Laboratory testing for biomarkers of alcohol exposure
Learning Objectives

• Describe the effects of ethanol on the human body

• Highlight the clinical need to test for alcohol exposure

• Review biomarkers commonly used to detect and monitor alcohol exposure

• Describe analytical methods to analyze alcohol biomarkers in biological matrices by LC-MS/MS
  • Urine
  • Whole blood
  • Serum
  • Meconium
  • Umbilical cord tissue
2017 – Alcohol Use Disorder - Worldwide

1.4% (~107 million) of the global population (~7.53 billion)
70% male (75 million) to 32 million females

https://ourworldindata.org/drug-use
2018 Alcohol (Ethanol) Facts and Statistics

• Alcohol use disorder (AUD)
  • 14.4 million adults ages 18 and older
  • 401,000 adolescents ages 12–17

• Leading cause of morbidity and mortality
  • 3rd Leading cause of preventable death
    • Ages: 15 – 45 yr

• 2018 – Centers for Disease Control and Prevention
  • Ethanol-induced deaths
    • > 88,000 deaths/ year
      • Ethanol and drug interactions – (cocaine, opiates, benzodiazepines, acetaminophen)
      • < 10,000 ethanol related traffic fatalities (all ages)
      • 39,921 deaths from alcohol liver disease (12 yr and older)

• Moderate drinker – 1-2 drinks/day
• Binge drinker – Males: ≥ 5 drinks
  Females: ≥ 4 drinks
• Heavy alcohol use – Binge drinking ≥ 5 days
What is a Standard Drink?

Standard Drink – 10-14 g of ethanol

The percent of "pure" alcohol, expressed here as alcohol by volume (alc/vol), varies by beverage.
Health Benefits and Adverse Effects of Alcohol

(> 40 – 60 g ethanol / day)

Possible long-term effects of Ethanol

Red - generally "bad"
Green - generally "good"

Small to moderate consumption

Systemic:
- Increases insulin sensitivity
- Lower risk of diabetes

Brain:
- Atrophy
- Reduce the number of silent infarcts
- Decrease risk of dementia

Blood:
- Increases HDL
- Decreases thrombosis
- Reduces fibrinogen
- Increases fibrinolysis
- Reduces artery spasm from stress
- Increases coronary blood flow

Skeletal:
- Higher bone mineral density

Effects linked with both small and large consumption

Joints:
- Reduced risk of rheumatoid arthritis

Gallbladder:
- Reduced risk of developing gallstones

Kidney:
- Reduced risk of developing kidney stones

(< 30 g ethanol / day)

http://1.bp.blogspot.com/-2gTViGCbkOI/UQvxi7Qcv9I/AAAAAAAAAc8/hG-c2pHKWY/s640/long+term+effects+alcohol.jpg
Mechanism of Action

• CNS Depressant
  • Mechanism is not completely elucidated
  • Involves GABA-mediated inhibitory response
  • Inhibits neuronal NMDA and kainate receptors (Excitatory)

• Heavy ethanol use
  • Cause up-regulation of NMDA receptors
  • Desensitization through phosphorylation of GABA and glutamate receptors
  • Physical abstinence syndrome from abrupt withdrawal
Ethanol Pharmacokinetics

- Ethanol is transported to liver
  - First-pass metabolism - ~85% of alcohol
    - rate of ~10 g/hour (70 kg adult)

- Ethanol Elimination in Blood
  - 0.015 g/dL/hr or ~0.01%/hr
  - Alcoholics have increased elimination rates due to enzyme induction

- 10% Excreted in the urine, breath and sweat
Ethanol Metabolism

Alcohol dehydrogenase (ADH)
Acetaldehyde dehydrogenase (ALDH)

Disulfiram - Symptoms of Elevated acetaldehyde
• increased flushing
• tachycardia (elevated heart rate)
• nausea, vomiting & hyperventilation

Goodman & Gilman’s The Pharmacological Basis of Therapeutics, 11th edition
Physiological Effects of Alcohol

**Ethanol affects men and women differently**

- Women have more body fat and less water than men
- Women have 50% less gastric *alcohol dehydrogenase* (ADH)
- Contribute to higher blood alcohol concentrations
- More vulnerable to impairment due to alcohol consumption

### MEN

**Know Your Limit**

**Approximate Blood Alcohol Content (BAC) In One Hour**

*Source: National Highway Traffic Safety Administration*

<table>
<thead>
<tr>
<th>Drinks</th>
<th>Body Weight In Pounds</th>
<th>Influenced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>120</td>
</tr>
<tr>
<td>1</td>
<td>.04</td>
<td>.03</td>
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<tr>
<td>2</td>
<td>.08</td>
<td>.06</td>
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<tr>
<td>3</td>
<td>.11</td>
<td>.09</td>
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<tr>
<td>4</td>
<td>.15</td>
<td>.12</td>
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<tr>
<td>5</td>
<td>.19</td>
<td>.16</td>
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<tr>
<td>6</td>
<td>.23</td>
<td>.19</td>
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<tr>
<td>7</td>
<td>.26</td>
<td>.22</td>
</tr>
<tr>
<td>9</td>
<td>.34</td>
<td>.28</td>
</tr>
<tr>
<td>10</td>
<td>.38</td>
<td>.31</td>
</tr>
</tbody>
</table>

Subtract .015 for each hour after drinking.

### WOMEN

**Know Your Limit**

**Approximate Blood Alcohol Content (BAC) In One Hour**

*Source: National Highway Traffic Safety Administration*

<table>
<thead>
<tr>
<th>Drinks</th>
<th>Body Weight In Pounds</th>
<th>Influenced</th>
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<tbody>
<tr>
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<td>.11</td>
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<tr>
<td>4</td>
<td>.18</td>
<td>.15</td>
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<tr>
<td>5</td>
<td>.23</td>
<td>.19</td>
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<tr>
<td>6</td>
<td>.27</td>
<td>.23</td>
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<tr>
<td>7</td>
<td>.32</td>
<td>.27</td>
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<tr>
<td>8</td>
<td>.36</td>
<td>.30</td>
</tr>
<tr>
<td>9</td>
<td>.41</td>
<td>.34</td>
</tr>
<tr>
<td>10</td>
<td>.45</td>
<td>.38</td>
</tr>
</tbody>
</table>

Subtract .015 for each hour after drinking.
# Intoxication Effects correlated to Blood Alcohol concentrations

<table>
<thead>
<tr>
<th>Blood Alcohol Concentration</th>
<th>Acute Intoxication Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02–0.05 g/dL 20–50 mg/dL</td>
<td>Most individuals experience <strong>mild euphoria</strong> but may have no obvious signs. Diminished fine motor function occurs.</td>
</tr>
<tr>
<td>0.05–0.15 g/dL 50–150 mg/dL</td>
<td>Euphoria with reduction in judgment, motor function and reaction time.</td>
</tr>
<tr>
<td>0.15–0.30 g/dL 150–300 mg/dL</td>
<td>Obvious signs of intoxication including impaired balance, speech, reaction time, emotional stability, vision and comprehension.</td>
</tr>
<tr>
<td>&gt;0.25 g/dL &gt;250 mg/dL</td>
<td>Marked loss in motor function, impaired consciousness may be associated with death in uncomplicated adult cases.</td>
</tr>
<tr>
<td>&gt;0.40 g/dL &gt;400 mg/dL</td>
<td>Respiratory depression. Blood concentration at which most fatalities occur.</td>
</tr>
</tbody>
</table>

100 mg/dL = 0.10%W/V = 0.1 g/dL
Why Should we Test for Ethanol Exposure?

- Trauma Centers/Emergency Department
- Department of Transportation/Highway Patrol
  - DUI and related-fatalities
- Child/Family Services
  - > 10% of children live with parent with AUD
  - Underage drinking
    - 7.1 million people ages 12–20
- Adherence Testing
  - Pain management
  - Addiction recovery
  - Alcohol abstinence
- Transplant Clinics
  - Surgery Prequalification testing
- Prenatal Care /Neonate Management
  - Neonatal abstinence syndrome

Alcohol Use in Pregnancy

• About 1 in 9 pregnant women
• About 1/3 of pregnant women who consumed alcohol engaged in binge drinking
• Pregnant women who reported binge drinking in the past 30 days reported an average of 4.5 binge-drinking episodes during that same time period
2015 National Survey on Drug Use and Health

Pregnant Women Aged 15 to 44, Overall and by Trimester

- **Any Alcohol Use**
  - All Pregnant Women: 9.3%
  - Women in 1st Trimester: 16.4%
  - Women in 2nd Trimester: 6.1%
  - Women in 3rd Trimester: 4.3%

- **Binge Alcohol Use**
  - All Pregnant Women: 4.6%
  - Women in 1st Trimester: 8.7%
  - Women in 2nd Trimester: 2.3%
  - Women in 3rd Trimester: 1.6%

- **Heavy Alcohol Use**
  - All Pregnant Women: 0.8%
  - Women in 1st Trimester: 1.5%
  - Women in 2nd Trimester: 0.1%
  - Women in 3rd Trimester: 0.8%

Past Month Substance Use among Pregnant Women

<table>
<thead>
<tr>
<th>Substance</th>
<th>2015</th>
<th>2016</th>
<th>2017</th>
<th>2018</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illicit Drugs</td>
<td>109K</td>
<td>143K</td>
<td>194K</td>
<td>128K</td>
</tr>
<tr>
<td>Tobacco Products</td>
<td>319K</td>
<td>239K</td>
<td>271K</td>
<td>233K</td>
</tr>
<tr>
<td>Alcohol</td>
<td>214K</td>
<td>187K</td>
<td>261K</td>
<td>233K</td>
</tr>
</tbody>
</table>

- * Estimate not shown due to low precision.
- + Difference between this estimate and the 2018 estimate is statistically significant at the .05 level.
Fetal Alcohol Spectrum Disorders

- Alcohol-related birth defects
  - Facial abnormalities

- Growth retardation
  - Low birth weight

- CNS impairment
  - Learning disabilities
  - Behavioral abnormalities

Prevalence – 1-2 cases per 1000 live births

Biomarkers to Assess Alcohol Exposure

**Ethanol**

- **95% Oxidative Metabolism**
  - Alcohol Dehydrogenase
  - CYP2E1
  - Catalase
  - Acetaldehyde
  - Aldehyde Dehydrogenase
  - Acetic Acid

- **< 5% Excretion**
  - Breath
  - Urine
  - Sweat

- **< 1% Non-oxidative Metabolism**
  - Sulfotransferase
  - Ethyl Sulfate (EtS)
  - Phospholipase D
  - Phosphatidylethanol (PEth)

- **FAEE Synthase**
  - Fatty Acid Ethyl Esters (FAEE)

- **UDP Glucuronosyltransferase**
  - Ethyl Glucuronide (EtG)
Specimens to test biomarkers for Alcohol Exposure

- Urine
- Breath
- Saliva
- Hair
- Whole blood/Serum/Plasma/DBS
- Meconium
- Umbilical Cord
Alcohol Concentrations in Different Specimen Types

- Alcohol is distributed throughout the body in proportion to the water content of the body fluid
  - Plasma and serum concentrations – 12-18% higher than whole blood
  - Saliva alcohol concentrations – 7% higher than whole blood
  - Urine alcohol concentration may be 30% higher than whole blood

- The laboratory report must indicate the specimen type
Limitations for Ethanol Detection

- Half-life (blood): 2-14 h
- Half-life (urine): <24h
- Unintentional exposure
  - Mouthwash, hand sanitizer
- Evaporation from specimen

- False Positives
  - Bacterial/Yeast fermentation of glucose in sample
  - Postmortem ethanol formation

- False Negatives
  - Dilute urine
  - Diuretics
  - Microbial consumption
## Specificity of Indirect Ethanol Biomarkers

<table>
<thead>
<tr>
<th>Marker</th>
<th>Sensitivity/Specificity (%)</th>
<th>Clinical Use</th>
<th>Potential for False Positives</th>
<th>General Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma-glutamyl transferase (GGT)</td>
<td>64% / 72%</td>
<td>Heavy Alcohol Use</td>
<td>Not specific – liver, diabetes, biliary disease, obesity, and medications can increase enzymes</td>
<td>Elevations caused by excessive drinking (100g/day) for up to 14 – 26 days</td>
</tr>
<tr>
<td></td>
<td>Cutoff: 30 U/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine Aminotransferase (ALT)</td>
<td>32% / 90%</td>
<td>Chronic Alcohol Abuse</td>
<td>Not specific – liver, biliary disease, obesity and medications can induce increase in enzymes</td>
<td>AST/ALT &gt; 2.0 suggests ethanol-related liver disease</td>
</tr>
<tr>
<td>Aspartate Aminotransferase (AST)</td>
<td>68% / 80%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cutoff: 35 U/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean corpuscular volume (MCV)</td>
<td>44% / 98%</td>
<td>Heavy alcohol use</td>
<td>Not specific – hemolysis, anemia, liver disease, Vitamin B12 and folate deficiency, medications, leukemia, monoclonal gammopathies</td>
<td>MCV increases with excessive ethanol intake Cause by drinking 60g/day for &gt; 2 wk</td>
</tr>
<tr>
<td></td>
<td>Cutoff: 96 fL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbohydrate-deficient transferrin (CDT)</td>
<td>84% / 92%</td>
<td>Heavy alcohol use</td>
<td>Not specific – iron deficiency, fulminant HCV, Inborn Errors of Glycogen metabolism, pregnancy, oral contraceptives, immunocompromised patients</td>
<td>Altered form of iron transport protein when drinking is continued for &gt;2 weeks. Caused by drinking 60g/day for &gt; 2 wk</td>
</tr>
<tr>
<td></td>
<td>Cutoff: 2.4%</td>
<td>Indicator of relapse</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Carbohydrate Deficient Transferrin

• Serum marker of long-term, heavy alcohol use (≥40 g/day for up to 2 weeks) or relapse.
• Concentrations correlate with an individual’s drinking pattern, especially during the preceding 30 days
• Useful indirect marker for long-term abstinence monitoring.
• Factors that affect CDT levels include body mass index (BMI), female sex, and smoking.
• CDT testing cannot be used in individuals suspected of having congenital glycosylation disorders.
Ethyl Glucuronide / Ethyl Sulfate

- Non-volatile, water-soluble, direct minor metabolite of ethanol
- Excreted in the urine
- Appears 1 h after ethanol ingestion
  - Window of detection – up to 120 hr
- Detects recent ethanol ingestion
  - EtG Concentration range: up to 300 mg/mL
  - EtS concentration range: up to 61.0 mg/mL
- Stable marker of recent ethanol ingestion
  - Not produced from bacterial formation
  - Not sensitive to bacterial hydrolysis

Detection of EtG and EtS for Recent Exposure

<table>
<thead>
<tr>
<th>EtG and EtS – present after recent use</th>
<th>EtS Positive</th>
<th>EtS Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtG Positive</td>
<td>86</td>
<td>3</td>
</tr>
<tr>
<td>92.5% (both)</td>
<td>3.2% (only EtG)</td>
<td></td>
</tr>
<tr>
<td>EtG Negative</td>
<td>4</td>
<td>261</td>
</tr>
<tr>
<td>4.3% (only EtS)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Single Detected Metabolites

- Genetic polymorphisms
  - UDP-glucuronosyltransferases (UGT1A)
    - Gilbert’s Disease
    - Criglar-Najar Syndrome
  - Sulfotransferases (SULT)

- False Negatives (EtG)
  - Bacterial hydrolysis
    - β-glucuronidase (*E. coli*)

- False Positives (EtG)
  - Bacterial production (*E. coli*)

Reisfield et. Al, 2011
Helander and Beck, 2005
Helander and Dahl, 2005
Confirmation testing by LC-MS/MS

- Presence of both EtG and EtS
  - Accurate indicators of recent ingestion

- Quantitative
  - Distinguish between high and low ethanol exposure

- AMR range:
  - 100 – 10,000 ng/mL
  - Consumption of >24 g ethanol
    - > 10,000 ng/mL EtG (w/i 24h)

Dahl et al., 2011
# Hand Sanitizer Exposure

## Table IV. Summary of Literature Reports of EtG and/or EtS After Passive Exposure

<table>
<thead>
<tr>
<th>Reference</th>
<th>Agent (ethanol)</th>
<th>Max EtG (ng/mL) (per g creatinine)</th>
<th>Max EtS (ng/mL) (per g creatinine)</th>
<th>Max Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosano and Lin (8)</td>
<td>Avagard D (61%)</td>
<td>114</td>
<td>NM*</td>
<td>NM</td>
</tr>
<tr>
<td>Rogrig et al. (5)</td>
<td>Germ-X (62%)</td>
<td>62</td>
<td>NM</td>
<td>ND*</td>
</tr>
<tr>
<td>Helliker (7)</td>
<td>Purell (62%)</td>
<td>770</td>
<td>NM</td>
<td>NM</td>
</tr>
<tr>
<td>Jones et al. (9)</td>
<td>Purell (62%)</td>
<td>713 (799)</td>
<td>51 (29)</td>
<td>ND</td>
</tr>
<tr>
<td>This report</td>
<td>Purell (62%)</td>
<td>2001 (1998)</td>
<td>83 (94)</td>
<td>ND</td>
</tr>
</tbody>
</table>

* NM, not measured and ND, not detected.

**LC-MS/MS Detection**

- EtG – cutoff 500 ng/mL
- EtS – cutoff 100 ng/mL

Reisfield et al. 2011
Hydrolysis of EtG by E. coli in urine specimens

Helander and Dahl, 2005
Phosphatidylethanol

- Long-term direct biomarker of alcohol use
- Formed in the presence of ethanol
- PEths are incorporated into the membrane of erythrocytes
- PEth half-life of 4±0.7 days
- Specificity: ~100%

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol (POPEth)  1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanol (PLPEth)
Detection Window for PEth Homologs

![Graph showing PEth concentration over days for different homologs.](image-url)
Alcohol Biomarkers in Neonatal Specimens

• Ethyl glucuronide (EtG) and ethyl sulfate (EtS)
  • Umbilical cord tissue

• Phosphatidylethanol (PEth)
  • 1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanol (POPEth)
  • Whole blood
  • Dried blood spots

Alcohol Biomarkers in Neonatal Specimens

• Fatty Acid Ethyl Esters (FAEE)
  • Direct marker of heavy alcohol use
  • Chemical reaction of fatty acids and alcohol by FAEE synthase
  • Formed in Liver and Pancreas and released into circulation

• Specimens for testing
  • Hair Cutoff: 0.5 ng/mg
  • Meconium Cutoff: 500 ng/g
  • Blood – up to 24 hr after last drink

• Sensitivity: >90% / Specificity: >90%
Analytical methods – Breathalyzer

• Based on Alcohol Blood:Breath partition ratio
  • 2100 mL breath : 1 mL of blood
• Platinum electrode in Fuel Cell – oxidizes alcohol $\rightarrow$ acetic acid, $H^+$, $e^-$
• Electrons flow through wire from electrode to current meter
• The more alcohol present $\rightarrow$ the greater the electrical current
Analytical methods – Enzymatic Assay

**Advantages**
- Rapid, easy to use kits
- Quantitative
- Stat testing within 1 hr
- Specimens - serum/plasma, urine

**Disadvantages**
- Not able to detect methanol and isopropanol overdoses

\[
\text{CH}_3\text{CH}_2\text{OH} + \text{NAD} \rightarrow \text{CH}_3\text{CHO} + \text{NADH} + \text{H} 
\]
Enzymatic Oxidation Assay

**Advantages**

- Rapid, easy to use kits
- Quantitative
- Stat testing within 1 hr
- Widely used (95% of labs from CAP survey)
  - Kits can be adapted to various chemistry analyzers
- Testing for serum/plasma and urine

**Disadvantages**

- Not specific for ethanol
  - Cross-reactivity with n-Propanol
- Not able to detect methanol and isopropanol overdoses
Analytical Methods – Gas Chromatography - Flame Ionization Detector (GC-FID)

https://blogreu.files.wordpress.com/2015/06/11.gif
## Gas Chromatographic Techniques

### Advantages
- Specificity for ethanol
- Quantitative assay
- Testing for serum, plasma, blood and urine
- Ability to quantify
  - Methanol
  - Isopropanol
  - Ethylene glycol

### Disadvantages
- Equilibration time
  - (15–30 min) delays turnaround time
- Requires specialized instrumentation (GC)
- Requires highly trained technical staff
- Analysis slower than enzymatic assay
Commercial EtG Screening Assay

- Cutoff – 500 ng/mL

No Cross-reactivity to EtS
EtG EIA vs LC-MS/MS

\[ Y = 0.96 \text{ (LC/MS/MS)} - 0.104 \]

EIA cutoff: 500 ng/mL

<table>
<thead>
<tr>
<th>EIA</th>
<th>LC/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

EIA
- + 151 5
- - 2 242

Bottcher et al. 2008
Liquid Chromatography Tandem Mass Spectrometry

Umbilical Cord
Testing for EtG and EtS

Urine
Testing for EtG and EtS

Whole blood
Testing for Phosphatidylethanol

Sample extraction
Sample
HPLC column

Abundance
m/z

Photo: ARUP ClinTox3 Protocol
### What do these results mean?

<table>
<thead>
<tr>
<th>Ethyl glucuronide and Ethyl sulfate</th>
<th>Phosphatidylethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EtG</strong> &gt; 10,000 ng/mL  <strong>EtS</strong> &gt; 10,000 ng/mL</td>
<td><strong>&lt;20 ng/mL</strong>  Abstinence or light drinking (&lt; 2 drinks per day for several days a week)</td>
</tr>
<tr>
<td>• Recent exposure (w/i 24h)</td>
<td><strong>20–200 ng/mL</strong>  Moderate drinking (to 4 drinks per day for several days a week)</td>
</tr>
<tr>
<td><strong>EtG</strong>: 1359 ng/mL    <strong>EtS</strong>: &lt; 100 ng/mL</td>
<td><strong>&gt;200 ng/mL</strong>  Heavy drinking (at least 4 drinks per day several days a week)</td>
</tr>
<tr>
<td>• Not considered to be recent exposure (&gt;24h)</td>
<td></td>
</tr>
<tr>
<td>• Possible hand sanitizer exposure</td>
<td></td>
</tr>
<tr>
<td><strong>EtG</strong>: &lt; 100 ng/mL    <strong>EtS</strong>: 187 ng/mL</td>
<td></td>
</tr>
<tr>
<td>• Possible bacterial degradation of EtG</td>
<td></td>
</tr>
</tbody>
</table>
Summary

• Testing for alcohol exposure is important to manage the health of patients with alcohol use disorders

• Testing for alcohol biomarkers provides opportunities to identify and evaluate alcohol use and exposure
  • Direct markers are preferred over indirect markers
  • Phosphatidylethanol has high specificity for acute and chronic alcohol exposure

• Testing for alcohol biomarkers should be aligned with clinical needs/expectations