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Graphical Abstract

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Oxidative and Non-Oxidative Metabolomics of Ethanol

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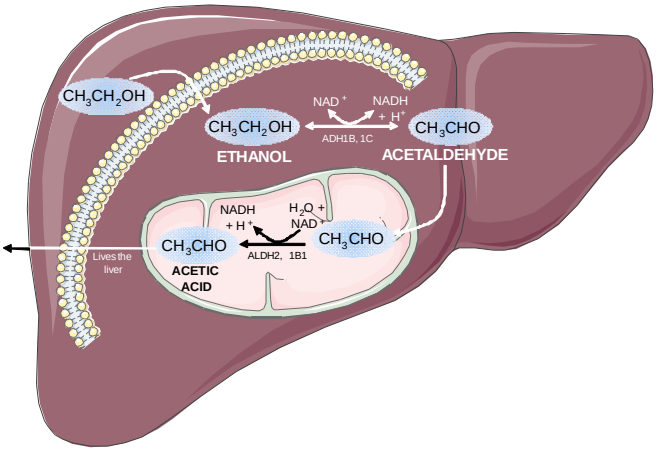

The main metabolic pathway of ethanol metabolism is the bioactivation to acetaldehyde catalyzed by cytosolic alcohol dehydrogenase and the detoxification to acetate catalyzed by mitochondrial aldehyde dehydrogenase.

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Oxidative and Non-Oxidative Metabolomics of Ethanol

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Abstract: ***Background:*** It is well known that ethanol can cause significant morbidity and mortality, and much of the related toxic effects can be explained by its metabolic profile.

Objective: This work performs a complete review of the metabolism of ethanol focusing on both major and minor metabolites.

Method: An exhaustive literature search was carried out using textual and structural queries for ethanol and related known metabolizing enzymes and metabolites.

Results: The main pathway of metabolism is catalyzed by cytosolic alcohol dehydrogenase, which exhibits multiple isoenzymes and genetic polymorphisms with clinical and forensic implications. Another two oxidative routes, the highly inducible CYP2E1 system and peroxisomal catalase may acquire relevance under specific circumstances. In addition to oxidative metabolism, ethanol also originates minor metabolites such as ethyl glucuronide, ethyl sulfate, ethyl phosphate, ethyl nitrite, phosphatidylethanol and fatty acid ethyl esters. These metabolites represent alternative biomarkers since they can be detected several hours or days after ethanol exposure.

Conclusion: It is expected that knowing the metabolomics of ethanol may provide additional insights to better understand the toxicological effects and the variability of dose response.

Keywords: Ethanol, metabolomics, oxidative, non-oxidative, major metabolites, minor metabolites, biomarkers.

INTRODUCTION

Ethanol abuse is associated with significant morbidity and mortality [1, 2]. Blood ethanol concentrations are the result of upper gastrointestinal absorption by passive diffusion, uniform distribution in body water, metabolism and unchanged excretion [3]. Ethanol absorption is a fast process since it is a small molecule highly soluble in water, but not in fat [4]. Twenty percent of ethanol is absorbed in the stomach and 80% in the upper small intestine. In the gastric mucosa (and not in the small intestine), 5-10% of ethanol is metabolized by alcohol dehydrogenase isoform (or isozyme) 7 (ADH7) and this is called gastric first pass metabolism of ethanol [3, 4]. The remaining ethanol accesses the liver via the portal vein where it is partially metabolized, since some flows out of the liver without being metabolized. Peak blood ethanol concentrations usually occur within 30-90 minutes of ingestion. Ninety percent of ethanol is metabolized in the liver after multiple passages but the lungs (especially via CYP2E1) also contribute to metabolism. Unchanged ethanol is eliminated in small quantities (approximately 5% of orally absorbed ethanol) by the kidneys (0.5–2%), lungs (1.6–6%) and skin (<0.5%) [4].

Fifty years ago, it was believed that the metabolism of ethanol involving multiple forms of ADH was the only significant pathway [1]. Indeed, ethanol is primarily bioactivated (92-95%) by cytosolic (especially hepatic) ADH1B into acetaldehyde, which has proved to have mutagen and carcinogen effects [1]. At high blood ethanol levels, the microsomal ethanol-oxidizing system (CYP2E1 isoform, located in the smooth endoplasmic reticulum) also has an important role. Ethanol itself induces the activity of CYP2E1, and therefore also influences the metabolism of other xenobiotics (*e.g.*, paracetamol and cocaine). Through CYP2E1, reactive oxygen species (ROS) are produced and lipid peroxidation may occur. Catalase is of minor importance (responsible for approximately 5%) in the metabolism of ethanol. Acetaldehyde is subsequently detoxified to acetate, mainly by the mitochondrial enzyme aldehyde dehydrogenase (ALDH2) [5]. In addition to oxidative metabolism, ethanol also originates minor metabolites such as ethyl glucuronide (EtG), ethyl sulfate (EtS), ethyl nitrite (EtN), ethyl phosphate (EtP) phosphatidylethanol (PEth) and fatty acid ethyl esters (FAEE; *e.g.*, ethyl palmitate, ethyl oleate, and ethyl stearate), which can be detected several hours or days after ethanol exposure, representing alternative biomarkers of exposure [6, 7]. This non-oxidative metabolism is less useful in explaining toxic effects of ethanol, but is highly important for monitoring consumption due to longer half-lives. Indeed, ethanol has a short half-life, which means that it possess a reduced value as an exposure biomarker.

This work reviews all known oxidative and non-oxidative metabolic routes of ethanol, focusing on both major and minor metabolites with forensic and clinical toxicology.

ALCOHOL DEHYDROGENASE

Ethanol metabolism involves primarily multiple isoforms of cytosolic ADHs and it is the rate-limiting step. These belong to the medium chain dehydrogenases/reductases (MDRs), which typically catalyze oxidation of simple alcohols (such as methanol and ethanol) to aldehydes (namely, formaldehyde and acetaldehyde), coupled with the reduction of NAD^+ to NADH (Fig. **1**) [1]. Besides xenobiotics, ADHs also metabolize endobiotics such as dehydrogenation of hydroxysteroids, oxidation of intermediary alcohols and *m*-oxidation of fatty acids [8]. There are seven human ADHs, and these are divided into five classes (I-V) based on patterns of tissue-specific expression, catalytic properties, and amino acid sequence (Table **1**) [9, 10]:

a) Class I – comprises ADH1A or α-ADH, ADH1B or β-ADH, and ADH1C or γ-ADH (formerly known as ADH1, ADH2, and ADH3, respectively) with homodimeric and heterodimeric forms of the 3 subunits (*e.g.*, αα, αβ, ββ, βγ, γγ, etc.);

b) Class II – comprises ADH4 with 2 pi subunits (ππ);

c) Class III - comprises ADH5 with 2 chi subunits (XX);

d) Class IV - comprises ADH7 with 2 sigma subunits (σσ);

e) Class V - comprises ADH6 (for which there is no subunit designation).

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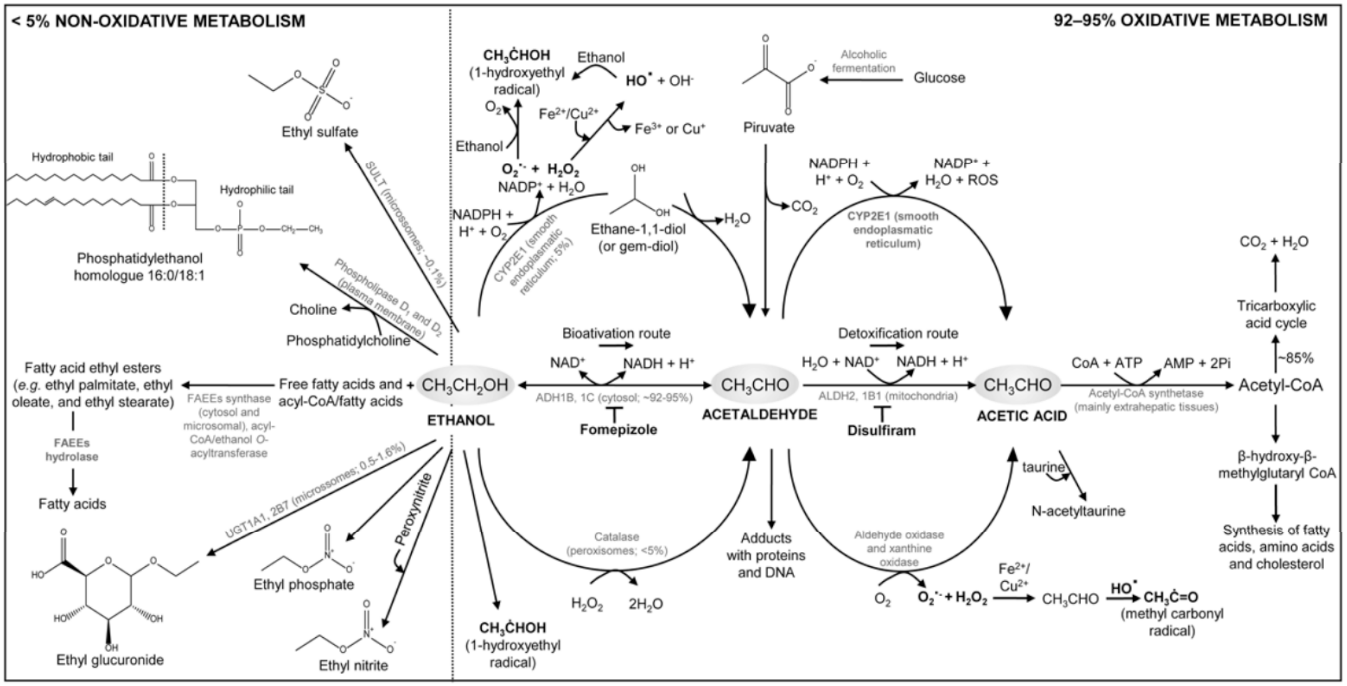

Fig. (1). Oxidative and non-oxidative metabolism of ethanol. Approximately 5% of ethanol is excreted unchanged in urine (0.5-2.0%), sweat (<0.5%) and breath (1.6-6.0%). In gray color are the enzymes that catalyze the respective reactions.

Table 1. Principal characteristics of ADH isoforms [9, 118].

Are Zn metalloenzymes
Cytosolic enzymes present in tissues such as the liver (which has the highest levels), kidneys, lungs, and gastric mucosa
Are dimeric proteins consisting of two ~40-kDa subunits α, β, γ, π, σ, X (formerly known as μ), or ADH6 (there is no subunit term)
7 AHD isoforms: Class I (ADH1A, ADH1B and ADH1C), Class II (ADH4), Class III (ADH5), Class V (ADH6), Class IV (ADH7)

The need to clear the body of small amounts of ethanol produced by fermentation in the gut may explain its utility [11]. Ethanol is not capable of inducing its own metabolism via ADH.

Class I ADH isozymes are responsible for the oxidation of ethanol and other small aliphatic alcohols, and are strongly inhibited by pyrazole and its 4-alkyl derivatives (*e.g.*, 4-methylpyrazole or fomepizole). They exhibit high affinity (low K_m; reacting at a relatively low concentration) but low capacity (high V_{max}) for ethanol. High levels of class I ADH isozymes are present in the liver (especially in the centrolobular area) and adrenals, with lower levels in the kidneys, lungs, blood vessels (in the case of ADH1B), gastric mucosa (in the case of ADH1C), and other tissues, but not in the brain or placenta. It is worth noting that the liver expresses a very large amount of ADHs (approximately 3% of all soluble protein) and also the widest variety of isozymes [9].

Genetic polymorphisms have been well described for class I ADH isozymes, resulting in different affinities (K_m) and/or capacity (V_{max}) for oxidizing ethanol to acetaldehyde. There are at least 3 allelic variants of ADH1B (*i.e.*, *1, *2, and *3), with a single amino acid change at position 48. The homodimer, $\beta_2\beta_2$, and heterodimers of at least one β_2 subunit (*i.e.*, ADH1B*2) are particularly active in the oxidation of ethanol. Indeed, ADH1B*2 (formerly known as ADH2*2) is known as "atypical" ADH, and is responsible for the unusually rapid metabolism (40 times more active than the enzyme encoded by the ADH1B1 allele) of ethanol to acetaldehyde in up to 90% of the East Asian population, whereas only ~10% of Caucasians express this allele. ADH1B*3 is relatively common in individuals of African descent. The latter two alleles have greater activity toward ethanol than the ADH1B1*1 allele [12]. These population differences in ADH1B allelozyme expression contribute to ethnic differences in ethanol consumption and toxicity. Indeed, since individuals with the ADH1B2 allele produce relevant amounts of acetaldehyde and experience its toxic effects (*e.g.*, flushing, tachycardia, diaphoresis, nausea and vomiting), they tend to avoid ethanol consumption. Unlike the allelic variants of ADH1B, the allelic variants of ADH1C do not differ markedly in their ability to oxidize ethanol. Nevertheless, it has been demonstrated that the ADH1C1 allele encodes for an enzyme that is 2.5 times more active in producing acetaldehyde than ADH1C2 [13].

The class II enzyme ADH4 (π-ADH) is mainly expressed in the liver (and to some extent in other gastrointestinal tissues), where it preferentially oxidizes larger alcohols but may play some role in ethanol oxidation, especially at high concentrations [12]. Indeed, ADH4 has a relatively high K_m and negligible the fomepzole inhibition. Some studies support a role for polymorphisms of ADH4 in susceptibility to alcoholism [12].

The class IV enzyme ADH7 (σ-ADH; also referred to as the μ subunit) is a low-affinity but high-capacity enzyme for oxidizing ethanol. Indeed, among the human ADH forms, ADH7 has the highest activity toward ethanol [14]. It is the main ADH expressed in human gastric mucosa (also ADH1C and ADH3) and other cells in the upper gastrointestinal tract (*e.g.*, esophagus, pharynx, gingiva, mouth, and tongue), and in the cornea [14]. This localization is of considerable interest in the gastric first pass metabolism of

ethanol since it may explain the development of cancer in the gastrointestinal tract due to acetaldehyde exposure [1, 4]. In contrast to the other ADHs, ADH7 is not expressed in the adult human liver [9]. Its contribution to ethanol metabolism is no more than 5-10% *in vivo* but may be significantly influenced by a number of factors [15] (Table **2**). The role of a protective effect against alcoholism of high-activity ADH7 polymorphisms remains to be fully clarified, with some studies showing a positive association [14], and others no association [16, 17]. However, A92G SNP (rs1573496; C → G) in ADH7 has been found to result in a reduced risk of squamous cell carcinoma of the head and neck in Caucasians [18].

Several experiments carried out with human volunteers have found that plasma levels of acetaldehyde are higher in alcoholics than non-alcoholics, probably due to the vicious cycle manifested by the toxic effects of acetaldehyde on mitochondrion [19].

CYP2E1

A second pathway of ethanol metabolism, the microsomal ethanol oxidizing system (MEOS), was first described almost fifty years ago. CYP2E1 utilizes the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, and also requires O_2, which is reduced to water during catalysis. The K_m of CYP2E1 for ethanol is 10 mM, which is roughly one order of magnitude higher than that of ADH1. Therefore, at low blood ethanol levels, the contribution of CYP2E1 to its oxidation is limited, acquiring an important role only at high levels of ethanol consumption [20].

Unlike the other two major pathways, a 4-10-fold increase of CYP2E1 expression has been associated with ethanol abuse [21, 22]. This induction explains tolerance to the desirable/expected effects of ethanol after chronic consumption and cross-tolerance with other CYP2E1 substrates [23]. Indeed, in addition to CYP1A2 and CYP3A4, CYP2E1 is also recognized as a key enzyme involved in the bioactivation of paracetamol to its liver reactive metabolite N-acetyl-*p*-benzoquinone imine (NAPQI) [24, 25]. CYP2E1 also metabolizes a large number of volatile halogenated alkanes (*e.g.*, chloroform, carbon tetrachloride, vinyl chloride, and many more), halogenated anesthetics (*e.g.*, halothane, enflurane, and others), small aromatic and nitrogen aromatic compounds (*e.g.*, benzene, styrene, toluene, pyridine, pyrazole, and others), alkanes (ethane-hexane), other alcohols besides ethanol (*e.g.*, primary aliphatic alcohols such as methanol and propanol, secondary alcohols such as isopropanol and tertiary alcohols such as *t*-butanol), cocaine, the colon carcinogen azoxymethane, and numerous mutagenic/carcinogenic nitrosamines [26].

CYP2E1 is expressed in the liver (*i.e.*, the smooth endoplasmic reticulum of the centrolobular area) and several extrahepatic tissues, such as the kidneys, pancreas, brain, lungs, intestine, nasal epithelium, bone marrow, and lymphocytes. Low levels of CYP2E1 are also expressed in the human fetal liver and prenatal brain, suggesting ROS involvement in fetal alcohol syndrome [27]. Numerous genetic polymorphisms in the CYP2E1 gene have been identified but none gives rise to a poor- or ultra-metabolizer phenotype [26]. In rat liver, approximately 40% of CYP2E1 is phosphorylated and localized to the inner membrane of mitochondria [28].

Compared with other isoforms of P450, CYP2E1 exhibits a higher rate of oxidase activity [29]. From this activity, ROS are produced even in the absence of substrate, similarly to CYP1A2 (the other high-spin enzyme) [30-32]. During its catalytic cycle CYP2E1 is prone to uncoupling, so that, during the metabolism of ethanol, approximately 50% of the cycles result in the production of superoxide anion ($O_2^{\cdot-}$) and related ROS instead of metabolite formation [33, 34]. Accordingly, several studies have demonstrated that antioxidants can protect against ethanol-related toxic effects [35].

The CYP2E1-catalyzed metabolism of ethanol produces an unstable intermediate (*i.e.*, a gem-diol) that decomposes to acetaldehyde. Moreover, since ROS are produced, CYP2E1 indirectly catalyzes the formation of a radical species from ethanol itself (*i.e.*, 1 (or α)-hydroxyethyl radical - $CH_3\dot{C}HOH$), which also contributes to oxidative damage [36]. The detection of the 1-hydroxyethyl radical has been suggested as a useful proof of oxidative stress *in vivo* as a consequence of ethanol exposure [37]. Although not yet completely understood, two mechanisms of hydroxyethyl radical formation have been proposed:

a) NADPH-oxidase activity of CYP2E1 is responsible for the formation of $O_2^{\cdot-}$and H_2O_2. The interaction of transition metals

Table 2. Factors that may influence the gastric first pass metabolism of ethanol [15, 109, 119-122].

The manner of consumption: large doses over a short time (*e.g.*, shots) produce high ethanol concentrations in the stomach, which compensate for the high K_m of gastric ADH7
Age and gender: young women have lower gastric ADH7 activity than men. ADH7 activity in men decreases and reaches the level in females at the age of 65 or older
Alcoholics: gastric ADH7 activity tends to be lower (some alcoholic women have no detectable gastric ADH7, and blood levels of ethanol after oral consumption of alcohol are the same as those that are obtained after intravenous administration
Fasting: decreases ADH7 activity (ethanol is more intoxicating when consumed on an empty stomach)
Noncompetitive inhibitors: commonly used drugs [*e.g.*, cimetidine (inhibits ADH7 activity), ranitidine (enhanced gastric emptying with a decreased contact time of ethanol with gastric ADH7), tramadol, and aspirin (injure gastric mucosa leading to a decrease in gastric production] increase the systemic availability of ethanol, although the effect is too small to have serious medical, social, or legal consequences
Ethnicity: 30% of Asians appear to be genetically deficient in ADH7
Rate of gastric emptying: if delayed, increases gastric first pass metabolism and vice-versa. This may be of relevance when ethanol is taken together with food (fat delays gastric emptying) or in diabetic patients with gastroparesis
Gastric morphology: atrophic gastritis or gastric atrophy decreases gastric parietal cell mass and therefore the amount of ADH in the stomach
Helicobacter pylori: decreases first pass effect. Although this bacteria has ADH activity and produces acetaldehyde from ethanol (as other microorganisms may do) this effect is counteracted by the damaging effects of the bacteria on gastric mucosa
Polymorphism of ADH1C

(*e.g.*, Fe^{2+} and Cu^{+}) with H_2O_2 produces $HO^{\cdot}$ that, by reacting (scavenging) with ethanol, may give rise to less reactive hydroxyethyl free radicals;

b) Direct one-electron oxidation of ethanol by $O_2^{\cdot-}$ at the catalytic site of the enzyme may also account for the formation of ethanol-derived radical species as a result of interaction of ethanol with the ferric cytochrome P450-oxygen complex (CYP2E1-$Fe^{3+}O_2^{-}$).

Interestingly, it has been shown that acetaldehyde may also be oxidized by CYP2E1 to acetate, a reaction that will be always negligible [38] if it occurs at all.

CYP2E1 can be inhibited by 4-methylpyrazole (fomepizole), which also inhibits ADH (class I ADH), and by disulfiram (Antabuse), which also inhibits ALDH (ADH2). Males exhibit higher activity of CYP2E1 and this isoenzyme is induced by fasting and prolonged starvation [39-41].

NITRIC OXIDE SYNTHASE

Nitric oxide synthase (NOS), a member of the same superfamily of enzymes [42] that includes cytochrome P450, oxidizes *l*-arginine to *l*-citrulline and nitric oxide (NO) [43]. This enzyme can also transfer electrons to O_2 to form $O_2^{\cdot-}$ and H_2O_2. Porasuphatana *et al*. [44] demonstrated that, in the presence of *l*-arginine, NOS1 (neuronal) and NOS2 (inducible) can metabolize ethanol to a 1-hydroxyethyl radical. Authors have also observed that once this free radical is formed, it is metabolized to acetaldehyde.

CATALASE

It is also known that the peroxisomal catalase is capable of oxidizing ethanol in the presence of H_2O_2 [45]. Nevertheless, under physiological conditions, catalase seems to play an insignificant role [46]. Indeed, it is estimated that <5% of an ethanol dose is metabolized through this pathway [4, 46]. In this reaction, ethanol functions as an electron donor for the reduction of H_2O_2 to water. Thus, the capacity of this pathway is limited due to the low levels of H_2O_2 and, like CYP2E1, is also associated with the generation of ROS.

Several polymorphisms in the catalase gene have been described, but these do not alter enzymatic activity [47]. It has also been suggested that acetaldehyde produced in the brain (where ADH is inactive) due to metabolism of ethanol by catalase plays a role in the development of tolerance and in the positive reinforcing actions of ethanol [48].

ALDEHYDE DEHYDROGENASE

ALDHs (especially ALDH2 but also ALDH1B1 and ALDH1A) oxidize acetaldehyde to acetic acid [1, 49]. This pathway generally uses NAD^{+} as the cofactor that is reduced to NADH. ALDH enzymes are involved in the oxidation of other aldehydes, such as those formed from allyl alcohol, carbon tetrachloride, cyclophosphamide, and ifosfamide [46].

Besides ADH, relevant polymorphism with toxicological significance involves the mitochondrial ALDH2, with the ALDH2*2 isoform showing low or no catalytic activity (the wild type variant is ALDH2*1) [50]. ALDH2 is expressed at the highest levels in the liver, although it can also be found in other tissues such as kidney, skeletal, cardiac muscle, and mammary tissues. Since ALDH2*2 is found in 40%-50% of East Asians but is absent in Caucasians, this may explain the low incidence of alcoholism in the Asian population [51]. As mentioned above, Asians also have a high incidence of the "atypical" active form of ADH1B (*i.e.*, ADH1B*2), which means that they rapidly bioactivate ethanol to acetaldehyde but slowly detoxify acetaldehyde to acetic acid. Moreover, they also have a relatively high prevalence of ADH7 deficiency, which reduces the gastric first pass metabolism of ethanol. Collectively these data may protect the Asian population against heavy drinking and alcoholism [1].

ALDH1B1 (mitochondrial) and 1A1 (cytosolic) presented higher K_m than ALDH2. However, these enzymes may still have physiological relevance under certain conditions, especially in ALDH2 deficient individuals, and also in the gastrointestinal tract, where high levels of acetaldehyde can be generated as a result of ethanol metabolism by microorganisms [20].

Finally, it is important to mention that acetaldehyde may be present in alcoholic drinks (especially spirits) as a result of the action of yeast and bacteria, and also autooxidation [52].

ACETATE

The oxidation of ethanol by ADH and ALDH leads to the formation of acetic acid. Elevated acetate blood levels have been proposed as an indicator of alcoholism [53]. Most of the acetate produced leaves the liver, since the need to oxidize the NADH generated by ADH and ALDH uses 70% to 75% of the oxidative capacity of the organ [54]. The principal isoform of acetyl-CoA synthetase (ACS I) in the liver is a cytosolic enzyme that generates acetyl-coenzyme A, which is used for fatty acid and cholesterol synthesis. Nevertheless, most acetate enters the systemic circulation and is then taken by several tissues such as heart, skeletal muscle, and brain, which have a high concentration of mitochondrial acetyl-CoA synthetase isoform (ACS II) [55]. CoA is then rapidly oxidized to carbon dioxide and water in the Krebs cycle, or is used as a substrate for protein acetylation [56]. AMP is also produced from acetyl-CoA synthetase activity and is further hydrolyzed by 5'-nucleotidase to adenosine, which is a powerful physiological vasodilator [57]. Thus, while acetate is generally not considered a toxic compound, it can itself have important effects on the body, including increased portal blood flow in the liver and central nervous system depression, and seems to play a role in hangovers [58, 59].

Notably, acetate is also the major anion product of intestinal formation, resulting especially from decarboxylation of pyruvate and further acetyl-CoA oxidation by *Bacteroids*, *Clostridium* and *Enterobacterium*, and by acetylphosphate oxidation as in *Bifidobacterium* [60].

XANTHINE OXIDASE AND ALDEHYDE OXIDASE

Besides CY2E1 and ALDH, two metalloflavoproteins, xanthine oxidase and aldehyde oxidase, have also proved to oxidize acetaldehyde present in tissues [61, 62]. Moreover they are also capable of producing $O_2^{\cdot-}$ and H_2O_2 in cells exposed to ethanol. Indeed, ethanol stimulates the conversion of xanthine dehydrogenase to $O_2^{\cdot-}$-producing oxidase [63]. H_2O_2 in the presence of transition metals (*e.g.*, Fe^{2+} and Cu^{+}) can lead to the formation of $HO^{\cdot}$, radicals that are responsible for attacking another molecule of acethaldehyde giving rise to methyl carbonyl radical ($CH_3\dot{C}{=}O$) [64, 65].

ETHYL-GLUCURONIDE (EtG) AND ETHYL-SULPHATE (EtS)

EtG and EtS are direct, non-volatile metabolites of ethanol with relevant clinical and forensic applications [66]. EtG and EtS are phase II metabolites of ethanol, catalyzed by the enzymes UDP-glucuronosyltransferase (UGT) superfamily (it utilizes UDP-glucuronic acid as a cofactor) or sulfotransferase (it utilizes 3'-phosphoadenosine-5'-phosphosulfate as a cofactor), respectively. Less than 2% of the ethanol ingested is metabolized through this nonoxidative route [67]. Multiple isoforms of UGT are involved in catalyzing the formation of EtG, but only UGT1A1 and UGT2B7 seem to play a major role [68]. Much less kinetic data is available for EtS.

Peak EtG serum concentrations typically occur about 4 hours after ingestion [69] and after heavy ethanol consumption. EtG and EtS may be detectable for up to 8 and 80 hours in blood and urine,

respectively [69, 70]. Moreover, within just one hour of consumption of low amounts of ethanol, EtG and EtS are already positive in urine and, due to their longer half-life, they are still present in this matrix even when ethanol is negative [71]. Therefore, EtG and EtS are useful biomarkers for the detection of recent ethanol exposure and for abstinence monitoring [7, 72, 73].

However, false positive EtG results may occur, if collected urine contains bacteria (*e.g.*, *Escherichia coli*) and ethanol, which might be produced from glucose (*e.g.*, glycosuria in diabetics) and yeast by alcoholic fermentation [74]. On the other hand, EtG false negative results may be also recorded for urine samples (especially if transported and stored improperly without cooling) since this metabolite is sensitive to hydrolysis by β-glucuronidase present in high levels in *Escherichia coli*, a common cause of urinary tract infection) [75-77]. Therefore, refrigeration or frozen storage is recommended to improve EtG stability in these specimens [75]. Neither postsampling formation nor degradation has been documented in urine samples for EtS [76]. Some studies have reported EtS biodegradation only under high bacterial density conditions [77]. Finally, genetic polymorphisms in UGT and SULT may justify inter- and intra-individual differences in the EtG and EtS levels [46].

PHOSPHATIDYLETHANOL

Other non-oxidative pathway involves the formation of phosphatidylethanol (PEth) in the cell membranes. When present, ethanol acts as a co-substrate in the transphosphatidylation reaction catalyzed by the enzyme phospholipase D (PLD), which inserts ethanol in place of choline in phosphatidylcholine [78-80]. PLD has a high K_m for ethanol, and therefore the reaction would be most important predominantly in high circulating ethanol concentrations. Two isoforms of phospholipase D have been characterized in humans. Phospholipase D_1 has a perinuclear distribution and requires phosphokinase C activation, whereas phospholipase D_2 is located in the cellular membrane and is constitutively active. However, both isoforms of phospholipase D catalyze the formation of PEth in human erythrocyte membranes [81].

PEth is not a single molecule but a group of abnormal phospholipid homologues. It is formed by a glycerol molecule with two fatty acid chains (typically containing 14 to 22 carbon atoms with 0 to 6 double bonds) in the *sn*-1 and *sn*-2 position and with phosphoethanol as the head group [82]. At least 48 homologues of PEth have been identified in blood [82]. The structure of the most common PEth homologue is 16:0/18:1 (1-palmitoyl-2-oleosyl-sn-glycero-3-phosphoethanol).

Compared with its formation, the elimination half-life of PEth from human blood (mainly in erythrocytes) is slow (approximately 4 days) [7, 83]. It has been detected in the blood of ethanol abusers even up to three weeks after withdrawal [83]. Moreover, PEth concentration is highly correlated with the amount of ingested ethanol [83]. These facts show that PEth may constitute a promising biomarker for the detection of ethanol abuse and for monitoring of abstinence [84].

False PEth positive results have been reported, since it can be formed in blood samples containing ethanol at -20ºC and at room temperature [85, 86].

FATTY ACID ETHYL ESTERS (FAEEs)

FAEEs are also minor lipophilic nonoxidative products of ethanol metabolism [87]. They are formed in almost all tissues by esterification of endogenous fatty acids or fatty acyl coenzyme A with ethanol, catalyzed by FAEEs synthases. Carboxyl ester lipase and glutathione S-transferase have FAEEs synthase activity [87]. Triglycerides, lipoproteins and phospholipids (*i.e.*, myristate, palmitate, oleate and stearate) have also been reported to contribute to FAEEs formation [68].

Elevated levels of FAEEs are present in blood soon after ethanol exposure and may remain detectable for 24 hours to four days (especially in heavy drinkers) due to its accumulation in several organs and consequent long half-life (approximately 16 hours) [7, 88, 89]. Moreover, FAEEs analysis of hair and meconium have been suggested as suitable biomarkers for documenting long-term and prenatal ethanol exposure [7, 90]. Ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate have been more frequently analyzed [91].

Excessive ethanol intake is regarded as one of the major causes of acute pancreatitis [1, 92] and FAEEs and fatty acids (and not ethanol and acetaldehyde) have been implicated in Ca^{2+}-dependent necrosis of pancreatic acinar cells [93]. It has been shown that FAEEs stimulates the Ca^{2+}release from the endoplasmic reticulum, possibly by activating IP_3 receptors and fatty acids (released from the deesterification of FAEEs by intracellular hydrolases) predominantly inhibit mitochondrial ATP synthesis, which would inhibit both Ca^{2+} uptake into the endoplasmic reticulum and Ca^{2+} extrusion across the cytoplasm membrane [93, 94]. The consequent global elevation of the cytosolic Ca^{2+} concentration triggers necrosis [95]. Interestingly, early *postmortem* studies shown that FAEEs are present in the pancreas, at higher concentrations than in any other organ, of patients intoxicated with alcohol at the time of death [96].

ETHYL PHOSPHATE (EtP) AND ETHYL NITRITE (EtN)

Tomaszewski and Buchowicz [97] first reported the formation of EtP in the liver and coined it as another potential biomarker of ethanol exposure. Authors hypothesized that EtP may originate from ethanolysis of endogenous phosphate esters. Nevertheless, very few studies of EtP have been published and it is not yet considered to be a reliable biomarker [98]. The same is true for EtN, which was found *in vivo* when ethanol and tobacco were consumed together [99, 100]. Based on these results, authors suggested that EtN is formed as a consequence of ethanol nitrosation by NO, most likely by peroxynitrite ($ONOO^-$), a potent oxidizing formed by the reaction of NO and $O_2^{\cdot-}$ [101]. More recently, Deng *et al*. [102] also proved the formation of EtN *in vivo* after ethanol administration.

BACTERIAL METABOLISM OF ETHANOL

Microbial ADHs have several physiological (*e.g.*, metabolism of endobiotics and xenobiotics) and industrial (*e.g.*, production of alcoholic beverages, solvents or vinegar) roles [103]. Under anaerobic conditions microorganisms are capable of producing energy through fermentation [104]. In the specific case of alcoholic fermentation, acetaldehyde is reduced to ethanol by microbial ADHs [105]. Therefore, endogenous ethanol may be produced in the gastrointestinal tract and blood, especially in patients with high carbohydrate intake and under cimetidine therapy, which allows bacterial overgrowth by increasing gastric pH [106-108]. Besides producing endogenous ethanol, microbial ADHs also metabolize ingested ethanol to acetaldehyde in the oral cavity, stomach and large intestine. This fact may explain the increased incidence of carcinogenesis in the gastrointestinal tract of alcoholics [1, 13, 108] and of gastritis, especially if *Helicobacter pylori* and *Neisseria* species are colonizing the gastric wall [109].

CONCLUDING REMARKS

Although moderate ethanol consumption has many health benefits, heavy consumption has toxic effects. Indeed, moderate ethanol consumption can increase longevity and reduce the risk of heart disease, stroke, certain types of cancer and the risk of neurodegenerative diseases such as Alzheimer's. Conversely, ethanol consumption in excess has been implicated in more than 60 types of diseases, and it is a contributing cause in 200 others [1, 2]. In order to understand the toxic effects of ethanol and its mechanisms of production, it is fundamental to recognize the importance of metabolism and the multiple routes described above. As a good example,

alcoholic liver disease remains a major cause of morbidity and mortality worldwide and its mechanisms of production can be explained at least in part by oxidative metabolism [110]. On the other hand, minor non-oxidative metabolic pathways of ethanol, such as the single-molecules EtG and EtS and the group of molecules FAEE and PtE, are direct biomarkers of exposure, and used in various clinical and forensic settings, such as workplace and military testing, monitoring consumption if suspected in pregnant women, and in ethanol rehabilitation programs.

Finally it is important to highlight the effect of age on ethanol metabolism since the percentage of individuals aged over 65 is steadily increasing. Indeed, it is clear that the elderly exhibit higher blood ethanol concentrations, and ethanol metabolism is reduced with age due to lower activities of ADH, CYP2E1 and ALDH [111, 112]. Although not fully clarified, it has been suggested that this decrease in metabolic capacity can be explained by liver size reduction (*i.e.*, reflecting fewer hepatocytes), reduced availability of NAD^+ (*i.e.*, a cofactor for ADH), chronic atrophic gastritis (*i.e.*, which influences the activity of ADH7), reduced function of the smooth endoplasmic reticulum (and therefore CYP2E1 activity), and alterations in mitochondrial transport and number (affecting the activity of ALDH) [113-116]. Reduced mitochondrial function may lead to increased acetaldehyde levels in the liver, and may also explain enhanced fat accumulation in the elderly following ethanol ingestion [112]. In addition, blood flow to the liver and the distribution volume for water soluble substances, such as ethanol, decrease with age [111].

All known ethanol metabolites have been reviewed in this work, also emphasizing their contributing toxicological effects. With the advent of metabolomics, researchers are expecting to study the whole "picture" of ethanol metabolism as well as associated metabolic alterations [49, 117]. Perhaps further metabolites formed in a time- and dose-dependent manner and new, different pharmacodynamic targets will be discovered, and these may offer additional insights into the pathways involved in ethanol toxicity and the variability of response.

CONFLICT OF INTEREST STATEMENT

Author declare any conflict of interest, particularly any financial and personal relationships with other people or organizations that could inappropriately influence (bias) this work.

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