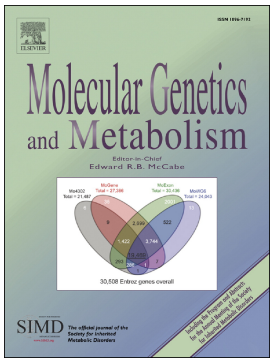

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Breath Biopsy in Inborn Errors of Metabolism: A Proof-of-Principle Study in Propionic Acidemia

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Synopsis

A proof-of-principle study putatively identifies 3-pentanone in exhaled breath as a correlate of the clinical and biochemical outcomes in propionic acidemia.

Abstract

Background: Impaired oxidation of branched chain amino acids may give rise to volatile organic compounds (VOCs). We hypothesized that VOCs will be present in exhaled breath of participants with propionic acidemia (PA), and their relative abundance would correlate with clinical and biochemical characteristics of the disease.

Methods: We enrolled 5 affected participants from a natural history study of PA (ClinicalTrials.gov ID NCT02890342) plus five age- and sex-matched unaffected controls. We collected exhaled breath using a non-invasive breath sampling platform paired with thermal desorption-gas chromatography-mass spectrometry. Clinical and biochemical parameters were correlated with the relative abundance of VOCs.

Results: Unbiased screening identified several candidate VOC biomarkers of PA. One candidate putatively identified as 3-pentanone was the most abundant (45-fold higher in cases *vs.* controls, p-value < 0.05). 3-Pentanone abundance positively correlated with plasma propionylcarnitine ($p = 0.01$), plasma 2-methylcitrate ($p < 0.05$), 3-OH-propionate ($p < 0.01$), full scale IQ ($p < 0.01$), and showed a statistical trend with height z-scores ($p = 0.08$). It inversely correlated with the whole-body *in vivo* oxidation of 1-^{13}C-propionate ($p < 0.05$). In a participant who received an orthotopic liver transplant, 3-pentanone levels were lower and segregated with "mild" PA.

Conclusion: Non-invasive breath sampling is a promising method to identify and quantitate VOCs that correlate with the clinical and biochemical parameters of PA. Our proof-of-principle findings may have wide implications for the diagnosis and severity stratification of inborn errors of metabolism affecting oxidation of amino acids which might be monitored in a similar fashion.

Keywords: propionic acidemia, volatile organic compounds, breath biopsy, organic acidemias, 3-pentanone.

Introduction

Odors have played a significant role in diagnostic medicine throughout history, dating back to ancient times.[1] With the development of quantitative chemistry in the 19th century, the study of odors and their relationship to health became more precise, with the diagnosis of diabetes through the smell of ketones being one such example.[2] In biochemical genetics, unusual odors of volatile chemicals have played a diagnostic role in a wide range of disorders mostly related to abnormal amino acid metabolism. For example, a "sweet" smell of sotolone can be detected in maple syrup urine disease, an "acrid" odor of isovaleric acid in isovaleric acidemia, or a disagreeable "rancid" odor of 3-hydroxyisovaleric acid in severe 3-methylcrotonyl-CoA carboxylase (3-MCC) deficiency.[3-5] Tanaka [1990] described "two smell specialists" who helped characterize odor emanating from two siblings who presented in metabolic crisis as the smell of a short chain fatty acid, later confirmed to be isovaleric acid, thus leading to the diagnosis of isovaleric acidemia.[5] It is perhaps not surprising that many examples of diagnostic odors are linked to the disorders of impaired oxidation of branched chain amino acids (BCAA), given their low molecular weight and complex catabolism which has the potential to generate volatile organic compounds (VOCs).

Disorders of BCAA oxidation represent a large group of inborn errors of metabolism (IEM) characterized by a range of clinical manifestations ranging from life-threatening (e.g., methylmalonic and propionic acidemias) to apparently benign biochemical traits (e.g., mild deficiencies of 3-methylcrotonyl-CoA carboxylase, isobutyryl-CoA dehydrogenase, and short/branched chain acyl-CoA dehydrogenase).[6] Readily identifiable through universal newborn screening, many of these disorders have limited or no effective means to monitor their clinical course in the outpatient setting, stratify their severity, or detect impending metabolic decompensations.

Propionic acidemia (PA) is a prominent example of a severe disorder linked to the BCAA oxidation pathway. It is characterized by the deficient activity of propionyl-CoA carboxylase, an enzyme that catalyzes the conversion of propionyl-CoA to D-methylmalonyl-CoA derived from the BCAAs isoleucine and valine, as well as other sources of odd-chain carbon. Biochemically, PA is characterized by accumulation of propionate metabolites, most notably 2-methylcitrate and 3-hydroxypropionate, in body tissues. Some intermediates in the BCAA oxidation pathway

possess properties of VOCs, including derivatives of carboxylic acid, low-weight ketoacids, and short-chain fatty acids.[7] These intermediates can undergo additional non-enzymatic and enzymatic chemical modifications such as condensation, conjugation and oxidation leading to the formation of additional VOCs such as aldehydes, dialkyl ketones as well as methylated and omega-oxidized short-chain fatty acids. [8-10]

Detection of VOCs in breath represents a promising new approach that can facilitate the diagnosis and prognostication of disease outcomes.[11, 12] A number of analytical technologies have attracted attention as low-cost, non-invasive, and efficient techniques to analyze VOCs in exhaled breath.[13] The range of exhaled VOCs detectable by these technologies encompasses alcohols, aldehydes, heterocyclics, ketones, aldehydes and low-weight fatty acids, among others.[13, 14] Systems which allow the collection of exhaled breath while limiting environmental gas contamination are especially well suited to extract endogenously-produced VOCs in a clinical setting.[13, 14]

Metabolomic studies have shown that a deficiency of a single enzyme in the BCAA oxidation pathway can result in the increased accumulation of multiple upstream metabolites of clinical and diagnostic significance.[15] We hypothesized that enzymatic defects in the BCAA oxidation pathway can result in the rise of VOCs, with accumulation in exhaled breath proportionate to the severity of the underlying disorder. In this proof-of-principle work, we provide evidence that elevated VOCs accumulate in patients with PA and correlate with laboratory and clinical parameters.

Methods and Materials

Participants

We enrolled five participants from the natural history study of PA at the National Institutes of Health (NIH), (https://www.clinicaltrials.gov/study/NCT02890342, amended for VOC collection; sample collection date range, Oct 2019 – Dec 2019, see *Clinical Description of the Affected Study Participants* in **Supplemental Methods and Materials**) and five age-, sex-, and BMI-matched healthy controls (a sample collection date range, June 2021 – July 2022). Given the rarity and heterogeneity of PA, the cohort was designed to include cases representing a range of clinical and biochemical manifestations, from mild to severe, plus one liver transplanted

participant with PA.[16] Exclusion criteria included the presence of halitosis, untreated cavities, dental or gingival problems; pre-existing respiratory diseases (e.g., asthma or recent history of COVID19); use of tobacco products (e.g. cigarette smoking or chewing tobacco); use of electronic nicotine delivery systems (e.g. e-cigarettes or vaping devices). Five affected participants had undergone deep phenotyping via a dedicated natural history study that included clinical, imaging and biochemical parameters.[17, 18] *In vivo* whole-body 1-^{13}C-propionate oxidation and quantitative analysis of 2-methylcitrate and propionylcarnitine have been described elsewhere.[17-19] Plasma lactate, pyruvate and 3-OH-propionate were measured by GC-MS in a commercial testing laboratory (Mayo Clinic, Rochester, MN).

Collection of Samples for VOC Analysis

Breath samples were collected using The ReCIVA® Breath Sampler (Owlstone Medical Ltd, Cambridge, UK) through a single-use mask as previously described.[20] The sampling device was disinfected between collections according to the experimental protocol independently evaluated by a microbiological testing laboratory.[21] The breath biopsy cartridge was comprised of four Tenax TA/Carbograph™ 5TD sorbent tubes (Markes International, Bridgend, UK). Real-time CO_2 and pressure sensors monitored subjects' breathing patterns, and dynamically determined gates triggered the sampling pumps to collect breath. The collection method was designed to capture a typical tidal breath volume, with the start collection threshold set at a CO_2 level of ~0.6%. Breath from both the upper and lower airways was sampled. Each of two independent pumps drew pressure-gated exhaled breath through two sorbent tubes, collecting 1473 mL of breath in each tube. Samples were stored at 4°C at the collection site until shipment to Owlstone Medical within 7-12 days of collection. Finally, a pair of sample tubes were combined to form a single sample for thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) analysis.

Quality Controls (QCs) and Calibration Standards

An assessment of the quality of the breath sample and the volume collected was performed by Owlstone Medical. A total of 10 participant breath samples were analyzed across two sequences, with 5 samples in each sequence as described in **Supplemental Table 1**. In each

sequence, 5 calibration standards were included that contained 52 VOCs at a range of concentrations in ng/mL (i.e., at one of five custom levels of standard, L1-L5, **Supplemental Table 2**). In each sequence, a QC standard at the same level as the middle calibration standard (L3) was run before and after every 2-3 participant breath samples. Due to the difficulty of obtaining bulk blank breath matrix, both calibration standards and QC samples consisted of the VOCs in solvent, injected on a single Tenax TA/Carbograph 5TD sorbent tube via a flow stream of 100 ml min^{-1} dry nitrogen (N5.5 grade or above). The preparation of calibration standards and QCs followed the Owlstone Medical standard operating procedure for calibration standards preparation and validation. Prior to analysis, deuterated internal standards were added to each sorbent tube (**Supplemental Table 3**).

VOC Analysis

Breath samples were analyzed at the Breath Biopsy Laboratory of Owlstone Medical Ltd. using the Breath Biopsy® OMNI® assay.[22] The tubes were dry purged with helium gas using a TD100-xr thermal desorption autosampler (Markes International, Bridgend, UK) to remove excess water and stored at -60°C until the final cohort had been assembled. A programmed temperature ramp was used to chromatographically separate chemicals in the samples using a TraceGOLD™ TG-624SilMS column, 1.4µm film thickness, 0.25mm I.D., and 30m length (Thermo Scientific, Waltham, MA). Q Exactive™ GC Orbitrap™ high resolution accurate mass (HRAM) spectrometer (Thermo Scientific, Waltham, MA) was used to acquire mass spectral data using electron ionization. Raw chromatograms were imported into Compound Discoverer version 3.2 (Thermo Fisher Scientific) for feature extraction. Thermo Fisher Scientific proprietary algorithms were used to perform deconvolution. Deconvolution results in a list of molecular features (MFs), with each feature consisting of mass spectral ions with similar chromatographic characteristics. Key feature extraction parameters included a total ion chromatogram threshold, ion overlap window, dot product, smoothing, mass tolerance, peak signal-to-noise, spectrum signal-to-noise, and gap filling.

Prior to collection of data, a hypothesis-driven list of candidate VOCs (compounds related to metabolism of BCAAs, levocarnitine, and ketones; propionate conjugation products; ammonia) likely to be appear in PA cases was submitted for targeted analysis. Some of these candidates (short-chain fatty acids, propionate conjugation products, and ammonia) were deemed

not optimal for detection using Owlstone Medical's methodology and were not expected to be present in this dataset. Compounds potentially related to oxidation of BCAAs, volatile aldehydes and ketones, or levocarnitine breakdown products were more suitable for investigation with Breath Biopsy. An unbiased analysis of TD-GC-MS spectra from the tidal breath was performed to identify additional VOC candidates. Initially, each molecular feature was assigned a unique numerical identifier comprising its retention time (RT) and the mass-to-charge ratio of the quantifying ion (m/z) – for example, mf_9.219_56.02561. Candidate VOCs were then identified by matching their spectral features against VOC profiles listed in the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) and Owlstone Medical's High Resolution Accurate Mass (HRAM) reference libraries. To establish the identity of the top hit, compound mf_9.219_56.02561, its retention time and spectral information were compared to the purified reference standard using GC Deconvolution software (Thermo Fisher Scientific Inc., Waltham, MA).

Data Preprocessing and Statistical Analysis

Procedures for feature extraction, imputation and normalization are described in the **Supplemental Methods and Materials**. Due to the limited sample size, a t-test was performed to compare the PA cohort to the control cohort with an unadjusted p-value < 0.05 considered as the threshold for significance. To give a higher level of confidence in the significance of some VOCs, p-values were then adjusted using the Benjamini-Hochberg procedure. The correlation between each pair of variables (biochemical parameters or clinical endpoints *vs*. the relative abundance of VOCs represented by an area under the curve for each peak) was calculated using appropriate correlation measures, such as Matthew's coefficient of correlation for binary-binary correlation, Spearman's rank correlation coefficient for continuous-continuous correlation, or the correlation coefficient from a linear regression for continuous-binary correlation. Full scale IQ and height z-score from the transplanted participant was not included in the correlation analysis of clinical outcomes, since the potential change in biomarker levels may not be accompanied by clinical changes soon after transplant.

Results

Clinical Characteristics of the Participants

Participants' demographics and select clinical characteristics are summarized in **Supplemental Methods and Materials**. In summary, three participants in the VOC study were classified as affected with the "mild" form and one participant with the "severe" form of PA.[17] One participant with a "severe" form of PA underwent an orthotopic liver transplant prior to their evaluation at NIH – data points from this participant were used to help understand potential effects of the restored hepatic activity on VOCs. Select biomarkers from the NIH PA cohort have been published elsewhere.[17, 18]

Unbiased Screening Identifies VOC Candidates

The initial TD-GC-MS analysis of tidal breath from affected participants and controls identified tentative candidates with features matching NIST and HRAM reference profiles of propionate derivatives as well as volatile aldehydes and ketones (**Supplemental Table 4**). Candidate compounds related to oxidation of BCAAs (e.g., propionate, acetate, 2-propanol, methylpropanols, and acetoacetate), volatile aldehydes (propionylaldehyde, isovalerylaldehyde, and butanal), and products of levocarnitine breakdown (e.g., trimethylamine) were not identified (**Supplemental Table 4**). Compounds exhibiting at least a two-fold difference between groups and an adjusted statistical significance of $p < 0.05$ are highlighted in the volcano plot. (**Figure 1A**). Since the effect of liver transplantation on VOCs in PA has not been characterized before, the same analysis was performed on the 4 non-transplanted cases showing similar results (**Supplemental Table 4**). Using a spectral comparison to NIST reference profiles, a VOC that was 45-fold higher in the affected participants compared to unaffected controls was initially tentatively identified as 3-pentanone using a spectral comparison to NIST (**Figure 1B**). The spectra of compound mf_6.267_88.05193 closely matched that of 3-methyl propionate. Given the instability of its p-value in different cohorts, and a relatively small fold elevation, we did not pursue further efforts to confirm the identify of this compound.

A purified 3-pentanone standard was run on the same instrument using the same analytical method that was used to analyze the breath samples. The retention time and spectral information of the standard were then compared with compound mf_9.219_56.02561. However, drifts in retention times between the sequences containing the samples and the purified standard did not allow a direct comparison. A related compound, 2-pentanone, was a calibration standard run with both the 3-pentanone standard and the breath samples and was used to estimate the retention time

drift of 3-pentanone. The retention time drift of 2-pentanone was 0.23 min. The retention time difference between mf_9.219_56.02561 and the 3-pentanone standard was also 0.23 min.

To determine the quality of the match between the spectrum of the 3-pentanone standard and that of mf_9.219_56.02561, the standard spectrum was added to an in-house library using NIST Mass Spectral (MS) Search Program software (v2.3). The spectra of mf_9.219_56.02561 from the three breath files with the highest intensity peaks were then searched against the library. The returned SI matching scores were between 893 - 911, indicating a good match. All major ion fragments of mf_9.219_56.02561 were found in the 3-pentanone spectrum. Based on these observations, it was concluded that mf_9.219_56.02561 is likely to be 3-pentanone.

3-Pentanone Correlates with the Clinical and Biochemical Severity of PA

After putatively confirming the identity of 3-pentanone, we compared its abundance to the biochemical and clinical outcomes in PA. 3-Pentanone directly correlated with plasma propionylcarnitine (R^2 = 0.89, p-value = 0.01), total 2-methylcitrate (R^2 = 0.85, p-value < 0.05), 3-OH-propionate (R^2 = 0.92, p-value <0.01), lactate (R^2 = 0.78, p-value < 0.05), and pyruvate (R^2 = 0.83, p-value < 0.05) (**Figure 3A-E**). It inversely correlated with the whole-body *in vivo* oxidation of 1-^{13}C-propionate (R^2 = 0.78, p-value < 0.05) (**Figure 3F**). Study parameters in a participant with severe PA who underwent an orthotopic liver transplant segregated with the "mild" biochemical manifestations (**Figure 3A-F,** marked in red and an arrow). 3-Pentanone did not correlate with the alanine aminotransferase (ALT), creatinine- and cystatin C-based estimated glomerular filtration rates (eGFR) (p-value = 0.97, 0.28 and 0.44, respectively; data shown for eGFR in **Figure 3G**). 3-Pentanone also correlated with full-scale IQ (R^2 = 0.98, p-value < 0.01) and showed a statistical trend for age- and sex-adjusted height z-scores (R^2 = 0.84, p-value = 0.08) (**Figure 3H, I**).

Discussion

Using a proprietary breath sampling platform paired with TD-GC-MS, we performed an unbiased and highly sensitive analysis of VOCs in exhaled breath. We demonstrate that a compound putatively identified as 3-pentanone is significantly elevated in PA and appears to be quantitatively linked to the underlying severity of PA. Specifically, 3-pentanone was found to be significantly associated with the known biomarkers including propionylcarnitine, 2-

methylcitrate, 3-OH-propionate, lactate and pyruvate in blood, *in vivo* whole-body oxidation of labelled 1-^{13}C-propionate to $^{13}CO_2$, and appeared to be associated with parameters of clinical significance, intellectual and growth outcomes (**Figure 3A-I**). 3-Pentanone did not correlate with eGFR suggesting that when compared to blood propionylcarnitine and total 2-methylcitrate, its appearance in breath may be less affected by renal dysfunction (**Figure 3G**) – an important characteristic for a candidate biomarker in PA patients with advanced kidney disease.[17] We also demonstrate that biochemical improvements in a study participant who had undergone liver transplantation prior to participating in the study clustered with the biochemical outcomes of study participants affected by the "mild" form of propionic acidemia. Although this participant's pre-transplant values are unknown, and the biochemical response to transplant is difficult to assess, we speculate that lower 3-pentanone could in part reflect restored hepatic oxidation of propionate.

Detection of 3-pentanone has been reported in two previous VOC studies linking small elevations to non-specific causes like liver disease, liver cirrhosis, and inflammation [23, 24]. Several lines of evidence suggest that the magnitude of 3-pentanone elevation in PA stems from causes other than hepatic dysfunction. We found no statistical correlation between ALT and exhaled 3-pentanone (p-value = 0.97, data not shown), suggesting that in PA, 3-pentanone metabolism is likely driven by causes other than liver dysfunction. More likely, a 45-fold elevation in cases *vs*. controls suggests a direct biochemical link between 3-pentanone and propionyl-CoA. Indeed, dialkyl ketones including 3-pentanone have been reported in urine samples of PA patients in the 1960-70s.[25, 26] Although the mechanism of 3-pentanone formation remains unclear, two pathways have been proposed. One involves condensation of methylmalonyl-CoA and propionyl-CoA.[25] This mechanism is unlikely in PA, since methylmalonyl-CoA formation in PA patients should be drastically reduced. The more likely mechanism involves the formation of 3-keto-2-methylvaleric acid through condensation of two molecules of propionyl-CoA, followed by its decarboxylation. Consistent with this hypothesis, variable excretion of 3-keto-2-methylvaleric acid has been demonstrated in PA patients.[27] The plausibility of 3-pentanone being directly derived from a propionate-related compound was directly established by Truscott et al. [1979]: incubation of 3-keto-2-methylvaleric acid in a urine sample (5 mM, pH 5.1, 37^{o}C) led to the accumulation of 3-pentanone within hours. In addition,

decarboxylation of 3-keto-2-methylvaleric acid might occur enzymatically, presumably through catalysis by acetoacetate decarboxylase.[28]

Our study has limitations and caveats. A small number of observations will need to be extended by enrolling a larger number of participants across the PA spectrum. Our analysis was likely underpowered to detect significant elevations of other VOCs of interest. For example, the spectra of compound mf_6.267_88.05193 was most consistent with methyl propionate. In the PA and MMA literature, methyl propionate in blood and/or urine has been mentioned once as a biomarker inconsistently tracking with the clinical course of these disorders.[29] Additionally, 3-pentanone, which was noted to elevated in the patients, was not part of the calibration standard and did not serve as an internal standard. A larger cohort will be required to follow up to confirm the role of tentatively identified methyl propionate as a severity biomarker. All PA patients were studied under conditions of clinical stability, and repeating this investigation in a less controlled setting may reveal wider VOC variability. Although 3-keto-2-methylvaleric acid can degrade *ex vivo*, it is unlikely to be a significant source of 3-pentanone due to its low vapor pressure and predicted low volatility.[30] Therefore, we believe that *in vivo* formation of 3-pentanone is much more likely reflective of the *in vivo* metabolism of propionyl-CoA.[26, 28] Since 3-pentanone has occasionally been reported in trace amounts in some foods (Amaranthus, guava, and roasted coffee), we reviewed participants' 3-day diet recalls.[31-33] Their records were negative for consumption of Amaranthus or guava. One participant with low values of 3-pentanone consumed one coffee beverage per day. The highest level of 3-pentanone was found in participant whose diet almost exclusively consisted of medical foods. Although this dietary pattern did not affect out conclusions, a follow study will need to consider the intake of these foods during collection of breath samples. A correlation of 3-pentanone with select clinical outcomes prompted us to review literature revealing that 3-pentanone can be toxic to exposed mucocutaneous surfaces and well as nervous and respiratory systems.[34] Whether there is a mechanistic link between life-long chronic exposure to endogenous 3-pentanone and adverse outcomes in PA will be the subject of future studies.

In summary, this proof-of-principle study demonstrates that non-invasive breath sampling paired with TD-GC-MS appears to be promising method, capable of identifying VOCs that correlate with the clinical and biochemical parameters of propionic acidemia. Confirmation of

VOCs as monitoring and response biomarkers in IEMs of amino acids, coupled with device miniaturization, could enable a platform for real time assessments in selected IEMs, and perhaps the early detection of metabolic decompensations, especially at home.

Legends

Figure 1: **A**. Top hits were differentiated by the adjusted p-value and fold change in 4 non-transplanted cases *vs.* controls. Top ten candidates were consistent with features of the following compounds: 3-pentanone; phenol, 2,6-bis(1,1-dimethylethyl)-4-(1-methylpropyl)-; methyl propionate; hexadecane, 2-methyl-; undecane, 4-methyl-; 1,1'-bicyclooctyl; compound mf_3.53_263.98679; compound mf_31.36_103.02106; decane, 3,8-dimethyl-; 2-butanone. Except for 3-pentanone, no additional steps were taken to confirm identities of other top candidates. **B**. Compound mf_9.219_56.02561 whose spectra was most close to 3-pentanone was significantly higher in cases *vs.* controls. **C**. An example of an annotated ion chromatogram showing relative abundances of volatile organic compounds detected in breath of an affected participant (case subject 4 denoted by a black line) overlaid with that of an unaffected participant (control subject 8, denoted by a red line). *denotes findings with nominal statistical significance; **denotes a statistically significant finding after correction for multiple testing.

Figure 2. The spectral information of compound mf_9.219_56.02561 in exhaled breath closely matched that of the standard 3-pentanone.

Figure 3. A-F, Abundance of 3-pentanone directly correlated with plasma levels of propionylcarnitine (**A**) total 2-methylcitrate (**B**), 3-OH-propionate (**C**), lactate (**D**), pyruvate (**E**), and inversely with the *in vivo* 1-^{13}C-propionate oxidation (**F**). A study participant who received a liver transplant prior to testing clustered with the "mildly" affected PA participants, as marked in red with the arrow. **G,** In this series, 3-pentanone did not correlate with the cystatin C-based eGFR. **H** and **I**, 3-Pentanone correlated with neurocognitive outcomes as measured by the full-scale IQ and showed a statistical trend for the linear growth outcomes; full scale IQ and height z-score from a transplanted participant was not included in the correlation analysis with clinical outcomes. A blue solid line represents linear regression. Dotted lines denote a 95% confidence interval. Biochemical and clinical outcomes were collected during the same visit when VOC

samples were collected. eGFR – estimated glomerular filtration rate; PA – propionic acidemia; LT – liver transplant.

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Conflict of Interest

Owen Birch, Huw Davies, Ace Hatch, and Robert Mohney are employees of Owlstone Medical Ltd, a company that develops tests for precision medicine and early detection of disease, and, as such, have affiliations with or financial involvement with Owlstone Medical Ltd. Irini Manoli and Charles P. Venditti are inventors on patents related to isotopic biomarkers in organic acidemias filed by the NIH on their behalf. Other authors have no conflicts of interest to declare.

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