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Changes in breath carbon isotope composition as a potential biomarker of inflammatory acute phase response in mechanically ventilated pediatric patients

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Sepsis is a leading cause of mortality in the intensive care unit. Animal studies have shown exhaled breath carbon isotope delta value (BDV, i.e., $^{13}$CO$_2$/^{12}$CO$_2$ delta value) is a marker for the inflammatory acute phase response (APR). The purpose of this study was to determine baseline variability of BDV in mechanically ventilated pediatric patients with and without systemic inflammatory response syndrome (SIRS) and to correlate the BDV to clinical course over-time. The study was an observational pilot study in a pediatric intensive care unit at an urban, tertiary care children’s hospital. Seventeen mechanically ventilated pediatric patients underwent measurement of exhaled BDV every 8 hours for 72 hours. The BDV was not statistically different between SIRS, No-SIRS and SIRS with shock. The mean BDV was significantly lower in subjects with active sepsis or trauma/post-op status compared to subjects with No-Infection/Trauma/Surgery (N-ITS) or septic shock. Trend analysis over time revealed that the No-ITS and ITS in recovery groups had positive slopes. Subjects who developed infections during the study, and subjects who underwent shock had a negative trend over-time. These results indicate the BDV does not correlate well with SIRS status. However, when patients are classified based on their inflammatory APR the BDV correlates with the severity of systemic inflammation. When monitored over-time, changes in the BDV may correlate to changes in physiology related to fractionation during the APR to infection, trauma or due to altered macronutrient oxidation during episodes of septic shock.

Introduction

The systemic inflammatory response to a wide variety of insults has been termed systemic inflammatory response syndrome (SIRS). SIRS is nonspecific and develops following a severe biological insult such as infection, trauma, surgery, pancreatitis, or burns.\textsuperscript{1} SIRS occurs in 82% of patients admitted to pediatric intensive care units (PICUs), including 23% with sepsis (SIRS in the presence of or as a result of suspected or proven infection).\textsuperscript{2} Sepsis is the most common cause of death in infants and children in the world. In the United States, there were more than 47,000 cases of severe sepsis in children aged 19 years or less in 1999. Hospital mortality among US children with severe sepsis was 9% in 1999. Estimated annual total costs were $2.3 billion in children in the US alone.\textsuperscript{3}

Early detection of bacterial sepsis followed by immediate intervention is critical for successful outcomes to patient care.\textsuperscript{4} Current clinical methods are often unreliable in detecting early bacterial sepsis; accordingly, there is great interest in identifying laboratory methods for early detection of sepsis. Although several biomarkers are potentially useful in the diagnosis of bacterial sepsis, all of them have limitations. All blood based biomarkers require blood be drawn, a procedure that is somewhat invasive. White blood cell count (WBC) and absolute neutrophil count are inexpensive but lack adequate sensitivity and specificity to detect bacterial sepsis.\textsuperscript{5} Measurement of C-reactive protein (CRP) is readily available and relatively inexpensive compared to measurement of procalcitonin (PCT), but CRP measurements are not sensitive enough to detect bacterial sepsis early in its clinical course.\textsuperscript{6} PCT is a sensitive and early indicator of bacterial sepsis. However, measurement of PCT is expensive and requires a blood sample.\textsuperscript{7, 8} Frequently PCT and CRP tests are used for predictors of sepsis outcome rather than as a means for early detection of infections.

Breath based markers, such as the carbon isotope breath delta value (BDV), may serve as better infection surveillance tools since they can be easily and non-invasively monitored. The BDV (i.e., $^{13}$CO$_2$/^{12}$CO$_2$ delta value) has been shown to be a valuable biomarker of bacterial sepsis in different animal models.\textsuperscript{9} BDVs begin dropping immediately after administration of lipopolysaccharide (LPS) to experimental animals. The inflammatory APR induced by bacterial antigens initiates a complex cascade of events that result in the natural change in ratio of $^{13}$CO$_2$/^{12}$CO$_2$ (termed fractionation) due to the kinetic isotope effect (Figure 1). This fractionation results in measurable changes in BDV, which provides an avenue for early and non-invasive detection of sepsis through real-time monitoring.\textsuperscript{9, 11} Furthermore, the change in BDV precedes other physiological changes associated with infection, such as drop in
mean arterial pressure, hypoperfusion of the gut or alterations in blood oxygenation, carbon dioxide, and pH.\textsuperscript{12, 13}

There are no published studies evaluating the BDV as a marker of acute inflammation in humans. The purpose of this observational pilot study was to determine baseline variability of exhaled BDV in a population of mechanically ventilated pediatric patients and to correlate the trends in BDV over-time to severity of inflammation and clinical course.

**Experimental Methods**

The Institutional Review Board at University of Wisconsin-Madison approved the study. All patients in the PICU at American Family Children’s Hospital were prospectively screened for participation in the present study. Informed consent was obtained for all participants. We enrolled mechanically ventilated (orotracheal, nasotracheal, tracheotomy) patients ages 1 month to 18 years who were expected to remain ventilated for at least 72 hours. Seventeen subjects were enrolled and classified into groups (SIRS, no-SIRS and SIRS with shock) based on widely accepted criteria for pediatric SIRS \textsuperscript{14} and by severity of their underlying inflammatory APR at enrollment: No infection, trauma or surgery (No-ITS; n=6); trauma or post-operative status (n=5); active sepsis (SIRS in the presence of suspected or proven infection; n=3); and shock (n=3). Shock was defined as subjects who have undergone hemodynamic dysfunction, such as vascular hypotension, with multiple organ failure \textsuperscript{15}. Subjects who have undergone a shock response were considered separately from active sepsis and/or SIRS due to a known alteration in the physiology of oxygen consumption at the cellular level \textsuperscript{15} that could impact macronutrient utilization and thus the BDV \textsuperscript{13, 16, 17}

All patients enrolled in this study underwent measurement of exhaled BDV at study enrollment and every 8 hours thereafter for a total of 72 hours. Expiratory breath samples were collected from the ventilator exhaust by affixing a modified Rushe adaptor that created minimal positive pressure to fill the breath sample bag with expired air. Every effort was made to ensure that no changes from the standard of care in ventilator function occurred. The breath samples obtained were considered as the average breath sample from the expiratory side of the ventilator circuit.

**Carbon isotope measurement**

Cavity ring-down spectroscopy (CRDS) was used to determine the BDV for all samples in the study. CRDS is a well-established technique for detecting trace quantities of gases \textsuperscript{14}. The key element in CRDS is a precisely aligned optical cavity with three very high reflectance mirrors (>99.995%). Briefly, when light from a continuous wave laser that matches the resonance frequency of the cavity enters the cavity, the energy levels in the cavity increase over time. When the laser is turned off, the energy decays (“rings down”) exponentially over microseconds; this decay is measured using a photodiode. CRDS measures the length of the ring down time over different wavelengths, because the laser is tuned for the analyte gas’ molecular signature. A strongly absorbing gas will have a relatively short ring down time compared to the empty cavity. Each gas has its characteristic concentration-dependent ring down time at different wavelengths. Figure 2 depicts a ring-down curve, showing the changing light intensity inside the cavity as a function of time after the laser is shut off. CRDS generates a detailed profile, area and height of an individual feature (f = 1/τ); this decay is measured using a photodiode. CRDS measures the length of the ring down time over different wavelengths. Figure 2 depicts a ring-down curve, showing the changing light intensity inside the cavity as a function of time after the laser is shut off. CRDS generates a detailed profile, area and height of an individual feature (f = 1/τ); this decay is measured using a photodiode. CRDS measures the length of the ring down time over different wavelengths, because the laser is tuned for the analyte gas’ molecular signature. A strongly absorbing gas will have a relatively short ring down time compared to the empty cavity. Each gas has its characteristic concentration-dependent ring down time at different wavelengths. Figure 2 depicts a ring-down curve, showing the changing light intensity inside the cavity as a function of time after the laser is shut off. CRDS generates a detailed profile, area and height of an individual feature (f = 1/τ); this decay is measured using a photodiode. CRDS measures the length of the ring down time over different wavelengths, because the laser is tuned for the analyte gas’ molecular signature. A strongly absorbing gas will have a relatively short ring down time compared to the empty cavity. Each gas has its characteristic concentration-dependent ring down time at different wavelengths. Figure 2 depicts a ring-down curve, showing the changing light intensity inside the cavity as a function of time after the laser is shut off. CRDS generates a detailed profile, area and height of an individual feature (f = 1/τ); this decay is measured using a photodiode.

**Figure 2:** Ring-down trace showing exponential decay of the optical signal with and without analyte gas.

**Figure 3:** Panel A: Loss spectrum from WS-CRDS. The curve represents the cavity loss over a spectral line for a single analyte, for example $^{13}$CO$_2$. Panel B: Box plot of the analyzer delta value precision for two reference samples.
referred to Pee Dee Belemnite (PDB). Data are expressed as delta values or parts per mil (%e):

$$\delta = \frac{^{13}CO_{2}/^{12}CO_{2} \text{(sample)} - ^{13}CO_{2}/^{12}CO_{2} \text{(PDB)}}{^{13}CO_{2}/^{12}CO_{2} \text{(PDB)}} \times 1000$$

The precision obtained for $^{13}$CO$_2$/$^{12}$CO$_2$ using the Picarro 2101i isotopic CO$_2$ CRDS instrument used in this study is better than 0.3% with CO$_2$ concentrations between 380 and 500ppm CO$_2$ corresponding to a precision of 10ppb $^{13}$CO$_2$ and 200ppb for $^{12}$CO$_2$ individually. Figure 3, panel B depicts isotopic precision (in box plots) of CRDS for determination of isotope ratio of two 5% CO$_2$ reference gasses (Cambridge Isotope Labs, Tewksbury, MA) diluted to 400 ppm CO$_2$ with Zero Air (i.e. Air containing >0.001ppm CO$_2$).

Breath sample analysis

Isomark, LLC analyzed all carbon samples. The expired breath was placed in a gas-tight bag with a de-identified sample number by a respiratory therapist at the point of care, and transported to Isomark for analysis. Breath sample bags were constructed of an edge sealed multilayered plastic bag consisting of Nylon/Polyethylene-EVOH co-extrusion with an integrated high volume lure-valve for filling and sealing the bag. The bag was verified to maintain the carbon stable isotope ratio for greater than 72 hours. To ensure accurate measurements all breath samples were analyzed within the first 48 hours of collection, typically less than 24 hours from time of collection. At the time of sample measurement some of the gas was withdrawn from the gas-tight bag and diluted to about 500ppm CO$_2$ concentration using CO$_2$ free air. The stable isotopes of carbon in CO$_2$ were determined by an isotopic CO$_2$ cavity ring-down spectrometer as above.

Statistics

The BDV was analyzed in two ways, first by assessing baseline variation in BDV at enrollment, and second by assessing trends in the BDV over time. To assess baseline variation subjects were classified SIRS (n=5), No-SIRS (n=8) or SIRS with shock (n=4), or by the severity of their underlying inflammatory APR at enrollment based on the following categories: No infection, trauma or surgery (No-ITS); (n=6); trauma or post-operative status (n=5); active sepsis (n=3); and shock septic (n=3). The mean, range and interquartile range were calculated for each category. ANOVA with least significant difference post-hoc analysis was applied individually to both sets of diagnostic classification. To assess trends in the data over time the subjects were classified based on their clinical course with the following categories: Stable with No-ITS, ITS with improving clinical condition, developing infection, and septic shock with organ failure. Best-fit linear trends were calculated using Microsoft Excel and only subjects who provided at least 9 consecutive samples were considered. The mean and standard deviation of the slope of the line were calculated and ANOVA with least significant difference post-hoc analysis was applied to the slope of the line. Additionally the trends in the BDV and daily SIRS criteria were assessed using the SAS statistical analysis package using the mixed model for repeated measures; least square means were used to compare each category to the stable No-ITS group. Differences were considered significant with a p-value ≤ 0.05.

Results

We enrolled 17 mechanically ventilated pediatric patients ranging in age from 1.5 months to 17.5 years between July 2011 and January 2012 (table 1; supplementary table 1). Nine of 17 patients met SIRS criteria at the time of enrollment of 4 of which had undergone shock. The mean BDV was lower, though not significantly different, in the SIRS group compared to the non-SIRS and SIRS with shock groups at the time of enrollment (mean BDV in SIRS -22.7‰ SE 1.1; No-SIRS -21.1‰, SE 0.8; SIRS with shock -19.5‰ SE 0.9 p=0.14; Figure 4, panel A). However, the mean BDV was significantly lower in subjects with active sepsis or trauma/post-op status compared to subjects with No-ITS or septic shock (mean BDV of -23.0‰ SE 1.3, -23.1‰ SE 0.5, -19.7‰ SE 0.7, and -19.1‰ SE 1.1 respectively; p=0.01 Figure 4, panel B). There was no statistical difference in the mean breath delta value between patients with No-ITS and patients with septic shock (Figure 4, B).

Table 1

<table>
<thead>
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<tr>
<td>Gender</td>
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<td></td>
<td>17.6% Decline to report</td>
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</table>

Figure 4: Shows the mean breath delta value (BDV) at the time of study enrollment of subjects classified as systemic inflammatory response syndrome (SIRS) or no systemic inflammatory response syndrome (No-SIRS) or SIRS with shock (Panel A), and the mean BDV at the time of study enrollment of subjects classified based on the severity of their underlying inflammatory acute phase response (APR) (Panel B). Data are presented as mean with interquartile range with error bars representing the range. ANOVA with least significant differences post-hoc analysis was used to analyze the data, and letter superscripts a and b represent significant differences with p<0.05.

Since it is hypothesized that the trend in the BDV over time may correlate to changes in physiology during the onset of infection, sepsis and septic shock we analyzed the trend in the BDV over the course of study participation. Only subjects who were able to provide at least 9 consecutive samples were included in trend analysis. The BDV was plotted over time for each subject, and the slope of the linear trend line was assessed.
The subjects were grouped as before in the No-ITS, ITS in a recovery trend, subjects who developed an infection during the study, and subjects who underwent septic shock (Figure 5). The slope of the line was averaged for each group and analyzed by ANOVA. The mean slope of the line was positive for No-ITS subjects (0.33, 0.35 SD) and ITS subjects in a recovery phase. The mean slope of the line was negative for patients who developed infections during the study (-0.56, 0.13 SD), and for subjects who underwent septic shock (-0.45, 0.76 SD). When analyzed over time the No-ITS group had a stable BDV between -18 to -19‰. The ITS subjects in recovery had a significantly more negative BDV at the start of the study that trended in a positive direction towards the No-ITS group and was no longer significantly different than the No-ITS group by the final sample. Subjects that underwent septic shock had a generally negative, but variable response in the BDV trend over time, however, these subjects were not significantly different from the No-ITS group. Subjects that developed infections during the study were initially not significantly different from the No-ITS group, but as subjects developed infections the BDV trended in a negative direction and became significantly more negative than the No-ITS group.

**Discussion**

Sepsis leading to shock is a major cause of mortality in adult and pediatric intensive care units. Early detection and prompt treatment of sepsis is critical for survival and optimal outcomes. Based on preclinical animal studies, we hypothesized that the breath delta value would be a sensitive marker of the inflammatory APR to infection in mechanically ventilated patients and may be marker for changes in metabolism during the onset of infection and septic episodes. We examined the relationship between the breath delta value and a diagnosis of SIRS and SIRS with shock. We found a lower, though not statistically significant, BDV in subjects with SIRS compared to subjects with no-SIRS or SIRS with shock. The pediatric definition of SIRS is based on clinical parameters (principally body temperature and WBC count) that are not specific to infection. Spikes in temperature may be related to infection, but may also be related to trauma, burns or other diagnoses. As infections progress increasing body temperature and changing WBC counts are reliable markers, however, by the time changes in body temperature and WBC counts can be observed the infection has had systemic effects. The changes in macronutrient oxidation that occur as a result of changing physiology during sepsis and septic shock can cause dramatic changes in the BDV. During severe sepsis peripheral tissues do not utilize oxygen well even with an abundant supply, thus increases in plasma lactic acid are a hallmark sign of sepsis. The effect on whole body macronutrient metabolism is that lipid oxidation is limited and anaerobic glycolysis dominates. During normal metabolism lipids oxidation may contribute between 40 to 60% of the body’s caloric need. The isotopic signature of lipids is much lighter than protein or carbohydrate due to an enzymatic discrimination against C-structures by pyruvate dehydrogenase, thus reduced lipid oxidation causes a positive shift in the BDV. This effect has been observed in animal studies 13, 22, and is evident in the SIRS with shock group (Figure 4 panel A). An individual case of sepsis transitioning to septic shock and death is presented in the supplementary materials (Figure S4) where the BDV can be seen transitioning from -25.2‰ during sepsis to -16.9‰ just prior to death due to septic shock. These data are consistent with impaired oxidative metabolism, particularly in hemodynamically unstable subjects who die within the first 24 hours of septic shock. Significantly lower breath delta values were observed in subjects with active sepsis without shock compared with those with No-ITS (Table 1). This difference in breath delta value could be explained in part by fractionation of carbon during the catabolic inflammatory APR to infection (Figure 1). Invading pathogens stimulate the innate immune response by activating tissue macrophages. Inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin 1 (IL-1) are released by macrophages into the circulation. TNF and IL-1 induce catabolism and amino acid release from skeletal muscle and stimulate acute phase protein synthesis in the liver.
The BDV does not correlate well with SIRS status. However, when patients are classified based on their inflammatory APR the breath delta value correlates with the severity of systemic inflammation. When monitored over-time, changes in the BDV may correlate to changes in physiology related to fractionation during the APR to infection, trauma or due to altered macronutrient oxidation during episodes of septic shock.

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Notes and references

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This work was performed at the University of Wisconsin-Madison.

†Conflicts of Interest and Sources of Funding:

Daniel E. Bütz, Mark E. Cook, and Warren P. Porter have an ownership interest in Isomark, LLC, which has licensed the technology reported in this publication. Juan P. Borriosi received funding from Isomark, LLC to conduct this study. For the remaining authors none were declared.

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