Breath ammonia and ethanol increase in response to a high protein challenge


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Abstract
Quantifying changes in ammonia and ethanol in blood and body fluid assays in response to food is cumbersome. We used breath analysis of ammonia, ethanol, hydrogen (an accepted standard of gut transit) and acetone to investigate gastrointestinal physiology. In 30 healthy participants, we measured each metabolite serially over 6h in control and high protein trials. Two-way repeated measures ANOVA compared treatment (control versus intervention), change from baseline to maximum and interaction of treatment and time change. Interaction was significant for ammonia (p<0.0001) and hydrogen (p<0.0001). We describe the dynamic measurement of multiple metabolites in response to an oral challenge.

Introduction
Ammonia, a by-product of protein metabolism, and ethanol, a by-product of carbohydrate metabolism, are important metabolites. Ammonia is relevant to nutrition physiology, exercise and aging as well as numerous disease states, including hepatic encephalopathy in patients with liver cirrhosis and urea cycle disorders (Auron & Brophy, 2012; Häberle, 2013; Jover-Cobos et al., 2014; Sturgeon & Shawcross, 2014). Interest in endogenous ethanol has been growing as it has been implicated in the pathophysiology of fatty liver and the metabolic syndrome (Abu-Shanab & Quigley, 2010; AronWisnewsky et al., 2013; Cope et al., 2000; Volynets et al., 2012). The major source of acetone in humans is either lipolysis or amino acid degradation (Kalapos, 2003).

As the physiology of these metabolites is complex, there are many unknowns, even regarding basic questions (Adeva et al., 2012). For example, because in humans, bacteria produce ethanol and the gut, specifically the distal small bowel and colon, hosts the largest bacterial community, this compartment has generally been assumed to be the most significant source of endogenous ethanol (Nair et al., 2001; Visapää et al., 1998). However, the source of endogenous ethanol has not been rigorously tested and, by published reports, is virtually untestable.

Ammonia and ethanol are measurable in blood and other body fluids. However, physiological research is hindered by the reactivity of ammonia (Amann & Smith, 2013), and even blood assays are variable and fraught with technical errors (Blanco Vela & Bosques Padilla, 2011; Goggs et al., 2008). Furthermore, research based on blood or body fluid assays is limited by the nature of episodic sampling (e.g. phlebotomy via limb venipuncture).

Perhaps one of the most important unmet needs for understanding both ammonia and ethanol physiology is to determine an individual’s response to food intake. This is because food significantly influences the daily ongoing production of these metabolites and is modifiable, including its quantity, composition and timing. To illustrate with ammonia only, a better understanding of an individual’s ammonia response to protein could lead to improved personal dietary guidance in the management of many disease and wellness states. Common clinical scenarios, for example, would include a patient with cirrhosis and renal failure experiencing both recurrent hepatic encephalopathy and progressive sarcopenia, a child with a urea cycle disorder on a prescribed amino acid regimen or an elderly patient striving to lose weight and maintain muscle mass.

Presently available assays for blood and urine (Friedlander et al., 2014) are poorly equipped for this purpose, because none can reliably measure gastrointestinal food bolus transit time. Collecting blood samples at multiple time points from healthy human volunteers presents difficulties for approval by Institutional Review Boards. And, the number of sequential urine samples that can be collected is limited by human physiology.
There is an opportunity for multi-metabolite breath analysis to address this need. Breath measurement is non-invasive and safe, which enables repeated measures. Furthermore, engineering advances have resulted in monitors that are ever more accurate, faster and more portable (Dweik, 2011). Finally, breath analysis is ideally suited for nutritional and gastrointestinal research because breath hydrogen measurement can serve as a unique and essential timing marker for food bolus transit to the small bowel and colon (Saad & Chey, 2014; Simrén & Stotzer, 2006).

Two factors, however, have limited enthusiasm for this approach. First, as with blood assays, volatile metabolite measurement is technically challenging, especially for ammonia (Hibbard et al., 2013; Schmidt et al., 2013). This has led to ongoing controversy regarding best measurement methodology and data interpretation (Wang et al., 2008). Second, study design has often been suboptimal. Most published breath studies are small, uncontrolled observational studies lacking a physiologic intervention.

Herein, we sought to build confidence in the potential for breath analysis to evaluate nutrition and gastrointestinal physiology. We measured ammonia, ethanol, acetone and hydrogen repeatedly in response to an oral high protein challenge compared to a negative control day. Protein was used to provoke an increase in ammonia, whereas lactulose, an unabsorbed sugar, was used to provoke an increase in hydrogen production by gut bacteria (Saad & Chey, 2014). We measured breath acetone in order to assist in the interpretation of the ammonia response; hypothesizing that amino acid degradation increases both ammonia and acetone. Finally, we tested the hypothesis that exhaled breath ethanol is derived from bacteria in the distal gut.

Our approach compared the concordance between the times of peaks in the maximum concentrations of breath ammonia and ethanol in response to a high protein oral challenge versus the hydrogen peak in a negative control oral challenge. To the best of our knowledge, this is the first experience to describe the dynamic measurement of these four metabolites. This is also the first report describing repeated measures of ammonia and ethanol in response to an oral intervention.

Materials and methods

Study participants

Participants were recruited via flyers and advertisements. All eligible participants provided informed consent as required by the St. Luke’s University Hospital Institutional Review Board. Thirty healthy volunteers, without periodontal, liver or kidney disease or report of tobacco use, fasted 12 h prior to presentation. The absence of liver and kidney disease was self-reported. Subjects with obvious halitosis were excluded. Volunteers abstained from exercise the morning of the study and brushed their teeth at least 1 h before arrival.

Study protocol

We measured the end-tidal portion of the exhaled breath for ammonia (Rice Monitor), ethanol (Sionex Metabolite Monitor), acetone (Sionex Metabolite Monitor) and hydrogen (Quintron BreathTracker SC) with three different devices serially over 6 h on 2 days in each subject. Each day began with 30 min of baseline breath collection. Baseline measurement consisted of: three ammonia measurements taken 10 min apart and two measurements for ethanol, acetone and hydrogen each taken 15 min apart. After baseline measurements, the study subjects drank an oral challenge of either Gatorade® or a high protein and lactulose beverage. On Day #1 (control trial), we mapped trends of ammonia, ethanol, acetone and hydrogen in response to the oral ingestion of Gatorade® (95 mL containing: 836.8 kJ, 52 g sugar, 0 g fiber, 0 g protein and 0 g fat). On Day #2 (intervention trial), we measured breath ammonia, ethanol, acetone and hydrogen in response to the consumption of a high protein challenge (Rockin’ Refuel Muscle Builder shakes containing: 1590 kJ, 12 g sugar, 6 g fiber, 60 g protein and 9 g fat) augmented with lactulose (10 g). The oral intervention was followed by a 30 s water rinse to flush any residue from the mouth. Breath samples for ammonia, ethanol, acetone and hydrogen were taken every 30 min for 5 h following the rinse.

Determination of breath ammonia

The participants were required to exhale for at least 10 s in a defined manner via a restrictor, and each exhalation constituted one sample. Since each study subject was his/her own control, consistent breath sampling technique was critical. A specially designed breath sampler (Loccioni, Angeli di Rosora, Italy) was used to monitor breath exhalation in a manner similar to the American Thoracic Society/European Respiratory Society recommended breath collection protocol for analyzing breath nitric oxide (FiNO: Anon, 2005). This breath sampler monitors, displays, prompts and archives real-time measurements of mouth pressure and the concentration of CO2 for each breath sample. Ideal mouth pressure for a sample is 10 cm of water maintained at least 10 s. This mouth pressure coupled with the flow restrictor corresponds to a flow rate of 50 mL/s. For all breath sampling, a disposable one-way in-line valve was used on the mouth port of the breath sampler. Latex gloves were worn when inserting this valve into the breath sampler in order to prevent contamination with ammonia from the skin. Each breath was sampled continuously via a 50 cm long inlet line (Teflon) heated to 55°C and the concentration of ammonia was determined with a novel, sensitive, selective and fast quartz enhanced photoacoustic spectrometer (Rice Monitor; Rice University, Houston, TX; Lewicki et al., 2009) as previously described (Solga et al., 2013, 2014). Plateau breath ammonia concentrations measured during the phase III portion of the exhalation profile were reported in parts per billion (ppb). Real-time ammonia concentrations determined by the ammonia sensor are displayed on the breath sampler and archived.

Determination of breath ethanol and acetone

Ethanol and acetone were measured by thermal desorption-capillary gas chromatography-differential ion mobility spectrometry (microAnalyzer, Sionex Inc, Bedford, MA). This instrument was coupled to a breath sampler (Loccioni Monitor) that prompts controlled, constant breath exhalation in a manner that exceeds the American Thoracic
Normalization by correction CO₂ factor

The ammonia, ethanol and acetone values in ppb were normalized by employing the corresponding sample CO₂ value. The corrected breath value in pmol/mL CO₂ was calculated by the following equation: corrected value = (sample breath value in ppb/24.45) x (760/sample CO₂ pressure in Torr). The MicroLyzer protocol also normalized hydrogen with an approximately alveolar CO₂ pressure of 40 mmHg (Torr) by: corrected hydrogen value = sample hydrogen value x (40/sample CO₂ pressure in Torr).

Statistical analysis

We compared baseline versus post-rinse maximum ammonia, hydrogen, ethanol and acetone values for each subject in control versus intervention trials. Due to skewed distributions, medians and interquartile ranges (Q1–Q3) were reported. We graphed ammonia versus hydrogen, ethanol versus hydrogen and acetone measured over 12 time points: baseline at time₀ followed by 11 subsequent time points. Data were log transformed prior to analysis (Limpert & Stahel, 2011; Sorrentino, 2010). ANOVA for treatment type (control versus challenge), change from baseline to maximum and the interaction of treatment type and change in metabolite level from baseline to maximum was performed. The inclusion of an interaction term in the statistical model allowed for the evaluation of the relationship between the type of treatment received (control versus challenge) and the effect of elapsed time (baseline versus post-rinse maximum) on the measured amount of metabolite (ammonia, hydrogen, ethanol or acetone). All statistical analyses were conducted with SAS version 9.2 (SAS Institute, Inc., Cary, NC). For all tests, p value of <0.05 was considered significant.

Results

The demographics for the 30 study participants, mean age was 24 years (SD = 7 years), 47% were men (14/30) and mean body mass index was 24.2 kg/m² (SD = 4.0). Table 1 lists median and interquartile range (Q1–Q3) values for ammonia (pmol/mL CO₂), hydrogen (ppm), acetone (pmol/mL CO₂) and ethanol (pmol/mL CO₂). The increase from baseline to maximum was significantly different when comparing measured metabolite results of control versus oral challenge intervention trials for both ammonia (p < 0.0001) and hydrogen (p < 0.0001).

Thirty participants contributed to the ammonia analysis. A total of 24 participants contributed to ethanol and acetone analyses. Four participants did not produce measurable breath ammonia for intervention trials for both ammonia (Figure 1a), hydrogen (Figure 1b), and ethanol (Figure 1c) are shown. Figure 2(a) illustrates the median ammonia and hydrogen calculated at each time point, with maximum median ammonia occurring at 330 min and maximum median hydrogen at 270 min. Both metabolites rose during the first 4 h on the intervention day. Figure 2(b) illustrates median ethanol and hydrogen for each time point: ethanol peaked 30 min after the oral challenge. The median values and interquartile range for acetone in control and intervention trials are shown for each time point in Figure 2(c).
Table 1. Median values and interquartile range (Q1–Q3) for ammonia, hydrogen, ethanol and acetone.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Baseline</th>
<th>Oral challenge</th>
<th>Treatment*</th>
<th>Baseline versus maximum*</th>
<th>Treatment × Baseline versus maximum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia (pmol/mL CO₂)</td>
<td>606 (406–699)</td>
<td>621 (433–823)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hydrogen (ppm)</td>
<td>4 (3–8)</td>
<td>8 (4–14)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ethanol (pmol/mL CO₂)</td>
<td>8 (6–14)</td>
<td>8 (4–14)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Acetone (pmol/mL CO₂)</td>
<td>201 (75–488)</td>
<td>293 (122–476)</td>
<td>0.39</td>
<td>0.0001</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Calculation of ammonia values (n = 30). Calculation of hydrogen values (n = 28) excludes two participants (#1 and #2). Calculation of ethanol values (n = 24) excludes six participants (#4, #5, #21, #22, #28, #29). Calculation of acetone values (n = 24) excludes six participants (#4, #5, #21, #22, #28, #29).

ANOVA analysis results, p value < 0.05 considered significant.

Discussion

Physiologic considerations in breath analysis

Our results show that ammonia significantly increases over time in response to a high protein oral challenge compared to a negative control oral challenge, and we hypothesize that exhaled breath ammonia represents systemic ammonia. The main purpose of the intervention day was to establish that increases were due to the physiologic effect of the high protein oral challenge. This is important because some literature indicates that mouth exhaled ammonia only measures the activity of mouth flora and salivary urea, and cannot reflect systemic ammonia. Since our ammonia peaks occurred hours after ingestion and are contemporaneous with the hydrogen peak, we believe our data represent systemic and not oral ammonia.

We used a control day to establish confidence that increases on the intervention day are not due to the influence of time of day or chance. This was an important factor to address because physiologic variance is common to many metabolites, including ammonia. Due to the essential need to establish each of the above points, the control day was specifically designed to differ from the intervention day, e.g. different amount of protein, calories and fiber. We believe the inclusion of this control day, which to the best of our knowledge has not previously been employed in breath research, is a strength of this study.

A unique aspect of this study, with respect to the interpretation of the data, is the use of breath hydrogen. In contrast to ammonia and ethanol, hydrogen is easy to measure, inert and its source is relatively non-controversial. It is produced when the food bolus residue encounters the bulk of bacteria in the distal small bowel and, to a greater degree, colon. It therefore serves as a distinctive and essential timing marker. This is important when examining serial data after an oral challenge because gut transit time is variable and unpredictable (Huizinga & Lammers, 2009). Since “endogenous ethanol” has been postulated to be derived from this same microbial community (Zhu et al., 2013), then these peaks should be approximately concurrent.

However, on both control and intervention trials, ethanol peaked earlier than hydrogen, suggesting that the source of the ethanol peak is unrelated to the direct impact of the food bolus residue entering the distal small bowel or colon.

Both the source of this early peak and the absence of a later peak coinciding with the hydrogen peak were unexpected, and not easily explained. The presence of an early peak has been appreciated by other breath researchers (Cope et al., 2004; Smith et al., 1999). It is possible, therefore, that “gut derived” endogenous ethanol is produced by bacteria in the relatively sterile stomach or proximal small bowel.

Finally, we note more variability in ethanol response compared to ammonia. Since alcohol dehydrogenase is inducible and differentially expressed in various tissue beds including the gastrointestinal tract and liver (Engelend & Maret, 1993), increased variability may be expected. Endogenous ethanol produced in the distal small bowel and colon may not be measurable in breath due to rapid clearance and first pass metabolism in the liver. Our ethanol results are generally consistent with recent work that has explored the use of breath ethanol (often coupled with acetone) to determine blood glucose among diabetics (Galassetti et al., 2005).

Technical considerations in breath analysis

Importantly, we measured the phase III portion of the breath and reported an immediate decrease in breath ammonia before a steady increase to above baseline seen in samples collected over 5 h. We propose that a recovery period after rinsing is necessary. If measurements are recorded too soon after rinsing or not followed for a sufficient duration, recorded breath ammonia values may be lower as maximum levels have not yet been achieved. Some investigators may have recorded low breath ammonia levels due to this methodological difference. For example, Adrover et al. (2012) report that ammonia samples collected 15 min after tooth brushing are much lower than those reported in the literature.

As an element to explore diurnal variation, we compared intervention to control trials. During control trials, we found a modest increase in ammonia. This has been previously reported (Ribbard & Killard, 2011; Schmidt et al., 2013). In our study, by comparing breath ammonia levels measured after a control trial to levels after high protein intervention, each participant served as their own control, and breath ammonia was significantly increased after high protein intervention when compared to the control.
Our mean ammonia value is comparable to those measured by other groups (Schmidt et al., 2013; Smith et al., 1999; Turner et al., 2006, 2008). Some groups have concluded that exhaled breath ammonia measured via the mouth may be contaminated by oral bacterial products (Schmidt et al., 2013; Spanèl et al., 2013) and some investigators have recommended measurement of nose-exhaled breath (Schmidt et al., 2013; Wang et al., 2008). Others incorporated a urea mouth wash to evaluate the potential for oral contamination and found supra-physiologic ammonia levels (4500 ppb) seen in mouth-exhaled breath of a single subject (Smith et al., 2013).

The differences between groups may be due to protocols followed during breath collection, mouth- versus nose-exhaled breath or differences in devices used to collect samples. Therefore, future research must clearly report the method of breath exhalation: nose- or mouth-exhaled, especially since the evidence indicates that nose-exhaled ammonia values are consistently lower than mouth-exhaled samples. This may be

Figure 1. Baseline to maximum breath ammonia of individual participants. Data shown are each individual’s baseline linked to their maximum post-intervention breath sample. The baseline is the mean of the pre-intervention breath samples. (A) Breath ammonia (pmol/mL CO₂) in control versus intervention groups. (B) Breath hydrogen (ppm) in control versus intervention groups. (C) Breath ethanol (pmol/mL CO₂) in control versus intervention groups.
due to the greater surface area of the nasal cavity compared to the oral cavity to act as an ammonia sink. In that reproducible ammonia levels have been published in the literature, we propose that consistent use of either method will allow for the study of systemic ammonia.

Sourcing considerations in breath analysis

The study does not identify the source of these metabolites definitively. However, this is rarely the case for these metabolites using any modality. To illustrate with ammonia, landmark studies evaluating the gut contribution of ammonia to systemic blood ammonia used blood sampling on euthanized dogs and humans during laparotomy; even then, there was no intervention, the data was collected during highly un-physiologic circumstances, and their data could not discriminate between, for example, ammonia derived from gut microbes versus small bowel enterocyte activity. Hence, while we acknowledge this limitation, it is essential to note that it is shared by virtually all previous human research on these metabolites.

Putative mechanisms of increased breath ammonia include increased activity of gut flora and/or increased activity of small bowel or kidney glutaminase promoted by amino acid absorption. Kidney-derived ammonia-genesis may also contribute because gut-derived ammonia does not increase systemic ammonia in healthy persons without porto-systemic shunting (van de Poll et al., 2008; Yang et al., 2000). Moreover, a recent review cites multiple studies implicating the kidney as a key organ in ammonia homeostasis (Mpabanzi et al., 2011). We also note that the kidneys return blood to the right heart and pulmonary circulation via the renal veins and inferior vena cava, thereby bypassing the liver. Accordingly, renal ammonia-genesis may be relatively easy to detect by breath analysis. Finally, we note that ammonia rises somewhat earlier than hydrogen. This finding is expected, as amino acids are absorbed in the proximal and mid-small bowel, whereas increased hydrogen identifies the time at which the food bolus residue enters the distal small bowel and colon.

The differential response of acetone to the oral challenges lends additional biological plausibility. We found that acetone increased after the high protein intervention. We believe this increase may have been due to ketogenic amino acid breakdown. In contrast, acetone decreased on the negative

Figure 2. Medians ± interquartile ranges (IQR) for ammonia (pmol/mL CO₂), ethanol (pmol/mL CO₂), hydrogen (ppm) and acetone (pmol/mL CO₂). Baseline (time zero) was calculated by the mean of the three pre-intervention breath samples. The arrow represents time of intervention. Open markers indicate control trial results and closed markers indicate intervention trial results. (A) Median and IQR for ammonia (N = 30). Ammonia is depicted by circle markers. Hydrogen, depicted by square markers, is included to serve as a timing comparator (N = 28). (B) Median and IQR for ethanol (N = 24). Ethanol is depicted by circle markers. Hydrogen, depicted by square markers, is included to serve as a timing comparator (N = 28). (C) Median and IQR for acetone (N = 24). Open circle indicates control acetone; closed circle indicates intervention acetone.
control day which may be attributable to reduced lipolysis. This decrease would be expected and has previously been demonstrated in fasting healthy subjects in response to an oral sugar challenge (Galassetti et al., 2005; Mpabanzi et al., 2011). Thus, given that our high protein challenge contained sugar and glucogenic amino acids, the increase in acetone after the high protein challenge is notable and supports the hypothesis that breath ammonia is sourced from amino acid absorption and subsequent degradation.

Finally, regarding the source of exhaled breath ammonia, we note recent work demonstrating that a significant amount of exhaled ammonia is derived from hydrolysis of urea present in saliva (Chen et al., 2014). Our results do not disagree, and it is possible that some of our observed increase in ammonia after the high protein challenge could be the result of increased systemic urea rather than increased systemic ammonia. Regardless, the totality of the data, including the hydrogen and acetone results, is indicative of systemic protein metabolism. Future studies may further elucidate sources.

Although this study lacks data from blood and urine assays, this is not necessarily an important impediment for the study of ammonia or ethanol. With ammonia, our breath assays measure exhaled NH$_3$ presumably derived from the lungs while venipuncture measures the protonated ammonium ion (NH$_4^+$) derived from a limb. As already noted, both approaches have the potential for significant variability and error. In the case of blood assays, this concern has been repeatedly reviewed (Blanco Vela & Bosques Padilla, 2011). Therefore, it may not be possible or even necessary for exhaled NH$_3$ (derived from pulmonary and/or oral-pharyngeal sources) to correlate precisely with NH$_4^+$ via limb venipuncture (derived from systemic venous circulation) for either to be considered valid markers of systemic ammonia. In fact, DuBois et al. (2005) used fiber optic sensors to detect breath ammonia and compared it to arterial ammonium in 15 cirrhotic individuals and found no correlation. Nevertheless, our own preliminary work on this comparison using a separate cohort suggests a fair correlation between breath and blood ammonia (unpublished results). This current study provides an internal control by comparing repeated levels of breath ammonia in participants after an oral control intervention and a treatment intervention. Even exceptional studies using blood assays do not generally have this asset.

Our results also demonstrate early peaks in breath ethanol in both the control and intervention groups. This is consistent with the concept of gut-derived ‘endogenous ethanol’, which has previously been shown in several small studies using highly sensitive blood assays in response to food (Sarkola & Eriksson, 2001; Watanabe-Suzuki et al., 1999) as well as prior breath research using a murine model (Cope et al., 2000) and humans (Nair et al., 2001). Endogenous ethanol is postulated to contribute to the pathogenesis of non-alcoholic fatty liver and perhaps the metabolic syndrome (Cope et al., 2000; Volynets et al., 2012).

Our study is limited by small sample size and single-center experience. As our monitors are unique prototypes, our results may not be generalizable; this is a common problem for trace breath analysis research. Furthermore, we are not able to verify the exact source of either ammonia or ethanol in our study. As noted above for ammonia, trace breath metabolite measurement is difficult and this is true for acetone as well as ethanol (Anderson et al., 2006; Yang et al., 2000). It is noted that lactulose is used clinically as an ammonia lowering therapy, though its exact mechanisms of action, dose and time-response are unknown. Therefore, its inclusion in this study may have served to lower systemic ammonia. However, any possible lactulose effect would have lowered ammonia on the intervention day only.

Conclusion
This study highlights a key strength of breath analysis: the ability to evaluate an individual’s response to a physiologic challenge non-invasively. Each individual may therefore serve as his or her own control, and multiple data points can be easily obtained over several hours and testing days.

Another important strength of both our work and breath analysis in general is the capability to evaluate gut flora activity in real time through a combination of biomarkers. While each metabolite offers distinct information, when combined they offer insights into digestion and metabolism not matched by other methods. In this instance, ammonia and ethanol represent by-products of protein and carbohydrate metabolism, respectively. Hydrogen serves as a reliable marker to time the passage of bolus through the gut. Acetone, a product of the decarboxylation of acetoacetate, may serve to identify protein breakdown.

To the best of our knowledge, no previous human breath study has measured these metabolites together in response to a physiologic oral challenge compared to a negative control day. We believe this approach holds great promise and is timely, given the worldwide effort to evaluate the impact of the gut microbiome on health and metabolism (Owyang & Wu, 2014).

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Declaration of interest
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