolastici 2003-2004 e 2004-2005 ed è stato svolto in collaborazione tra ricercatori americani, molti dell'Università del New Mexico (Albuquerque), e italiani dell'Università "La Sapienza" (Roma). Sono stati contattati 1086 alunni delle classi di prima elementare delle scuole presenti nel territorio. Vengono discusse le considerazioni circa la percentuale di risposte e le implicazioni in termini di prevenzione dovute al processo attivato da tale studio.

*Parole chiave:* sindrome fetoalcolica, studio clinico epidemiologico, Italia.

## INTRODUCTION

Fetal alcohol spectrum disorders (FASD) is a cluster of abnormalities occurring in children born to mothers with histories of alcohol drinking during pregnancy, while fetal alcohol syndrome (FAS) is the full-blown syndrome. The diagnosis of FAS needs the existence of specific signs in each of the following categories: growth retardation, central nervous system involvement and the characteristic face.

In Italy, little is known about FAS and FASD. The teratogenic effects of alcohol were first reported in France in by Lemoine in the 1968 [1] but it remained substantially ignored until, in the '70, it was described by Jones *et al.* in 1973 [2-3]. Up to today, the awareness of FAS problem is poor in Italy both among experts (doctors, psychologists, social operators and teachers) and laymen.

In US, the prevalence of FAS is 1-3‰ [4] and the highest prevalence in the world was found in South Africa, where 46‰ of children were affected by FAS even if the real prevalence could be even higher, according to studies in progress [5]. High prevalence was found also in ethnic minorities.

No reliable estimate of FAS prevalence in Italy: epidemiological studies using active case ascertainment are lacking, as in the other countries of Western Europe. Indeed, while in ICD-9 the 760.71 code for FAS was introduced in 1976, the prevalence of FAS was severely underestimated in hospital diagnosis at birth, since most of the abnormalities may be related to other birth defects or other mechanisms (malnutrition, genetic disorders), or may be detected only when the child ages.

Only few case reports were published about children born to Italian mothers, with symptoms like microcephaly, hypoplasia of corpus callosum, hyperactivity, low IQ, not different from FAS symptoms registered in other countries [6-10].

Conflicting results are reported in the few Italian studies about the effect of prenatal exposure to alcohol: in some studies, negative effects are reported, as spontaneous miscarriage, low weight at birth, jaundice, premature delivery; in other studies, no harmful effects of alcohol drinking during pregnancy was reported. This last finding is very surprising, since in the study of Primatesta *et al.* [11], 9% of women enrolled used to have more than 11.5 drinks a week, at risk drinking during pregnancy for most scientists.

Unfortunately, this finding is perfectly in agreement with the alcohol-drinking pattern for most Italian people. In Italy, binge drinking is fairly rare, while a steady moderate drinking at meals is very common, and several women do not change their habits during pregnancy, which is often unplanned: thus, some women (mostly poorly educated) drink alcohol during the first months of pregnancy, since they are not aware of their condition. The spread of this dangerous behavior in Italy is confirmed by a transversal study performed by our group in Rome in 2003. Drinking habits were investigated in 122 pregnant women by a semi-structured interview: 62.1% of the women used to drink alcohol before pregnancy, and 52.6% during pregnancy; thus, only 10% of the women quit drinking when pregnant. While the 68.4% of the women reduced or quit smoking, only 21.5% reduced or quit drinking. The 11.7% of pregnant women on study used to have more than 7 drinks a week. Moreover, 2 women started alcohol drinking during pregnancy, probably because popular ideas about safety and even usefulness of moderate alcohol drinking during pregnancy are still widespread in Italy where, several years ago, beer drinking was considered a lactation-enhancer by common people, and this idea is still present, mostly in uneducated people [12].

In this situation, a nation-wide effort for prevention is needed. Whereas in US the National Health Institute officially advised women that drinking alcohol (any amount) during pregnancy is highly dangerous for the fetus and must be avoided, in Italy FAS prevention from official health agencies is lacking and the risk of fetal damage related to prenatal alcohol exposure is under-evaluated.

This lack of correct information about FAS risk is present even among midwives and obstetricians, who should be the advisers of pregnant women, and even in some manuals for medical and nursing schools.

Taking into account the lack of awareness of the FAS risk, the popular ideas about drinking in pregnancy, the behavior of pregnant women and the large number of alcohol abusers and alcoholics (about 4 000 000 out of 57 000 000 inhabitants), the actual prevalence of FAS in Italy could be relevant. A successful prevention needs a careful assessment of the real prevalence of FAS and FASD, using extensive outreach methods.

## EXPERIENCE IN THE FIELD. THE LAZIO STUDY

This in-field study was performed in the years 2003-2004 and 2004-2005 in cooperation with American researchers, mainly from University of New Mexico, Albuquerque, and Italian researchers University "La Sapienza", Rome. It was aimed to the assessment of the prevalence of FAS and FASD in the area on study, and the evaluation of the drinking pattern of mothers of the enrolled children. The study was approved by the Ethic Committees of the Aziende Sanitarie Territoriali (ASL) involved in the study, and by the Ethic Committee of the University of New Mexico. The schools of the districts of two Territorial Health Agencies (in Italy, Aziende Sanitarie Locali, ASL), including several small towns and rural areas, were selected for the study. Many people were commuters to Rome, while others were local agriculture workers. Among the primary schools that volunteered to participate to the study, 25 schools were randomly selected. The first grade students of these schools were enrolled if permission was granted by their guardians by signing a permission form. Only 543 children out of 1086 participated in the study and underwent the screening protocol.

As the cooperation of the schoolteachers and the children's mothers was crucial, several informative meetings with the Italian researchers were needed. Briefly, the questionnaire for the mothers' interview employed in the South-African study was translated and modified for the needs of the Italian study, and was used to assess the life-style of the mothers, before and during pregnancy and at present. The questions about alcohol drinking were included in a large series of questions about health, food consumption and life-style, to increase the reliability of the answers. The one week - day by day method was used. The areas investigated in the questionnaire are reported in *Table 1*.

**Table 1** | *Maternal interview. Areas investigated*

1. Social and demographic variables
2. Mother's health lifelong: general aspects
3. Mother's conditions during the pregnancy and after delivery, regarding physical, psychological and behavioral problems.
4. Food intake at present and during the pregnancy. The three trimesters of pregnancy were investigated separately
5. Alcohol intake at present and during the pregnancy. The three trimesters of pregnancy were investigated separately
6. Use of tobacco and other psycho-active drugs at present and during the pregnancy. The three trimesters of pregnancy were investigated separately
7. Information, suggestion, advises received during the pregnancy
8. Quality of the family environment where the child is living
9. Information about any disease, inherited or not, present in the child's family

*Anything noteworthy observed by the interviewer was reported. "Pregnancy" is referred to the pregnancy which gave birth to the child on study.*

In the screening first step, the children with impaired growth (height, weight, cranial circumference  $\leq 10\%$ ) or impaired performance at school (learning deficit and/or attention and hyperactivity, assessed by the Parent/Teacher Disruptive Behavior Disorder Rating Scale Pelham's test [13] and the Problem Behavior Checklist (PBLC) test [14], in Italian translation), were selected for further tests. School performance and behavior were assessed by the teachers, previously briefed by the psychologists of the team, filling a form based on the tests employed. Also the children's parents were asked to fill the same form about attention and hyperactivity.

In the second step, the children were examined by two American dysmorphologists and by two Italian pediatricians, working blinded to child's history. The children with FAS-related morphological abnormalities were tested with the Ravens Coloured Progressive Matrices [15] and the Rustioni Test of Language Comprehension [16], to assess their psychological and developmental levels.

In the diagnosis step, at present still in progress, each case is discussed by the whole team, collecting and discussing all the information obtained by different sources: the parent's questionnaire, the report from the teachers, the mother's interview, the morphological assessment and the neuro-psychological testing. The diagnosis of FAS or FASD was established, according to the diagnostic criteria of the Institute of Medicine (IOM) [17].

The parents of each child were informed about the results of the screening in a meeting with the psychologists: the problems of the child, if any, were discussed with the parents, as well as the guidelines of following interventions, if needed.

### PRELIMINARY RESULTS

At present, only data about the compliance of the family are available, suggesting some considerations about the feasibility of these studies, and of their implications for prevention and health promotion.

Among the schools in the chosen districts, 70% voluntarily agreed to enter the study.

A limit of the study is the low participation rate of the families: only 50% of parents agreed the participation of their children to the study. During preparatory meetings with the parents, most of them were reluctant to submit their children to an unnecessary medical visit; many parents said they were perfectly satisfied with the state-granted pediatric care, which in Italy is free and capillary widespread in the field. On the contrary, the parents who thereafter accepted the study found useful a further check of the psycho-physical health of their children, and liked the idea of helping the scientific progress in this field. Moreover, the poor cooperation of families may be related to the choice of the study design, as suggested by the study of Clarren *et al.* [18], in two US counties: when passive parental recruitment was employed, the participation rate was very high, whereas was low when active recruitment (the parents

had to sign an acceptance form) was employed. In Italy, because of legislation and high sensitivity about privacy and human rights, only active parental recruitment was possible.

During the first year of the study, the teachers were questioned about the possibility that a problematic child was not included in the study (because of the parents' refusal), and 71.4 of the teachers gave a positive answer. This fact raises several doubts about the efficacy of in-school studies in reaching all the population worth of interest, but an easy solution of this problem is difficult to find. According to the preliminary data available, 33.0% of the children participating to the first screening had low weight or height, or reduced cranial circumference, or learning and behavioral problems.

### IMPLICATION OF THE STUDY FOR FAS PREVENTION

As the effect of prenatal exposure to alcohol on the psycho-physical development of the child is dramatic, and no effective treatment is available, FAS prevention is a worthwhile task. In our country, the risk of FAS is increased by the common pattern of alcohol drinking (a large number of reproductive women drinking moderately during meals, even when pregnant), by the poor awareness and the under-evaluation of the FAS risk. The awareness of the risk may be increased only by a careful assessment of FAS and FASD risk in our country, which is unknown, and by a nation-wide effort for a campaign of information and education, for the promotion of the health of pregnant woman and unborn child.

Thus, this first Italian study in a large group of children is a significant step for FAS prevention. Moreover, the awareness of the problem among the teachers and the health workers (doctors, psychologists, staff) employed in the study and the children's families is increased. The awareness of the of the behavior disorders in problematic children is certainly increased among the teachers participating in the study, beyond the FASD children, and their effectiveness in coping with problematic children is further enhanced. All the activities for people information and for involvement of the whole community will be passed to common people in the neighborhood, further increasing the awareness in the area interested by the FASD Lazio project, obtaining an effective health promotion.

The data from the interviews of 519 mothers may be very helpful for understanding the drinking habits of Italian women, during and after pregnancy. Nearly all of them were drinkers at present: 100% of the mothers of control health children, but, surprisingly, about 90% of mothers of FASD children; the veracity of their answers seems questionable, as some of the answers were false, because of the guilty feeling and the fear of social blame in heavy drinkers. The same mechanisms may be responsible of the low participation rate, as discussed before. An alternative explanation of the relatively high rate of non drinkers among the FASD mothers may be that some of them were heavy drinkers, and they quit drinking, after the birth of the child on study,

being worried about their health. Two-thirds of women reported alcohol drinking during pregnancy, without difference between the mothers of FASD children and of controls; the veracity of these answers seems even more questionable.

Also the formation of an Italian team able in diagnosing FAS and FASD subjects is a crucial step for the treatment, but is helpful also for prevention, since the awareness of the problem will be further increased. Moreover, the parents of a FASD child will be informed of the risks of prenatal exposure to alcohol in a further pregnancy: this is a relevant goal, since the probability of FASD in children from further pregnancies is increased in mothers of a FASD child, if they do not quit drinking.

More relevant is the opportunity of helping the children resulting affected by disorders related to prenatal exposure to alcohol, as happened for the two children evaluated in this screening. The Medicina Scolastica (School Medicine) Service accepted the indication from the University team, and both the children have a support teacher. According to the most recent research, this is not the best approach for the needs of a FAS child: if he is placed with age peers, he may have a wide range of abilities that may not all fall in the range of abilities compared to his classroom peers [19]. Unfortunately, personalized teaching is not available at present in Italian school.

A crucial point for the prevention of FAS/FASD is to avoid unintended pregnancies in heavy-drinking women: obviously, women, ignoring that they are pregnant, drink as usually and alcohol can cause fetal damage. Unfortunately, at-risk sexual intercourse is common in alcoholics, and unwanted pregnancies are frequent in female alcoholics and are an increasing problem in general people.

An interesting, positive side-effect of this study is the improvement of the teachers' and parents' awareness of behavioral and learning problems of the children. In the first year of the study, 11.4% of the children seemed affected by attention deficit, 13.4% by hyperactivity, 13.4% seemed to have a delay in the acquisition of learning abilities. The diagnosis of FASD was not confirmed in all the cases, but in most cases the awareness of the child's problems lead to a modification of the family and school environment and to an improvement of the attitude of the parents toward their child. Indeed, the teachers found a support in their evaluation of children's behavior and

could improve their attitude towards problematic children. The final meeting with the parents was performed according to the basal principles of professional counseling.

All the information obtained in the study are available from the Territorial Health Services participating in the study, and may be used for the implementation of further projects in the field. The institutions and services participating in the study could acquire a new experience about a poorly known problem, and this information will be shared with the whole institutions. Thus, the improvement of the awareness and the knowledge of the effects of prenatal exposure to alcohol are more far-reaching than the present results.

## CONCLUSIONS

At present, FAS is the more frequent syndrome of mental impairment completely preventable. We hope that in Italy clinicians and psychologists will be advised of the need of taking a thorough history to determine alcohol use in all women of childbearing age and to provide information regarding FASD prevention.

Screening tests to detect at risk pregnancies may be helpful, and also some biological markers of alcoholism may be useful [20]: moreover, the assessment of fatty acid ethyl esters (FAEE) in the newborn meconium seems the most reliable test [21]. Programs and interventions to help the women to quit drinking in pregnancy may be implemented. Some experimental studies suggest that the administration of antioxidants and free-radicals scavengers to pregnant women unable or unwilling to quit drinking may reduce fetal damage, but further research in humans is needed [22].

Moreover, the procedures for an early diagnosis of FAS should be improved, to obtain a FAS/FASD diagnosis in the newborn, to set up an early treatment, if possible, and to prevent further at risk pregnancies in the same family.

FAS primary prevention is a major target, since no effective treatment is at present available for the effects of prenatal exposure to alcohol. Also the formation of skilled health operators may be very useful for a better care of FASD children.

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# Educational planning for children with fetal alcohol syndrome

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**Summary.** It has now been thirty-two years since Jones and Smith first identified fetal alcohol syndrome (FAS) in the United States. Since then, numerous epidemiology studies have been conducted to determine prevalence rates of this disorder. More recently, the research focus has concentrated on studies to elucidate a neurobehavioral phenotype for the alcohol-exposed population. As a result, the FAS field has learned what types of neurobehavioral issues occur most frequently with these children. This paper discusses the results of neurobehavioral research with alcohol exposed children and how that information can be used to inform school assessment, intervention planning, and support.

*Key words:* fetal alcohol syndrome, neurobehavioral research, intervention planning.

**Riassunto** (*La progettazione educativa per i bambini affetti da sindrome fetoalcolica*). Sono trascorsi trentadue anni da quando Jones e Smith identificarono per la prima volta la sindrome fetoalcolica (fetal alcohol syndrome) negli Stati Uniti. Da allora sono stati condotti molti studi epidemiologici allo scopo di determinare la prevalenza di tale disturbo. Più di recente la ricerca si è concentrata su studi che potessero isolare un fenotipo neurocomportamentale tipico nella popolazione di bambini esposti all'alcol in utero. Grazie a questi studi la ricerca del settore ha potuto evidenziare quali sono le problematiche di tipo neurocomportamentale che ricorrono più di frequente in tali bambini. Questo lavoro esamina i risultati della ricerca neurocomportamentale sui bambini esposti all'alcol e come tali informazioni possano essere utilizzate nella valutazione scolastica, nella pianificazione degli interventi e nell'assistenza.

*Parole chiave:* sindrome fetoalcolica, ricerca neurocomportamentale, piani di intervento.

## INTRODUCTION

July 2003, marked the 30<sup>th</sup> anniversary of the introduction of the term fetal alcohol syndrome (FAS) in the United States [1, 2]. Kenneth Jones and David Smith, along with a group of astute nurses and psychologists from The University of Washington, recognized and described a group of Washington State children who had similar facial dysmorphology. On closer scrutiny, that group of children had all been exposed to excessive amounts of alcohol *in utero*. Common among the children was a distinct constellation of physical abnormalities, growth retardation, central nervous system damage, and prenatal alcohol exposure. It was determined that all of these children had suffered teratogenic damage as a result of maternal alcohol ingestion during the gestational period. Consequently, the term FAS was introduced and these children were assigned the diagnosis.

In the subsequent 30 years, through animal and human population studies, much has been discovered about the effects of prenatal alcohol exposure. For example, the developmental outcome of children exposed to alcohol prenatally is dependent on a wide range of factors including; the quantity and timing of the alcohol exposure, maternal age, nutritional status of the mother, and parents' intelligence and level of education. Therefore, the

effect of prenatal alcohol exposure on a child's development is highly variable, and not all children who have been exposed to substantial amounts of alcohol display all of the features of full-blown FAS.

In addition, researchers have had difficulty determining an alcohol consumption threshold that causes adverse neurobehavioral findings. Some researchers, though, suggest that seven standard drinks per week may be enough to cause neurobehavioral challenges for a child [3]. Because the factors contributing to developmental issues are so complex and the developmental outcomes so variable, the field of FAS study has begun to discuss the continuum of effects that occurs with children who have had prenatal exposure to alcohol. The preferred nomenclature is now fetal alcohol spectrum disorders (FASD).

## USING A HOLISTIC MODEL AS A BACKDROP FOR PROVIDING INTERVENTION SERVICES TO CHILDREN WITH PRENATAL ALCOHOL EXPOSURE

In the United States, as well as Italy, educational mandates promote the inclusion of children with special needs within regular classrooms whenever possible (educating children within the least restrictive en-



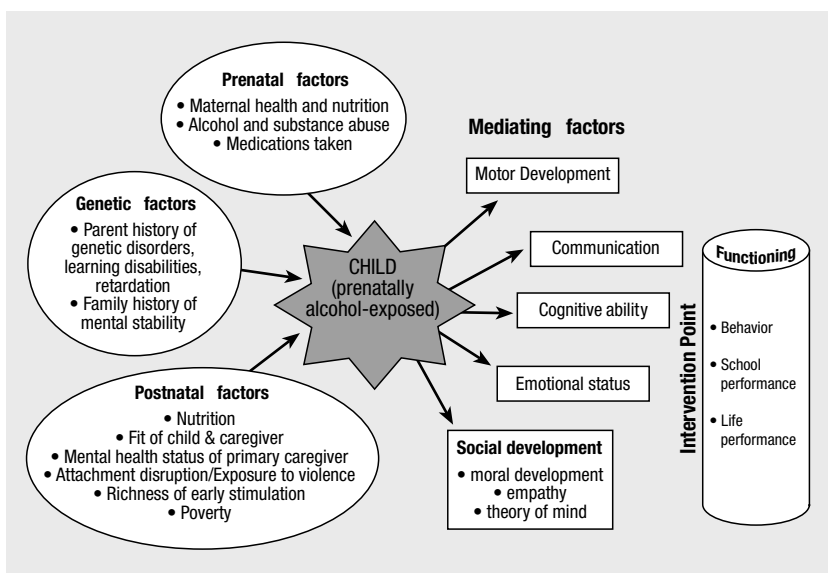
vironment) [4, 5]. This means that the individual needs of a child with disabilities must be addressed within the context of the regular classroom. Although these educational mandates are country-specific, culturally-specific, and historically-specific events, one salient question remains: How can we best serve children with FAS or FASD in their schools, community, and homes? The overall goal of intervention is to provide the necessary academic and functional supports so that the child grows into an independent adult who can have a positive life experience. In order for this to happen, intervention must begin as early as possible and focus on developing both the academic and functional abilities of the individual.

The following model (Fig. 1) illustrates the factors that must be considered when planning interventions for alcohol-exposed children. Because the goal of any intervention is to promote the general well-being of the child within his school, home and community, a complex set of factors must be considered. The diagnosis of FASD presents an interface between the health community and the educational community and all aspects of both must be considered in the planning of educational and social interventions for the affected child. The health community and educational community are inextricably connected. This model considers the child within the context of his/her community, social milieu, family, and culture. The promise of this model includes cross discipline sharing of resources, greater access to community health personnel, a greater opportunity for coordinated curriculum development, improved teaching methods, and realistic understanding of the child's individual educational needs.

**GENERATING A LEARNING PROFILE**

Because there are few medical doctors who feel confident in making the diagnosis of FAS or any other level of FASD or who are trained appropriately

to do so, children may come into an educational setting with or without a specific diagnosis of FASD. If there is a diagnosis, the school may or may not be aware of it. In most cases, however, children who were prenatally exposed to alcohol come to the attention of the educational system because of the learning issues they display. In either case, the schools generally will initiate an assessment of their own to determine the learning profile of an identified child. While standardized IQ measures are helpful, neuropsychological testing, achievement testing, contextual observations, and behavioral assessments provide the most useful information for developing programming for children with FASD. Often children with a diagnosis of FASD are ineligible for special services because their intellectual abilities fall within the average range of intelligence. Studies have shown that the average intelligence scores of children with prenatal alcohol exposure fall two standard deviations below the mean. Approximately fifty percent of children with FAS are mentally retarded, however, IQ scores range from severely retarded to high average in this population [6]. Because of the variability of IQ scores in this population and the known deficits in higher order cognitive functioning found in this population [7-11], an individual learning profile must be developed for each child. Children who have average intelligence and are prenatally alcohol-exposed benefit most from a battery of tests that can best elucidate issues of attention, verbal learning and recall, verbal memory, auditory memory, spatial memory, auditory processing and verbal processing. In a typical school process, children will be given the following standard battery of tests: an IQ measure, achievement measure, and an adaptive measure. We propose the use of neurobehavioral testing to better elucidate the individual learning profile of affected children.



**Fig. 1 | Factors influencing the life functioning of children prenatally exposed to alcohol.**

***Utilizing neuropsychological testing results to develop a learning profile for individualized programming***

Although a typical neurobehavioral phenotype of FAS has not yet been described, the field is moving toward the articulation of such a phenotype. Some researchers have promoted the concept of identifying a set of core deficits in these children. The Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD) has been funded by the United States, National Institute on Alcohol Abuse and Alcoholism (NIAAA) to, “coordinate basic, behavioral and clinical investigators in a multidisciplinary research project to better inform approaches aimed at developing effective intervention and treatment for FASD. Input and contributions are coming from basic researchers, behavioral scientists, and clinical investigators who are willing to utilize novel and cutting edge techniques, not simply to replicate previous or ongoing work, but rather to move the field forward in a rigorous fashion” [12]. This consortium is an international effort that includes studies of children in Finland, Russia, Ukraine, South Africa, United States, and Italy.

Children exposed to moderate amounts of alcohol may not show all of the features of the full syndrome but may still display neurobehavioral issues, particularly in executive functioning abilities [3]. Executive functioning refers to an individual’s cognitive ability to plan and sequence behavior to efficiently achieve a goal. Neuroscientists evaluate this ability by presenting testing tasks that involve effortful, deliberate actions requiring working memory (holding and manipulating information mentally in order to complete the task successfully). In addition, attentional abilities of the child are also evaluated as part of this rubric.

Executive functioning has been the focus of a number of studies with children exposed to alcohol during gestation. Findings have revealed that those individuals with FAS and individuals with known exposure but without a diagnosis of FAS exhibit executive functioning difficulties. Mattson *et al.* reported executive functioning deficits in children with known prenatal alcohol exposure with and without the diagnosis of FAS [9]. These executive functioning deficits were also seen in children whose intelligence fell in the average range of functioning. Specifically these children demonstrated marked difficulty in complex working memory related tasks and shifting sets (in both cognitive and emotion-based tasks), planning ability, cognitive flexibility, selective inhibition, and concept formation and reasoning [8]. In addition, executive functioning deficits in alcohol-exposed children have been found to closely correlate with reported behavioral issues in these children.

The information derived from neuropsychological testing of executive functioning in affected individuals can be useful in two specific ways. First, the neurobehavioral effects of alcohol-exposure on developmental outcomes are thereby better understood for each child. And, secondly, results of executive functioning testing provide valuable information regarding attention,

memory, problem-solving and inhibitory control, all of which are extremely useful in tailoring interventions to suit the needs of a child.

The usefulness of neurobehavioral test protocols involving executive functioning tasks is apparent. This type of testing assists the family, medical provider, and classroom teacher with a clearer understanding of the issues that interfere with learning and behavior in the classroom, home and community. The next section of this paper will outline the empirically determined deficits often seen in alcohol exposed children and the functional deficits observed as a result.

***Executive functioning***

As described, executive functioning difficulties are common among children who were prenatally exposed to alcohol [7-11]. Deficits in this area interfere with successful completion of some of the simplest tasks of daily living, academic achievement and problem solving. The types of functional issues that may be seen as a result of executive functioning deficits fall into two broad categories: cognition-based difficulties and emotion-related difficulties [8].

*Cognition-based* executive functioning limitations may manifest in the child’s inability to understand and hold in memory the specific steps of a given task or sequence. For example, children with this type of difficulty struggle with following sequences that are inherent in a typical daily routine, the steps of social exchange, and typical learning sequences. Daily routines are apparent to most of us and do not require much extra cognitive effort to hold in our understanding. Similarly, most learn the steps of appropriate social exchange by observing and being guided by the adults and children with whom we interact. Typically, it is not necessary to teach these steps explicitly. However, for children with prenatal alcohol exposure, the steps of appropriate social interaction are not so apparent and easily understood. Therefore, the steps of social engagement must be taught by rote and eventually learned through repetition.

Learning sequences can also be very difficult for children with FASD to grasp. In the process of learning, many tasks require the use of cognition-based executive functioning abilities. One good example of this is arithmetic. Arithmetic is a subject that requires a clear understanding of the relationship between the specific order and function of the numbers with which one is working to derive a correct answer on a given computation. If the order and function of that task are not cognitively held in working memory, and if the specific steps to correctly solve the problem are not understood, then the child will not be successful in completing the arithmetic problem.

*Emotion-related* executive functioning deficits may manifest themselves in the inability to inhibit responses. This can be seen behaviorally when children speak or act out inappropriately, or when a child’s behavior is impulsive or overly active. In the classroom, a child may speak out before thinking about what is acceptable in that situation. He or she also may have difficulty controlling his actions when he/she is upset.

### **Functional issues related to executive functioning deficits**

*Social pragmatics.* - Acting before considering the consequences of the behavior is a hallmark of children with FAS [13]. For example, children with FAS often are socially intrusive to those around them. They will encroach on the personal space of peers, have difficulty inhibiting themselves to wait their turn, and blurt out inappropriate communication. These are examples of the challenges this population has with social pragmatics (the rules and steps of social interaction). Logically, these types of deficits interfere greatly with an affected child's ability to make friends and maintain relationships.

*Memory and attention.* - As discussed previously, children with executive functioning difficulties have trouble holding information in memory for later use (working memory) in solving novel problems, planning a task's trajectory, and maintaining attention to complete a goal. In the classroom this deficit may manifest itself in the child's inability to follow directions, retain information previously presented, generalize information from one situation to another, or organize events into a logical sequence or timeline. General organizational abilities also present a challenge to a student who has been exposed to alcohol prenatally. It may be very difficult for that child to organize and keep track of his personal belongings and school materials, independently organize a learning task into a meaningful sequence for completion, and grasp that most tasks contain a beginning, middle, and an end. In addition, working memory deficits interfere with academic readiness often progress much more slowly for children with FAS. As a result, foundational concept acquisition such as shapes, letters, numbers and words present more of a challenge.

Using Mirsky's model of attention, Claire Coles *et al.* [14] compared children diagnosed with FAS with those children who were diagnosed with ADHD. Mirsky's [15] model defines attention using a four part theoretical model: Focused attention, Maintenance of attention, Ability to Shift attention, and Ability to Encode new information. Her research revealed that children with FAS have the most difficulty shifting their attention and encoding new information whereas children with ADHD have more difficulty focusing and sustaining attention. When a child has issues with shifting attention, there is greater potential for that individual to perseverate on a given task. He or she may have extreme difficulty moving from one topic to another, become confused by a change in the routine, and become resistant to transitioning from one place to another. Additionally, life changes such as family membership, moving from one school to another, and changing from one grade to the next may all be highly frustrating and perplexing to a child with this type of attentional challenge.

Willford *et al.* [11] looked at verbal and visuospatial learning and memory with children with moderate prenatal alcohol exposure. They found that moderate prenatal exposure was associated with a generalized deficit

in learning (encoding) and memory, impaired learning (encoding) and memory performance in the auditory/verbal domain, and impaired encoding/storage and retrieval processes. In other words, these children have a hard time taking in new verbal information through auditory channels and holding that information in memory for use at a later time. This was consistent with previous studies done with children who had been exposed to substantial amounts of alcohol [9, 16-18]. However, a more recent study revealed that, although children with FASD have more difficulty encoding new information, once they have learned or encoded the information, they are able to retain that information and recall it for use later [19]. These difficulties functionally translate into problems adequately learning (encoding) information initially, recalling auditory verbal information, following directions presented in verbal form, and the ability to generalize information from one application to another. These characteristics of the fetal alcohol exposed child lead to what can be termed the Speech/Comprehension Paradox. Often children with FAS have relatively good vocabularies and are loquacious. However, because of the deficits in verbal memory, most often the comprehension level lags behind the expressive ability and makes the child appear to be more capable than is actually the case.

### **Working in concert with the family to develop educational programs**

The ultimate goal of educational planning and community responsiveness to individuals with FAS is to work toward a respectable and desired quality of life for that individual. The way that is defined and how that might look will be different for each individual because of the unique nature of that child's experiences, degree of need, family culture, and community culture.

The family of the child is instrumental in defining and guiding the school program for the child with FAS. Professionals are educated and trained in a variety of disciplines to assist children in their development of skills in all areas of life; social, academic, physical, and emotional. However, professionals move in and out of a child's life over the educational career and are financially compensated for their services. In most cases, a professional will not follow a child through his life span. Therefore, the professional must recognize and regard the hopes, wishes, and desires families hold for their children with FAS.

The parents and primary caregivers of the child must play an integral part in the educational planning process so that the wishes of the family for their child and the motivations of the child become the basis for goals set for the child. Ideally, the school team and the family will work in concert to develop, assess, and redefine a child's school program over his school career. It is helpful to encourage families to think about the long term goals they hold for their child (*i.e.*, goals to set over the course of the child's lifetime). These long term goals transcend the yearly goals set for children in the educational process. The yearly goals that are set by

the school and family team become the stepping stones toward the achievement of the desired long term goals. Often the long term goals shift as time passes and development of the child takes its course, however, this is only revealed in the cooperative process over time. One good resource to help schools work with families to set long term goals is a educational planning tool created by Michael Giangreco titled Choosing Outcomes and Accommodations for Children (COACH) [20].

### DIAGNOSIS VERSUS ASSESSMENT, MAKING THE DISTINCTION

Once the diagnosis is made, the real work begins to determine the individual needs and the differences that are specific to each child. These individual differences are determined through assessment processes that are ongoing. The assessment(s) will determine what special supports and modifications will be required for each student. The importance of the assessment process is that it feeds directly into the actual goals and objectives that will be included on the individualized plan for instruction in the classroom [21]. Often schools leap from the eligibility evaluation straight to the goals and objectives of the individualized plan without fully assessing the current skill levels of the child and the specific needs the child has in various school settings. There are many different ways a comprehensive school assessment can be accomplished. Ideally, the assessment should contain information from a myriad of sources: parents, previous teachers, and observation of the child in a variety of settings. The primary components of the assessment process should lead to useful information to answer the following questions:

- 1) *Is a particular skill present or not, and if present, at what competence level is it observed and in what form(s)?* For example, when assessing reading readiness, is the child able to recite the alphabet, is the child able to write the letter of the alphabet, is the child able to write all letters of the alphabet in capital and lower case forms, is the child writing his or her name, is the child combining letters to form words?
- 2) *What potential does the child have for developing that skill?* For example, if the child is delayed in his acquisition of reading readiness skills, how delayed is he, and is there anything that will ultimately prevent him from acquiring those skills?
- 3) *What type of organization and structure will be needed for the child to develop the ability to independently display that skill?* For example, will the child who is displaying delays and difficulties acquiring reading readiness skills require specific structuring tools, systematic teaching methods, or increased repetitions to help him become competent in that area?

Based on what we know empirically about children with FASD (their possible cognitive, behavioral, and academic challenges), we begin to create an individual profile for each child to determine the best school programming and intervention methods to apply. It is

important to reiterate consideration of each individual child and his or her unique learning profile in the process of planning interventions. Each child with FASD is an individual and the kinds of support the child needs will reflect the particular situation.

### FUNCTIONAL CLASSROOM ASSESSMENTS

Using the above described questions as a guide, functional or real life abilities of the child must be assessed [22, 23]. This will supplement the diagnostic testing (IQ, academic achievement, behavioral, and neuropsychological) results, family information, and, actual school achievement information. How does one embark on this functional assessment process? First, the assessment process must include comprehensive observation of the child and it must be done in a variety of natural settings. This helps the observer see how the child does in different settings and assess the impact of the environmental conditions on his abilities. Observations should be conducted on at least two to three different occasions in several different settings so that the observer can account for setting-triggered events and assess the child over time. It is very important to conduct observations across settings to determine where identified behaviors occur as well as where those behaviors are absent. Multiple observations allow the observer to determine current abilities and establish reasonable expectations. This process also enhances one's understanding of what conditions may be necessary for the child to perform optimally. It also helps to determine what conditions might disrupt effective functioning. The following key factors should be observed during the observation time: 1) skills; 2) attention; 3) independence; 4) social interactions; 5) functional language; 6) strengths and interests; and 7) behavior.

#### *Skill acquisition*

First, the actual skills that the child displays should be observed and noted. What *skill does* the child have or not have, and what skills are emerging? How do the skills of the observed child compare to the skills of the other children in the classroom? As part of this process it is important to note the child's ability to understand the verbal and written directions that are given during the observation time. Does the child understand what is expected in the classroom?

#### *Attention*

Another key factor to observe is the child's *attention* to specific expectations in the environment [24, 25]. How well does the child take in and use new information (encode)? How does the child do when required to shift attention from one task to another? When transitioning from one setting to another or from one topic to another, how well does the child follow and cope with the change? Also important to note is the child's ability to focus on a task. We know that children focus better and attend longer to tasks that are interesting and motivating. Children also are able to focus their attention better to tasks that provide the "just right" developmental chal-

lenge. In other words, the educator can determine if a task is developmentally appropriate if the child is able to maintain focus on the task. Finally, the observer should watch the child's ability to sustain attention during a task. This includes determining what is termed a "distractibility quotient". For example, does the child turn to every noise? Does the child spend excess time looking out the window? Do the activities and movement of others in the class take him off task?

#### ***Ability to work independently***

The next factor to observe is the child's *ability to work independently*. Inherent in these observations is the need to assess the level and types of assistance the child requires to complete a task. Also, the observer must determine how well the child can independently set up and organize an activity. Because children with FASD have extreme difficulties with executive functioning, one must determine how much additional structure and organization he may need in the environment to successfully complete a given task. Children with FASD may only display skills when an appropriate amount of structure is provided for them. If a task is organized and structured for the child to help him independently complete the task and he still cannot complete it successfully, then he may not have the skills to complete the tasks (*i.e.* the task may be outside his developmental skill level). When assessing this, start with minimal amount of structure and add more as necessary until you provide the exact amount of structure needed for success.

#### ***Interactional abilities***

Another area to assess in children with FASD is the nature of their *interactions* with other students as well as the teacher [26]. It is beneficial to determine if the child prefers to interact with adults or peers. In addition, the observer should note how frequently or infrequently the child attempts to initiate interactions with other. How well does he respond to the overtures of others to interact with him? When the child does attempt to interact with others, watch for how well he navigates the pragmatics of that interaction. The pragmatics of social interaction involves how well the child can follow the accepted rules of social engagement. For example, is the child appropriate in his interactions or does he engage in rude behavior? Is he able to stay on topic? Is he overbearing or overly intrusive into another's personal space. It is also good to note his social preference. Does the child have the ability to share space with another or is the child resistant to sharing space? Can the child work or play side by side with another child without interacting or intruding with another child? Is the child able to freely share materials? How well does he cooperate? Can she take turns or does his behavior interfere with his ability to wait for a turn?

#### ***Functional language***

Although formal language testing is most likely available for the child you are observing, it is informative to assess the *child's language* informally in the

context of real life situations at home, at school and in the community. Noting the amount of language a child processes and understands (receptive), the way(s) a child communicates and the reasons why the child communicates (expressive), and the pragmatics the child uses in real life contexts should all be included in the process of the informal assessment [27-29].

#### ***Strengths and interests***

Another component of a functional assessment involves the discernment of the child's *strengths and interests* [30]. This is key to successful school programming. By watching what a child is interested in, we can determine what might provide the "hook" to motivate the child's learning. This is done by discussing the child's interests and strengths with the child's family as well as observing what attracts the child in the school setting. Assessment of a child's choices helps determine what kind of learner he is: visual, tactile, auditory, or proprioceptive.

#### ***Behavior***

Specific target *behaviors* may be interfering with the learning path of a child with FASD [31]. When a problematic behavior is observed, a functional behavioral assessment (FBA) is suggested to identify what conditions exist that are perpetuating the target behavior [32, 33]. Functional behavior assessments provide a critical link between topographical descriptions of behavior and intervention planning. FBA considers the biological, social, affective, and environmental factors as potential contributors to problem behavior. When a child's behavior is impeding learning, a FBA followed by positive behavioral supports and interventions can often remediate the situation and address the behavior. Functional behavioral assessment is appropriate for any behavior that interferes with a student's education or that of the child's peers.

Once all informal assessment is completed and/or a functional behavioral assessment is completed, the school team, including the family, can utilize the information to create a specific learning profile for the child. The information gained through formal testing and these assessment processes provides a comprehensive packet of information from which initial program planning can begin [34]. The child's needs as well as the child's strengths will be revealed in the process of the formal evaluation and the informal assessment. This information is then pieced together like a puzzle to reveal a specific *learning profile* for each child. Although there may be similarities among profiles, each profile should reflect the unique picture of the particular child with whom you are working. No two learning profiles are ever the same.

### **DEVELOPING INDIVIDUALIZED INTERVENTIONS WITH THE LEARNING PROFILE AT THE CORE**

It is important to understand the child's present levels of performance when planning your instructional activities so that those activities are appropriate for his

developmental abilities. If a child with FASD is placed with age peers, he may have a wide range of abilities that may not all fall in the range of abilities compared to his classroom peers. In addition, one must carefully consider the modifications and supports that will be necessary in the environment to support the child. It is helpful to think of the environment as an external nervous system of the child, a place where supports can be implemented to bolster the deficit areas of the child. Because of the learning differences that exist with many children who have FASD, structure is a very helpful environmental support. Placing appropriate structure in the environment is an imperative for success with children who have FASD [13]. Structuring the teaching environment helps the child know what is expected of him. Although structuring is helpful, it is important that adults are mindful when structure is appropriate and when structure turns to control. At times, a child's escalating behavior can make an adult seek more control over the child. When this scenario presents itself, it is important, as the teacher, to know when structure turns to control. At this juncture it is advisable to re-assess what is not working and restructure the environment accordingly [24].

#### ***Structure and systematic teaching for children with fetal alcohol syndrome***

Environmental structure (functional routines and structured teaching) and systematic teaching are excellent tools to use with the FASD population. Functional routines occur naturally for all individuals and provide a structure that lends predictability and a clear understanding of what activities will happen and in what sequence to complete a routine [35]. Functional routines provide opportunities for parents and teachers to provide systematic instruction. Teaching functional routines requires identifying skills, routines or activities that can be taught through routine practice such as dressing, getting ready for bed, bathing, etc. A teaching plan must be created for teaching a functional routine. The teaching plan will include developing strategies/objectives, deciding where an activity will be taught, what materials will be needed, the steps involved, the cues that will indicate the beginning of the routine, and what responses will indicate correct and incorrect performance (the reinforcement procedures promoting independence). Teaching functional routines early in a child's life provides clarity and organization on which the child comes to depend [13].

Structured teaching, developed by the Division of TEACCH in the Department of Psychiatry of the University of North Carolina School of Medicine, aims to understand the child's unique learning challenges and to develop environmental support to compensate. Once there is an understanding of the individual needs of the child, an intervention program is built around the child's strengths and needs. The visual structure provided for the child makes the environment and learning tasks predictable and visually clear. Predictability helps the child feel more comfortable and safe. Building structure into a child's day not only makes life more

predictable but it provides external supports that assist the child toward better organization. External structuring techniques provide compensatory tools to aid the child's deficit areas (e.g. executive functioning, set shifting, working memory and attention).

#### ***Visual structure***

To begin developing structuring tools, it is useful to think of the various aspects of structure. One aspect of structure is visual structure. *Visual structure* includes physical structure of the environment that decreases both visual and auditory distraction, the use of individualized daily schedules, incorporates routines, and includes tasks structure that provides visual organization, clarity, and instructions [36].

Visual structure can provide organization, clarity and instructions. Some examples of visual organization include using containers to separate materials, taping off sections of the room for specific activity centers, and using assigned carpet squares for circle time. Visual clarity is achieved through highlighting relevant and important information pertinent to a task or activity, color coding each content area, and labeling tasks or work centers. Visual instructions provide the child with a clear visual cue regarding the sequence to complete a task. Some examples of visual instructions include placing arrows to direct the student, numbering the steps of a given sequence, providing written steps of an instruction, and providing a finished example of the assigned task so the child can see what is expected.

When a visual structuring tool is being developed, ask the following questions:

- 1) how might a visual support tool benefit the child?
- 2) will the visual tool assist in teaching a skill?
- 3) will it provide clarification?
- 4) could it support memory deficits?
- 5) might it assist in the student's problem solving? or
- 6) would it help the student to manage time (transition from one task to another, within the group, or work independently)?

A widely used example of a visual structuring tool is a schedule. Schedules can be used for many purposes. Almost everyone uses some kind of daily schedule to get through their day or week. Some make lists, others fill in a calendar, and still others use a daily planner. These visual schedules help indicate what will occur during the day and in what sequence events will happen. Children with FASD benefit from visual schedules in that they help to alleviate anxiety during transitions, give information that helps them anticipate and predict what will happen next and in what sequence. They also help to provide motivation by giving the child a concrete reference for how long his day will be. Because the schedule can be changed as the needs of the environment change, these schedules often help build flexibility into the child's thinking. These tools help the child learn to work more independently in that they help the child rely on the schedule (a thing) rather than the teacher (a person).

Schedules are set up in a number of ways. They may be arranged top to bottom or left to right, depending upon which is most comfortable for the child. It is helpful if

the schedule can be manipulated by the child to indicate progress or when an activity is finished (*i.e.*, crossing off tasks as they are completed, checking off tasks, moving a task icon off the schedule when it is complete).

### **Environmental structure**

Environmental structure helps provide the best conditions for learning as well as define what occurs in a particular location [37]. For example, a child with FASD may be able to complete difficult academic activities that require vigilant concentration and attention if they are provided a space that is clear from distractions. Thinking about the task and the setting in which the child can best accomplish that task is key to the child's success. Children with FASD are often distracted by visual clutter. Therefore, keeping the environment simple with a minimum of decorations can be helpful. Another example of environmental structure involves clearly defining work centers for the child. For example, there may be a place in the classroom for arithmetic, another place for reading, another for the computer, etc. It is worth spending the time to determine the best environmental structure for children with FASD in the classroom. This enhances the student's understanding of their environment and what the expectations are, minimizing the potential for behavior challenges.

### **Task structure**

Specific task structuring can be very beneficial to the child with FASD in that tasks structuring provides a clear system for the child to follow. A task can be structured so that the child understands what task expectations there are, how many tasks need completing, when one task is finished, and what task comes next.

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## **CONCLUSION**

For children with FASD, the school environment can be difficult to traverse and schooling may become a negative experience. The keys to success for these children are properly assessing the child and the environments in which he or she will function and to then develop structures and routines that create a sense of safety and comfort so the child will be more inclined to step out and take risks. With risk-taking comes increased skill development. With increased skill development comes a greater sense of competence and ultimately an enhanced quality of life.

Once those educating and supporting the child with FASD understand the specific learning challenges of each student, appropriate structure can be applied to the environment and clear multi-modal environmental cues implemented to help assist the child toward a better educational outcome.

Armed with adequate diagnostic and assessment information, a school team can utilize that information to create a positive school program for a child [38]. The intervention tools described in this paper are meant to provide an overview of the external supports needed for most children who have experienced prenatal exposure to alcohol. As these intervention tools are created and tailored to the individual learning needs of each child, it is necessary to assess how well they are working and if the tools need to be adjusted or changed in any way. Utilizing these types of interventions with students who have FASD is not static but instead, a dynamic ongoing process.

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