Synthesis of phosphatidylethanol—a potential marker for adult males at risk for alcoholism

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ABSTRACT   Phosphatidylethanol, whose synthesis is catalyzed by a phospholipase D in a transphosphatidylation reaction, is a unique metabolite of ethanol. Phorbol 12-tetradecanoate 13-acetate, a tumor-promoting phorbol ester and stimulator of protein kinase C, activates this enzyme in peripheral blood lymphocytes. This system has been developed into an assay for measuring the potential of this pathway in human subjects. A pilot study of phosphatidylethanol synthesis in lymphocytes of adult males who have both an alcohol dependency and a family history of alcoholism has revealed that the average potential for phosphatidylethanol synthesis in this population is significantly elevated over that of control subjects.

In the course of investigating the effects of the tumor-promoting phorbol ester, phorbol 12-tetradecanoate 13-acetate (TPA) on the metabolism of [³H]arachidonic acid during the mitogenic activation of lymphocytes, Wrighton *et al.* (1) discovered that a class of unusual lipid metabolites were rapidly formed in response to TPA. These products were subsequently characterized to be glycerolphospholipids in which the typical head group (i.e., choline, ethanolamine, inositol, or serine) had been replaced by ethanol (2) (Fig. 1). The unchanged [³H]arachidonic acid was present as an acyl group in position 1 or 2 of the phospholipid. The formation of this class of acidic lipids, phosphatidylethanols, depended on both the treatment of the cells with an active phorbol ester (or another activator of protein kinase C) and the presence of exogenous ethanol in the culture medium (2, 3). Recent studies of Pai *et al.* (4) and Tettenborn and Mueller (5) have also shown conclusively that the observed phosphatidylethanols are formed by a transphosphatidylation reaction that is catalyzed by membrane-associated phospholipase D; this involves an exchange of ethanol for choline of preexisting phosphatidylcholine (Fig. 1).

The enzymatic pathway for phosphatidylethanol synthesis appears widespread in animal tissues with significant TPA-induced responses being observed in bovine lymphocytes, human peripheral lymphocytes, human mammary cells, human colon cancer cells, and HL-60 cells (2, 3). The *in vivo* production of phosphatidylethanol in kidney, liver, and brain tissues has also been reported independently by Alling *et al.* (6) for rats receiving toxic doses of ethanol.

Our finding that the pathway for phosphatidylethanol synthesis could be activated by TPA in human lymphocytes from peripheral blood has opened the way to exploring whether the ability of human subjects to synthesize this unique metabolite of ethanol correlates in any way with the individual's dependency on alcohol. To explore this question, an assay was developed for measuring the induced synthesis of phosphatidylethanol in short-term cultures of

human peripheral blood lymphocytes. This test has been used to assess the ability of two groups of males to synthesize phosphatidylethanol: group 1 consisted of 24 nonalcoholic males with no family history of alcoholism in any first degree relatives; and group 2 consisted of 25 males who met the DSM-III (Disease Statistical Manual, version 3, American Psychiatric Association) criteria for alcohol dependence and who also reported an alcoholic parent who met the DSM-III criteria for abuse and/or dependence (7).

The results of this pilot study show that adult males with both a personal history of alcohol dependence and a family history of alcoholism (DSM-III criteria) have a significantly greater average ability to synthesize phosphatidylethanol than do their control counterparts. These preliminary findings provide the first evidence that the synthesis of phosphatidylethanol and the regulation of this phospholipase D-catalyzed pathway may play an important role in alcohol dependency—and may contribute as well to the development of alcohol-related pathology. A molecular and genetic exploration of the components in this pathway is expected to provide new approaches to the therapy and prophylaxis of alcoholism in humans.

MATERIALS AND METHODS

Assay of Potential for Phosphatidylethanol Synthesis by Using Human Lymphocytes. Approximately 20 ml of venous blood was drawn from the test subject into Vacutainer tubes containing 143 units of sodium heparin. After mixing with 0.5 vol of sterile Dulbecco's Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), 7 ml of the diluted blood was carefully layered over 4 ml of Histopaque-1077 (8) in a 15-ml disposable centrifuge tube and centrifuged at 500 × *g* for 30 min. The buffy cell layer was removed and diluted with 25 ml of PBS, and the cells were pelleted by centrifugation at 350 × *g* for 10 min. The cells were then resuspended in 10 ml of PBS by gentle shaking. A small sample of the suspension was diluted with an equal volume of Eagle's HeLa medium containing 10% bovine serum, and the cell number was determined in a hemocytometer. The volume of the cell suspension in PBS, containing the required number of cells, was then sedimented at 300 × *g* for 10 min and the cell pellet was resuspended in phosphate-free Eagle's HeLa medium containing 10% bovine serum at a density of 2.5 × 10^6 cells per ml.

For studies of the induced synthesis of phosphatidylethanol, duplicate 3.5-ml cultures (8.75 × 10^6 cells) were set up in 15-ml screw cap conical glass centrifuge tubes and prelabeled for 18 hr with $^{32}P_i$ (20 µCi/ml; 1 Ci = 37 GBq) by incubation at 37°C in an atmosphere of 95% air/5% CO_2. TPA (100 nM) in dimethyl sulfoxide (final concentration, 0.2%), along with ethanol (final concentration, 0.5%), were then

Abbreviation: TPA, phorbol 12-tetradecanoate 13-acetate.
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$$CH_3CH_2^{*}OH$$

$$+$$

PHOSPHATIDYLCHOLINE

PHOSPHOLIPASE D
ACTIVATION BY TPA →

$$CH_3 \text{/\/\/\/} C-O-CH \quad O$$
$$H_2C-O-\overset{O}{\overset{\|}{C}} \text{/\/\/\/} CH_3$$
$$H_2C-O-\overset{}{\underset{OH}{P}}-O-CH_2CH_3^{*}$$
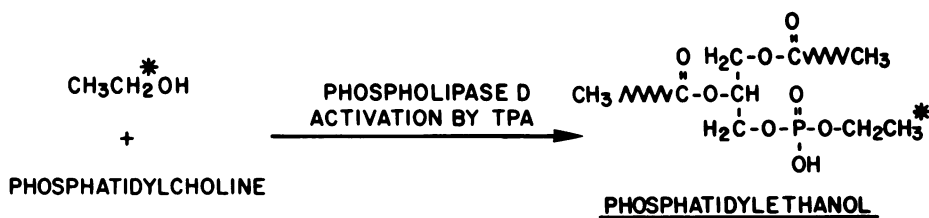
PHOSPHATIDYLETHANOL

FIG. 1. Formation of phosphatidylethanol. Phospholipase D catalyzes the exchange of ethanol for choline in phosphatidylcholine.

added to the cultures and the incubations continued for 180 min.

Incubations were terminated by sedimenting the cells, washing one time by suspension in 5 ml of cold PBS, and initiating the lipid extraction. The lipid analysis was carried out according to Tettenborn and Mueller (3). The lipids were extracted with chloroform/methanol (1:1, vol/vol), taken to dryness *in vacuo*, and the lipid residues were chromatographed on plastic-backed silica gel Kodak chromagram TLC plates using the organic phase of a mixture of ethyl acetate/ 2,2,4-trimethylpentane/acetic acid/H_2O (110:50:20:100, vol/vol) as the developing agent. The phosphatidylethanol and polar lipid regions were visualized by iodine vapor staining and the appropriate regions of the chromatograms were cut and assayed for ^{32}P-labeled lipids by liquid scintillation spectrometry. The results are expressed as the percentage of lipid phosphorus that is accounted for as phosphatidylethanol.

Selection and Classification of Human Subjects. Test subjects for this pilot study were drawn from alcoholic and nonalcoholic adult males ranging in age from 18 to 52 years (mean age, 34 years) who utilize an alcohol treatment center (Madison Family Institute or the University of Wisconsin Family Medicine Clinics). The study was limited to males in that the familial nature of alcoholism, as documented by Goodwin *et al.* (9), Cotton (10), and Schuckit (11), suggests a male influence in the genetic transmission from parent to offspring. The interviews were conducted by a graduate assistant with experience in conducting alcohol and drug abuse (AODA) assessments. Patients were classified as alcoholic or nonalcoholic by an independent panel of three researchers with experience in AODA by the criteria described below. All classifications were performed by the panel without knowledge of phosphatidylethanol production data. Similarly, the laboratory personnel who performed the assays of induced phosphatidylethanol synthesis were blinded to the alcohol group status of the subjects throughout the assays.

Group 1 consisted of 24 nonalcoholic males who had no history of alcoholism in any first degree relatives. They ranged in age from 20 to 48 years (mean age, 34 years); 23 subjects were White and nonsmokers. Alcohol consumption in the 6 months prior to the study ranged from 0 to 21 drinks per week (mean, 4.4). Group 2 consisted of 25 males who met the DSM-III criteria for alcohol dependence and who reported an alcoholic male parent who met DSM-III criteria for abuse and/or dependence. They ranged in age from 18 to 52 years (mean age, 34 years); all subjects were White, and 4 of the 25 subjects were nonsmokers. Alcohol consumption in the 6 months prior to the study ranged from 0 to 189 drinks per week (mean, 81.4).

Materials. The following special reagents were purchased commercially: Histopaque-1077 from Sigma; TPA was from LC Services (Woburn, MA); dimethyl sulfoxide from Aldrich; and ^{32}P$_i$ (carrier free) was from New England Nuclear.

RESULTS

Properties of the Assay System. The enzymatic pathway assayed in this study involves a transphosphatidylation

reaction catalyzed by phospholipase D as depicted in Fig. 1. Recent studies of Pai *et al.* (4) and Tettenborn and Mueller (3, 5) have revealed that phosphatidylcholine is the primary donor substrate in the TPA-stimulated system and that a variety of simple alcohols in addition to ethanol can function as the acceptor. This enzymatic pathway also appears to reside in an inactive or cryptic state until activated by an exogenous agent. Phorbol esters that are active as tumor promoters, telocidin, and bryostatin—all agents that activate protein kinase C—are highly effective stimulators of the *in vivo* system. In addition, phospholipase D has been shown to be activatable by the chemotactic peptide *N*-formyl-Met-Leu-Phe (4), vasopressin (12), and the G-protein activating nucleotide GTP[γS] (5, 12).

In the assay used in the present study, the lymphocytes were cultured for 18 hr in the presence of ^{32}P$_i$ to prelabel the pools of phospholipids. To initiate the assay, TPA (100 nM) along with 0.5% ethanol were added to the prelabeled cells and the production of phosphatidylethanol was followed over a 180-min period. The TPA and ethanol levels were chosen to give a maximal response in lymphocytes. A typical thin-layer chromatogram of the lipid products of control and TPA-treated lymphocytes is shown in Fig. 2. In the absence of TPA, the cells failed to make a measurable level of phos-
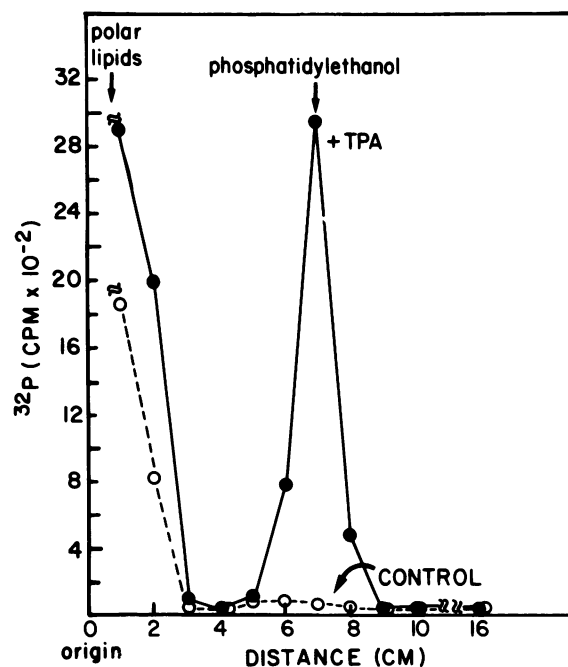

FIG. 2. TPA induces the synthesis of phosphatidylethanol in human lymphocytes. Lymphocytes (8.75 × 10^6 cells per culture) were prelabeled 18 hr with inorganic ^{32}PO$_4$ (20 μCi/ml). After the prelabeling interval, ethanol (final concentration, 0.5%) and TPA (100 nM) or the control vehicle dimethyl sulfoxide were added and phosphatidylethanol synthesis was measured over 180 min. Cell lipids were extracted and resolved by TLC. The peak of ^{32}P-labeled lipids in sections 6–8 of the chromatogram corresponds to [^{32}P]phosphatidylethanol. Note the lack of label in this region in the absence of TPA treatment.

phatidylethanol. Exogenous ethanol was also absolutely required for phosphatidylethanol production (data not shown). The total yield of phosphatidylethanol was also directly proportional to the level of the cells in the culture.

Assay of the Ability of Alcoholic and Nonalcoholic Subjects to Synthesize Phosphatidylethanol. The results of a pilot study are revealed in Fig. 3. In this study, the TPA responses ranged from 0.4% to 4.0% of the lipid phosphorus being accounted for as phosphatidylethanol. For purposes of plotting the data, the full range of phosphatidylethanol production by all subjects was divided into 25 equal intervals; each interval corresponded to a 0.13% increment in the amount of total lipid phosphorus that could be accounted for as phosphatidylethanol; the lowest production interval was 0.42–0.55% and the highest interval was 3.78–3.91%. The number of subjects of each group that fell into the indicated intervals is plotted as a histogram.

Inspection of this chart reveals that 23 of the 24 nonalcoholic subjects are clustered in intervals 4–12. By contrast, 12 of the 25 alcoholic subjects exhibited potentials for phosphatidylethanol production in intervals 15–25, a production range that was above all nonalcoholics. The striking difference in the distribution of these two populations is better visualized when the data are plotted as the percentage of test subjects accounted for with increasing levels of phosphatidylethanol production (Fig. 4).
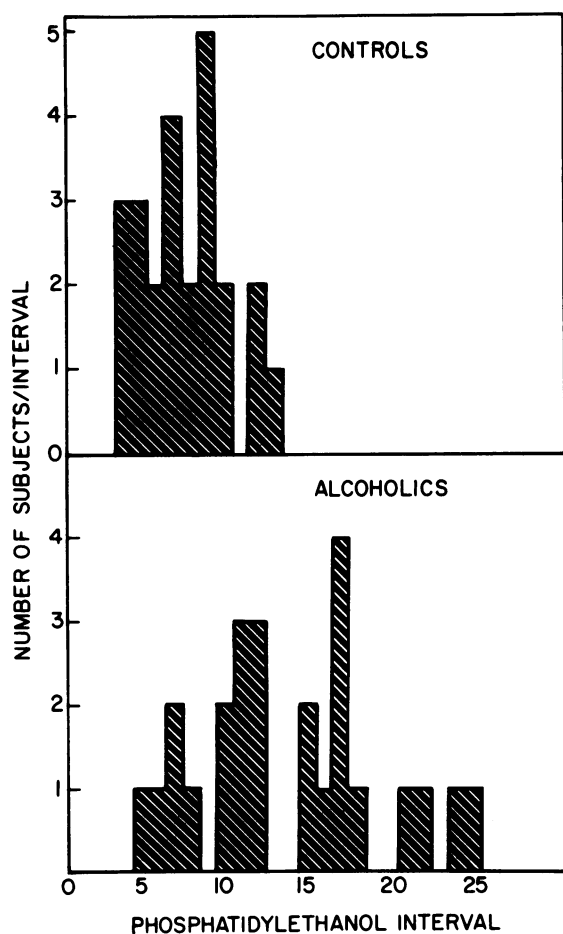

Fig. 3. Synthesis of phosphatidylethanol by control and alcohol-dependent subjects. The potential for phosphatidylethanol synthesis was measured in control and alcohol-dependent subjects. Phosphatidylethanol production in this study ranged from 0.42% to 3.91% of the total ^{32}P-labeled phospholipids; this range of production was divided into 25 equal incremental intervals corresponding to 0.13% of the ^{32}P-labeled lipid. The number of subjects having phosphatidylethanol production in a given interval is plotted as a histogram for each group of subjects.

A comparison of the two groups, using the two-sided Wilcoxon rank sum test (13), revealed that phosphatidylethanol production by the alcoholics (mean, 2.26% ± 0.77% of ^{32}P$_i$-labeled lipids) was significantly greater than among the controls (mean, 1.44% ± 0.37%) ($P < 0.0001$). As might be expected from the classification determinants, the alcoholics also consumed significantly more alcohol (mean, 81 ± 62 drinks per week) than the controls (mean, 4.4 ± 5.2 drinks per week) ($P < 0.0001$). The two groups, however, did not differ significantly in age distribution ($P = 0.87$).

The sensitivity (100 – percent of false negatives) and specificity (100 – percent of false positives) of the phosphatidylethanol production test were determined by using a cut-off score of 1.81 (interval 10). In this calculation, the 8 of 25 alcoholic subjects that registered phosphatidylethanol production levels below the cut-off score correspond to a false-negative rate of 32% and a sensitivity of 68%. In the control group of nonalcoholic males, the 3 of 24 subjects with levels above 1.81, correspond to a false-positive rate of 12% and a sensitivity of 88%.

With respect to the alcoholic subjects whose phosphatidylethanol production potential overlaps the levels exhibited by the controls (i.e., intervals 4–12), it can be added that recent experiments have revealed that phosphatidylethanol is subject to a unique metabolism in certain cell systems. This metabolism is carried out by a steroid-inducible pathway, yields chemically reactive derivatives of phosphatidylethanol, and may be important to the regulation of phosphatidylethanol levels under conditions of endogenous stimulation. Since this pathway could also play a role in the development of alcohol-related pathology, it will be of interest to assess this pathway in control and alcoholic subjects in the near future. Perhaps this analysis will provide the perspectives that are required for discrimination between the alcoholic and nonalcoholic populations in the region of overlap.

Status of Other Correlations. While the number of test subjects is too few to permit a detailed assessment of other variables that will be addressed subsequently in a larger survey, it can be projected that subject age and recent alcohol consumption may not be important parameters. Using Kendall's rank correlation test (13), it was found that phosphatidylethanol production did not vary significantly with age or alcohol consumption (phosphatidylethanol vs. age; $P = 0.09$ for controls and 0.81 for alcoholics) (phosphatidylethanol vs. alcohol consumption; $P = 0.28$ for controls and 0.61 for alcoholics).

Smoking, however, remains as a potential confounding variable since practically all of the alcoholics were heavy smokers (≈ 2 packs of cigarettes per day), whereas the controls were largely nonsmokers. While there is no sound scientific reason for anticipating that smoking contributed to our results, an assessment of this potential confounding variable requires an expansion of our present study to include properly matched populations.

DISCUSSION

The results of this pilot study provide strong support for the view that adult males with both a personal and a family history of alcohol dependence have, in general, a higher potential to synthesize phosphatidylethanol—a unique metabolite of ethanol. In fact, the average level of phosphatidylethanol production in TPA-treated lymphocytes of alcoholic subjects was approximately twice that of the control population. Since this synthesis is mediated by phospholipase D, this relationship raises several interesting possibilities: (i) that alcoholics may have a different phospholipase D in their lymphocytes than nonalcoholics—one that is on average twice as active per unit of enzyme protein; (ii) that cells of alcoholics contain the same enzyme but carry, on the
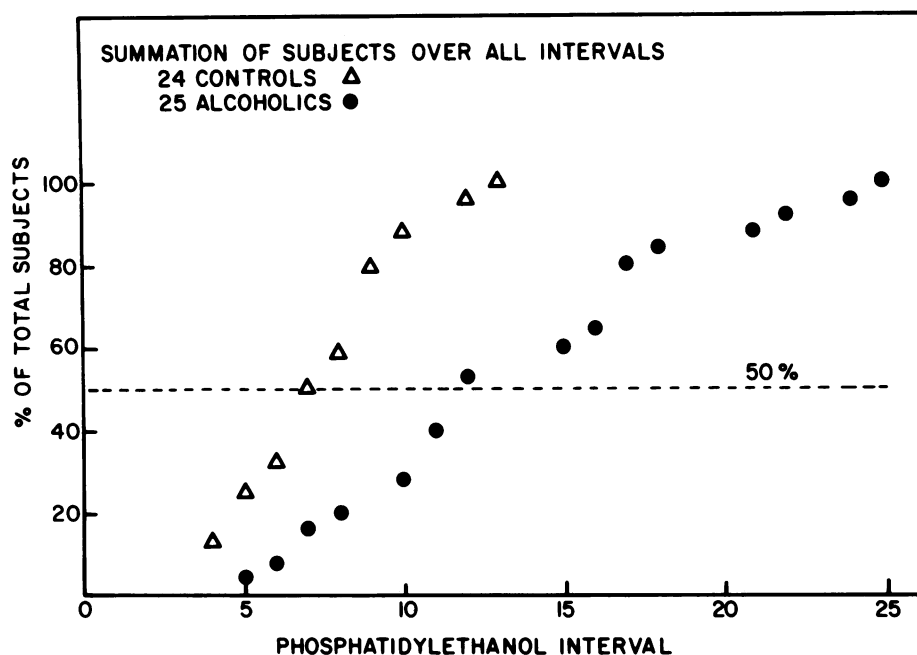
FIG. 4. Percent of total control and alcohol-dependent subjects accounted for with increasing intervals of phosphatidylethanol production. Data from Fig. 3 have been summed according to the patients' increasing abilities to produce phosphatidylethanol and are plotted as accumulation curves.

average, twice the level in a cryptic state; or (*iii*) that the cells of alcoholics have a system for down-regulation of phospholipase D activity that is only one-half as effective as that of control subjects. To explore these possibilities as well as others, it will be necessary to isolate phospholipase D and study its regulation by molecular genetic approaches in properly matched sets of family members for each class of subjects. Such studies are expected to establish whether or not there is a fundamental genetic basis for the present results.

The observation that phosphatidylethanol is not synthesized until the cells are exposed to the phorbol ester suggests that the enzyme resides in a cryptic state—awaiting activation by an exogenous or endogenous factor. Accordingly, a study of the levels of potential activators of phospholipase D—as well as the responsiveness of this enzyme in the cells of control and alcoholic subjects—will be of considerable interest. The remarkable responsiveness of the system to phorbol esters, implying that protein kinase C is a major regulator of this pathway, points to a need to assess the role of certain nutrients, growth factors, drugs, and hormones (i.e., agents that mediate their effects through protein kinase C) in alcoholism. This view is also supported by the observation that bryostatin and teleocidin, also activators of protein kinase C, can stimulate this pathway in other cell systems (2, 3).

In addition to being regulated directly or indirectly by protein kinase C, the pathway also appears to be responsive to factors that interact with cell-surface receptors and propagate their effects through GTP-binding proteins. For example, Bocckino *et al.* (12) found that vasopressin stimulates phospholipase D in hepatocyte preparations from rat liver. They also showed that this cell-surface peptide receptor mechanism could be bypassed in hepatocyte fragments by GTP[γS], a nonhydrolyzable GTP analog that can activate G-protein systems. In our own laboratory, C. S. Tettenborn and G.C.M. (unpublished data) have shown the activation of phosphatidylethanol production in lysates of HL-60 cells by GTP[γS]. In a similar type of study, Pai *et al.* (4) showed that N-formyl-Met-Leu-Phe, a chemotactic peptide, produced a rapid but transitory activation of phospholipase D in macrophages arising from the differentiation of HL-60 cells. These

observations, like those showing the role of protein kinase C in the regulation of this pathway, point to the need of screening neuropeptides and related factors in the present system and for possible effects that may relate to the alcoholism problem.

The present pilot study has used peripheral blood lymphocytes with an aim to establishing a relatively noninvasive assay for testing the status of human subjects for possible risk for alcoholism. It seems appropriate in the near future to extend the studies and epidemiological correlations to other tissues. In view of the remarkable responsiveness of the system to phorbol esters and the knowledge that these agents work through the activation of the protein kinase C class of enzymes, it will be of interest to assess the level of the different protein kinase C isozymes since they vary remarkably in different tissues and have different phosphorylation targets. Perhaps additional perspectives on the correlations between phosphatidylethanol production and risk status for alcoholism will emerge with such studies. In addition, a correlation of levels of different receptor systems for peptide hormones, adrenergic agents, and growth factors with the responsiveness of the phosphatidylethanol synthesis pathway in such cells is considered a high priority objective in future studies.

In summary, the studies of phosphatidylethanol synthesis by phospholipase D and the regulatory systems that govern the activity and responsiveness of the enzyme system in control and alcohol-dependent subjects are expected to provide a new scientific basis for assessing factors relevant to the problem of alcoholism. The new data are also anticipated to be of value in identifying subjects at risk, in guiding genetic counseling, and in developing effective therapies for alcohol-dependent subjects.

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