changes at the 69th or 70th amino acids. Theoretical analysis of the monoisotopic $[M+7H]^{+7}$ A70T compared to the wild-type yielded a predicted difference of m/z 4.2871, which is within approximately 0.5 ppm mass error of the experimental data. We have also confirmed the A70T amino acid substitution (c. 208G>A; p.70A>T) on the genetic level in 3 patients who had sufficient residual serum sample to allow extraction of enough cell-free DNA for PCR and Sanger DNA sequencing (data not shown). The A70T single nucleotide polymorphism (SNP) is catalogued in the NCBI SNP database (rs151098426) *(3)*.

In conclusion, we have identified a fairly common variant of IGF-1, A70T-IGF1, of unknown clinical significance in our clinical LC-MS HRAM assay, which cannot be distinguished from wild-type by the current market-leading immunoassay. When known pathogenic mutations exist or discordant results with established immunoassays are discovered, variant protein sequences should be considered and evaluated. The corollary is also true. When mass spectrometric assays give unexpectedly low results, one should contemplate the possibility of the presence of variants and scan for them. One reason for doing this is that a "missed" pathogenic variant would give the clinically correct result for the wrong reason, which might result in a failed opportunity for definitive genetic family testing *(4, 5)*. The other reason is that several of the variants are either nonpathogenic or of uncertain significance, leading to a potentially falsely low IGF-1 result being reported.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *No authors declared any potential conflicts of interest.*

Acknowledgments: We thank Dr. David Barnidge, Kendall Cradic, and Amy Gorsh of Mayo Clinic's Department of Laboratory Medicine and Pathology for their input and contributions.

References

1. Algeciras-Schimnich A, Bruns DE, Boyd JC, Bryant SC, La Fortune KA, Grebe SKG. Failure of current laboratory protocols to detect lot-to-lot reagent differences: findings and possible solutions. Clin Chem 2013;59:1187–94.
2. Bystrom CE, Sheng S, Clarke NJ. Narrow mass extraction of time-of-flight data for quantitative analysis of proteins: determination of insulin-like growth factor-1. Anal Chem 2011;83:9005–10.
3. dbSNP: short genetic variations [Database]. Bethesda (MD): National Center for Biotechnology Information, National Library of Medicine. http://www.ncbi.nlm.nih.gov/SNP/ (Accessed June 2015). See dbSNP build ID: 142.
4. Walenkamp MJE, Karperien M, Pereira AM, Hilhorst-Hofstee Y, van Dorn J, Chen JW, et al. Homozygous and heterozygous expression of a novel insulin-like growth factor-I mutation. J Clin Endocrinol Metab 2005;90:2855–64.
5. Netchine I, Azzi S, Houang M, Seurin D, Perin L, Ricort J-M et al. Partial primary deficiency of insulin-like growth factor (IGF)-I activity associated with IGF1 mutation demonstrates its critical role in growth and brain development. J Clin Endocrinol Metab 2009;94:3913–21.

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Previously published online at
DOI: 10.1373/clinchem.2014.234799

Phosphatidylethanols in Breath: A Possible Noninvasive Screening Test for Heavy Alcohol Consumption

To the Editor:

Several biological specimens are available for toxicological investigations. Analyzing exhaled breath for the presence of alcohol has a long tradition as a preliminary screen in roadside sobriety testing and is also used in medical and workplace settings. For detection of chronic heavy alcohol consumption, measurement of blood biomarkers is the standard. Traditionally, this has involved measurement of liver function tests such as γ-glutamyltransferase, but today measurement of an alcohol-induced change in serum transferrin glycosylation, known as carbohydrate-deficient transferrin (CDT),[1] offers a more specific biomarker for heavy drinking *(1)*. Another specific and seemingly more sensitive blood test used for the same application is the measurement of phosphatidylethanols (PEth), which are degradation products of membrane phosphatidylcholines and ethanol formed by enzymatic action *(2)*. On abstinence from alcohol after a period of heavy drinking, CDT and PEth concentrations could remain increased for several weeks up to approximately 1 month.

For drugs-of-abuse testing, urine is the principle specimen. However, because supervised sampling (used to minimize the risk of adulteration or substitution) is considered personally sensitive, specimens allowing for noninvasive sampling, such as oral fluid and hair, have been introduced. Breath testing has also been successfully explored as an alternative method for drugs of abuse, since low molecular

[1] Nonstandard abbreviations: CDT, carbohydrate-deficient transferrin; PEth, phosphatidylethanol; BAC, blood alcohol concentration.

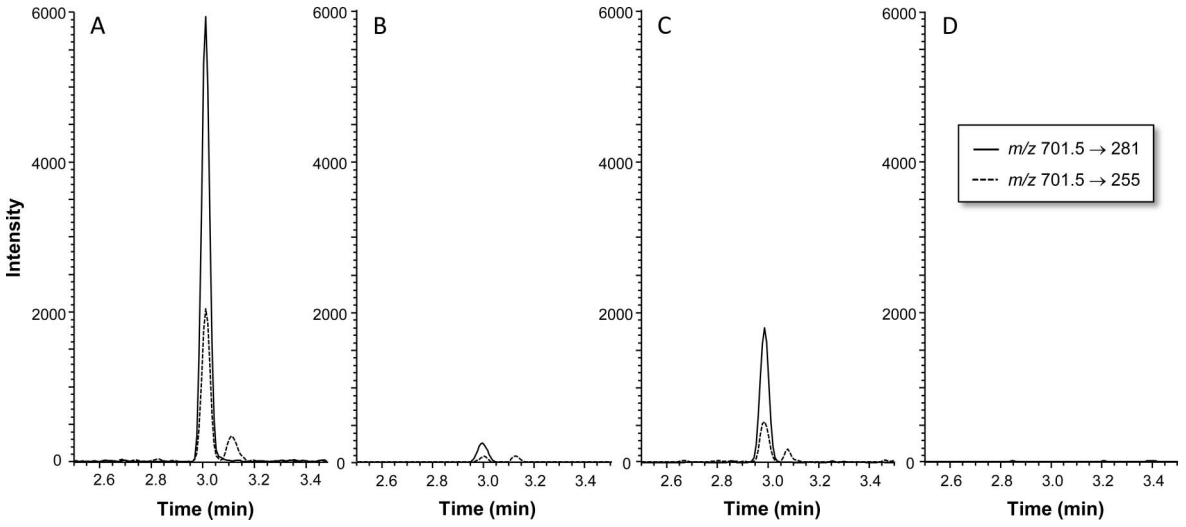

Fig. 1. Chromatograms for PEth 16:0/18:1 in standard samples at 100 pg (A) and 5 pg (B) PEth 16:0/18:1 spiked onto a filter containing PEth-negative breath matrix, and authentic breath samples collected from a heavy drinker (~30 pg/filter) (C) and a non-drinking control subject (D).

Results are shown for the qualifier (*m/z* 701.5 → 255) and quantifier (*m/z* 701.5 → 281) ion transitions. Quantification was based on measuring peak areas relative to the internal standard.

weight substances circulating in the body are to some extent exhaled in breath as part of aerosol particles *(3)*. A variety of abused substances, including amphetamines, benzodiazepines, cannabis, cocaine, and opiates, are detectable in breath samples *(4, 5)*.

Exhaled breath contains phospholipids such as phosphatidylcholines *(3)*, suggesting it may also be a useful matrix for PEth measurement and hence for noninvasive screening of moderate to heavy drinking. To explore this possibility, breath samples were collected from 12 male and female patients (aged 35–71 years) with self-reported heavy drinking seeking treatment at the Stockholm Dependence Treatment Centre, half of them being in a state of intoxication [blood alcohol concentration (BAC) range 0.01–0.30%, median 0.08%] according to a breathalyzer test. Twelve laboratory colleagues (25–59 years) with self-reported alcohol abstinence or regular low drinking but no alcohol intake in the previous 2 days served as controls (ethics permission 2008/1347–31 from the Stockholm Regional Ethics Board).

A breath sample was collected from all subjects, using a commercial disposable device (SensAbues) *(4)*. The specimen consists of aerosol microparticles originating mainly from the airway lining fluid (surfactant) *(3)*, and each sample corresponds to approximately 30 L exhaled air. Samples were prepared for LC-MS/MS analysis, as detailed elsewhere *(4)*. Briefly, the device was uncapped and 2 mL methanol carefully applied directly onto the filter, avoiding contact with the wall. After 5 min, a further 4 mL methanol was added and the effluent processed for analysis by evaporation.

LC-MS/MS analysis of 2 PEth molecular forms (16:0/18:1 and 16:0/16:0) was done by electrospray ionization and monitoring negative ions in selected reaction monitoring mode *(4)*. PEth 16:0/18:1 was chosen because it is the major form in blood *(2)*, and PEth 16:0/16:0 because the corresponding phosphatidylcholine is the major airway surfactant phospholipid *(3)*. Analyte identification was based on correct retention time and product ion ratio relative to standards (PEth 16:0/18:1 from Med Chem 101; PEth 16:0/16:0 from Avanti Polar Lipids) at 5–5000 pg/sample and a deuterated internal standard (PEth 16:0/18:1-d_{31} from Avanti Polar Lipids). The limits of detection (signal:noise >3) were approximately 2 pg/filter, and the lower limits of quantification (signal:noise >10, CV <20%) were approximately 5 pg/filter, as determined by spiking filters containing PEth-negative breath matrix with standards.

All 12 breath samples collected from alcohol-using patients contained measureable concentrations of PEth 16:0/18:1 (range 20–77, median 45.5 pg/filter), whereas the 16:0/16:0 form was not detected. The PEth 16:0/18:1 concentrations were 4–15 times higher than the limit of quantification, and all showed a correct ratio between qualifier (*m/z* 701.5 → 255) and quantifier (*m/z* 701.5 → 281) ion transitions (ratio range 0.28–0.35, median 0.32) (Fig. 1). In contrast,

neither PEth form was detected in the samples from control subjects (Fig. 1).

The PEth 16:0/18:1 concentrations in breath samples from alcohol-using patients did not correlate with the BAC ($P = 0.660$, Spearman), nor was there any statistically significant difference in PEth 16:0/18:1 concentration between BAC-positive (median 50.5 pg/filter) and BAC-negative (median 45.5 pg/filter) patients ($P = 0.699$, Mann–Whitney).

Phospholipids are a main constituent of exhaled breath particles originating from the surfactant *(3)*. The findings of this study extended this knowledge by demonstrating that abnormal phospholipids formed in blood following heavy alcohol consumption (i.e., PEth) *(2)* are also measurable in breath. Whether PEth has any influence on surfactant or respiratory function or is involved in alcoholic lung injury is not known. However, our results support PEth measurement in exhaled breath as offering a novel screening method for excessive drinking and, as such, a noninvasive alternative to blood biomarkers. Because there was no difference between intoxicated and sober patients, the breath PEth test apparently reflects chronic heavy drinking and not acute intoxication. The exact relationship between alcohol consumption and PEth in breath, and the association between concentrations in breath and blood, remains to be elucidated.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: O. Beck, SensAbues AB.
Honoraria: None declared.
Research Funding: None declared.
Expert Testimony: None declared.
Patents: None declared.

References

1. Weykamp C, Wielders J, Helander A, Anton RF, Bianchi V, Jeppsson J-O, et al. Harmonization of measurement results of the alcohol biomarker carbohydrate-deficient transferrin by use of the toolbox of technical procedures of the International Consortium for Harmonization of Clinical Laboratory Results. Clin Chem 2014;60:945–53.
2. Helander A, Zheng Y. Molecular species of the alcohol biomarker phosphatidylethanol in human blood measured by LC-MS. Clin Chem 2009;55:1395–405.
3. Almstrand A-C, Josefson M, Bredberg A, Lausmaa J, Sjövall P, Larsson P, Olin A-C. TOF-SIMS analysis of exhaled particles from patients with asthma and healthy controls. Eur Respir J 2012;39:59–66.
4. Beck O, Stephanson N, Sandqvist S, Franck J. Detection of drugs of abuse in exhaled breath using a device for rapid collection: comparison with plasma, urine and self-reporting in 47 drug users. J Breath Res 2013;7:026006.
5. Himes SK, Scheidweiler KB, Beck O, Gorelick DA, Desrosiers NA, Huestis MA. Cannabinoids in exhaled breath following controlled administration of smoked cannabis. Clin Chem 2013;59:1780–9.

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Previously published online at
DOI: 10.1373/clinchem.2015.239848

Interference from 3-*O*-Methyldopa with Ultra–High Performance LC-MS/MS Measurements of Plasma Metanephrines: Chromatographic Separation Remains Important

To the Editor:

Wright et al. *(1)* recently described the use of multiple reaction monitoring with multistage fragmentation (MRM^3), for which the conventional product ion produced by collision-induced fragmentation of the precursor ion $[M+H^+]$ is further fragmented in an ion trap to produce a "second generation product ion." This approach can eliminate occasional interferences during measurements of metanephrines from unknown substances in plasma samples. We outline here interference from an endogenous analyte present in all plasma samples, 3-*O*-methyldopa, which cannot be eliminated by MRM^3. The interference affects measurements of methoxytyramine, the *O*-methylated metabolite of dopamine, important for diagnosis of chromaffin cell tumors and identification of metastatic disease *(2, 3)*.

3-*O*-Methyldopa is formed by the action of catechol-*O*-methyltransferase (COMT) on L-dopa, the immediate precursor of dopamine. 3-*O*-methyldopa is present in plasma at 50–300 nmol/L (10–63 ng/mL) concentrations *(4)*, more than 10-fold higher than L-dopa, reflecting slow renal clearance. In contrast, concentrations of methoxytyramine normally do not exceed 0.11 nmol/L (18 pg/mL), with cutoffs of 0.20–0.41 nmol/L (33–69 pg/mL)