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Volatile Decay Products in Breath during Peritonitis Shock are Attenuated by Enteral Blockade of Pancreatic Digestive Proteases --Manuscript Draft--

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Abstract:	<p>There is a need to develop markers for early detection of organ failure in shock that can be non-invasively measured at point of care. We explore here the use of volatile organic compounds (VOCs) in expired air in a rat peritonitis shock model. Expired breath samples were collected into Tedlar gas bags and analyzed by standardized gas chromatography. The gas chromatograms were digitally analyzed for presence of peak amounts over a range of Kovach indices. Following the induction of peritonitis, selected volatile compounds were detected within about one hour, which remained at elevated amounts over a 6 hours observation period. These VOCs were not present in control animals without peritonitis. Comparisons with know VOCs indicate that they include 1,4-diaminobutane and trimethylamine N-oxide. When pancreatic digestive proteases were blocked with tranexamic acid in the intestine and peritoneum, a procedure that serves to reduce organ failure in shock, the amounts of VOCs in the breath decreased spontaneously to control values without peritonitis. These results indicate that peritonitis shock is accompanied by development of volatile organic compounds that may be generated by digestive enzymes in the small intestine. VOCs may serve as indicators for detection of early forms of autodigestion by digestive proteases.</p>
Response to Reviewers:	Editor: We uploaded the manuscript without markings and with reference list adjusted to ten author names followed by et al.

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4 Volatile Decay Products in Breath during Peritonitis Shock are
5 Attenuated by Enteral Blockade of Pancreatic Digestive Proteases
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27 **Keywords:** Peritonitis shock, pancreatic digestive enzymes, breath, volatile organic compounds
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50 **Competing interests:** F.A.D., and G.W.S.S. own equity in InflammaGen, a company by Leading
51 Bioscience Inc., which develops therapy for shock patients.
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5 **Abstract**
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8 There is a need to develop markers for early detection of organ failure in shock that can be non-
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10 invasively measured at point of care. We explore here the use of volatile organic compounds
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12 (VOCs) in expired air in a rat peritonitis shock model. Expired breath samples were collected into
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14 Tedlar gas bags and analyzed by standardized gas chromatography. The gas chromatograms
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16 were digitally analyzed for presence of peak amounts over a range of Kovach indices. Following
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18 the induction of peritonitis, selected volatile compounds were detected within about one hour,
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20 which remained at elevated amounts over a 6 hours observation period. These VOCs were not
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22 present in control animals without peritonitis. Comparisons with know VOCs indicate that they
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24 include 1,4-diaminobutane and trimethylamine *N*-oxide. When pancreatic digestive proteases
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26 were blocked with tranexamic acid in the intestine and peritoneum, a procedure that serves to
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28 reduce organ failure in shock, the amounts of VOCs in the breath decreased spontaneously to
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32 development of volatile organic compounds that may be generated by digestive enzymes in the
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34 small intestine. VOCs may serve as indicators for detection of early forms of autodigestion by
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36 digestive proteases.
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Introduction

One of the major requirements for effective intervention in shock is early detection of signs for progression towards organ failure. There are a large number of blood indicators for shock which range from monitoring of small molecules, like blood oxygen values, to detection of elevated blood plasma values of lactate or inflammatory markers such as complements and cytokines (1-3).

Selected combinations of lower molecular weight (up to 1.5kD) metabolomic markers (e.g. organic acids, lipids, amino acids, carbohydrates, peptides, vitamins, steroids, xenobiotics) in exhaled breath condensates were proposed to characterize patients with shock and respiratory failure (4).

But there is a need to explore non-invasive early indicators for end organ damage that serve to minimize delay in diagnosis and facilitate interventions to attenuate progression into multi-organ failure.

Volatile organic compounds (VOCs) have been identified as possible approaches for non-invasive detection of several diseases, e.g. cancers, acute lung injury, tuberculosis, liver disease, myocardial infarction (5-11). VOCs include acetone, acetaldehyde, alkanes, amines, benzenes, pentane, sulfur compounds, and many others (12). After endotoxic administration in the rat, compounds like propanol, butanal, acetophenone, butandiol, hexanone, dimethylether, pentafluoropropionamide, xylene, propanol, toluene and others have been proposed to serve a multivariate diagnostic analysis of shock versus control animals (13). Alcohols and ketones (e.g. propanol, pentanone, acetone, hexanone) were reported to decrease over a six hour period in cecal ligation and endotoxin shock but not in hemorrhagic shock (14). In a study with Wiggers hemorrhagic shock 16 different VOCs changed in shock compared to controls, five of which had higher intensities, nine had lower intensities during hypotension and 9 hours thereafter, and two increased only during the hypotensive period (15). It is largely uncertain what mechanisms are

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4 involved in the formation of VOC and whether their detection may serve as a diagnostic tool to
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6 evaluate the effectiveness of therapy. This is the objective of the current report.
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10 Our increasing evidence suggests that pancreatic digestive enzymes in the small intestine and
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12 digestive fragments they produce may play a central role in the development of organ failure by
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14 escape from the lumen of the intestine after breakdown of the mucosal barrier (16). As digestive
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16 enzymes enter into the wall of the intestine, they break down the mucosal lining (17) and start an
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18 autodigestion process, which leads to multi-organ failure and death if uninterrupted (18). During
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20 this process not only the food content of the intestine, as is the case during normal digestion, but
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22 also autologous tissue is degraded to the point that morphological features associated with the
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24 intestinal mucosa are destroyed. The process starts at the tip of villi in the small intestine already
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26 within minutes of an ischemic insult (19) and can lead to complete destruction of the villi (20-23).
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33 Introduction of ischemia causes formation of VOCs detected by gas chromatography (24). But
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35 their source in the tissue remains uncertain. We hypothesize that in experimental peritonitis shock
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37 distinct VOCs are generated during proteolytic degradation of the small intestine and are
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39 detectable in the breath. Accordingly, we present measurements of VOCs before and after
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41 peritonitis shock without and with blockade of the pancreatic proteases in the lumen of the intestine.
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4 **Methods**
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8 Animals
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13 The animal protocol was reviewed and approved by the UC San Diego Animal Subjects Committee.
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15 Young mature Wistar rats (age 15 to 20 weeks, 350-450 grams body weight, Harlan Sprague
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17 Dawley) were maintained on a regular diet (Harlan Teklad Rodent diet (W) 8604, 0.29% sodium by
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19 weight) without restriction and water ad libitum. After general anesthesia (pentobarbital sodium, 50
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21 mg/kg, Abbott Laboratories, i.m.), the body temperature was maintained at 37°C by keeping the
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23 animals on a water-heated support and by use of a heat blanket. At the end of the six-hour
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25 observation period the animals were euthanized (120 mg/kg, Beuthanasia-D Special, Merk Animal
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27 Health).
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33 Peritonitis Shock
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38 In this shock model, cecal material (900 mg/kg derived from a mixture of multiple donor rats,
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40 diluted by addition of 1 ml of 5% glucose saline solution to 1 gm cecal material) was injected into
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42 the peritoneal cavity and gently spread by mild skin compression over the abdomen. Control
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44 animals without peritonitis were administered the glucose saline solution without cecal material.
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48 Digestive Enzyme Blockage
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53 The digestive enzymes were blocked at 60 min after cecal material administration as described
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55 (16). Briefly, to block digestive enzymes in the lumen of the intestine, a mid-line incision (2.5 cm)
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57 was made to temporarily expose the small intestine. Tranexamic acid (127 mM, Pfizer Inc.)
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59 dissolved in GoLYTELY (60 g/L PEG-3350 and electrolytes for oral solution; Braintree Laboratories,
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4 Inc) was injected with a needle (BD Sub-Q 26G 5/8, Becton Dickinson & Co) into the lumen of the
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6 intestine from the duodenum to the cecum. Untreated shock controls received GoLYTELY without
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8 the protease inhibitor. In a 350 gm rat a total fluid volume of 15 ml (at 39°C) was injected into the
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10 lumen of the small intestine spaced over 8 injections in intervals of about 5 cm and 2 ml was
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12 injected into the cecum. The intestine was returned into the peritoneal cavity and the incision
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14 closed by sutures.
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20 In addition to the enteral protease inhibition, a mixture was also administered as lavage into the
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22 peritoneum composed of tranexamic acid (2 ml; 127 mM dissolved in saline), an antibiotic
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24 (ciprofloxacin, 10 mg/ml, Hospira, Inc), and albumin (40 mg/ml, Sigma-Aldrich Co) at one hour
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26 after placement of cecal material. The mixture serves to inhibit not only digestive enzymes, but
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28 also bacteria and lipid cytotoxic mediators (e.g. unbound free fatty acids) that were administered
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30 by the cecal material into the peritoneum. The untreated peritonitis shock group received the same
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32 volume of saline into the peritoneum and the same volume of GoLYTELY into the lumen of the
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34 intestine but without additives. To determine the role of the enteral treatment in VOC generation,
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36 we also studied a group of shock rats (n = 3) with peritoneal treatment only (using the same
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38 mixture) but without enteral treatment.
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46 Breath Analysis

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51 To collect exhaled air, a tracheotomy was carried out for placement of a two-way non-rebreathing
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53 valve. Exhaled breath samples were collected in Tedlar gas sample bags (1 L, Jensen Inert, Coral
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55 Springs, Florida) placed at the end of the tracheostomy tube. A two-way non-rebreathing valve
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57 (series 2300 T-shape; Hans Rudolph, Inc., Shawnee, Kansas) was used for collection of exhaled
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59 air into gas sampling bags. Control breath samples were collected before laparotomy to block
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4 digestive enzymes. Room air samples were collected as controls for inspired air. Samples were
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6 collected in intervals of 30 min over the first hour and then hourly for 6 hours.
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10 The gas sample bags were kept at room temperature and analyzed within a period of two hours
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12 after collection. Repeat measurements of the same air sample over a period of seven days
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14 showed that there was no significant loss of VOCs in the bags, for example due to absorption to
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16 the Tedlar bags.
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22 The presence of VOCs was detected by use of gas chromatography (Z-Nose Ultra-Fast GC, Model
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24 4200, MicroSense 5.44 GC analysis software, Electronic Sensor Technology, Newbury Park, CA).
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26 The settings on the gas chromatography were standardized (sensor temperature 30°C, column
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28 temperature 40°C, valve temperature 140°C, inlet temperature 200°C, trap temperature 250°C,
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30 flow 3.0 ccm--helium carrier gas; sample time = 30 seconds; temperature ramp = 200°C at
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32 5°C/sec). Before measurements, the gas chromatography chamber was cleaned by heat (205 °C)
33
34 running helium through the system in a continuous mode until a background was reached with little
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36 activity. For calibration of the Kovach index (KI), an alkane calibration (n-alkane vapors C6-C14;
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38 Electronic Sensor Technology) solution was tested five consecutive times. The chromatography
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40 profile was digitally scanned and peak amounts were determined with peak-detection software
41
42 provided with the Z-nose. The values are expressed in form of “amount” (i.e. digital detector GC
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44 output).
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51 A list of 25 VOCs was tested with the same GC settings to determine their associated KI values
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53 (acetone; amine; n-butyl acetate; 2-butanone; t-butanol; butyraldehyde; benzaldehyde; cadaverine;
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55 carbon disulfide; chlorobenzene; dimethyl sulfide; 1,4-diaminobutane; 1,5-diaminopentane; 1,2-
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57 dichloroethane; ethanol; indole; limonene; 6-methyl-5-hepten-2-one; 4-methylphenol; pentanoic
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59 acid; 1-propanol; putrescine; methylamine N-oxide, TMAO; o-xylene). The list was derived from
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4 literature values for gastrointestinal, respiratory and cancer patients and associated in part with
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6 tissue decay (6, 11, 25-32). Each compound was dissolved in distilled water and the supernatant
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8 tested in the GC. The KI values for these compounds were compared with the KI values
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10 determined in the breath samples of the rats.
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20 Statistics

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23 All measurements are summarized as mean \pm standard deviation. Repeated measures ANOVA
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25 followed by Bonferroni post-hoc test was used to compare sham controls with untreated and
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27 treated shock groups. Comparison between treated and untreated groups was carried out by
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29 Student's t test. $P < 0.05$ was considered significant.
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39 **Results**

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45 The control breath samples had a gas chromatography profile that was similar among the control
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47 animals with relatively small variation among rats (typical variance about 10%). During peritonitis
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49 shock breath samples exhibited shifting peak amounts at distinct KI values, some of the peak
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51 amounts increased and some also decreased (Figure 1).
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4 Analysis of changes in peak amounts over a range of KI values between about 600 and 2000
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6 showed that the peak amounts at KI 720, 900, and 1550 in the breath (Figure 2) exhibited
7
8 significant differences between sham control and shock. While in sham controls the peak amounts
9
10 did not rise significantly over the 6-hour period of the current experiments, in peritonitis shock the
11
12 peak amounts at KI 720, KI 900 and KI 1550 monotonically and significantly rose. The rise
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14 occurred already within the first hour of the peritonitis and there was no trend for a reduction of
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16 peak amounts over the course of the experiment.
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24 In contrast, after the combined enteral/peritoneal treatment and blockade of the digestive enzymes
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26 with tranexamic acid at 1 hour, the peak amounts started to decrease and after 6 hours fell to
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28 values that were not different compared to non-shock controls (Figure 2). The decreases in peak
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30 amounts were only observed after treatment. The *enteral* treatment is essential for the reduction
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32 of the VOC amounts. Without enteral administration and only peritoneal treatment in shock
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34 animals, there was no reduction of the peak amounts and their values without significantly
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36 difference compared to the untreated peritonitis group (Figure 2, legend).
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44 Peak amounts at KI values, other than at 720, 900 and 1550 exhibited no significant changes in
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46 the current exhaled gas analysis, although several peak amounts reached close to significant
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48 values.
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55 Among the set of VOCs we tested (see Methods), 1,4-diaminobutane (Lot# STBC1987V, SIGMA-
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57 Aldrich Co.) had a KI at 720 ± 5 and trimethylamine *N*-oxide (TMAO, 317594, SIGMA-Aldrich Co.) a
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59 KI of 900 ± 5 . We could not find in this list a compound that had a peak amount at KI 1550. All
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4 other VOC peak amounts fell at least by a value of ± 50 outside the KI values observed in the
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6 breath samples. Both 1,4-diaminobutane and TMAO have a strong foul odor.
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10 11 12 **Discussion** 13

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15 The current results indicate that in experimental peritonitis the breath contains within less than an
16 hour elevated levels of VOCs over several hours that can be inhibited by enteral protease inhibition
17 in the small intestine, but not without it. These measurements are in line with qualitative
18 observations during a previous study with an identical peritonitis shock model (16) as well as other
19 forms of shock (including hemorrhagic and endotoxic shock) in which we noticed foul volatile odor
20 originating from the small intestine. Without exception (18 out of 18 rats examined) the odor was
21 noticed in those animals that succumb to the insult, but less so in surviving animals. In addition to
22 mortality the presence of the odor was accompanied by escape of digestive proteases into the wall
23 of the intestine, lesion formations in the small intestine and the lung. All animals in these studies
24 were maintained with food and water at libitum at the time before shock. Therefore the small
25 intestines were in part filled with food items so that the pancreatic digestive enzymes in the
26 partially digested food and/or the wall of the intestine could serve as a source of the VOCs (16).
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46 A combination of enteral and peritoneal placement of tranexamic acid, a serine protease inhibitor,
47 e.g. for pancreatic trypsin (17), served to reduce the VOC amounts. The protection provided by
48 inhibition of the digestive proteases in the intestine and peritoneum includes beside a reduction of
49 the mortality also a significant reduction of the lesions in the small intestine, heart and lung,
50 including a reduction of the protease activity in the wall of the small intestine and in plasma.
51 Inhibition of the pancreatic digestive enzymes in hemorrhagic shock also reduces the development
52 of insulin resistance by proteolytic cleavage of the ectodomain of the insulin receptor (33). This
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4 evidence suggests that the powerful digestive enzymes in the small intestine may be a major
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6 producer for the VOCs that eventually appears in expired air. The VOCs appear to be carried in
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8 the plasma and diffuse into the airways.
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15 The VOCs found in this study could be detected without the use of a chemical concentrator during
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17 sample collection. They are in sufficient concentrations to be detectable by unaided human smell
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19 sensor. Among specific VOCs with equal KI values as seen in the breath samples were 1,4-
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21 diaminobutane, TMAO and a volatile compound whose identity remains to be identified. TMAO is a
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23 foul smelling compound with below body temperature melting points. It is a metabolite from dietary
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25 phosphatidylcholine (e.g. lecithin) and can be generated by certain intestinal bacteria (e.g. by
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27 generation of trimethylamine which can be transformed into TMAO) (34). Elevated levels of TMAO
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29 have been associated with cardiovascular risk (25, 35).
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35 The mechanism by which the TXA protease inhibition blocks the formation of TMAO, as seen in
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37 the current experiments, remains to be identified. TXA was administered enterally and by lavage
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39 into the peritoneal cavity around the intestine. The fact that it serves to acutely reverse VOCs
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41 formation indicates that their formation is ongoing and protease dependent. The fact that the
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43 peritoneal without enteral blockade is inadequate to block the formation of the VOCs (Figure 2,
44
45 legend) suggests that the digestive enzymes in the lumen of the small intestine are centrally
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47 involved. A source for the odor may be food material in the lumen of the intestine, as seen in fecal
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49 material (12), but could also include intestine itself, since digestive enzymes enter the wall of the
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51 intestine in shock. TXA serves to preserve the mucosal barrier. If a source of the foul smelling
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53 VOCs is in the lumen of the intestine, then the preservation of the barrier may reduce entry of the
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55 VOC into the circulation and into the breath. If the source of the VOCs is in the wall of the intestine,
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57 the mode of action by TXA is to prevent proteolytic degradation of intestine and also other tissues
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4 into which digestive protease leak during peritonitis shock.
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9 TXA is recognized and marketed as an anti-fibrinolytic agent, able to block formation of plasmin
10 when administered i.v.. But its actions as protease inhibitor appear to be broader. In the current
11 application it was used to block serine proteases (e.g. trypsin) in the lumen of the small intestine
12 without i.v. infusion. Enteral blockade of digestive enzymes serves to attenuate a variety of tissue
13 injuries, organ dysfunctions and failures in multiple models of shock (16, 33), and thus the current
14 observation for attenuation of VOCs is in line with the evidence for other forms of protection from
15 digestive enzymes.
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26 We consistently smelled VOCs from the intestine in non-surviving animals that were subject to
27 hemorrhagic shock, a model without the use of cecal material with bacteria or an antibiotic agent in
28 the peritoneum. While measurements of VOCs with and without antibiotic treatment (as compared
29 to enteral protease inhibition) in shock remain to be carried out, this evidence suggests that VOCs
30 can be formed without a major bacterial load.
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41 Key questions to be investigated are whether VOCs in shock patients detected by an electronic
42 breath sensor are similar to those seen in experimental models and if present whether they serve
43 to predict impending organ failure before it reaches an irreversible stage associated with actual
44 organ failure. The current evidence indicates that VOC generation is an early event in a peritonitis
45 induced shock model but the amounts detected in the breath may depend on the extent of
46 intestinal damage. In the current model the major length of the small intestine wall tissue is subject
47 to infiltration by digestive enzymes (16) and a potential source for VOCs. If in other situations
48 shorter segments of the small intestine become ischemic and are infiltrated by digestive enzymes
49 the magnitude of the VOCs detected in the breath may be lower. This situation in ICU patients is
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largely unknown and will require clinical measurements from the time of earliest access to breath samples.

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4 **Figure Legends**
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11 **Figure 1.** An example of GC profile for control without peritonitis (Control group), untreated
12 peritonitis (Shock group) and peritonitis with treatment by inhibition of the digestive proteases with
13 TXA (Shock *Treated*, see Methods). Note elevation of peak amounts, e.g. at KI 720, 900, and
14 1550.
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27 **Figure 2:** The amounts of VOCs at KI (a) 720, (b) 900, and (c) 1550 measured over a period 6
28 hours from rat breath samples after exposure to peritonitis shock without (Shock group) and with
29 enteral protease inhibition with TXA (Treated group) at 1 hour after initiation of the peritonitis.
30 Control represents a sham group without peritonitis. n = 6 to 8 rats in each group. Amounts of
31 VOCs in a separate group (n = 3 rats) with peritonitis shock but with treatment in the peritoneum
32 only (with same mixture, see Methods) and without enteral treatment gave values that were not
33 significantly different compared to the untreated Shock group. At 6 hours the amounts of VOCs in
34 this group with peritoneal treatment only at KI 720, 900, and 1550 were 315 ± 43, 117 ± 34, 483 ±
35 61, respectively. *p<0.05 Control vs Shock group; †p<0.05 Shock vs Treated group; *p<0.05
36 Control vs Treated group.
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4 Volatile Decay Products in Breath during Peritonitis Shock are
5 Attenuated by Enteral Blockade of Pancreatic Digestive Proteases
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9 Frank A. DeLano, Jason Chow, Geert W. Schmid-Schönbein

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23 **Running Title:** Volatile Organic Breath Compounds in Shock
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27 **Keywords:** Peritonitis shock, pancreatic digestive enzymes, breath, volatile organic compounds
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50 **Competing interests:** F.A.D., and G.W.S.S. own equity in Inflammagen, a company by Leading
51 Bioscience Inc., which develops therapy for shock patients.
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5 **Abstract**
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8 There is a need to develop markers for early detection of organ failure in shock that can be non-
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10 invasively measured at point of care. We explore here the use of volatile organic compounds
11
12 (VOCs) in expired air in a rat peritonitis shock model. Expired breath samples were collected into
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14 Tedlar gas bags and analyzed by standardized gas chromatography. The gas chromatograms
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16 were digitally analyzed for presence of peak amounts over a range of Kovach indices. Following
17
18 the induction of peritonitis, selected volatile compounds were detected within about one hour,
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20 which remained at elevated amounts over a 6 hours observation period. These VOCs were not
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22 present in control animals without peritonitis. Comparisons with know VOCs indicate that they
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24 include 1,4-diaminobutane and trimethylamine *N*-oxide. When pancreatic digestive proteases
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26 were blocked with tranexamic acid in the intestine and peritoneum, a procedure that serves to
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28 reduce organ failure in shock, the amounts of VOCs in the breath decreased spontaneously to
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30 control values without peritonitis. These results indicate that peritonitis shock is accompanied by
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32 development of volatile organic compounds that may be generated by digestive enzymes in the
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34 small intestine. VOCs may serve as indicators for detection of early forms of autodigestion by
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36 digestive proteases.
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Introduction

One of the major requirements for effective intervention in shock is early detection of signs for progression towards organ failure. There are a large number of blood indicators for shock which range from monitoring of small molecules, like blood oxygen values, to detection of elevated blood plasma values of lactate or inflammatory markers such as complements and cytokines (1-3).

Selected combinations of lower molecular weight (up to 1.5kD) metabolomic markers (e.g. organic acids, lipids, amino acids, carbohydrates, peptides, vitamins, steroids, xenobiotics) in exhaled breath condensates were proposed to characterize patients with shock and respiratory failure (4).

But there is a need to explore non-invasive early indicators for end organ damage that serve to minimize delay in diagnosis and facilitate interventions to attenuate progression into multi-organ failure.

Volatile organic compounds (VOCs) have been identified as possible approaches for non-invasive detection of several diseases, e.g. cancers, acute lung injury, tuberculosis, liver disease, myocardial infarction (5-11). VOCs include acetone, acetaldehyde, alkanes, amines, benzenes, pentane, sulfur compounds, and many others (12). After endotoxic administration in the rat, compounds like propanol, butanal, acetophenone, butandiol, hexanone, dimethylether, pentafluoropropionamide, xylene, propanol, toluene and others have been proposed to serve a multivariate diagnostic analysis of shock versus control animals (13). Alcohols and ketones (e.g. propanol, pentanone, acetone, hexanone) were reported to decrease over a six hour period in cecal ligation and endotoxin shock but not in hemorrhagic shock (14). In a study with Wiggers hemorrhagic shock 16 different VOCs changed in shock compared to controls, five of which had higher intensities, nine had lower intensities during hypotension and 9 hours thereafter, and two increased only during the hypotensive period (15). It is largely uncertain what mechanisms are

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4 involved in the formation of VOC and whether their detection may serve as a diagnostic tool to
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6 evaluate the effectiveness of therapy. This is the objective of the current report.
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10 Our increasing evidence suggests that pancreatic digestive enzymes in the small intestine and
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12 digestive fragments they produce may play a central role in the development of organ failure by
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14 escape from the lumen of the intestine after breakdown of the mucosal barrier (16). As digestive
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16 enzymes enter into the wall of the intestine, they break down the mucosal lining (17) and start an
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18 autodigestion process, which leads to multi-organ failure and death if uninterrupted (18). During
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20 this process not only the food content of the intestine, as is the case during normal digestion, but
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22 also autologous tissue is degraded to the point that morphological features associated with the
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24 intestinal mucosa are destroyed. The process starts at the tip of villi in the small intestine already
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26 within minutes of an ischemic insult (19) and can lead to complete destruction of the villi (20-23).
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33 Introduction of ischemia causes formation of VOCs detected by gas chromatography (24). But
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35 their source in the tissue remains uncertain. We hypothesize that in experimental peritonitis shock
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37 distinct VOCs are generated during proteolytic degradation of the small intestine and are
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39 detectable in the breath. Accordingly, we present measurements of VOCs before and after
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41 peritonitis shock without and with blockade of the pancreatic proteases in the lumen of the intestine.
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4 **Methods**
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8 Animals
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13 The animal protocol was reviewed and approved by the UC San Diego Animal Subjects Committee.
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15 Young mature Wistar rats (age 15 to 20 weeks, 350-450 grams body weight, Harlan Sprague
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17 Dawley) were maintained on a regular diet (Harlan Teklad Rodent diet (W) 8604, 0.29% sodium by
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19 weight) without restriction and water ad libitum. After general anesthesia (pentobarbital sodium, 50
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21 mg/kg, Abbott Laboratories, i.m.), the body temperature was maintained at 37°C by keeping the
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23 animals on a water-heated support and by use of a heat blanket. At the end of the six-hour
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25 observation period the animals were euthanized (120 mg/kg, Beuthanasia-D Special, Merk Animal
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27 Health).
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33 Peritonitis Shock
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38 In this shock model, cecal material (900 mg/kg derived from a mixture of multiple donor rats,
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40 diluted by addition of 1 ml of 5% glucose saline solution to 1 gm cecal material) was injected into
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42 the peritoneal cavity and gently spread by mild skin compression over the abdomen. Control
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44 animals without peritonitis were administered the glucose saline solution without cecal material.
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49 Digestive Enzyme Blockage
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54 The digestive enzymes were blocked at 60 min after cecal material administration as described
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56 (16). Briefly, to block digestive enzymes in the lumen of the intestine, a mid-line incision (2.5 cm)
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58 was made to temporarily expose the small intestine. Tranexamic acid (127 mM, Pfizer Inc.)
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60 dissolved in GoLYTELY (60 g/L PEG-3350 and electrolytes for oral solution; Braintree Laboratories,
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4 Inc) was injected with a needle (BD Sub-Q 26G 5/8, Becton Dickinson & Co) into the lumen of the
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6 intestine from the duodenum to the cecum. Untreated shock controls received GoLYTELY without
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8 the protease inhibitor. In a 350 gm rat a total fluid volume of 15 ml (at 39°C) was injected into the
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10 lumen of the small intestine spaced over 8 injections in intervals of about 5 cm and 2 ml was
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12 injected into the cecum. The intestine was returned into the peritoneal cavity and the incision
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14 closed by sutures.
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20 In addition to the enteral protease inhibition, a mixture was also administered as lavage into the
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22 peritoneum composed of tranexamic acid (2 ml; 127 mM dissolved in saline), an antibiotic
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24 (ciprofloxacin, 10 mg/ml, Hospira, Inc), and albumin (40 mg/ml, Sigma-Aldrich Co) at one hour
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26 after placement of cecal material. The mixture serves to inhibit not only digestive enzymes, but
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28 also bacteria and lipid cytotoxic mediators (e.g. unbound free fatty acids) that were administered
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30 by the cecal material into the peritoneum. The untreated peritonitis shock group received the same
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32 volume of saline into the peritoneum and the same volume of GoLYTELY into the lumen of the
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34 intestine but without additives. To determine the role of the enteral treatment in VOC generation,
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36 we also studied a group of shock rats (n = 3) with peritoneal treatment only (using the same
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38 mixture) but without enteral treatment.
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46 Breath Analysis

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51 To collect exhaled air, a tracheotomy was carried out for placement of a two-way non-rebreathing
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53 valve. Exhaled breath samples were collected in Tedlar gas sample bags (1 L, Jensen Inert, Coral
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55 Springs, Florida) placed at the end of the tracheostomy tube. A two-way non-rebreathing valve
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57 (series 2300 T-shape; Hans Rudolph, Inc., Shawnee, Kansas) was used for collection of exhaled
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59 air into gas sampling bags. Control breath samples were collected before laparotomy to block
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4 digestive enzymes. Room air samples were collected as controls for inspired air. Samples were
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6 collected in intervals of 30 min over the first hour and then hourly for 6 hours.
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10 The gas sample bags were kept at room temperature and analyzed within a period of two hours
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12 after collection. Repeat measurements of the same air sample over a period of seven days
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14 showed that there was no significant loss of VOCs in the bags, for example due to absorption to
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16 the Tedlar bags.
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22 The presence of VOCs was detected by use of gas chromatography (Z-Nose Ultra-Fast GC, Model
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24 4200, MicroSense 5.44 GC analysis software, Electronic Sensor Technology, Newbury Park, CA).
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26 The settings on the gas chromatography were standardized (sensor temperature 30°C, column
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28 temperature 40°C, valve temperature 140°C, inlet temperature 200°C, trap temperature 250°C,
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30 flow 3.0 ccm--helium carrier gas; sample time = 30 seconds; temperature ramp = 200°C at
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32 5°C/sec). Before measurements, the gas chromatography chamber was cleaned by heat (205 °C)
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34 running helium through the system in a continuous mode until a background was reached with little
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36 activity. For calibration of the Kovach index (KI), an alkane calibration (n-alkane vapors C6-C14;
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38 Electronic Sensor Technology) solution was tested five consecutive times. The chromatography
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40 profile was digitally scanned and peak amounts were determined with peak-detection software
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42 provided with the Z-nose. The values are expressed in form of “amount” (i.e. digital detector GC
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44 output).
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51 A list of 25 VOCs was tested with the same GC settings to determine their associated KI values
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53 (acetone; amine; n-butyl acetate; 2-butanone; t-butanol; butyraldehyde; benzaldehyde; cadaverine;
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55 carbon disulfide; chlorobenzene; dimethyl sulfide; 1,4-diaminobutane; 1,5-diaminopentane; 1,2-
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57 dichloroethane; ethanol; indole; limonene; 6-methyl-5-hepten-2-one; 4-methylphenol; pentanoic
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59 acid; 1-propanol; putrescine; methylamine N-oxide, TMAO; o-xylene). The list was derived from
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4 literature values for gastrointestinal, respiratory and cancer patients and associated in part with
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6 tissue decay (6, 11, 25-32). Each compound was dissolved in distilled water and the supernatant
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8 tested in the GC. The KI values for these compounds were compared with the KI values
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10 determined in the breath samples of the rats.
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20 Statistics

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23 All measurements are summarized as mean \pm standard deviation. Repeated measures ANOVA
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25 followed by Bonferroni post-hoc test was used to compare sham controls with untreated and
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27 treated shock groups. Comparison between treated and untreated groups was carried out by
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29 Student's t test. $P < 0.05$ was considered significant.
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39 **Results**

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45 The control breath samples had a gas chromatography profile that was similar among the control
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47 animals with relatively small variation among rats (typical variance about 10%). During peritonitis
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49 shock breath samples exhibited shifting peak amounts at distinct KI values, some of the peak
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51 amounts increased and some also decreased (Figure 1).
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4 Analysis of changes in peak amounts over a range of KI values between about 600 and 2000
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6 showed that the peak amounts at KI 720, 900, and 1550 in the breath (Figure 2) exhibited
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8 significant differences between sham control and shock. While in sham controls the peak amounts
9
10 did not rise significantly over the 6-hour period of the current experiments, in peritonitis shock the
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12 peak amounts at KI 720, KI 900 and KI 1550 monotonically and significantly rose. The rise
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14 occurred already within the first hour of the peritonitis and there was no trend for a reduction of
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16 peak amounts over the course of the experiment.
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24 In contrast, after the combined enteral/peritoneal treatment and blockade of the digestive enzymes
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26 with tranexamic acid at 1 hour, the peak amounts started to decrease and after 6 hours fell to
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28 values that were not different compared to non-shock controls (Figure 2). The decreases in peak
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30 amounts were only observed after treatment. The *enteral* treatment is essential for the reduction
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32 of the VOC amounts. Without enteral administration and only peritoneal treatment in shock
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34 animals, there was no reduction of the peak amounts and their values without significantly
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36 difference compared to the untreated peritonitis group (Figure 2, legend).
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44 Peak amounts at KI values, other than at 720, 900 and 1550 exhibited no significant changes in
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46 the current exhaled gas analysis, although several peak amounts reached close to significant
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48 values.
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55 Among the set of VOCs we tested (see Methods), 1,4-diaminobutane (Lot# STBC1987V, SIGMA-
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57 Aldrich Co.) had a KI at 720 ± 5 and trimethylamine *N*-oxide (TMAO, 317594, SIGMA-Aldrich Co.) a
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59 KI of 900 ± 5 . We could not find in this list a compound that had a peak amount at KI 1550. All
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4 other VOC peak amounts fell at least by a value of ± 50 outside the KI values observed in the
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6 breath samples. Both 1,4-diaminobutane and TMAO have a strong foul odor.
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10 11 12 **Discussion** 13

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15 The current results indicate that in experimental peritonitis the breath contains within less than an
16 hour elevated levels of VOCs over several hours that can be inhibited by enteral protease inhibition
17 in the small intestine, but not without it. These measurements are in line with qualitative
18 observations during a previous study with an identical peritonitis shock model (16) as well as other
19 forms of shock (including hemorrhagic and endotoxic shock) in which we noticed foul volatile odor
20 originating from the small intestine. Without exception (18 out of 18 rats examined) the odor was
21 noticed in those animals that succumb to the insult, but less so in surviving animals. In addition to
22 mortality the presence of the odor was accompanied by escape of digestive proteases into the wall
23 of the intestine, lesion formations in the small intestine and the lung. All animals in these studies
24 were maintained with food and water at libitum at the time before shock. Therefore the small
25 intestines were in part filled with food items so that the pancreatic digestive enzymes in the
26 partially digested food and/or the wall of the intestine could serve as a source of the VOCs (16).
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46 A combination of enteral and peritoneal placement of tranexamic acid, a serine protease inhibitor,
47 e.g. for pancreatic trypsin (17), served to reduce the VOC amounts. The protection provided by
48 inhibition of the digestive proteases in the intestine and peritoneum includes beside a reduction of
49 the mortality also a significant reduction of the lesions in the small intestine, heart and lung,
50 including a reduction of the protease activity in the wall of the small intestine and in plasma.
51 Inhibition of the pancreatic digestive enzymes in hemorrhagic shock also reduces the development
52 of insulin resistance by proteolytic cleavage of the ectodomain of the insulin receptor (33). This
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4 evidence suggests that the powerful digestive enzymes in the small intestine may be a major
5
6 producer for the VOCs that eventually appears in expired air. The VOCs appear to be carried in
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8 the plasma and diffuse into the airways.
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15 The VOCs found in this study could be detected without the use of a chemical concentrator during
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17 sample collection. They are in sufficient concentrations to be detectable by unaided human smell
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19 sensor. Among specific VOCs with equal KI values as seen in the breath samples were 1,4-
20
21 diaminobutane, TMAO and a volatile compound whose identity remains to be identified. TMAO is a
22
23 foul smelling compound with below body temperature melting points. It is a metabolite from dietary
24
25 phosphatidylcholine (e.g. lecithin) and can be generated by certain intestinal bacteria (e.g. by
26
27 generation of trimethylamine which can be transformed into TMAO) (34). Elevated levels of TMAO
28
29 have been associated with cardiovascular risk (25, 35).
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35 The mechanism by which the TXA protease inhibition blocks the formation of TMAO, as seen in
36
37 the current experiments, remains to be identified. TXA was administered enterally and by lavage
38
39 into the peritoneal cavity around the intestine. The fact that it serves to acutely reverse VOCs
40
41 formation indicates that their formation is ongoing and protease dependent. The fact that the
42
43 peritoneal without enteral blockade is inadequate to block the formation of the VOCs (Figure 2,
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45 legend) suggests that the digestive enzymes in the lumen of the small intestine are centrally
46
47 involved. A source for the odor may be food material in the lumen of the intestine, as seen in fecal
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49 material (12), but could also include intestine itself, since digestive enzymes enter the wall of the
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51 intestine in shock. TXA serves to preserve the mucosal barrier. If a source of the foul smelling
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53 VOCs is in the lumen of the intestine, then the preservation of the barrier may reduce entry of the
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55 VOC into the circulation and into the breath. If the source of the VOCs is in the wall of the intestine,
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57 the mode of action by TXA is to prevent proteolytic degradation of intestine and also other tissues
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4 into which digestive protease leak during peritonitis shock.
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9 TXA is recognized and marketed as an anti-fibrinolytic agent, able to block formation of plasmin
10 when administered i.v.. But its actions as protease inhibitor appear to be broader. In the current
11 application it was used to block serine proteases (e.g. trypsin) in the lumen of the small intestine
12 without i.v. infusion. Enteral blockade of digestive enzymes serves to attenuate a variety of tissue
13 injuries, organ dysfunctions and failures in multiple models of shock (16, 33), and thus the current
14 observation for attenuation of VOCs is in line with the evidence for other forms of protection from
15 digestive enzymes.
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26 We consistently smelled VOCs from the intestine in non-surviving animals that were subject to
27 hemorrhagic shock, a model without the use of cecal material with bacteria or an antibiotic agent in
28 the peritoneum. While measurements of VOCs with and without antibiotic treatment (as compared
29 to enteral protease inhibition) in shock remain to be carried out, this evidence suggests that VOCs
30 can be formed without a major bacterial load.
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41 Key questions to be investigated are whether VOCs in shock patients detected by an electronic
42 breath sensor are similar to those seen in experimental models and if present whether they serve
43 to predict impending organ failure before it reaches an irreversible stage associated with actual
44 organ failure. The current evidence indicates that VOC generation is an early event in a peritonitis
45 induced shock model but the amounts detected in the breath may depend on the extent of
46 intestinal damage. In the current model the major length of the small intestine wall tissue is subject
47 to infiltration by digestive enzymes (16) and a potential source for VOCs. If in other situations
48 shorter segments of the small intestine become ischemic and are infiltrated by digestive enzymes
49 the magnitude of the VOCs detected in the breath may be lower. This situation in ICU patients is
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largely unknown and will require clinical measurements from the time of earliest access to breath samples.

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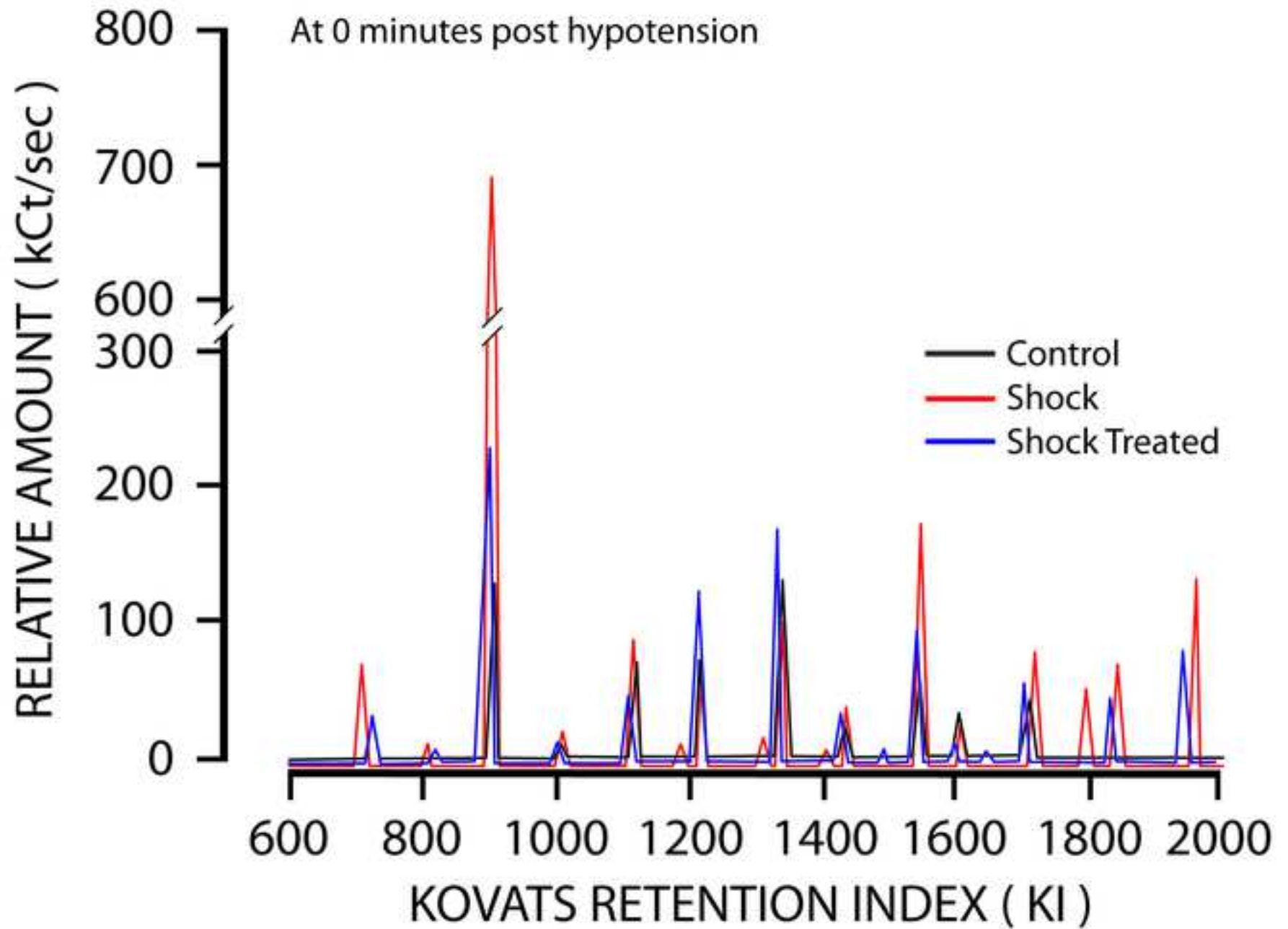
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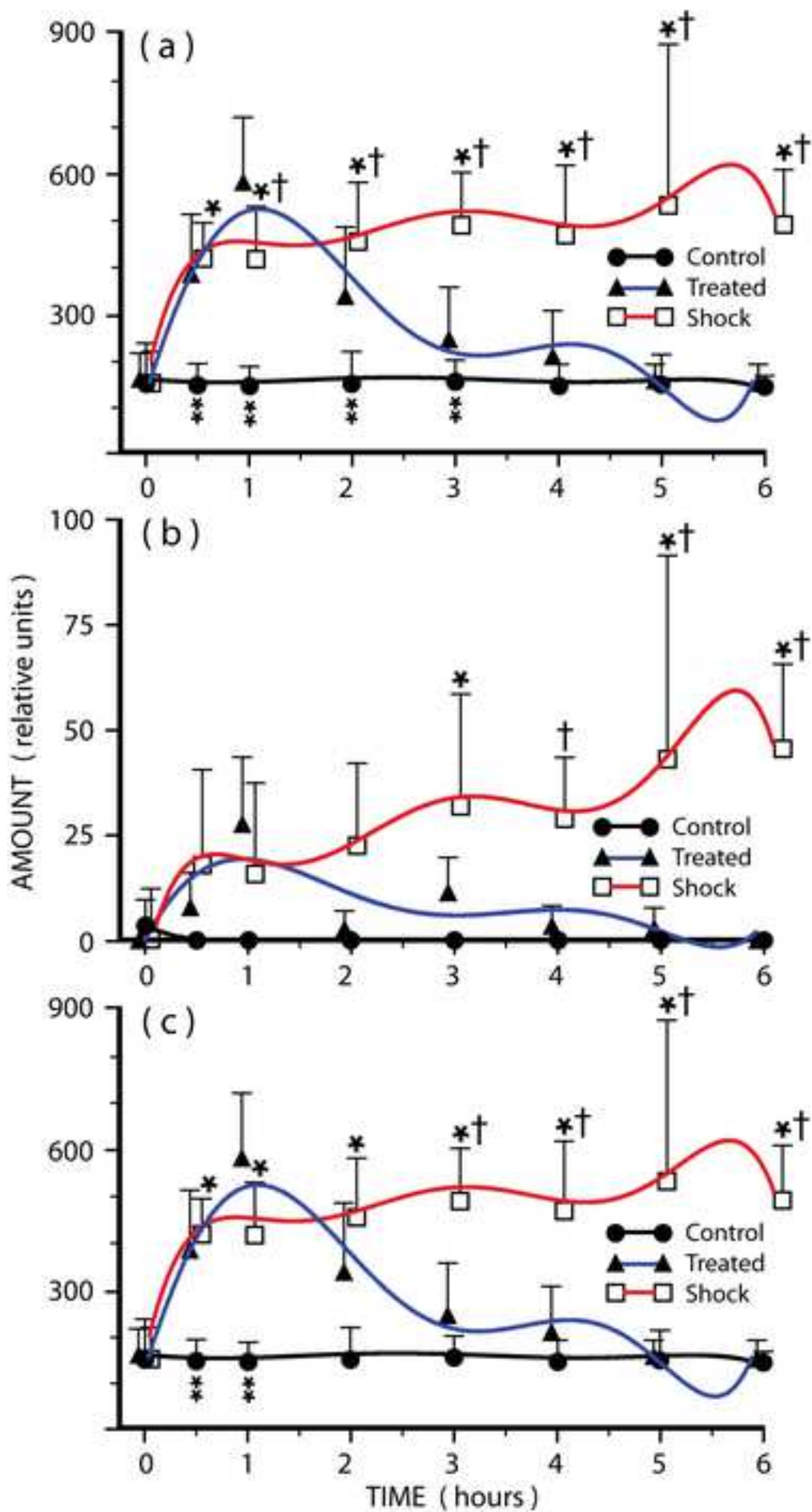
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4 **Figure Legends**
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11 **Figure 1.** An example of GC profile for control without peritonitis (Control group), untreated
12 peritonitis (Shock group) and peritonitis with treatment by inhibition of the digestive proteases with
13 TXA (Shock *Treated*, see Methods). Note elevation of peak amounts, e.g. at KI 720, 900, and
14 1550.
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27 **Figure 2:** The amounts of VOCs at KI (a) 720, (b) 900, and (c) 1550 measured over a period 6
28 hours from rat breath samples after exposure to peritonitis shock without (Shock group) and with
29 enteral protease inhibition with TXA (Treated group) at 1 hour after initiation of the peritonitis.
30 Control represents a sham group without peritonitis. n = 6 to 8 rats in each group. Amounts of
31 VOCs in a separate group (n = 3 rats) with peritonitis shock but with treatment in the peritoneum
32 only (with same mixture, see Methods) and without enteral treatment gave values that were not
33 significantly different compared to the untreated Shock group. At 6 hours the amounts of VOCs in
34 this group with peritoneal treatment only at KI 720, 900, and 1550 were 315 ± 43, 117 ± 34, 483 ±
35 61, respectively. *p<0.05 Control vs Shock group; †p<0.05 Shock vs Treated group; *p<0.05
36 Control vs Treated group.
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NOTE: If the "Gold" route has been selected, Section 3.b. of the Agreement will apply to the Work, and neither Section 3.a. nor Section 3.c. of the Agreement will apply to the Work. If the "Green" route has been selected, Section 3.c. of the Agreement will apply to the Work after an embargo, and neither Section 3.a. nor Section 3.b. of the Agreement will apply to the Work.

3. This Schedule B is inapplicable to the Work.

NOTE: If author has selected Item 3, Section 3.a. on the Agreement will apply to the Work, and neither Section 3.b. nor Section 3.c. of the Agreement will apply to the Work.

GOVERNMENT EMPLOYEES

4. This work was created in the course of an author's employment by the United States Government

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NOTE: If author has selected Item 4, Section 3. on the Agreement will not apply to the Work.

SIGNATURE PAGE

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