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TITLE: **COMBAT - A Prospective, Randomized Investigation of "Plasma First Resuscitation" for Traumatic Hemorrhage and Attenuation of Acute Coagulopathy of Trauma**

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14. ABSTRACT The COMBAT (Control of Major Bleeding After Trauma) study is a randomized clinical trial evaluating the early administration of plasma, compared to the standard crystalloid. Over the past year, we have worked on refining our procedures and workflows, collecting accurate and timely data, ensuring regulatory compliance, and communicating with all study team personnel at Denver Health and the University of Colorado Anschutz Medical Campus. We enrolled a total of 50 patients and collected over 6,000 samples this reporting year in addition to submitting one annual continuing review and publishing several papers.					
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[SF298] / Abstract

The COMBAT (Control of Major Bleeding After Trauma) study is a randomized clinical trial evaluating the early administration of plasma, compared to the standard crystalloid. Over the past year, we have worked on refining our procedures and workflows, collecting accurate and timely data, ensuring regulatory compliance, and communicating with all study team personnel at Denver Health and the University of Colorado Anschutz Medical Campus. We enrolled a total of 50 patients and collected over 6,000 samples this reporting year in addition to submitting one annual continuing review and publishing several papers.

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A. INTRODUCTION:

Bleeding is the most preventable cause of death in trauma patients. Coagulopathy has been documented in up to one third of trauma patients upon arrival to the emergency department. The mechanism of trauma induced coagulopathy (TIC) has yet to be elucidated. Presumptive early administration of plasma has been suggested to improved outcomes in observational studies, but no randomized clinical trial has been conducted to date comparing the administration of early plasma to the current standard crystalloid. In this research study, trauma patients who meet eligibility criteria, defined as field systolic blood pressure (SBP) ≤ 70 mmHg or 71-90mmHg with HR ≥ 108 bpm, will be randomized to receive plasma or intravenous crystalloid as the initial resuscitation fluid. Our hypothesis is that the administration of plasma early will attenuate TIC, leading to improved patient outcomes.

B. KEYWORDS:

Trauma; coagulopathy; trauma-induced coagulopathy; plasma; resuscitation

C. OVERALL PROJECT SUMMARY:

A. Sample Processing and Study Procedures

In July 2014, we began refining our operating procedures subsequent to the implementation of the study in April 2014. This past year, we have made minor changes to blood sampling methodologies and processes in order to make the most use of our time and resources.

B. Patient Enrollment and Sample Procurement

This quarter alone, we enrolled 17 patients and collected 2,703 plasma samples. Throughout the reporting year, we enrolled 49 patients and collected a total of 6,478 plasma samples (Table 1).

Table 1: Summary by Quarter, Year, and Total

Quarter	# of Patients Enrolled	# of Samples
July 2014 – September 2014	11	1717
October 2014 – December 2014	12	1533
January 2014 – March 2015	9	833
April 2015 – June 2015	17	2395
Total for Reporting Year	49	6478
Total Since Study Start	57	7306

During this reporting year, we sent 420 samples to the Trans-Agency for Trauma-Induced Coagulopathy (TACTIC). We also sent 278 samples to the Children's Hospital of Colorado for clotting analysis during the reporting year, including the 33 sent during the April – June 2015 quarter.

Also, a total of 66 samples were piloted to refine our proteomics and metabolomics research techniques.

C. Unanticipated Problems and Patient Withdrawals

During the reporting year, one patient was withdrawn from the study before any data was collected because the patient was transported to a hospital other than Denver Health. All appropriate regulatory agencies were notified, and our team worked with paramedic staff to ensure that appropriate measures are taken in the future should a similar situation arise.

Additionally, a total of three unanticipated problems arose during this reporting year, including one during the April-June 2015 quarter. We communicated with the COMIRB immediately after encountering each problem, and we submitted all documentation to each appropriate regulatory agency in a timely manner.

Due to the delay in initiating patient recruitment in April 2014 (as opposed to projected January 2013), we will request an extension to complete 3-year targeted enrollment by April 2017.

D. Paramedic Training and Continuing Education

During the April-June 2015 quarter, 20 paramedics were hired by the Denver Paramedic Division. Each new hire was trained for patient enrollment, study procedures, and other key information by a member of our study team. This reporting year, our team trained a 69 new paramedics and EMTs. Since the study began, we have trained approximately 287 paramedics and EMTs.

During the April-June 2015 quarter, we held a reception ceremony for paramedics to thank them for their hard work throughout the past year of study enrollment. We also used this as a time to provide continuing education with study procedures and materials, and we were available to answer questions from paramedics.

Throughout the reporting year, our study team is continuously trained on new procedures and consistently adapts to various situations as they arise.

E. Study Devices and Equipment

Quarterly maintenance continues to be performed on all our team's freezers by the contracted company, Tolin Mechanical. These freezers store FFP, cooler bottles, and banked plasma samples. Daily quality control checks are performed on the freezers to ensure study materials are functioning properly.

The ambulance fleet has been expanded by 5 units. We must outfit these ambulances, so as to not disrupt study design and randomization.

F. Problems/Issues

During the past year, we have encountered various logistical problems with study procedures and equipment. Each of these is addressed on a regular basis and in a timely manner, as described below:

- a. We have seen a decrease in the frequency of leaking Plasmatherm plasma-thawing device bags, attributed to the improved design of the bags.
- b. In our effort to resolve problems adequately, we sent 4 Plasmatherm devices to Germany for repairs in September 2014. While the devices were on their way back to Denver, they were held up at Customs, due to a clerical error. This was resolved in December 2014, and we have since put all devices back into the ambulance fleet. .

- c. Miscellaneous electrical issues, which are common to the Paramedic Garage and not study-specific, continue to cause ambulance fleet problems. As these electrical problems arise, they are dealt with as quickly as possible. We continue to work closely with the Paramedic Division to address any issues and modify procedures, as necessary.
- d. In certain cases the follow-up of the patients presents challenges (the absence of permanent addresses, disconnected telephone, etc.).
- e. Multiple problems with Thermo brand freezers were encountered during this reporting year. After actively communicating with service technicians and manufacturers to repair them when needed, we ultimately resolved this issue by purchasing new Helmer brand freezers to replace them.
- f. Several of our Plasmatherm units have had illegible faceplate screens. We believe this to be a result of the increased humidity in the area over the past few months. We are continuously monitoring this issue and ensuring that only units with legible screens are in service.

D. KEY RESEARCH ACCOMPLISHMENTS:

A. Regulatory Amendment Submissions, Continuing Reviews, and Protocol Modifications

- This reporting year, we submitted three amendments to further refine our study and to adapt to new situations regarding patient enrollment, data collection, and sample procurement. We also submitted our second annual continuing review. See Table 2 for more details.

Table 2: Regulatory Submissions

Quarter	Submission Type	Reason/Notes
July-September 2014	Amendment	To add Attachment K, allowing patients who are on police holds after enrollment to remain in the study
October-December 2014	Amendment	To add a Spanish version of the consent form, to update the protocol to include procedures for when multiple patients are enrolled at the same time, and to make personnel changes
April-June 2015	. Continuing Review . Amendment	a. Submitted and approved by COMIRB for study continuation b. To allow our team to utilize the “Short Form” when consenting patients who speak a language other than English or Spanish and to make personnel changes

B. Study Devices and Equipment

- April – June 2015 Quarter

- An HVAC system was installed in the Paramedic Garage to help offset the heat given out by the study freezers. We have paid for this unanticipated problem from our own funds (\$38,000).
- We purchased 3 new Helmer iSeries freezers to swap the Thermo brand freezers with after experiencing numerous complications with the Thermo freezers since the study began and as reported in the 10th Quarterly Technical Progress Report.

- We installed metal shelf-guides in each freezer to help organize cooler bottles and to better space them in each freezer to ensure adequate circulation of cold air
- The TC-Max software, utilized by the Vehicle Service Technicians in the Paramedic Garage, is now used by our team to track study cassettes. This system more efficiently helps our team know which units are expiring soon, which units were transfused, and where each unit is at any time.
- Several new ambulances were added to the Denver Health fleet; each ambulance was configured for study enrollment with the appropriate equipment.

- **Reporting Year**

- The manufacturer of the plasma thawing device improved the design of the plastic cushion bags based on field testing results and feedback from the study team.
- We established and modified procedures for operation and patient enrollment, including: Tracking plasma in the ambulance; notifying the hospital Blood Bank when a patient is enrolled in the experimental group; maintaining equipment; tracking specimens; checking freezers; performing quality controls at regular intervals. Each of these operating procedures has enabled our team to be consistent and has helped to minimize the occurrence of complications and problems.
- An agreement with Tolin Mechanical was signed for all service, quarterly maintenance, and repairs concerning our freezer units

C. Data Management

- REDCap data collection system is continuously being revised and improved to assist our team in collecting timely and accurate data.

D. Other Accomplishments

- Our original budget was for four Research Assistants. However, in order to maximize productivity and patient data capture, we have recruited three additional Research Assistants using non-DOD funds. The last individual was hired in July 2015.
- Personnel from the Department of Defense (Dr. Col. Anthony Pusateri) informally visited Denver Health and were shown our operating procedures and our facilities in June 2015. Other visitors included Dr. Ken Mann (PI: DOD-NHLBI consortium, TACTIC) and Dr. Irshad Chaudry (external reviewer TACTIC).
- Several journal articles were published (see Section 6)
- The Thermo freezers were found wanting and replaced by Helmer freezers
- We updated the HVAC system in the Paramedic Garage
- All Plasmatherm units passed their annual TSI tests, which were completed by Denver Health's Biomed Department during this reporting year
- We installed equipment in 2 new Denver Health ambulances, and we are in the process of obtaining supplies to complete installation of equipment into two other new ambulances.

E. CONCLUSION:

We continue to enroll patients in the study and are constantly adapting. We anticipate additional problems and/or issues to arise, but we plan to address them in a timely fashion.

Over the next few weeks, we will complete our study's interim analysis, following the enrollment of our 50th patient. We will also submit an annual report to the FDA in September, and we are working on an amendment that refines our data sharing agreement with the Trans-Agency for Trauma-Induced Coagulopathy.

We plan on training our new research personnel in the coming months as well as providing paramedics opportunities for continuing education study refresher sessions.

Due to the new and improved freezers purchased, we will be increasing the number of coolers and plasma/placebo units that go out onto the streets.

F. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

(1) Peer-Reviewed Scientific Journals:

Chapman, MP, Moore, EE, Chin, TL, Ghasabyan, A, Chandler, J, Gonzalez, E, Moore, HB, Banerjee, A, Silliman, CC, and Sauaia, A: COMBAT-Initial Experience with our Randomized Clinical Trial of Plasma-based Resuscitation in the Field for Trauma with Hemorrhagic Shock. Shock. 2015 Aug;44 Suppl 1:63-70. doi: 10.1097/SHK.0000000000000376.

D'Alessandro, A, Hansen, KC, Silliman, CC, Moore, EE, Kelber, M, and Banerjee, A: Metabolomics of AS-5 RBC Supernatants following Routine Storage. Vox Sang. 2015 Feb;108(2):131-40. doi: 10.1111/vox.12193. Epub 2014 Sep 9.

D'Alessandro, A, Moore, HB, Moore, EE, Wither, M, Gonzalez, E, Slaughter, AL, Hansen, K, Silliman, CC, and Banerjee, A: Early Hemorrhage Triggers Metabolic Responses that Build Up During Prolonged Shock. Am J Physiol Regul Integr Comp Physiol. 2015 Jun 15;308(12):R1034-44. doi: 10.1152/ajpregu.00030.2015. Epub 2015 Apr 15.

D'Alessandro, A, Travis, N, Kelher, M, West, B, Banerjee, A, Moore, EE, Silliman, CC, and Hansen, K: Routine Storage of Red Blood Cells in Additive Solution -3 -A Comprehensive Investigation of the RBC Metabolome. Transfusion. 2015 Jun;55(6):1155-68. doi: 10.1111/trf.12975. Epub 2014 Dec 30.

Moore, EE, Moore, HB, Gonzalez, E, Chapman, MP, Hansen, KC, Sauaia, A, Silliman, CC, and Banerjee, A: Postinjury Fibrinolysis Shutdown—Rationale for Selective Tranexamic Acid. J Trauma Acute Care Surg. 2015 Jun;78(6 Suppl 1):S65-9. doi: 10.1097/TA.

Peltz, ED, D'Alessandro, A, Moore, EE, Chin, TL, Silliman, CC, Sauaia, A, Hansen, KC: and Banerjee, A. Pathologic Metabolism: An Exploratory Study of the Plasma Metabolome of Critical Injury. J Trauma Acute Care Surg. 2015 Apr;78(4):742-51. doi: 10.1097/TA.0000000000000589.

(2) Presentations

Chin, TN, Moore, EE, Coors, M, Chandler, JG, Harr, JN, Ramos, C, Ammons, S, Banerjee, A, and Sauaia, A: Exploring Ethical Conflicts in Emergency Trauma Research- The COMBAT Study Experience (Presented at the Society of University Surgeons, New Orleans, LA, February, 2013). Surgery. 157:10, 2015

Moore, HB, Moore, EE, Gonzalez, E, Chapman, MP, Chin, T, Banerjee, A, Sauaia, A, and

Silliman, CC: Plasma is a Physiologic Buffer of tPA Mediated Fibrinolysis: Rationale for Plasma First Resuscitation after Life Threatening Hemorrhage. (Presented at the Surgical Forum of the American College of Surgeons, San Francisco, CA, October, 2014). JAm Coli Surg. 220: 872, 2015

Moore, HB, Moore, EE, Gonzalez, E, Hanson, KC, Chapman, M, Sauaia, A, West, B, Banerjee, A, and Silliman, CC: Hemolysis Exacerbates Hyperfibrinolysis while Plateletolysis Shuts down Fibrinolysis- Evolving Concepts of the Spectrum of Fibrinolysis in Response to Severe Injury (Presented as a Young Investigator Award at the Shock Society, Charlotte, NC, June 2014). Shock. 43: 39, 2015

Moore, HB, Moore, EE, Lawson, P, Gonzalez, E, Fragoso, M, Morton, A, Gamboni, F, Chapman, MP, Sauaia, A, Banerjee, A, and Silliman, CC: Fibrinolysis Shutdown Phenotype Masks Changes in Rodent Coagulation in Tissue Injury versus Hemorrhagic Shock (Presented at the Society of University Surgeons, Las Vegas, NV, February 2015). Surgery. 158: 386-92, 2015

Wiener, GD, Moore, HB, Moore, EE, Lawson, P, Gonzalez, E, Morton, AP, Sauaia, A, Banerjee, A, and Silliman, CC: Shock Releases Bile Acids Inducing Platelet Inhibition and Fibrinolysis. (Best Trainee Manuscript, Presented at the Academic Surgical Congress, Las Vegas, NV, February 2015). J. Surg Res. 15: 195, 2015

G. INVENTIONS, PATENTS AND LICENSES: Nothing to report

H. REPORTABLE OUTCOMES:

- **Reporting Year**

- The entire Denver Health ambulance fleet is equipped with Plasmatherm plasma-thawing devices. The devices are fully operational and regularly maintained.
- Larger surface-area bags that were designed to be rapidly thawed have been performing well in the field this reporting year
- Plasmatherm TSI procedures (required by the manufacturer in order to maintain our warranty) were performed by Denver Health's Biomed department. The procedures were completed for all of our devices.
- We worked to modify check-in and check-out procedures of plasma coolers in order to further minimize the time a cassette unit is out of the freezer

I. OTHER ACHIEVEMENTS: Nothing to report **J. REFERENCES:** Not applicable

K. APPENDICES: Appendices, copies of publications, will be mailed to Mr. Malloy in CD

format

COMBAT: INITIAL EXPERIENCE WITH A RANDOMIZED CLINICAL TRIAL OF PLASMA-BASED RESUSCITATION IN THE FIELD FOR TRAUMATIC HEMORRHAGIC SHOCK

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ABSTRACT—The existing evidence shows great promise for plasma as the first resuscitation fluid in both civilian and military trauma. We embarked on the Control of Major Bleeding After Trauma (COMBAT) trial with the support of the Department of Defense to determine if plasma-first resuscitation yields hemostatic and survival benefits. The methodology of the COMBAT study represents not only 3 years of development work but also the integration of nearly two decades of technical experience with the design and implementation of other clinical trials and studies. Herein, we describe the key features of the study design, critical personnel and infrastructural elements, and key innovations. We will also briefly outline the systems engineering challenges entailed by this study. The COMBAT trial is a randomized, placebo-controlled, semiblinded, prospective, phase IIB clinical trial conducted in a ground ambulance fleet based at a level I trauma center and part of a multicenter collaboration. The primary objective of the COMBAT trial is to determine the efficacy of field resuscitation with plasma first compared with standard of care (normal saline). To date, we have enrolled 30 subjects in the COMBAT study. The ability to achieve intervention with a hemostatic resuscitation agent in the closest possible temporal proximity to injury is critical and represents an opportunity to forestall the evolution of the “bloody vicious cycle.” Thus, the COMBAT model for deploying plasma in first-response units should serve as a model for randomized clinical trials of other hemostatic resuscitative agents.

KEYWORDS—Trauma-induced coagulopathy, plasma-first resuscitation, hemostatic resuscitation, COMBAT, randomized clinical trial, trauma, hemorrhage, hemorrhagic shock, plasma, FFP, FP24

INTRODUCTION

Trauma-induced coagulopathy (TIC) is present in up to one third of severely injured civilian trauma patients and is associated with a 4-fold increase in risk of mortality when present (1–7). Today, “hemostatic resuscitation” and “damage control” are watchwords for the trauma community, and the history of hemostatic resuscitation dates back at least as far as the use of plasma as a resuscitation fluid in World War II. Nevertheless, our understanding of how to intervene effectively with the massively bleeding trauma patient remains incomplete (8, 9). This understanding has taken numerous turns during the intervening decades since the 1940s. A key milestone was the recognition of the “bloody vicious cycle” of hemorrhagic shock,

hypothermia, acidosis, and coagulopathy (10, 11). This realization spawned the recommendation for empiric resuscitation with fixed ratios of plasma to packed red blood cells (PRBCs) as early as 1981 (12). Efforts since then have focused on early intervention with the intent to restore hemostasis chiefly with empiric use of various ratios of blood components.

The military experience with empiric resuscitation has strongly suggested that the administration of a 1:1 ratio of plasma to PRBCs yields a survival benefit (13–16). However, data from civilian trauma centers in the United States suggest that a ratio of 1:2 may be more beneficial in this setting and indeed highlight the potential dangers of excessive plasma use (17–23). Complicating the interpretation of the existing military and civilian studies of empiric plasma resuscitation strategies are the patients’ demographic dissimilarities, the differences in available interventions in terms of both blood products and transport time, and the necessary differences in study designs between urban trauma centers and battlefield environments (15, 16, 23, 24). Therefore, according the PICO (patients, interventions, comparators, and outcomes) format for applying the evidence of a study to care of one’s own patients, we remain uncertain as to how to interpret the existing studies of early plasma resuscitation (25, 26).

In addition, most existing studies are complicated by survivor bias, as most are retrospective (18, 27, 28). As Snyder et al. note of their own 2009 study of blood product ratios: “The nonsurvivors in our study population did not die because they got a lower plasma:PRBC ratio; they got a lower ratio because they died” (29, 30). Moreover, as most of the resuscitative efforts with severely injured patients have either succeeded or

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failed by 3 to 6 h after injury, most studies lack the detailed information about the early events in the patient's resuscitation to draw reliable conclusions about causality. Our recent study of goal-directed resuscitation using thrombelastography (TEG) highlights this difficulty: patients in this study were enrolled based on the criteria of initiation of a massive transfusion protocol and, by this time, valuable information about their physiologic and hematologic states from the time of injury onward was lost.

These confounders of existing studies notwithstanding, in aggregate, the existing evidence shows great promise for plasma as the first resuscitation fluid in both civilian and military trauma. We embarked on the Control of Major Bleeding After Trauma (COMBAT) trial with the support of the Department of Defense (DoD) to determine definitively if plasma-first resuscitation yields survival and hemostatic benefits. The considerations enumerated above prompted us to radically alter the approach to this clinical trial compared with previous ones we have engaged in. Our experience in the Polyheme trial gave us the necessary background to conduct a trial of intervention with blood products in the field using our ground ambulance fleet (31). This ability enables us to deliver plasma at the earliest possible time after injury, thus maximizing the potential benefit. Development of our Multiple Organ Failure database project during the past 22 years has given us experience in collecting and processing enormous amounts of patient data such as the COMBAT trial will generate (32). Lastly, our recent experience with our trial of TEG-guided resuscitation has built a team of personnel adept at the collection and handling of patient samples and data at numerous tightly clustered time points. Although the COMBAT trial goes beyond all of these studies in terms of the level of effort and detail required, they laid the essential groundwork.

MATERIALS AND METHODS

The methodology of the COMBAT study represents not only 3 years of development work but also the integration of nearly two decades of technical experience with the design and implementation of other clinical trials and studies. The detailed methods and procedures of the COMBAT trial comprise several hundred pages of standard operating procedures. Herein, we will attempt only to describe the key features of the study design, critical personnel and infrastructural elements, and key innovations. We will also briefly outline the systems engineering challenges entailed by this study.

Study design

The COMBAT trial is a randomized, placebo-controlled, semiblinded, prospective, phase IIB clinical trial based at Denver Health Medical Center's (DHMC) level I trauma center, with enrollment and interventions conducted in the ground ambulance fleet based at this center. The COMBAT study in Denver is one component of a larger DoD-funded multicenter trial of plasma-first resuscitation, including the University of Pittsburgh (principal investigator, Jason L. Sperry, MD) and Virginia Commonwealth University (principal investigator, Bruce D. Spiess, MD). Notably, the COMBAT study is the only center using a frozen plasma product, thawed at the scene of injury. Data from the multicenter study are also used by the Trans Agency Consortium for Trauma-Induced Coagulopathy (TACTIC) collaboration (principal investigator, Kenneth G. Mann, PhD), a large National Institutes of Health-supported collaborative effort with aims focused on elucidating the basic mechanisms driving TIC.

The primary objective of the COMBAT trial is to determine the efficacy of field resuscitation with plasma first compared with standard of care (normal saline). The primary end point is 28-day mortality, for which the study is powered to detect large differences (17% – 19%) between arms. Secondary end points include improvement in immediately postinjury coagulopathy and clot strength, markers of physiologic exhaustion (e.g., acidosis) and reduction in multiple organ failure and 24-h mortality. Projected enrollment is 150 individuals (75 per arm) at a rate of 50 per year.

Eligibility is determined at the scene of injury by the responding paramedics of the ground ambulance crew. Inclusion criteria are all traumatically injured adults with either a systolic blood pressure (SBP) of 70 mmHg or less or 71 to 90 mmHg, with an accompanying heart rate of 108 or more. Prisoners and visibly pregnant patients are excluded, as are those with isolated gunshot wounds to the head or requiring cardiopulmonary resuscitation in the field before enrollment. Patients may be enrolled at any time during their ambulance transport, but infusion of more than 800 mL of crystalloid before meeting criteria (e.g., in an initially normotensive patient who then decompensates and meets criteria) results in exclusion. The criteria are based on those developed by the Resuscitation Outcomes Consortium for their similarly structured trial of hypertonic saline as an initial resuscitation fluid (33). Based on pilot studies, the Resuscitation Outcomes Consortium group determined that these vital sign criteria captured the patient population most likely to be in hemorrhagic shock and thus with the greatest likelihood to benefit from the experimental intervention. Liberalizing the criteria to all patients with an SBP of 90 mmHg or less would only have increased enrollment by 25% while exposing many more minimally injured patients to the risks of the study intervention (34, 35). In the case of the COMBAT trial, the need for conservative enrollment criteria is even greater, as AB plasma is a scarce medical resource and the potential hazards of exposure to this therapy are greater than those of hypertonic saline.

Patients are enrolled under a waiver of informed consent, as permitted under US Federal Regulation 21 CFR 50.24. To comply with this regulation, the study was conducted after a process of community consent, wherein the local community has been informed via multiple media outlets and has been provided with the option to opt out by wearing a "No COMBAT" bracelet or dog tags. In addition, the waiver of consent element of the study design required that the study be conducted under US Food and Drug Administration (FDA) regulation as an Investigational New Drug study (IND no. 15216). The ethical and practical considerations involved in implementing a waiver of consent in the COMBAT trial are discussed in detail elsewhere (36).

The field blood sample is drawn immediately on enrollment before the administration of any therapy. Patients are then randomized by the act of opening a preloaded and sealed cooler that contains either two units of frozen plasma (FP24, ~250 mL each), which is immediately thawed and administered; or a dummy load, which prompts the paramedics to administer normal saline per the current standard of care. Plasma and dummy payloads are block randomized in lots of 20. These payloads are delivered to paramedic division staff in sealed aluminum cassettes by study personnel uninvolved in enrollment and data analysis, thus maintaining allocation concealment.

Thawing and administration of the first unit of plasma generally take a total 6 to 7 minutes, by which time the second unit is thawed and hanging. Generally, this means that patients in the plasma arm arrive at the emergency department (ED) with the second unit running and having received less than 250 mL (frequently zero) of crystalloid in the field. Patients in the control arm generally receive a comparable volume of normal saline, which, given our short transport times, is usually less than 800 mL, thus maintaining equipoise with respect to total prehospital volume of resuscitation. Blinding is partial, with the paramedics blinded to therapy (i.e., cooler contents) until after enrollment. Whereas we were unable to blind the admitting physician completely by using a placebo that resembles plasma (colorants in intravenous solutions are forbidden by the FDA), we have generally found that the "controlled chaos" of a trauma activation is sufficient to obscure the nature of the field therapy, as the paramedics do not report this information to the receiving staff. On arrival at the hospital, all study interventions are complete and the patient's ongoing care proceeds with all hospital staff blind to the field intervention (plasma or saline).

A key design feature of the COMBAT study is tightly clustered early time points for data and sample collection, again with the aims of removing survivor bias and detecting the most proximal effects of the study intervention before the effect is diluted by subsequent stochastic factors. Immediately on hospital arrival, collection of the next set of blood samples occurs, followed by samples at 2, 4, 6, 12, 24, 48, and 72 h and days 5 and 7 after injury (Fig. 1). Patients are followed for outcomes for 28 days or until discharged. This early granularity, coupled with long-term follow-up allows us to obtain a complete picture of the patients' clinical course as impacted by early intervention. Moreover, because most of the expected deaths and other measurable study objectives are expected to occur within the first 6 h, highly detailed analysis of the patients' physiologic and coagulation status within this period is critical to achieving its scientific aims.

Study metrics

In addition to the primary and secondary end points previously described, the COMBAT study is designed to capture detailed biochemical and coagulation data at each of the 11 time points, with the aim of describing the molecular natural history of TIC and the mechanism of response to plasma-first resuscitation therapy. At each time point, whole blood undergoes a battery of

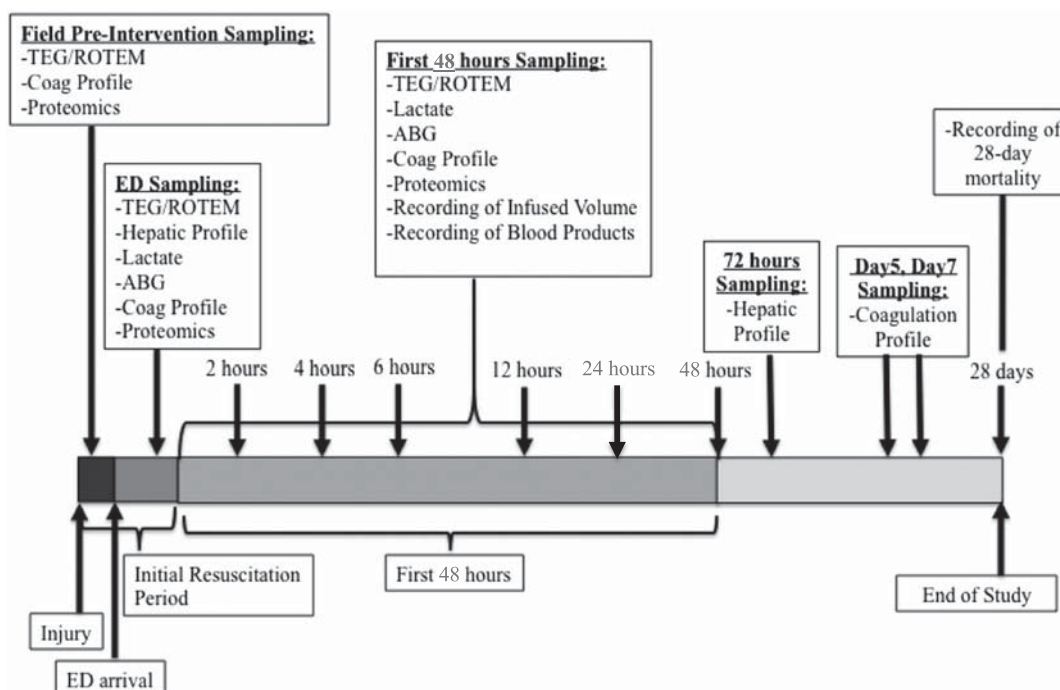


FIG. 1. **COMBAT biological sampling timeline.** Samples are drawn at 11 time points, starting in the field, immediately before randomization and the administration of the initial resuscitative fluids (FP24 or normal saline). Subsequent samples are drawn at ED arrival and hours 2, 4, 6, 12, 24, and 48 and days 5 and 7 after injury. The samples comprise a comprehensive battery of coagulation and other biochemical assays detailed in Figure 2 and are typical for each time point, with the exception of the field sample that lacks a lactate measurement (because of sample handling constraints) and the addition of a hepatic function panel at the 72-h time point. At 28 days after injury, mortality, complications, and other outcomes data are finalized.

12 channels of viscoelastic hemostatic assays on the TEG and ROTEM platforms, including TEG Platelet Mapping. In addition, whole-blood impedance aggregometry is performed with adenosine diphosphate and TRAP (thrombin receptor activating peptide) agonists on the ROTEM platelet platform. The remaining blood is immediately chilled, centrifuged to yield platelet-free plasma, and flash frozen in liquid nitrogen. These plasma samples are used for coagulation factor level tests (II, V, VII, X, and XIII), cytokine and chemokine panels, proteomics, metabolomics, and enzyme-linked immunosorbent assays for proteins of specific interest relevant to TIC, such as tissue plasminogen activator, plasminogen activator inhibitor-1, syndecan, and α -enolase, among others. An additional citrated blood tube and EDTA tube go to the DHMC central clinical laboratory for a complete blood cell count and conventional coagulation tests (prothrombin time, partial thromboplastin time, von Clauss fibrinogen level, and D-dimer level). A blood tube containing protease inhibitors is also collected and immediately chilled and centrifuged to yield plasma that, along with aliquots from the other tubes, is contributed to the TACTIC collaboration. The detailed sample processing procedure is described in schematic in Figure 2.

Statistical methods

The COMBAT trial is a phase IIB trial and is powered to detect large (17%–19%) differences in mortality or other binary outcomes (e.g., massive transfusion) between the two groups using Fisher exact test. Power analyses for the primary and secondary end points were performed with PASS 11 (PASS 11, NCSS, LLC; Kaysville, Utah). The study is adequately powered to detect small differences between groups with regard to continuous biological data relevant to coagulation. For example, sample sizes of 60 and 60 achieve 80% power to detect a difference of 1.18 dynes/cm² in viscoelastic clot strength (TEG G parameter) between the two groups (which is smaller than a clinically relevant difference) at a significance level (α) of 0.05 using a two-sided test. All power analyses account for two interim analyses for a total of three sequential equally spaced analyses using the O'Brien-Fleming method to calculate two-sided boundaries, assuming a 95% confidence, 80% power, and attrition rates of 0%, 10%, and 20%. The first interim analysis will be conducted when $n = 50$, the second at $n = 100$, and the final at end of the 3-year period of accrual.

An intent-to-treat approach will be used for all primary and secondary outcome analyses. All analyses will be conducted using SAS for Windows version 9.3 (SAS Institute Inc., Cary, NC) on deidentified data. Outcome and effect variables that are not normally distributed will be either categorized or analyzed using nonparametric methods. Missing data will be managed during analysis using the method proposed by Sauer et al. (37).

Effectiveness of randomization will be examined by comparing the two groups regarding demographic variables (age, gender), injury severity (Injury Severity Score, blunt versus penetrating mechanism), degree of shock (field SBP, field heart rate, field hematocrit), and a field coagulation measure (field international normalized ratio). We will adjust all analyses of end points for the covariates showing different distributions (either statistically significant difference at $P < 0.05$ or clinically significant difference as determined by the data safety monitoring board).

Mobile blood banking

Delivery of freshly thawed frozen AB (universal donor) plasma to trauma victims at the scene of injury was the greatest technical challenge faced during the design of this study. Early in the development of the study, we elected to use FP24, which is our locally available frozen plasma product and is functionally equivalent to fresh-frozen plasma (FFP) but conforming to a slightly different set of manufacturing standards. FP24 is plasma frozen with 24 h of collection, whereas FFP is required to be frozen within 8 h of collection. Although FP24 has slightly lower concentrations of coagulation factors than FFP, the difference is thought to be of minimal clinical impact, except with regard to factor VIII (which can be significantly lower in FP24), and the two products are generally used interchangeably, except for specific indications for factor VIII replacement (38). Moreover, in our specific case, the local AB FP24 product from Bonfils blood center is collected at the processing facility and packaged immediately and is thus functionally equivalent to FFP, although labeled as FP24.

As previously noted, the Denver COMBAT study is the only participant in the DoD's multicenter plasma-first study to use a frozen plasma product and thawed on demand at the scene of injury, whereas the other centers use prethawed plasma. Although prethawed plasma presents some logistical advantages, there are significant disadvantages to its use as well. Prethawed previously frozen plasma has a nominal shelf life of 5 days but has been shown to degrade rapidly in terms of both its hemostatic and anti-inflammatory potential (38–40). Moreover, as the targeted patient population presents infrequently (~50 cases per year at our center), equipping our entire fleet of 32 ambulances with thawed plasma at all times would result in the waste of nearly 7,000 units of AB plasma per year, lost to expiration. This would cost more than \$5 million in the course of a 3-year study and deplete our region of a precious medical resource, as AB donors comprise less than 3% of the donor pool. Lyophilized plasma and other room temperature-stable products are not available in the United States.

Therefore, a system to store, transport, and rapidly thaw plasma in our ambulance fleet had to be devised. The only existing methodology for rapid plasma

thawing was a microwave-based system. This system proved unacceptable on several counts. First, its thaw times were unpredictable ranging from 4 to 8 min and occasionally far longer depending on the shape and volume of the plasma unit. The microwave was difficult to load and use, and mishandling during the stress of a trauma response could result in burned plasma, partially frozen plasma, or punctured bags. The microwaves were also bulky and fragile and drew enormous amounts of electrical power, all of which rendered them unsuitable for installation in a vehicle. In short, the microwave system was neither sufficiently robust nor foolproof to be used in a mobile/first-response environment, and, above all, the system did not fail-safe.

Thus, we were compelled to invent an entirely new methodology for rapid plasma thawing, which in aggregate we refer to as the Field Plasma System (FPS),

a schematic of which is shown in Figure 3. A dry (i.e., contained circulation) warm water bath (the Plasmatherm, Barkey GmbH & Co., Leopoldshoehe, Germany) had recently been approved for the US market, and this system proved very simple to use and reliable and was easily adapted for vehicular use. However, it thawed FP24 units no more rapidly than any other conventional warm water bath: approximately 20 min. This exceeded our usual ground ambulance transport time but also meant that a patient in shock would require normal saline administration before we could thaw the first unit of plasma, undermining the central purpose of the study: "plasma first" resuscitation. This necessitated a complete redesign of our plasma packaging to increase the surface area-to-volume ratio. We worked with our local blood donation center (Bonfils Blood Center, Denver, Colo) to collect donated 250-mL plasma units directly into oversized 2-L bags. These

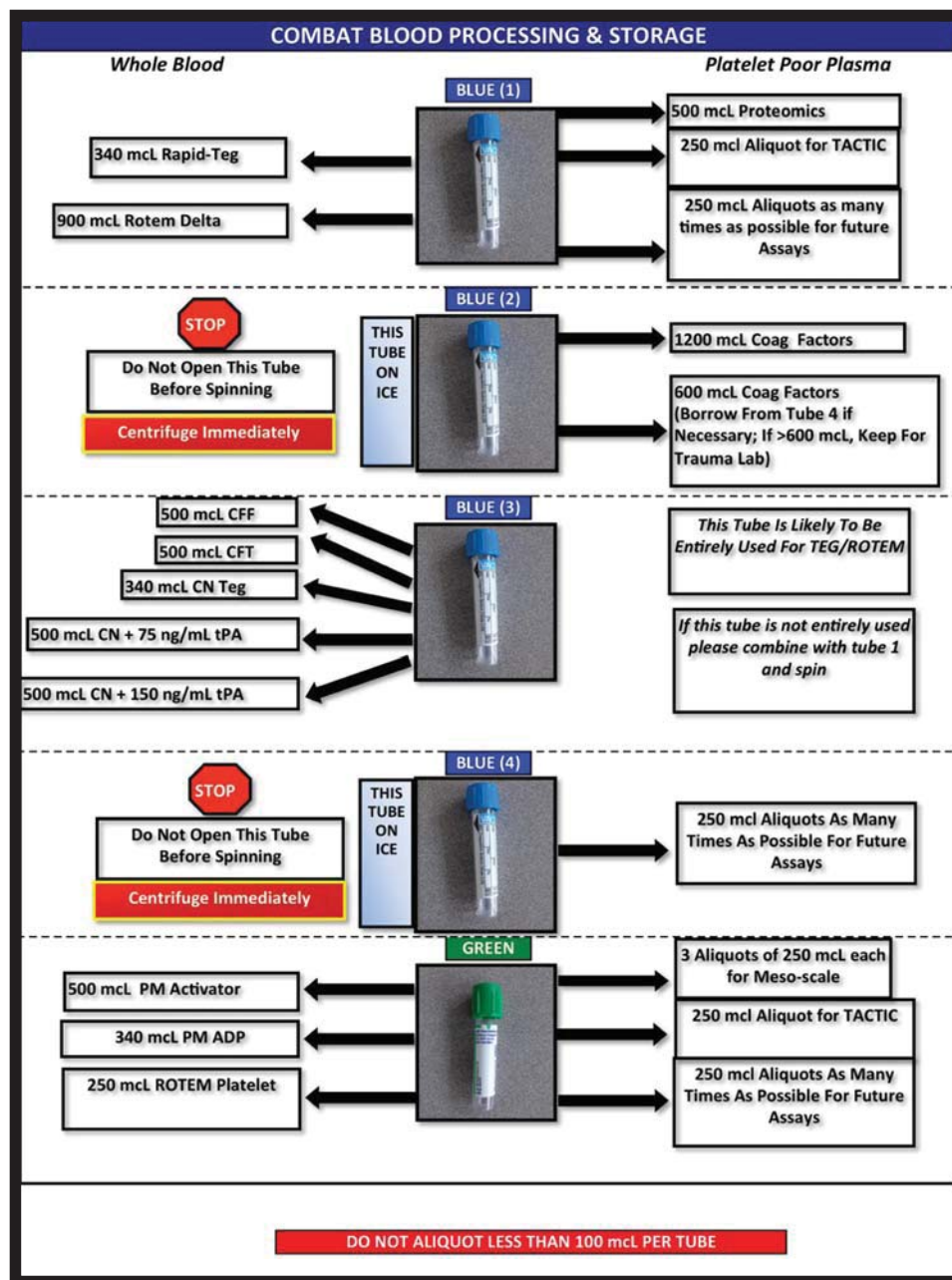


Fig. 2. **COMBAT biological sample processing chart.** This guide is carried by the COMBAT technical staff to aid in proper sample handling and allocation. Five sodium citrate (blue top), one lithium heparin (green), one EDTA, and one protease inhibitor vacuum tube are collected at each time point. One citrate and the EDTA tube are sent to the DHMC central clinical laboratory for a complete blood cell count and conventional coagulation tests. The protease inhibitor tube is immediately chilled and centrifuged to yield plasma. The remaining tubes are processed according to this chart. Whole blood undergoes a battery of 12 channels of viscoelastic hemostatic assays on the TEG and ROTEM platforms, including TEG Platelet Mapping as well as ROTEM platelet aggregometry. The remaining blood is immediately chilled, centrifuged to yield platelet-free plasma, and flash frozen in liquid nitrogen. These plasma samples are used for coagulation factor level tests (II, V, VII, X, and XIII), cytokine and chemokine panels, proteomics, metabolomics, and enzyme-linked immunosorbent assays for proteins of specific interest relevant to TIC, including tissue plasminogen activator, plasminogen activator inhibitor-1, syndecan, and α -enolase. Aliquots are also contributed to the TACTIC collaboration.



FIG. 3. **Field plasma system schematic.** FP24 is stored frozen at less than -18°C in a high-performance cooler (A) passively chilled with a phase change material. This cooler is unpacked at the time of randomization and, if it contains FP24 (instead of a dummy payload for the control arm), these units are transferred to the Plasmatherm dry warm water bath system (B) where they are thawed in approximately 3 min. The Plasmatherm is powered by a self-contained electrical system that is charged by connection to conventional 110 VAC mains power via a shore power coupling (C) when the ambulance is parked. A power inverter/battery charger (D) both charges a lithium-ion storage battery (E), which supplies power to the system while the ambulance is in the field, and converts the battery's DC power output to AC to run the Plasmatherm.

were then frozen under compression to yield plasma units with a thin flat form factor, which readily thawed in less than 3 min in the Plasmatherm. These units are, however, extremely fragile and must be transported in a padded rigid case within an outer insulated cooler.

Storing and transporting these plasma units also presented challenges. The FDA mandates that FP24 and FFP be stored at or below -18°C . We had to deliver reliably frozen plasma to up to 44 ambulance crew shifts per day, and this plasma might stay in the field for up to 16 h. To supply this demand, three -30°C blood bank freezers were installed in the equipment room of our ambulance garage. These freezers are stocked with sufficient units of FP24 to supply three consecutive fleet shifts of the ambulance service, as well as bottles of an aqueous salt solution that changes phase at -22°C . Half of the FP24 packages actually contain a dummy load for the control arm of the study. Both the FP24 units and the dummy loads are concealed within a protective aluminum cassette to maintain blinding during loading and unloading of the coolers. Two FP24 units (or a dummy load) and four 1-L bottles of frozen-phase change material are packed into specially designed coolers for each ambulance shift. These coolers consist of a 5-cm layer of pentane-blown rigid polyurethane foam insulation sandwiched between an inner shock absorbing cross-linked polyethylene foam liner and a highly durable steel outer shell constructed from a repurposed 30-mm ammunition can. These coolers are capable of storing the FP24 units below the mandated temperature of -18°C for at least 28 h even during summer weather conditions and can protect their payload from up to a 6-ft drop onto concrete. Integral with the physical elements of this system is the creation of an electronic (bar code-based) tracking system for the issuance and recovery of all fielded and consumable elements of the system. This system was built on the existing system for tracking the issuance of drugs and equipment to the ambulance crews. Additional record keeping and quality assurance duties (e.g., maintenance of freezer temperature logs), as would be associated with any blood bank, had to be implemented by the team of vehicle service technicians under the DHMC Paramedic Command, as well as by the professional research assistants of the DHMC/University of Colorado study team. A just-in-time supply system from Bonfils Blood Center to replace expended units within 24 h also had to be put in place, as we stock no working reserve, to retain the integrity of our block randomization.

Lastly, we had to provide reliable power to the Plasmatherm warm water bath. After field tests, where the enormous electrical draw of the microwave units damaged the ambulances' charging system, it was decided that the electrical power system for the study equipment should be entirely self-contained. Even after changing to the lower-current water bath system, this approach was maintained as an important safety feature, such that no possible failure of the FPS could render the ambulance inoperable. Wide safety margins were engineered into the FPS electrical components. The FPS is built around a 300-A-h 12-V lithium-ion battery (Smart Battery, model SB300, Tampa, Fla), which can supply power to the water bath for up to 36 h of idle operation plus the thawing of 4 units of FP24. A 2,000-W 200-A power inverter/charger (Magnum Energy, model MS2012-20B, Everett, Wash) converts the battery's DC power to AC to run the Plasmatherm (which draws up to 1,600 W) and charges the battery in less than 4 h even if completely drained and while running the Plasmatherm. Lastly, the ambulances and garage were fitted for connection to shore power (i.e., AC mains) at every parking spot, with a 20-amp 110-V AC service delivered to a GFCI-protected all-weather receptacle mounted at the end of a retractable cord reel and coupled to an automatic plug ejection system on the ambulance to

prevent accidental damage to the system by driving off with the shore line still connected. Ambulance crews and vehicle service technicians were all extensively trained on a "preflight" checklist for the FPS to ensure operability and safety during their shift in the field.

Systems and subsystems

Once the scientific aims were determined, the COMBAT trial fundamentally became a systems engineering project. The FPS and plasma storage systems detailed are the most innovative components of the study but represent only a small fraction of the systems, subsystems, and integration work, which comprise the whole of the COMBAT apparatus. In terms of sheer complexity, the COMBAT trial (in comparison with our previous trials at DHMC) is analogous to the moon landing of the Apollo program compared with the low Earth orbit of the Mercury program. Both human and materiel resources had to be organized into systems that interlocked and overlapped. Individual personnel frequently were made responsible for the use and maintenance of numerous systems, from the FPS to the ambulance itself, to the patients' blood samples. In terms of physical equipment alone, ranging from freezers to batteries to thromboelastographs and cryogenic storage equipment, more than 75 subsystems are specified in our procedures, each subdivided into numerous components in their own right. Figure 4 shows a highly abbreviated systems tree for the COMBAT study. In this view, only the "Plasma Thawing and Administration" subsystem is broken into third- and fourth-order details as an example. The impact of this fact is 2-fold. First, studies of this kind necessitate the inclusion of managerial personnel with systems engineering experience from their inception; and second, enormous amounts of manpower and extensive training of these personnel on all elements of the study are required for seamless and safe operation, as well as assuring maximization of enrollment.

RESULTS

The authors remain blinded to the treatment arm of each patient until our first interim analysis. Therefore, the results presented herein are restricted to the descriptive characteristics of the total enrolled population. To date, we have enrolled 30 subjects in the COMBAT study. The median patient age was 32 years (interquartile range [IQR], 24–48), and 27 (90%) were male. Ten (33%) had penetrating wounds. The median time from injury to arrival at the ED was 29 min (IQR, 22–32). The median New Injury Severity Score was 17 (IQR, 9–36); the median SBP in the field was 70 mmHg (IQR, 60–78). Seventeen patients displayed clinical signs of coagulopathy, and 15 patients required immediate transfusion of PRBCs, five of them massive (≥ 10 units in 6 h). Four patients required resuscitative thoracotomy in the ED. Three patients (10%) died, one from massive hemorrhage from a transecting thoracic aortic injury, one from unsurvivable brain injury, and one from multiple organ failure.

1. Human
 - 1.1. Professional research assistants/laboratory technicians (7 on 24/7 rotation)
 - 1.1.1. Training in all study-related tasks, including
 - 1.1.1.1. Patient enrolment, documentation and consent
 - 1.1.1.2. Laboratory instrumentation usage and sample handling
 - 1.1.1.3. Data entry, validation, storage, management, analysis and safety
 - 1.1.1.4. Specialized mobile study equipment maintenance and repair
 - 1.2. Paramedics (236 plus command staff)
 - 1.2.1. Training for study enrollment, blood product administration and field blood sampling
 - 1.3. Vehicle service technicians (9 on 24/7 rotation)
 - 1.3.1. Training for plasma and placebo storage, handling and disbursement
 - 1.3.2. Basic mobile study equipment maintenance and troubleshooting
2. Materiel and Infrastructure
 - 2.1. Field Plasma Delivery
 - 2.1.1. Plasma Packaging
 - 2.1.1.1. AB plasma supply (as FP24) and donor recruitment center
 - 2.1.1.2. Flat package freezing facilities and hardware
 - 2.1.1.3. JIT on-call delivery for replacement of expended plasma
 - 2.1.2. Plasma Storage and Transportation
 - 2.1.3. Plasma Thawing and Administration
 - 2.1.3.1. Plasma Thawing
 - 2.1.3.1.1. Dry warm water bath (Plasmatherm™)
 - 2.1.3.1.2. Self-contained mobile power system
 - 2.1.3.1.2.1. Shore power connectivity
 - 2.1.3.1.2.1.1. Auto-disconnect vehicle coupling (Kussmal™)
 - 2.1.3.1.2.1.2. AC mains electrical supply and terminal equipment
 - 2.1.3.1.2.1.2.1. GFCI breakered circuits, 32x 20 A service
 - 2.1.3.1.2.1.2.2. Retractable cord reels with all-weather receptacles
 - 2.1.3.1.2.2. Onboard power supply
 - 2.1.3.1.2.2.1. Lithium-ion battery, 12V, 300 AH
 - 2.1.3.1.2.2.2. Inverter Charger, 2000W, 20AAC, 200 ADC
 - 2.1.3.1.2.2.3. Internal chassis wiring and receptacles for ambulance
 - 2.1.3.2. Plasma Administration
 - 2.1.3.2.1. IV access kits
 - 2.1.3.2.2. Large bore blood product drip sets with hand pumps
 - 2.1.3.2.3. Monitoring equipment (e.g. sphygmomanometers, thermometers)
 - 2.1.3.2.4. Product administration documentation
 - 2.2. Laboratory Sample Collection
 - 2.2.1. Viscoelastic hemostatic assays
 - 2.2.1.1. 16 TEG channels
 - 2.2.1.2. 12 ROTEM channels
 - 2.2.2. Platelet aggregometers
 - 2.2.2.1. 4 Chrono-Log Channels
 - 2.2.2.2. 4 TEM Channels
 - 2.2.3. Conventional coagulation assays and factor levels (clinical core lab)
 - 2.2.4. Cryogenic plasma storage for protein analysis
 - 2.3. Data Collection
 - 2.3.1. CFR part 11(b) compliant database (Red Cap platform)
 - 2.3.2. Local instrumentation data storage and database upload tools
 - 2.3.3. Hospital electronic medical record
 - 2.3.4. Secure storage and handling facilities for hard copy data

FIG. 4. **Systems and subsystems of the COMBAT study.** This highly abbreviated systems tree for the COMBAT study is excerpted from the organizational documents used during development of the study. In this view, only the “Plasma Thawing and Administration” subsystem is broken into third- and fourth-order detail as an example. If all the nested systems, subsystems, and component descriptions were unpacked, the document would run to several hundred pages. Such organizational trees are essential to define and organize operational elements throughout the development of a study of this complexity and also aid in the process of quality assurance and improvement.

Sixteen patients were randomized to and received FP24 in the field, and 14 were randomized to and received standard of care treatment with normal saline. Five eligible subjects were not enrolled during this period. Two of these failures to enroll were attributable to response to the scene from the single older-model ambulance in our fleet, which was not able to be equipped with the FPS because of space constraints unique to its construction. One failure was caused by response from an ambulance whose Plasmatherm had been removed for maintenance immediately before the ambulance was put into service, with no spare equipment available at that time. One was not enrolled because of the proximity of the injury scene to the hospital, such that transport

time was insufficient for the randomization procedure, and the last was not enrolled because of the simultaneous transport of several patients from a multiple-casualty event. Notably, during the study period, to date, eight additional patients who did *not* meet the COMBAT enrollment criteria suffered massive hemorrhage because of trauma, four of whom required resuscitative thoracotomy, and only three of whom survived.

DISCUSSION

A prospective placebo-controlled randomized clinical trial (RCT) for the use of plasma as an initial resuscitation fluid in

trauma has been urgently needed to determine whether the civilian trauma population can indeed benefit from a plasma-first resuscitation strategy. The COMBAT trial was designed to answer this question. Through the methodology of this trial, we are giving the highest quality plasma product available in the United States (frozen) as close to the time of injury as is theoretically possible—faster, in fact, than if we were to use lyophilized plasma, which takes longer to reconstitute than our specially packaged FP24 units take to thaw. Indeed, the only two patients we were forced to exclude because of timing issues were a pedestrian struck by a car in front of the hospital who had a transport time of less than a minute and another patient whose prolonged extrication required that that patient receives more crystalloid in the field than allowed by study criteria.

This ability to achieve intervention with a hemostatic resuscitation agent in the closest possible temporal proximity to injury is critical to avoiding the survivor bias that has confounded previous similar studies. Moreover, it is intuitively evident in terms of achieving hemostasis in trauma, an ounce of prevention is worth more than a pound of cure, as to intervene early with a hemostatic agent represents an opportunity to forestall the evolution of the “bloody vicious cycle” of acidosis, hypothermia, and worsening coagulopathy and hemorrhagic shock. Thus, the COMBAT model for deploying plasma in first-response units should serve as a model for RCTs of other hemostatic resuscitative agents, both extant and on the horizon such as fibrinogen concentrates, novel platelet formulations and platelet-derived agents, prothrombin complex concentrates, and even antifibrinolytics. If these agents are indeed of benefit in preventing or forestalling TIC, then the best chance of proving their value is by using them in the mode of COMBAT: as early as possible at or *en route* from the scene of injury in a rigorously controlled RCT.

Several opportunities for improvement of the COMBAT model are, however, evident. The major limitation of the COMBAT model is that it is prone to type II error. This is chiefly caused by the fact that the response and transport times of our ground ambulance fleet in the Denver metropolitan area are so short (usually <30 min from injury to ED arrival) that there is less difference in time to first plasma between field and hospital administration than may be present in most parts of the country. This disparity in first response times is particularly evident in rural areas or urban trauma centers without a centralized professional ambulance system based out of their center. Our fortunate circumstances with regard to the organization and efficiency of our paramedic command and its ground ambulance service make the COMBAT study a logistical possibility, but the associated short transport times are a significant confounder. Now that we have established a safe and efficient system for delivery of frozen plasma by ground ambulance, it would be a logical next step to expand the study to encompass localities with far longer transport times, where the beneficial impact of early plasma may be more evident. This could potentially include other austere environments such as are seen in military applications and developing countries, although significant infrastructural hurdles would have to be overcome. One promising development that may aid in the generalizability of plasma-first is recent work demonstrating that group A plasma is a safe alternative to AB for unmatched transfusion, thus expanding the

available donor pool for emergency use plasma products by roughly 10-fold (41). The increasing availability of improved lyophilized plasma products both abroad and in the United States provides the best hope for future generalizability of the results of the COMBAT trial.

Although the COMBAT study is powered to detect changes in continuous variables such as clot strength, time to first PRBC requirement, and levels of specific coagulation factors, the infrequent nature of the very severely injured patients meeting the COMBAT enrollment criteria means that the study is not adequately powered to detect small benefits with regard to discrete variables such as mortality. Both from an ethical and scientific standpoint, it was an important and appropriate design consideration of this study to target only the highest-acuity trauma patients with the highest likelihood of death. These are the patients most likely to receive benefit from empiric plasma therapy and least likely to be put at increased risk by an intervention given under a waiver of consent.

Necessarily, however, the rigorous inclusion criteria, demanded by this design feature of the study, exclude many badly injured patients who could conceivably benefit from early hemostatic resuscitation. As noted above, during this initial period, wherein 30 patients were enrolled in the COMBAT trial, eight additional patients with hemorrhagic shock, but who did not quite meet the COMBAT criteria, were also admitted to our center from the catchment area of our ground ambulance fleet. These patients were, however, enrolled in our observational TAP (Trauma Activation Protocol) study, which, among other aims, will allow us to identify more accurate inclusion and exclusion criteria to target the enrollment of patients with significant traumatic hemorrhage (and specifically TIC) for future studies similar to the COMBAT trial. Such criteria may include better scoring systems for our first responders to apply, the inclusion of qualitative criteria, or possibly even the application of point-of-care laboratory testing such as viscoelastic hemostatic assays (e.g., TEG or ROTEM) in the field. To this end, we have embarked on an observational study, which runs in parallel to the COMBAT trial. The TAP study targets all high-level trauma activation patients who fail to meet the COMBAT criteria. Blood is collected in the field on these patients in an identical manner to the COMBAT trial, but no interventions are performed. The patients receive standard care throughout their hospital course. It is hoped that analysis of the outcomes, injury patterns, and coagulation laboratory results from the field blood samples of these patients will yield insights as to how to structure future RCTs of hemostatic resuscitation in the field to capture the patient population most likely to benefit from these interventions.

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Metabolomics of AS-5 RBC supernatants following routine storage

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Vox Sanguinis

Background and Objectives The safety and efficacy of stored red blood cells (RBCs) transfusion has been long debated due to retrospective clinical evidence and laboratory results, indicating a potential correlation between increased morbidity and mortality following transfusion of RBC units stored longer than 14 days. We hypothesize that storage in Optisol additive solution-5 leads to a unique metabolomics profile in the supernatant of stored RBCs.

Materials and Methods Whole blood was drawn from five healthy donors, RBC units were manufactured, and prestorage leucoreduced by filtration. Samples were taken on days 1 and 42, the cells removed, and mass spectrometry-based metabolomics was performed.

Results The results confirmed the progressive impairment of RBC energy metabolism by day 42 with indirect markers of a parallel alteration of glutathione and NADPH homeostasis. Moreover, oxidized pro-inflammatory lipids accumulated by the end of storage.

Conclusion The supernatants from stored RBCs may represent a burden to the transfused recipients from a metabolomics standpoint.

Key words: oxidative stress, red blood cell, storage, transfusion medicine.

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Introduction

Red blood cells (RBCs) are still the most widely transfused blood-derived therapeutic, with an annual estimate of approximately 90 million units being transfused worldwide [1, 2]. However, highly questioned retrospective clinical evidence [3] and laboratory investigations [4–6] have fueled the debate about the likely compromised safety and effectiveness of RBCs stored longer than 2 weeks. This is especially true when dealing with certain categories of recipients, such as the injured, critically ill or peri-operative patients [3]. While randomized clinical trials are currently underway [1], the application of Omics technologies (especially proteomics and metabolomics) to

transfusion medicine-relevant issues has endowed researchers with unprecedented tools to further dissect the major biochemical events that evolve during prolonged storage of RBC units in the blood bank [7, 8]. Indeed, laboratory studies have highlighted the storage-dependent accumulation of the so-called storage lesions, a long list of biochemical and morphological alterations to RBC integrity and functionality [8], targeting erythrocyte morphology [9], cytosolic and membrane [4, 10] proteome stability.

The documented storage lesions include the progressive metabolic deregulation and depletion of high energy phosphate compounds ATP and DPG, dysregulated cation homeostasis (increases in intracellular Ca²⁺ and extracellular K⁺), and the accumulation of reactive oxygen species (ROS), which is partially mitigated by prestorage leucoreduction [4–6, 10]. A compromised capacity to cope with oxidative stress has been associated with the progressive fragmentation and

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carbonylation of cytosolic and membrane protein components [4–12]. Storage promotes the vesiculation of irreversibly damaged proteins (i.e. fragmented/aggregated or no longer functional/oxidized) [11–13]. Vesiculation is associated with the fluid phase accumulation of potential pro-inflammatory or phagocytosis-stimulating proteins [11–14] and lipid mediators, including isoprostanes and long-chain fatty acids [15]. Stored RBCs are also characterized by decreased exposure of antiphagocytic signals such as CD47 on the membrane of [16], accompanied by the increased exposure on the outer membrane leaflet of pro-phagocytosis [17] markers such as phosphatidylserine [18] and band 3 clusters [10, 19].

Despite numerous shared features, RBCs stored in the presence of different additive solutions respond differently in terms of *in vitro* measurements and storage lesions to the proteome and metabolome [5, 6, 20–24]. Indeed, recent mass spectrometry-based investigations on the metabolome of RBCs stored in the presence of SAGM [5, 20], AS-1 [6], MAP [21], PAGGGM [22, 23] have highlighted a common trait related to a progressive impairment of energy metabolism in stored erythrocytes. Conversely, distinctive traits were also documented including the additive solution-dependent activation of the pentose phosphate pathway as to fuel antioxidant potential via the generation of NADPH, which drives the reduction of oxidized glutathione back to its reduced form [5, 6]. Only one metabolomics study has so far documented the likely changes to the metabolome of RBC supernatants during storage duration in the presence of CPD-SAGM [5]. Although CPD-SAGM and CPD-AS-5 share the same saline (NaCl 150 mM) and glucose (45 mM), AS-5 displays higher adenine (2.2 vs. 1.25 mM in SAGM) and mannitol (45.5 vs. 30 mM in SAGM) concentrations, which result in slightly lower pH (5.5 vs. 5.7) [24]. This is relevant in that differential composition of the additive solution is deemed to influence intracellular metabolism and, consistently, the metabolome of RBC supernatants, which indirectly mirrors the main intracellular storage-dependent catabolic and anabolic events. Therefore, to complement our recent proteomics observations on the very same biological matrix [13], we hypothesize that the metabolome of supernatants from RBCs stored in AS-5 will demonstrate the accumulation of complementary and unique metabolites due to the Optisol AS-5. The obtained results complement current knowledge on the metabolic alterations in the blood bank and pave the way for the designing of alternative additive solutions or provide a theoretical rationale for the implementation of innovative processing strategies [25, 26].

Materials and methods

Sample collection

One unit of whole blood (500 ± 50 ml) was collected from five healthy donors per AABB/FDA guidelines, using CPD with Optisol TM collection bag system (Teruflex; Terumo Corporation, Tokyo, Japan). Plasma was separated from RBCs by centrifugation followed by expression, employing an automated closed system, Compomat G4 (Fresenius-Kabi, Schweinfurt, Germany), and AS-5 (Optisol) was added to a final haematocrit of 50–60%. The estimated amount of residual plasma was 5–10 ml/unit [13]. RBC units were prestorage leucoreduced via filtration using a Pall BPF4 leucoreduction filter (Westbury, NY, USA) and stored at 1–6°C. Samples were obtained through sterile couplers on day (D)1 and D42 (the last day a unit can be transfused). The supernatant was isolated via centrifugation (5000 g for 7 min) followed by a second spin at 12 500 g for 6 min to sediment residual cellular material and contaminating platelets [13]. The supernatants were aliquoted and temporarily stored at –80°C prior to metabolomics analyses.

Metabolomics analyses

Extended details about the protocols adopted for metabolomics analyses are reported in Supplementary File S1. Briefly, samples were prepared using the automated MicroLab STAR[®] system from Hamilton Company and assayed by GC/MS and LC/MS/MS platforms (either a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization or Waters ACQUITY UPLC and a Thermo-Finnigan LTQ-FT mass spectrometer), run either in positive and negative ion modes with adequate buffer, column, phases and gradient adjustments (Supplementary File S1). Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities within a 5 ppm window range. Statistical significance was determined by calculating Welch's two sample t-test and random forest algorithms to determine significantly ($P < 0.05$) altered metabolic pathways.

Results

Supernatants of leucoreduced packed RBCs stored in AS-5 were assayed at day 1 (D1) from collection and after 42 days (D42) of refrigerated storage under blood bank conditions. A total of 348 distinct biochemicals were identified either via LC-MS, LC-MS/MS or GC-MS (Table 1). Statistical analyses highlighted the significance

Table 1 Concise report of the significant metabolic changes observed in RBC supernatants at storage day 1 and 42

Welch's Two Sample <i>t</i> -Test	PRBC-42 PRBC-1
Total number of biochemicals with $P \leq 0.05$	117
Biochemicals (↑↓)	101 16
Total number of biochemicals with $0.05 < P < 0.10$	15
Biochemicals (↑↓)	10 5

From a total of 348 named biochemical.

Welch's Two Sample *t*-Test.

Used to determine whether the means of two populations are different.

P-value: evidence that the means are different.

$P \leq 0.05$ was taken as significant.

P value trend of $0.05 < P < 0.10$ identified biochemicals approaching significance.

(P < 0.05) storage-dependent increases in the concentrations of 101 metabolites, while only 16 metabolites decreased in concentration, whereas 231 remained unchanged. In Figures from 1 to 5, results are graphed as

box-plots indicating median values (line), mean values ± upper/lower quartile distributions for D1 and D42 packed AS-5 RBC supernatants.

Additive solution nutritive components (glucose, Fig. 1; and adenine, Fig. 2) were progressively consumed during storage duration, while catabolic by-products were observed to accumulate in the supernatant (e.g. lactate, Fig. 1). The accumulation of ATP breakdown products AMP and free phosphate was consistent with a poorer energy state at D42(Fig. 2). Consistently, adenine was significantly consumed by the end of the storage period, and its by-products accumulated as a result (Fig. 2). These metabolites included purine catabolites (hypoxanthine, xanthine and inosine) either derived from adenine and adenosine deamination to hypoxanthine or inosine, respectively.

By-products of transamination reactions glutamate and alanine increased in the supernatants of stored RBCs, together with glutamate precursor glutamine and glutamate-derived transamination product ketoglutarate

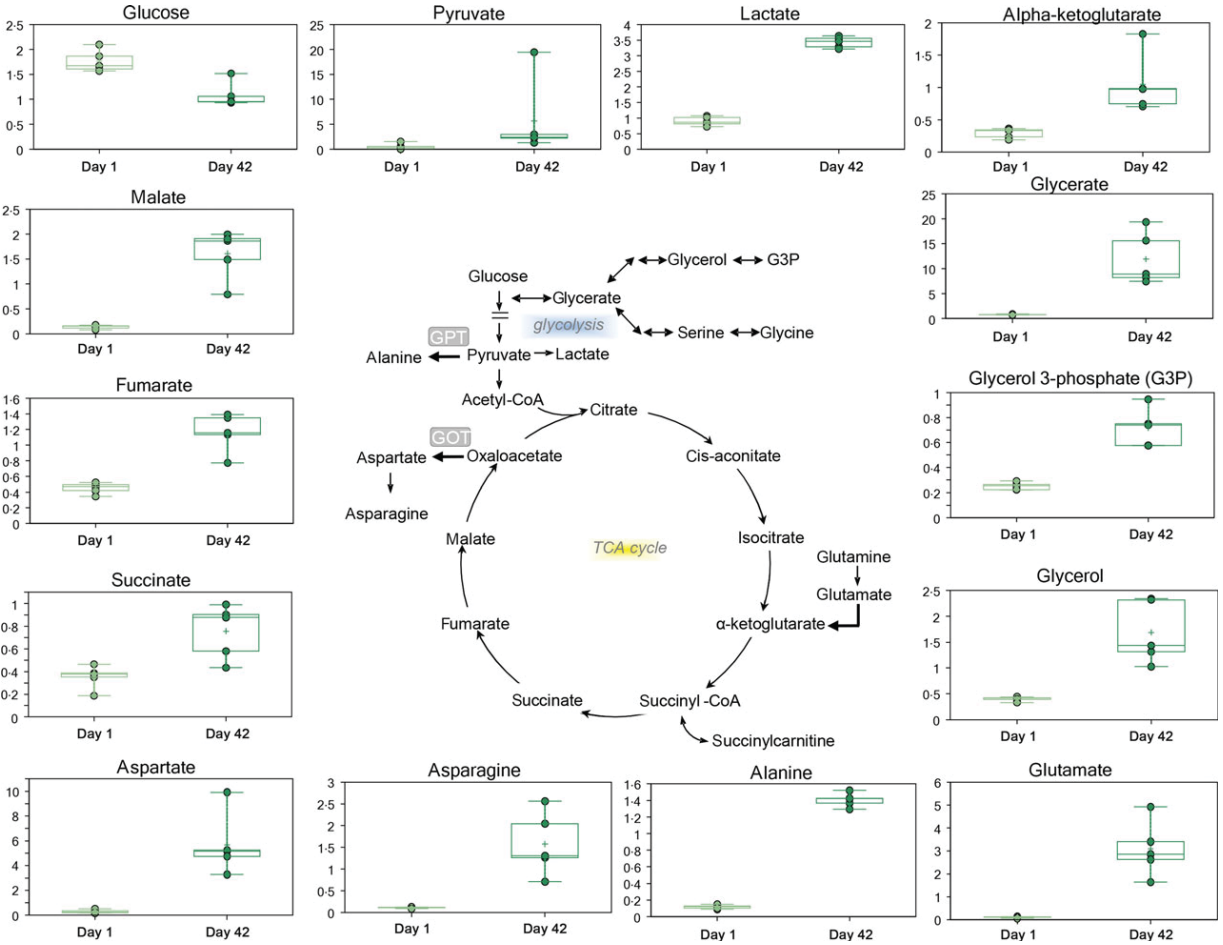


Fig. 1 An overview of glycolysis, cytosolic tricarboxylic acids, glycerol phosphate metabolism and transaminase.

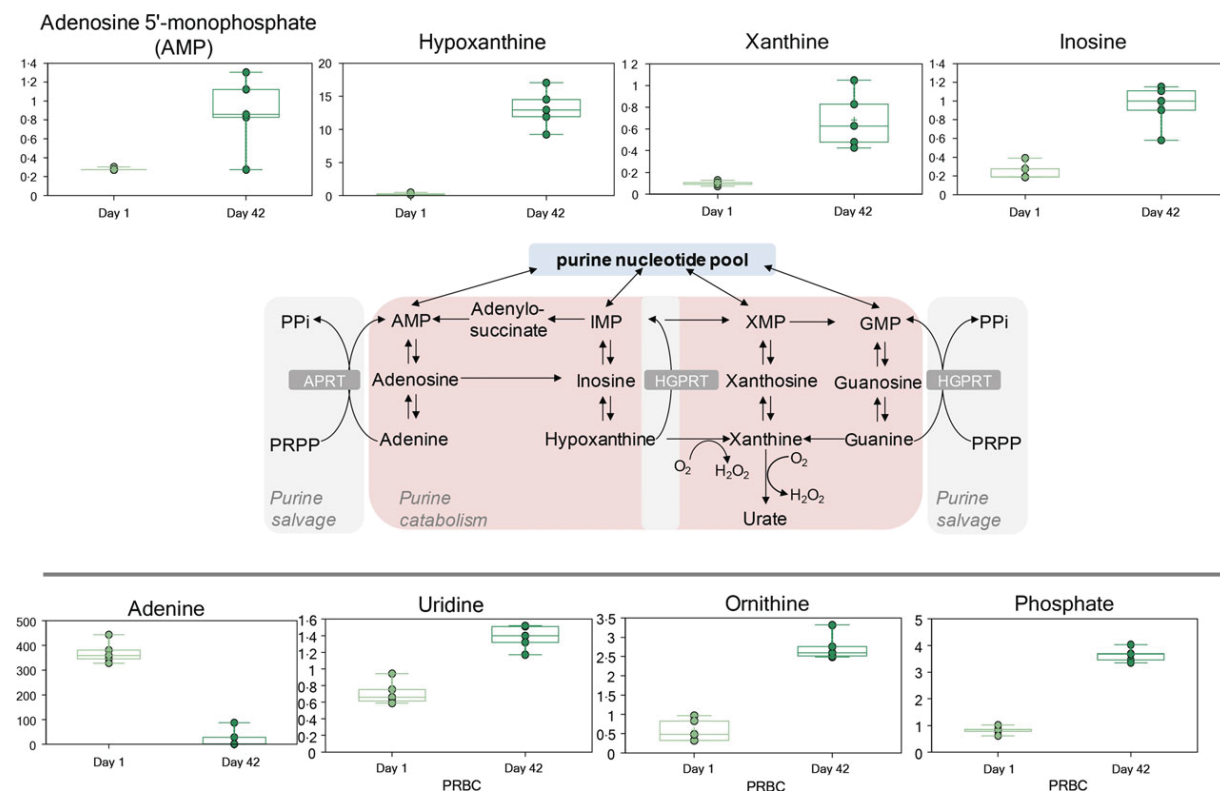


Fig. 2 An overview of purine metabolism.

(Fig. 1). Other tricarboxylic acids such as succinate, fumarate and malate were found to accumulate in the supernatants of stored packed RBCs (Fig. 1).

D42 supernatants also were characterized by increased levels of serine, together with its derivatives via one-carbon metabolism and cysteine biosynthesis/methionine trans-sulphuration, glycine and cysteine, respectively (Fig. 3). Several intermediates related to serine metabolism (e.g. serine, glycine, homoserine and threonine) and methionine salvage were more abundant at D42 and included homocysteine, intermediates to methyl donation by betaine (choline, betaine, dimethylglycine, sarcosine and glycine) as well as cysteine metabolites (cysteine, cystine and S-methylcysteine – Fig. 3). However, methionine was the only amino acid that decreased at the end of storage (Fig. 3).

Several metabolites associated with (oxidative) stress responses were elevated at D42 of storage, including 5-oxoproline, gluconate, cortisol, allantoin, cystine, erythronate and oxidized lipids (9 + 13-HODE, 2-hydroxystearate and 2-hydroxypalmitate-Fig. 4). In addition to hydroxylipids, other signalling lipids were also more abundant at D42. The inflammation signalling lipid arachidonate was elevated more than other free fatty acids; however, none of the lysophospholipids increased significantly during RBC storage (Fig. 4, Fig. S1).

Erythro-sphingosine-1-phosphate was more abundant at D42 (Fig. 4).

Various catabolites of membrane phospholipids were more abundant at D42 and included phospholipid head groups (ethanolamine and choline), glycerol, glycerol-3-phosphate and glycerophosphorylcholine (Fig. 1). Fatty acid catabolites including essential fatty acids, medium chain fatty acids (caproate, heptanoate, caprylate, pelargonate and caprate), long-chain fatty acids (LCFA: palmitate, margarate and stearate) and carnitine conjugates of LCFA (palmitoylcarnitine and oleoylcarnitine) were more abundant in storage fluid at D42 (Fig. S1).

Storage also promoted the progressive leaching from the plastic bags of the phthalate plasticizer (Fig. S2). Storage-dependent accumulation of heme and biliverdin was observed as well (Fig. S2).

Discussion

Storage corresponded to a progressive metabolic enrichment of the D42 RBC supernatants. This result was largely expected in the light of the simplicity of the composition of the Optisol AS-5 additive solution [24]. In this view, additive solution nutritive components (glucose – Fig. 1; and adenine – Fig. 2) were progressively consumed, while

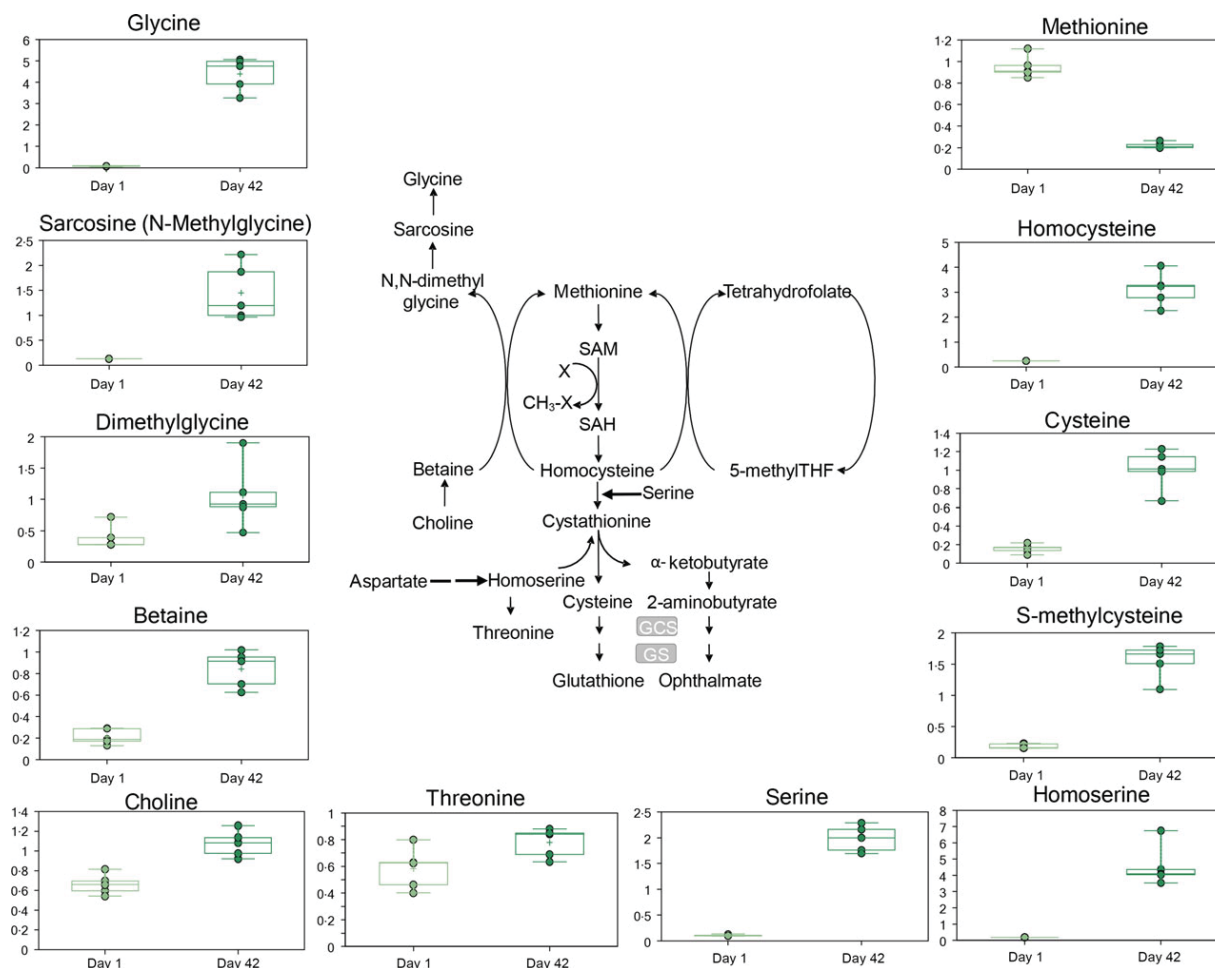


Fig. 3 An overview of one-carbon metabolism/serine biosynthesis and methionine metabolism.

catabolic by-products accumulated in the supernatant of stored RBCs.

Energy metabolism, detoxification routes

Previous studies on stored RBC metabolomics have documented a progressive deregulation of glycolysis in response to the acidification of the intracellular environment [5]. However, although glycolysis rate decreases as pH falls, pyruvate and lactate progressively accumulate during storage [5, 6, 22–24]. Intracellular lactate accumulation would result in increased levels of lactate in the supernatant as well (Fig. 1), since H^+ -monocarboxylate cotransporter-mediated efflux of lactate from RBCs is proportional to the intracellular levels of this metabolite [27].

In stored erythrocytes, lactate generation via the Embden-Meyerhof glycolytic pathway is not sufficient to sustain ATP production and 2,3-diphosphoglycerate levels [5, 6, 20–22]. This is relevant in the light of the role of

these compounds as energy tokens to be spent to promote cell survival and to stabilize the deoxygenated 'T' state of haemoglobin. In the present study, the accumulation of ATP breakdown products AMP and free phosphate was consistent with a poorer energy state at storage D42 (Fig. 2). Energy impairment during storage might be associated with the vesiculation of rate-limiting glycolytic enzymes (such as glyceraldehyde 3-phosphate dehydrogenase [28]). Alternatively, energy metabolism fluxes might decline in response to storage-dependent alterations of the multimeric organization of key enzymes, including glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase and biphosphoglycerate mutase [29].

From glycolysis to branching pathways

RBCs contain at least 2289 distinct proteins, and most are involved in energy and redox metabolism [30]. Maintenance of the redox poise in RBCs is mainly attributable

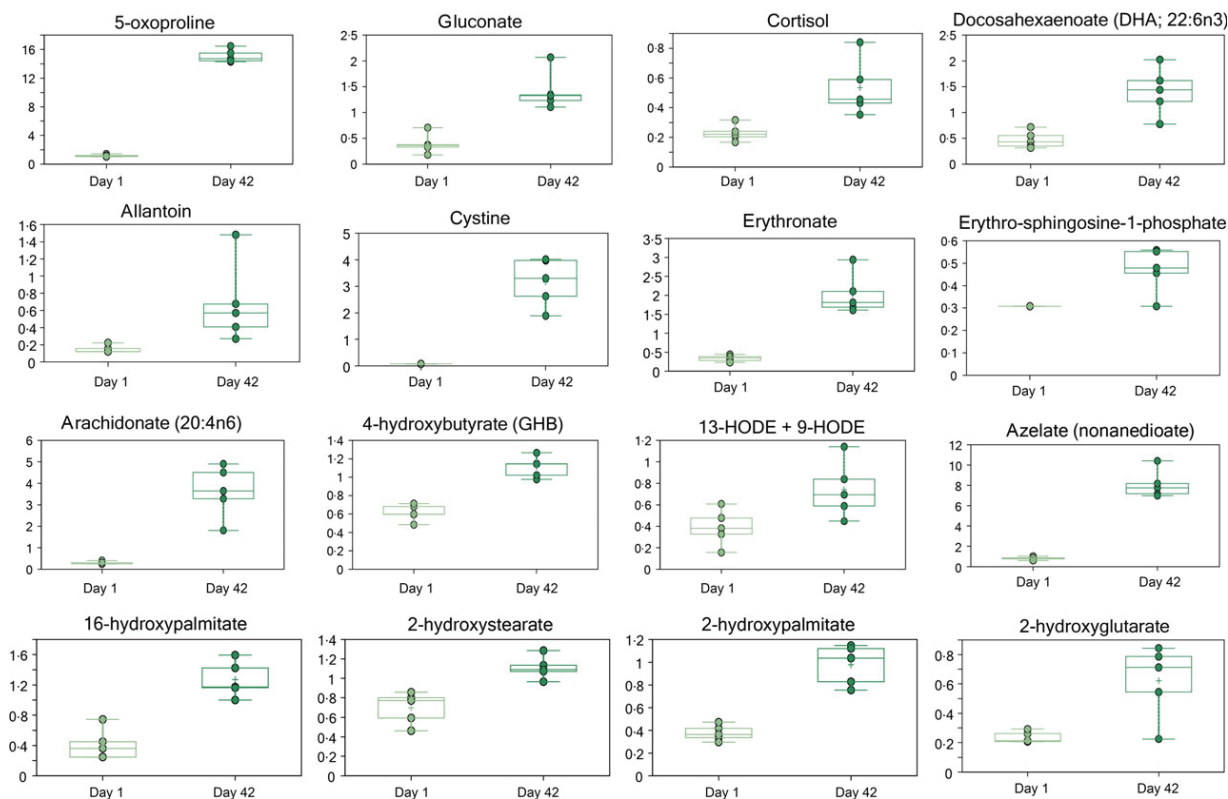


Fig. 4 Oxidative stress soluble and lipid markers.

to glutathione, a tripeptide of glutamate, glycine and cysteine whose synthesis is sustained in an ATP-dependent fashion to promote RBC survival in response to oxidative stress [7, 8, 20]. During prolonged storage, regeneration of reduced glutathione from its oxidized form is a key factor determining RBC survival [20]. Branching from glycolysis, the PPP generates NADPH and thus preserves glutathione homeostasis by balancing oxidized and reduced forms of the gamma-glutamylcysteine-glycine tripeptide. Fluxes to the PPP are modulated by competitive binding of glycolytic enzymes and deoxyhaemoglobin to the N-terminal cytosolic domain of band 3 [31]. Routine storage promotes a caspase and ROS-mediated fragmentation of this domain [8], thereby impairing RBC capacity to cope with oxidative stress by promoting NADPH generation via the PPP [5, 6, 28]. Therefore, stored RBCs are characterized by a significant accumulation of ROS and oxidative stress markers to proteins (carbonylation) and lipids (malondialdehyde) soon after the second week of storage [4, 5, 11].

Transaminases and the NADH reservoirs

Recent studies on RBCs stored in AS-1 (which contains more than twice as much glucose than SAGM or AS-

5:111 vs. 45 mM) demonstrated that additive solutions other than SAGM might better preserve energy modulation in stored erythrocytes, while fuelling metabolic pathways branching from glycolysis other than the hexose monophosphate shunt [5]. Biosynthesis of glutathione would benefit from a sustained accumulation of its precursors: glutamate, glycine and cysteine, the rate-limiting substrate. The presented data demonstrated a significant increase in glutamate (Fig. 1). Glutamate could be produced by glutamine metabolism or by the aspartate-dependent transamination of ketoglutarate by glutamate oxaloacetate transaminase (GOT). The presented data showed increased levels of all these metabolites in older units (Fig. 1). Increased levels of ketoglutarate despite increased glutamate concentrations can be attributed to the balancing activity of another transaminase. Indeed, glutamate can also result from the activity of glutamate pyruvate transaminase (GPT), either producing alanine and ketoglutarate (both increasing in Fig. 1) from pyruvate and glutamate or vice versa. Therefore, GPT would decrease the intracellular accumulation of pyruvate and relieve the burden on lactate dehydrogenase. In parallel, this mechanism would preserve a reservoir of NADH from lactate dehydrogenase-mediated oxidation to NAD^+ , which is relevant to the pivotal antioxidant activity of

NADH-dependent cytochrome b5 methaemoglobin reductase [7].

Although being devoid of mitochondria, RBCs still host cytosolic isoforms of NADP-dependent isocitrate dehydrogenase, and NAD-dependent malate dehydrogenase and fumarate hydratase [32]. Their activity promotes NADH and NADPH generation, and thus fuelling antioxidant pathways involving glutathione reductase and cytochrome b5 methaemoglobin reductase [7, 32]. The presented data suggest that stored RBCs could be capable of fulfilling the same goal in maintaining NADH homeostasis by coupling glycolytic fluxes to cytosolic TCA cycle enzymes. Further studies are mandatory within the framework of RBC storage for transfusion, since it is known that transaminase activities are negatively influenced by RBC age *in vivo* [32, 33].

These results should be also interpreted in the light of the fact that the TCA cycle is indeed present in the contaminating platelets and leucocytes, since prestorage leuco- and platelet reduction only removes 3.5 logs and ~2 logs, respectively. However, organic acids in RBC supernatants might play a role in positively influencing hypoxia-related responses as they can take part in the modulation of the life span of hypoxia inducible factor alpha (HIF α) by inhibiting its degradation via prolyl hydroxylase in the endothelial cells of the recipient [34] or rather contribute to ketoacidosis upon transfusion in trauma patients.

One-carbon and methionine metabolism to fuel cysteine and glycine generation as to sustain GSH biosynthesis

The supernatants of D42 RBCs also included increased levels of serine, as well as glycine and cysteine, which are derived from serine through one-carbon metabolism and cysteine biosynthesis/methionine trans-sulphuration pathways, respectively (Fig. 3). This finding has been reported in other cell models, such as tumour cells, in which one-carbon metabolism hyperactivity may occur in response to oxidative stress to fuel NADPH generation [35] and glutathione biosynthesis [36]. Analogously, Roback *et al.* [6] documented a storage-dependent increases in serine, cysteine and methionine-related metabolic pathways. Methionine may be generated by salvage from homocysteine, which uses betaine (Fig. 3) as a methyl donor or employs a reaction cycle that requires methyltetrahydrofolate and pyridoxal phosphate as cofactors in a NADPH generating pathway at the dihydrofolate reductase and methylenetetrahydrofolate reductase (MTHFR) activity steps.

Accumulation of metabolites involved in serine and cysteine metabolism (Fig. 3) may be partly attributable to

decreased methionine salvage and increased cysteine synthesis, in line with the posited increase of GSH biosynthesis during RBC storage AS-5[5, 20]. Lastly, Homocysteine accumulation (hyperhomocysteinaemia) is known to be caused by the decreased activity of MTHFR enzyme, or low levels of folate, B12 or B6, and predispose patients who receive stored blood to untoward vascular consequences [37].

Nucleotide metabolism

Red blood cells rely on salvage instead of *de novo* synthesis pathways to sustain nucleotide metabolism [7]. Consistently, despite AS5 formula containing a twofold higher dose of adenine than in SAGM, adenine in the supernatant was significantly consumed by the end of the storage period, and its deamination by-product, hypoxanthine, accumulated as a result (Fig. 2). These results are consistent with previous reports by Zolla's group on CPD-SAGM, RBCs stored under normoxic [5] or anaerobic conditions [38]. Purine catabolism can also contribute to oxidative stress through the activity of xanthine oxidase, which generates ROS [38].

Oxidative stress, plasticizer, haemolysis, fatty acids and vesiculation

Storage of RBCs promotes oxidative stress to the metabolome [4–6, 22–24], lipidome (isoprostanes, malondialdehyde) [5] and proteome (protein carbonylation, fragmentation, nonenzymatic glycation of haemoglobin and membrane proteins, relocation to the membrane of cytosolic antioxidant enzymes such as peroxiredoxin 2) [4, 7–11, 39, 40]. Several metabolites associated with stress responses and oxidative stress were elevated at D42 of storage, including 5-oxoproline. Oxoproline is involved in glutathione metabolism and was more abundant at D42 (Fig. 4) and recently reported to accumulate in SAGM-stored RBCs as a marker of impaired intra-cellular GSH homeostasis [20]. Gluconate accumulation reflects a ROS-dependent oxidation of glucose in the supernatant, which is consistent with previous observations in SAGM (Fig. 4) [5]. The stress steroid cortisol was more abundant at D42 (Fig. 4), probably as a result of the release from RBCs which can uptake cortisol while circulating *in vivo* [41]. The observed increases in allantoin (a purine catabolite made non-enzymatically by ROS in humans), cystine (the oxidized form of cysteine), erythronate (an oxidized aminosugar possibly derived from glycosylated proteins) and hydroxy-lipid signalling molecules (9 + 13-HODE, 2-hydroxystearate and 2-hydroxypalmitate) are also consistent with oxidative stress (Fig. 4).

Erythro-sphingosine-1-phosphate, a sphingolipid signalling molecule that regulates cell survival and inflammatory responses, was more abundant at D42 (Fig. 4). Collectively these changes document a significant increase in stress responses at D42 of storage.

In addition arachidonate increased significantly during storage of LR-RBC units identical to previous work [42]. Arachidonate has pro-inflammatory properties and demonstrated the ability to prime the NADPH oxidase of neutrophils and participate with the other neutral lipids, 5-, 12-, and 15- hydroxyeicosatetraenoic acid (HETE) that accumulate during routine storage of LR-RBCs in causing TRALI in a two-event animal model. In addition, lysophosphatidylcholines did not increase during storage of leucoreduced RBCs, in agreement with previous data, and likely due to the significant platelet reduction, 2 logs, by the prestorage leucoreduction filters (Haemonetics BPF4) employed in these experiments [43].

The accumulation of lipid pro-inflammatory mediators might hold potential pitfalls to certain categories of recipients and might thus indicate the necessity to consider supernatant washing prior to the administration of longer stored units [26].

Previous studies have documented storage-dependent progressive leaching from the plastic bags and intercalation in RBC membranes of the phthalate plasticizer (Fig. S2), a potential toxic compound whose detection in the urine has been recently proposed as a diagnostic test for the detection of illicit autologous blood doping practices in endurance sports [7].

Prolonged storage also results in the increased shedding of membrane portions in the form of micro and nanovesicles [12]. Such a vesiculation process ends up affecting RBC morphology during storage duration, as erythrocytes lose their discocyte shape and acquire a spherocytic phenotype [8, 9]. Such processes of membrane blebbing and vesiculation gradually compromise RBC capacity to cope with osmotic stresses [9]. Recent lipidomics studies have documented the preferential enrichment of membrane blebs with certain classes of lipids, especially ceramides (through the activity of sphingomyelinases), glycerophospholipids and sterols [15]. Glycerophospholipids, in particular, can be catabolized to generate signalling molecules and substrates that enter glycolysis in RBCs for energy production. In this study, the accumulation of several catabolites of membrane phospholipids in D42 supernatants indirectly testifies an exacerbated metabolism of these compounds, as gleaned from the accumulation of phospholipid head groups (ethanolamine and choline), glycerol, glycerol-3-phosphate and glycerophosphorylcholine (Fig. 1). These metabolites can be generated from phospholipase activity toward membrane phospholipids. Glycerate, either

generated from glycerol, or as a by-product of one-carbon metabolism from glycine, was also more abundant at D42 (Fig. 1).

While RBCs are devoid of mitochondria and are thus incapable of catabolizing fatty acids, they also show an incomplete *de novo* long-chain fatty acid biosynthesis enzymatic machinery [7]. Storage-dependent accumulation of fatty acid catabolites (including essential fatty acids, medium chain fatty acids, long-chain fatty acids and carnitine conjugates of LCFA) was observed in RBC supernatants (Fig. S1). Since they cannot derive from *de novo* synthesis, these molecules are likely to be released from RBC into the supernatant by means of the vesiculation process.

Finally, heme and biliverdin accumulated during routine RBC storage (Fig. S2), as if heme metabolism was blocked downstream to biliverdin reductase. These results are consistent with the age-dependent impairment in heme metabolism [7] and are consistent with the altered native multimerization of biliverdin reductase during storage progression, as recently gleaned via native preparative native 2D-CN-SDS-PAGE analyses (Pallotta *et al.*, paper in preparation).

Conclusion

In the present study, we performed metabolomic analyses of supernatants from RBCs stored in the presence of AS-5, as an indirect mirror of intracellular metabolism. As a result, we demonstrated the accumulation of pro-oxidant and pro-inflammatory compounds in the supernatants from stored units. Existing evidence regarding the progressive impairment of RBC energy metabolism by the end of the shelf life of packed RBCs was confirmed. At the same time, we present indirect evidence for altered NADPH and glutathione homeostasis, which in AS-5 RBCs could be promoted by the activity of transaminases, cytosolic isoforms of TCA cycle enzymes, serine/one-carbon and methionine trans-sulphuration metabolism.

These data explain how glycolysis, nucleotide and fatty acid metabolism were consistent with the progressive depletion of energy substrates from the additive solution and the accumulation of oxidized pro-inflammatory lipid derivatives, likely originating from membrane shed vesicles. While these results had been anticipated by metabolomics investigation on the RBC cytosolic fraction, no study had hitherto addressed the metabolome of the AS-5 RBC supernatants. The presented results also document that the supernatants of stored RBCs could represent a burden to the transfused recipients not only from a proteomics standpoint, as reported [13], but also from a metabolomics perspective.

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T32GM008315, and Grant #P50 GM049222 from NIGMS, NIH (CS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest

The authors disclose no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Free short, medium and long chain fatty acids.

Figure S2 Heme metabolism and phthalate levels.

Data S1 Materials and Methods extended version.

Early hemorrhage triggers metabolic responses that build up during prolonged shock

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¹Department of Biochemistry and Molecular Genetics, School of Medicine, University of Colorado Denver, South, Aurora, Colorado; ²Department of Surgery/Trauma Research Center, School of Medicine, University of Colorado Denver, Anschutz Medical Campus, Aurora, Colorado; ³Department of Surgery, Denver Health Medical Center, Denver, Colorado; ⁴Department of Pediatrics, School of Medicine, University of Colorado Denver, Aurora, Colorado; and ⁵Research Laboratory, Bonfils Blood Center, Denver, Colorado

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D'Alessandro A, Moore HB, Moore EE, Wither M, Nemkov T, Gonzalez E, Slaughter A, Frago M, Hansen KC, Silliman CC, Banerjee A. Early hemorrhage triggers metabolic responses that build up during prolonged shock. *Am J Physiol Regul Integr Comp Physiol* 308: R1034–R1044, 2015. First published April 15, 2015; doi:10.1152/ajpregu.00030.2015.—Metabolic staging after trauma/hemorrhagic shock is a key driver of acidosis and directly relates to hypothermia and coagulopathy. Metabolic responses to trauma/hemorrhagic shock have been assayed through classic biochemical approaches or NMR, thereby lacking a comprehensive overview of the dynamic metabolic changes occurring after shock. Sprague-Dawley rats underwent progressive hemorrhage and shock. Baseline and postshock blood was collected, and late hyperfibrinolysis was assessed (LY30 >3%) in all of the tested rats. Extreme and intermediate time points were collected to assay the dynamic changes of the plasma metabolome via ultra-high performance liquid chromatography-mass spectrometry. Sham controls were used to determine whether metabolic changes could be primarily attributable to anesthesia and supine positioning. Early hemorrhage-triggered metabolic changes that built up progressively and became significant during sustained hemorrhagic shock. Metabolic phenotypes either resulted in immediate hypercatabolism, or late hypercatabolism, preceded by metabolic deregulation during early hemorrhage in a subset of rats. Hemorrhagic shock consistently promoted hyperglycemia, glycolysis, Krebs cycle, fatty acid, amino acid, and nitrogen metabolism (urate and polyamines), and impaired redox homeostasis. Early dynamic changes of the plasma metabolome are triggered by hemorrhage in rats. Future studies will determine whether metabolic subphenotypes observed in rats might be consistently observed in humans and pave the way for tailored resuscitative strategies.

hemorrhagic shock; mass spectrometry; metabolomics; plasma; trauma

DESPITE DECADES OF ADVANCES in prehospital care, trauma remains the leading cause of death for individuals under the age of 40 (36). As much as 40% of injury-related mortality is attributed to uncontrollable hemorrhage (36), which in both civilian and military settings is the leading preventable cause of death after injury (40). Conspicuous factors associated with

early mortality in trauma patients include trauma-induced coagulopathy, hypothermia, and metabolic acidosis, a series of mechanisms referred to as the “bloody vicious cycle” and later renamed as the “lethal triad” (14). These concepts laid the foundation for “damage control surgery”, an approach aimed at minimizing operating time as to control sources of significant bleeding and gastrointestinal contamination, while prioritizing early management of coagulopathy, hypothermia, and metabolic acidosis (46).

Early descriptions of metabolic responses to trauma were documented by Cuthbertson, who characterized two distinct phases: the “ebb” and the “flow” (9). The former corresponds to an early hypometabolic state that may serve a protective role aimed at reducing posttraumatic energy depletion. The latter is accompanied by an increased metabolic rate (including increased energy expenditure and oxygen consumption) (14, 18, 24, 29). Other overlapping stages have been described over the years, such as the “ischemia-reperfusion,” “leukocytic,” and “angiogenic” (2), although the boundaries between these phases are rather labile (4, 24). The incomplete understanding of the metabolic stages seen during trauma and hemorrhage has hampered the capacity to significantly improve resuscitative strategies.

Advances in fields such as proteomics (10) and metabolomics (6, 8, 32, 42) offer big strides toward the understanding of the complex biochemistry underpinning metabolic responses to trauma/hemorrhagic shock. Mass spectrometry-based metabolomics has emerged as a more sensitive analytical approach in early “ischemia-reperfusion injury phase” responses (6), allowing detection of thousands of molecular features corresponding to hundreds of small molecules (<1.5 kDa) representative of key metabolic pathways (e.g., glycolysis, Krebs cycle, and ATP/purine catabolism).

Just as the “omics” era has enabled a more sophisticated approach to understanding changes in metabolism, viscoelastic assays have greatly expanded our understanding of the role of fibrinolysis in trauma-induced coagulopathy. The more severe forms of fibrinolysis (>15% of clot lysis 30 min after reaching maximum clot strength) can predict mortality, while fulminant lysis (complete clot degradation within 30 min) is associated with 100% mortality (26). Metabolic acidosis and coagulopathy are, indeed, deeply intertwined (14, 34). Recent studies identifying postinjury fibrinolysis using thrombelastography

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(TEG) have refined the degree of clot lysis that is associated with adverse outcomes from >15% to >3% (21). However, no observational evidence has been reported so far about the potential correlation of metabolome profiles and fibrinolysis. Identifying metabolites associated with fibrinolysis can lead to therapeutic interventions to attenuate impaired regulation preventing progression to hyperfibrinolysis.

In this study, we performed an ultra-high-performance liquid chromatography-mass spectrometric (UPLC-MS) analysis of the dynamic changes in the rat plasma metabolome following rapid hemorrhage and prolonged shock compared with sham controls. The purpose of this study is to document the metabolic responses of rapid near-lethal hemorrhage and prolonged hemorrhagic shock in a model that produces a hyperfibrinolytic coagulopathic phenotype. We hypothesize that metabolic changes that occur early during hemorrhage will increase during shock, in parallel to an increase in fibrinolysis (LY30) and plasma levels of the activator protein of fibrinolysis (tissue plasminogen activator).

METHODS

Animal model. The animal protocol (Fig. 1) was approved by the University of Colorado International Animal Care and Use Committee no. 90814. Juvenile male Sprague-Dawley rats (275–400 g) were induced with pentobarbital sodium followed by tracheostomy and femoral artery cannulation to measure blood pressure and induce hemorrhage. Prior to initiation of hemorrhagic shock, animals were allowed to recover from their initial surgery for airway and vascular access, including maintenance of normothermia (temperature >36°C), heart rate >240, and mean arterial pressure (MAP) >85 mmHg.

Sham. Blood draws were performed at baseline, after 1 and 30 min (consistent with the last time point of the shock group) to determine whether baseline values in the absence of hemorrhagic shock would result in plasma metabolic changes due to anesthesia and supine positioning. Blood samples obtained for the total duration of the experiment were less than 15% estimated blood volume (EBV).

Hemorrhage. Blood draws were adjusted for weight differences among rats, and the EBV was calculated from the animal weight employing the conversion of 0.06 ml of blood per gram conversion factor. The first blood sample obtained (baseline) consisted of 8% ($\pm 1\%$) of the total EBV. This was determined to be the minimal amount of blood obtained at a single time point to run TEG and have residual plasma for metabolomics analyses. This baseline blood draw was the first of a series of blood draws to induce hemorrhagic shock to attain a MAP of 25 (± 2) mmHg in 5 min. Blood volume (0.5 ml) from these

time points was $\leq 3\%$ EBV for the smallest animals. MAP was recorded at minute intervals correlating with 0.5-ml blood draws through minutes 1–5 with an additional blood draw (maximum 1.5 ml) if the animal's blood pressure did not drop sufficiently over the prescribed serial blood draws.

Shock. Animals were kept at MAP of 25 for 30 min, since the time window from injury to arrival at our hospital for most severely injured patients who underwent emergency department thoracotomies was 20–30 min (interquartile range, data not shown), with an average of 24 min. The degree of shock was selected on the basis of previous work with rodents that did not show changes in coagulation when blood pressure was >30 mmHg (47). Scheduled blood draws of 0.5 ml were drawn at 10 min and 20 min after the goal MAP was achieved. Additional blood was removed between these time points if the MAP exceeded 28 mmHg. At the end of 30 min, a final blood draw of 8% EBV was obtained, which was lethal in 100% of animals within a 10-min time frame.

Blood samples, rodent thrombelastography, and tissue plasminogen activator measurement. Whole blood was collected in 3% citrated at a 1:10 ratio, based on our previous experience with rodents and TEG (48). Individual Eppendorf tubes were pre-filled with citrate and marked to an appropriate fill level to ensure reproducible ratios of whole blood to citrate. Citrated native TEG assays were recalcified and run according to the manufacturer's instructions on a TEG 5000 Thrombelastograph Hemostasis Analyzer (Haemonetics, Niles IL). The following parameters were recorded from the tracings of the TEG: R time (minutes), angle (α , °), maximum amplitude (MA, mm), and lysis 30 min after MA (LY30, %). Whole blood was activated for TEG analysis with recalcification. Blood not used for TEG was spun to plasma for metabolomics analysis. Whole blood was centrifuged at 6,000 g for 10 min at 4°C. Plasma was removed and spun at 12,500 g for 10 min at the same temperature to remove contaminating platelets and acellular debris. The remaining plasma was flash frozen in liquid nitrogen and stored at -80°C until analyzed.

The levels of tissue plasminogen activator (tPA) were measured via ELISA, as previously reported (48).

Metabolomics analyses. Plasma samples (10 μl) were immediately extracted in ice-cold lysis/extraction buffer (methanol:acetonitrile: water 5:3:2) at 1:25 dilutions. Samples were then agitated at 4°C for 30 min and then centrifuged at 10,000 g for 15 min at 4°C. Protein and lipid pellets were discarded, while supernatants were stored at -80°C prior to metabolomics analyses.

Metabolomics analysis. Metabolomics analyses were performed as previously reported (12). Ten microliters of sample extracts were injected into an UPLC system (Ultimate 3000, Thermo, San Jose, CA) and run on a Kinetex XB-C18 column (150 \times 2.1 mm, 1.7- μm particle size; Phenomenex, Torrance, CA) at 250 $\mu\text{l}/\text{min}$ (mobile phase: 5% acetonitrile, 95% 18 mM H_2O , 0.1% formic acid). The

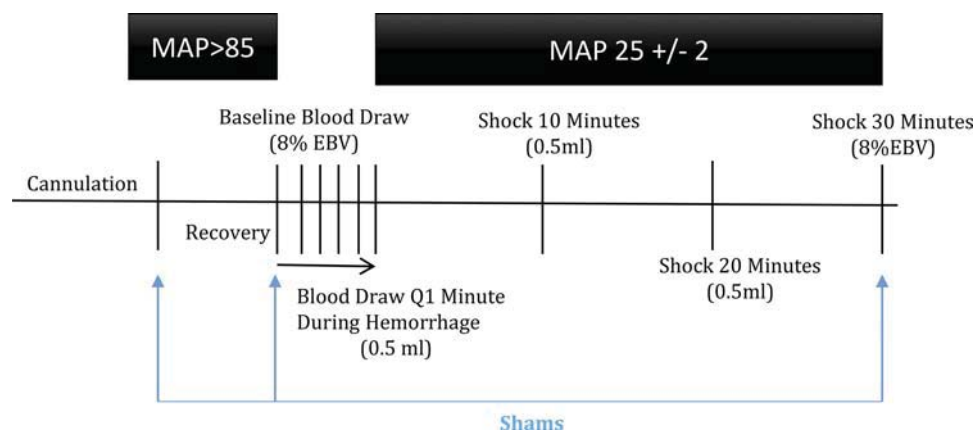


Fig. 1. An overview of the Sprague-Dawley rat animal model of hemorrhage adopted in this study.

UPLC system was coupled online with a QExactive system (Thermo, San Jose, CA), scanning in Full MS mode (2 μ scans) at 70,000 resolution in the 60–900 m/z range, 4-kV spray voltage, 15 sheath gas, and 5 auxiliary gas, operated in negative and then positive ion mode (separate runs). Calibration was performed before each analysis against positive or negative ion mode calibration mixes (Piercenet; Thermo Fisher, Rockford, IL) to ensure subparts per million error of the intact mass. Metabolite assignments were performed using the software Maven (7) (Princeton, NJ), upon conversion of .raw files into .mzXML format through MassMatrix (Cleveland, OH). The software allows for peak picking, feature detection, and metabolite assignment against the KEGG pathway database. Assignments were further confirmed against chemical formula determination (as gleaned from isotopic patterns and accurate intact mass), and retention times against a library of 619 standard compounds (Sigma-Aldrich, St. Louis, MO; MLSMS, IROATech, Bolton, MA).

Statistical analysis. Relative quantitation was performed by exporting integrated peak area values into Excel (Microsoft, Redmond, CA) for statistical analysis (repeated-measures ANOVA with Tukey multiple-column comparison test, significance threshold for P values < 0.05) and partial least square discriminant analysis (PLS-DA), calculated through the macro MultiBase (freely available at www.NumericalDynamics.com).

Hierarchical clustering analysis (HCA) was performed through the software GENE-E (Broad Institute, Cambridge, MA; freely available at <http://www.broadinstitute.org/cancer/software/GENE-E/>). Box and whisker plots were graphed through GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA), and figure panels were assembled through Photoshop CS5 (Adobe, Mountain View, CA).

RESULTS

Plasma metabolomics analyses were performed on seven Sprague-Dawley rats exposed to progressive hemorrhage (5 min: H1 through H5), or hemorrhagic shock for 10, 20, or 30 min (S10, S20; see *Shock* and Fig. 1). All of the rats involved in this study demonstrated systemic hyperfibrinolysis via TEG (LY30 >3%) after severe hemorrhagic shock (Supplemental Table S1). Overall, 118 metabolites were monitored throughout each stage, and results are reported in Supplemental Table S1, together with the metabolite name, KEGG pathway ID, super-pathway assignment (color-coded consistently throughout the paper), the polarity in which each metabolite has been detected, the experimentally observed mass-to-charge ratios, line plots showing the trends throughout each sample on the basis of the median values across each biological replicate, statistical analyses comparing late shock time points, specifically S10, S20, and Shock, to baseline values, together with fold-changes of median values from Shock rats against baseline parameters.

To highlight metabolic trends throughout the tested stages, results were elaborated for HCA, as reported in Fig. 2, whereby metabolites are grouped in a pathway-wise fashion (metabolite names are extensively reported in Supplemental Fig. S1, also including metabolite names and clustering patterns). Metabolite levels in each biological replicate during time course analyses were, thus, used to inform the elaboration of partial-least square discriminant analyses (PLS-DA), as reported in Fig. 3. Sample group clustering followed a clockwise distribution (PC1+PC2 accounting for almost the 50% of the total covariance, with PC1 explaining 45.8% of the covariance alone), a distribution that closely paralleled the phenotype (time-course measurements; Fig. 3A). The top 10 metabolites showing the highest covariances across the principal compo-

nents are labeled in Fig. 3B. Lactate was another key contributor to metabolic group clustering in response to hemorrhagic shock. Other key compounds include metabolites involved in the tricarboxylic acid (TCA) cycle, such as citrate and malate, metabolites involved in lipid metabolism (acyl-conjugated carnitines), purine/nitrogen catabolism substrates and by-products (nicotinamide, urate, and the polyamine spermidine), and glutathione homeostasis/oxidative stress (glutamate and glutathione disulfide, GSSG).

Although metabolic trends were consistent across biological replicates (Fig. 2), hive plots of key metabolites were graphed (Fig. 4) as to highlight twofold quantitative changes (either increases or decreases) that could not be appreciated through a linear color-coding of normalized values as the one used for heat maps. The choice of these metabolites was informed by PLS-DA analyses (Fig. 3) and line plot analyses of robust Z-score-normalized linear metabolic changes across metabolic stages, both in each independent rat and in the overall set of samples assayed in this study (Supplemental Fig. S2). These changes were observed in the hemorrhage/shock samples, albeit not in the sham group (Supplemental Fig. S3). As a result, we could highlight rat-specific patterns for some of these key metabolites, such as lactate, malate, and uracil, twofold decreasing in a subset of biological replicates at earliest hemorrhage time points (H1 through H5), while increasing twofold after 10, 20, or 30 min from hemorrhage through hemorrhagic shock (S10, S20, Shock). Other metabolites like glutamate, GSSG, and urate increased twofold in all of the samples during early hemorrhage (H time points), even before significant hemorrhagic shock could ensue (S10, S20, Shock). Again, these changes were absent in the sham group and only began to be visible (though remaining still significantly lower than in the hemorrhage/shock samples) for some metabolites (e.g., urate) at the 45-min draw in the sham plasma samples.

A pathway-wise elaboration of the results was performed as to gain mechanistic insights of the dynamic metabolic changes secondary to hemorrhage and hemorrhagic shock. Results are schematized from Figs. 5–7, either providing a snapshot of key intermediates of energy metabolism (glycolysis and TCA cycle, Fig. 5), fatty acid mobilization, transamination, glutathione homeostasis, and urea cycle (clockwise order, Fig. 6), and purine metabolism (Fig. 7). Most of the changes observed in response to hemorrhagic shock (S10, S20, Shock) are preceded by consistent trends (mostly toward increase) in the levels of most metabolites, trends beginning during early hemorrhage (H1 through H5). However, biological variability (exemplified by the hive plot in Fig. 4) negatively affected the calculation of statistical significance at these early stages.

Energy metabolism. Progressive plasma accumulation of glucose and fructose was observed upon hemorrhage, although significantly increased hyperglycemia was observed only upon hemorrhagic shock. Consistently, glycolytic metabolites, including hexose-phosphates and late by-products pyruvate and lactate, increased progressively from H3–H4 hemorrhage time points as to become significant after late hemorrhagic shock. Of note, lactate did decrease twofold during early hemorrhage (H1–H3) in three rats, before increasing twofold at the immediately subsequent hemorrhage time point in all the biological replicates (Fig. 4).

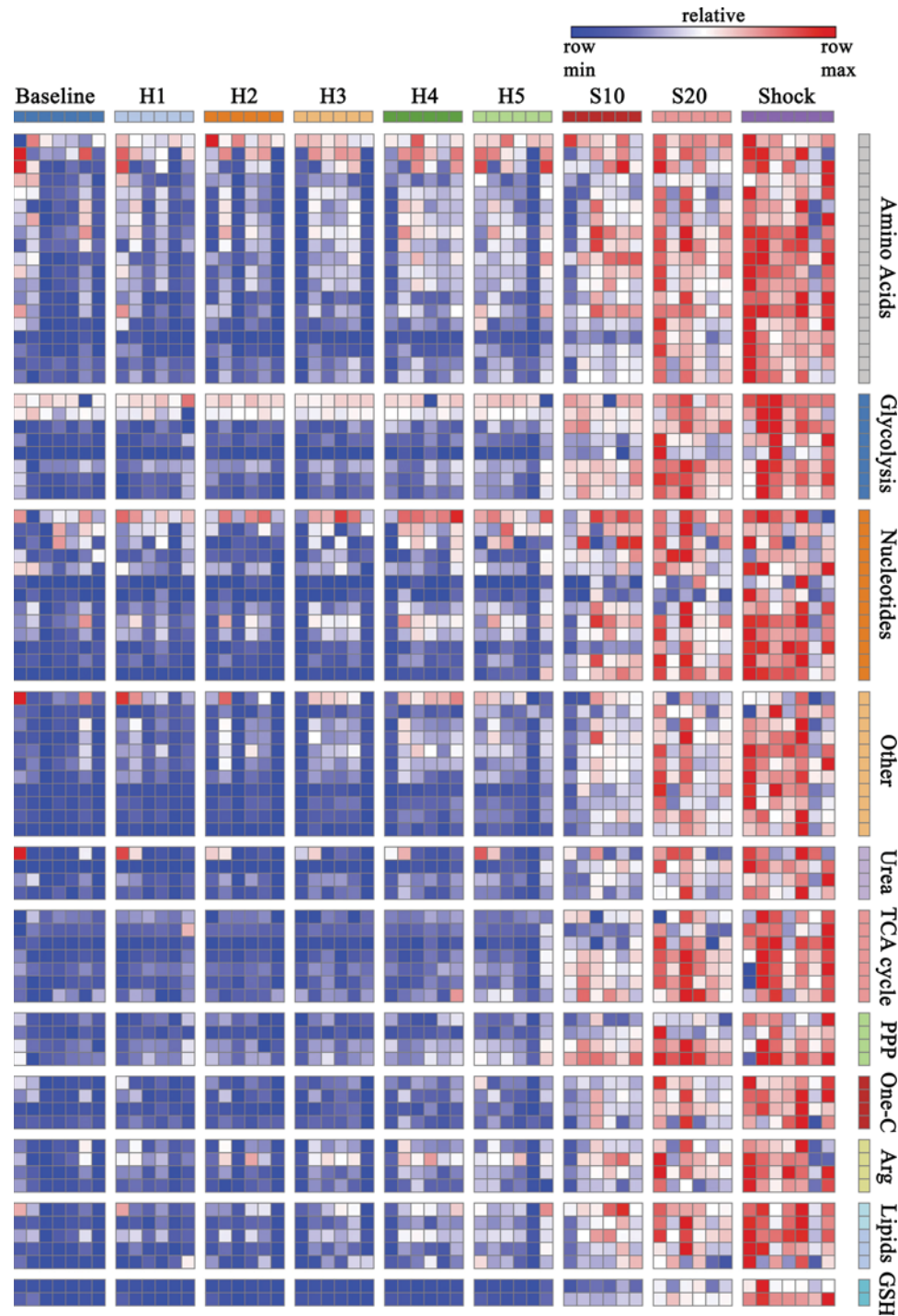


Fig. 2. Heat map of clustered (1-Pearson correlation) of metabolic changes in rat plasma upon exposure to gradual hemorrhage (H1 to H5), prolonged hemorrhage (S10, S20), and hemorrhagic shock (Shock) compared with the baseline. Each square is representative of the levels of that metabolite (names and hierarchical clustering are provided in Supplemental Fig. S1). Row values are Z-score-normalized for each metabolite across all biological replicates per each time point, while quantitative changes are color-coded from blue (low) to red (high). Pathways assignment is provided in the right y-axis of the map.

In addition, lipolysis and β -oxidation of lipids were suggested by the progressive accumulation of glycerophospholipid breakdown products (N-methylethanolamine phosphate, glycerol-3-phosphoethanolamine, ethanolamine phosphate), fatty acid-mobilizing acyl-carnitines (propanoyl-carnitine, butanoyl-carnitine), and ketone bodies (acetoacetate and hydroxyisobutyrate, Supplemental Table S1). Changes in lipolysis and β -oxidation were evident even before hemorrhagic shock and were statistically significant after it. On the other hand, the levels of these metabolites did not change in the sham group (Supplemental Fig. S3).

Hypercatabolism was also indicated by increased levels of all TCA cycle intermediates, from citrate to ketoglutarate, succinate, fumarate, malate, and oxaloacetate in the shock group, but not in shams (Fig. 5, Supplemental Fig. S3). Plasma levels of the carboxylic acid citramalate increased after hemorrhagic shock, while they did not in the sham group (Fig. 5, Supplemental Fig. S3).

Amino acids: proteolysis, transamination, and glutathione homeostasis. In the present study, all of the amino acid levels, except glycine, increased in response to early hemorrhage and shock (but not in sham animals) (Fig. 6, Supplemental Table

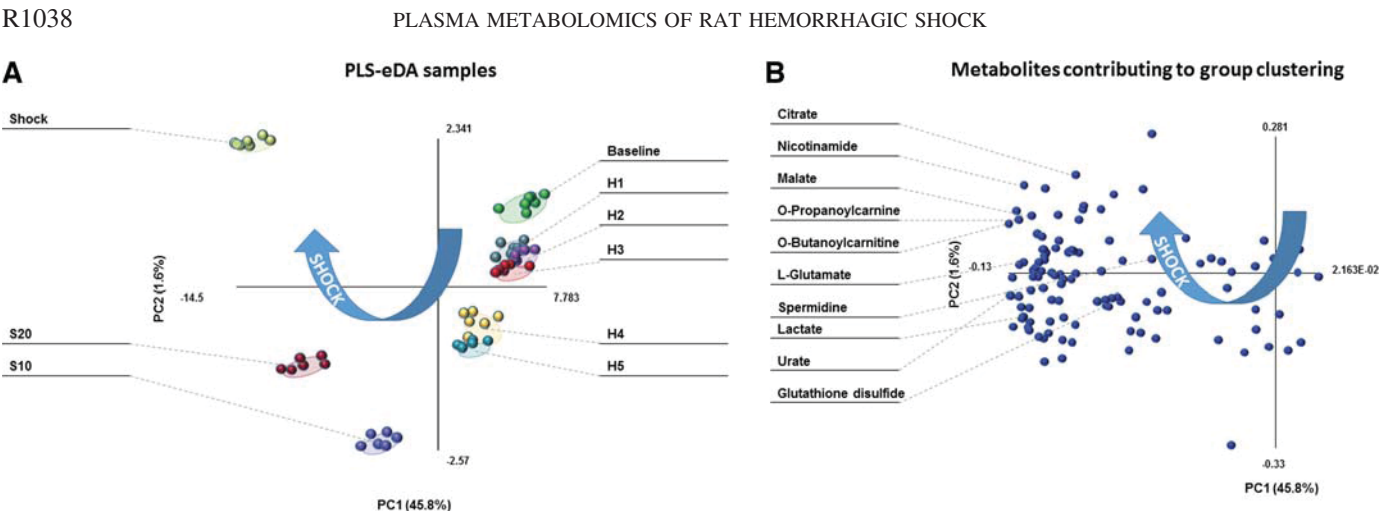


Fig. 3. Partial least square analysis of metabolic changes in rat plasma upon exposure to hemorrhage and hemorrhagic shock. In A, clusters are indicative of each sample group (from baseline to Shock). Cluster distribution followed a clockwise trend, paralleling the phenotype. In B, metabolites (variables) are graphed, contributing to maximize the covariance (mostly explained by PC1 = 45.8%, and to a lesser extent by PC2 = 1.6%) throughout all the samples (observations) in A. The top 10 metabolites contributing the most to covariance are highlighted in B.

S1). However, their relative fold-change increase was not distributed proportionally to their normal observed frequency in vertebrates (31), with glutamate and tyrosine increasing the most (5.6- and 3.48-fold change increase over the baseline levels; Supplemental Fig. S4). Glutamate anabolism interme-

diates aminobutanoate and succinate semialdehyde increased immediately after early hemorrhage (Supplemental Table S1). Increases in the levels of reduced and oxidized glutathione (GSH and GSSG) and 5-oxoproline were observed in the shock group only, although only the former started increasing during

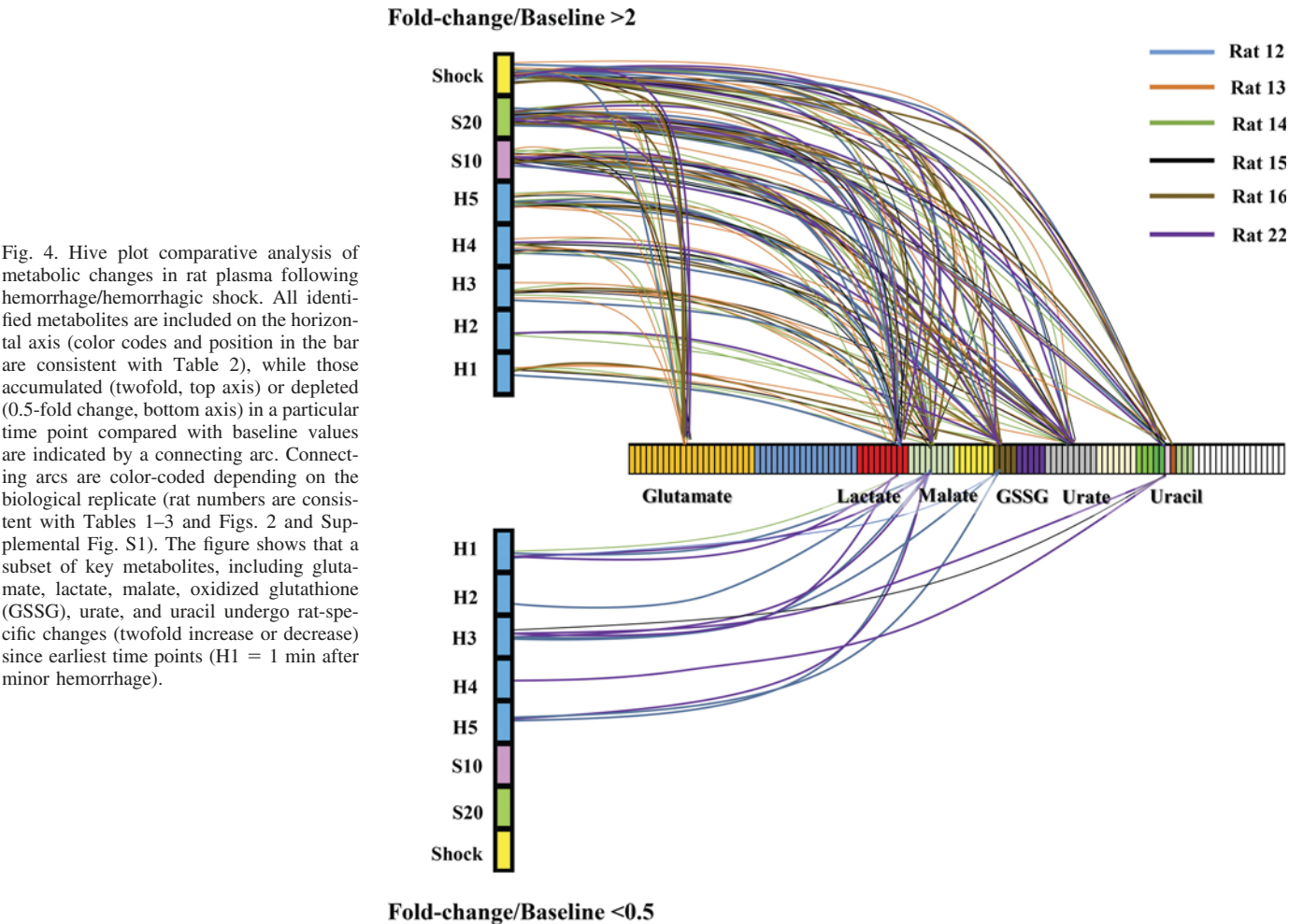


Fig. 4. Hive plot comparative analysis of metabolic changes in rat plasma following hemorrhage/hemorrhagic shock. All identified metabolites are included on the horizontal axis (color codes and position in the bar are consistent with Table 2), while those accumulated (twofold, top axis) or depleted (0.5-fold change, bottom axis) in a particular time point compared with baseline values are indicated by a connecting arc. Connecting arcs are color-coded depending on the biological replicate (rat numbers are consistent with Tables 1–3 and Figs. 2 and Supplemental Fig. S1). The figure shows that a subset of key metabolites, including glutamate, lactate, malate, oxidized glutathione (GSSG), urate, and uracil undergo rat-specific changes (twofold increase or decrease) since earliest time points (H1 = 1 min after minor hemorrhage).

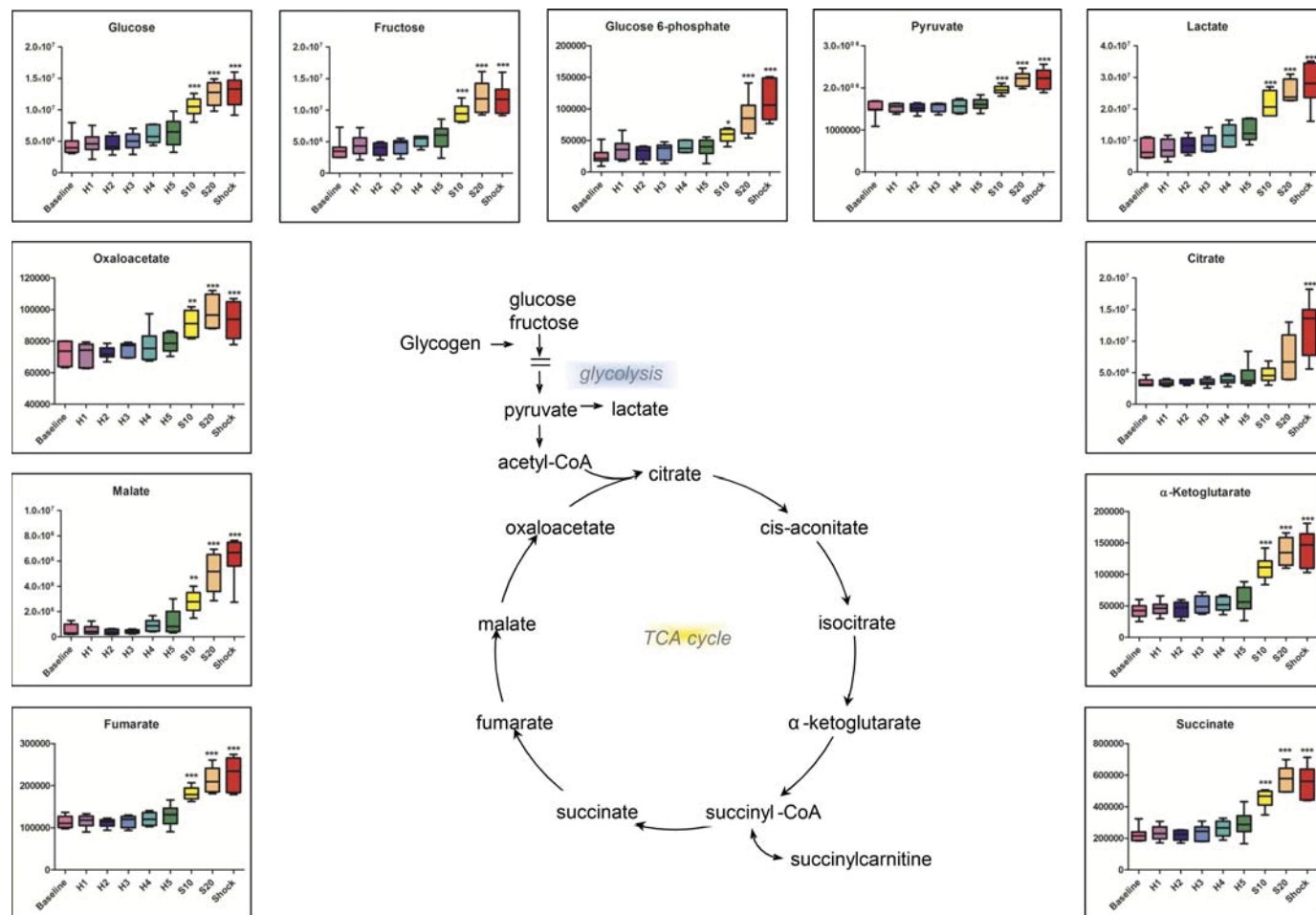


Fig. 5. An overview of glycolysis and the TCA cycle in plasma samples from rats undergoing progressive hemorrhage and hemorrhagic shock. Results are graphed as box plots, indicating median values (line), mean values (+), and upper/lower quartile distributions for each group. Asterisks indicate significance upon repeated-measures ANOVA test (* $P \leq 0.1$; ** $P \leq 0.01$; *** $P \leq 0.001$).

early hemorrhage (H3), and the latter started increasing only after prolonged hemorrhagic shock (Fig. 6).

Urea cycle and purine catabolism/salvage. Among all amino acids, arginine accumulation did not reach significance even upon late hemorrhagic shock (Fig. 6). Citrulline increased significantly upon late hemorrhagic shock (Fig. 6). The levels of urea cycle intermediates (e.g., arginine-succinate) did not increase significantly after hemorrhagic shock, while minor, but significant, late increase in the levels of ornithine was observed (Fig. 6). Polyamines accumulated since early stages of hemorrhagic shock, especially the late products of this pathway, spermidine and spermine, increasing up to 6.15- and 4.35-fold over the baseline values (Supplemental Table S1).

Purine catabolites urate, hydroxyisourate, and allantoin increased at H4 time points to become significant during prolonged hemorrhagic shock (Fig. 7). Nicotinamide increases were observed even later at S10, although they became significant only at S20 and Shock time points (Fig. 7). Immediate increases in the levels of adenosine followed a two-stage increase, with 1.4 increase ($P = 0.007$) immediately after hemorrhage (H1), and 2.17-fold increase after prolonged shock ($P = 0.004$). None of the metabolites described in this paragraph increased in sham animals (Supplemental Fig. S3).

DISCUSSION

Previous investigations on the metabolic response to trauma have consolidated the concept of trauma-induced hypercatabolism, resulting in an increased rate of proteinolysis of lean skeletal muscle sustained glycolysis, hepatic gluconeogenesis, and biosynthesis of acute-phase proteins (2, 3, 24, 45, 49). However, most of the evidence accumulated during the past decades has been based upon targeted biochemical assays, and no panoramic overview has been hitherto produced about the dynamic changes to the plasma metabolome induced by trauma/hemorrhagic shock. The advent of NMR-based metabolomics has allowed us to analyze tens of metabolites in a single analysis, thereby paving the way for a deeper understanding of the main metabolic changes secondary to traumatic injury (8, 32, 32, 42). Recent advances in the field of MS-based metabolomics have brought about a new era in the field of trauma metabolomics, even though reports available so far mainly focus on the “ischemia/reperfusion” stage (6). In the present study, we report the dynamic changes of the rat plasma metabolome in response to hemorrhage and sustained hemorrhagic shock, by exploiting a model of trauma-induced coagulopathy that results in the promotion of hyperfibrinolysis

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PLASMA METABOLOMICS OF RAT HEMORRHAGIC SHOCK

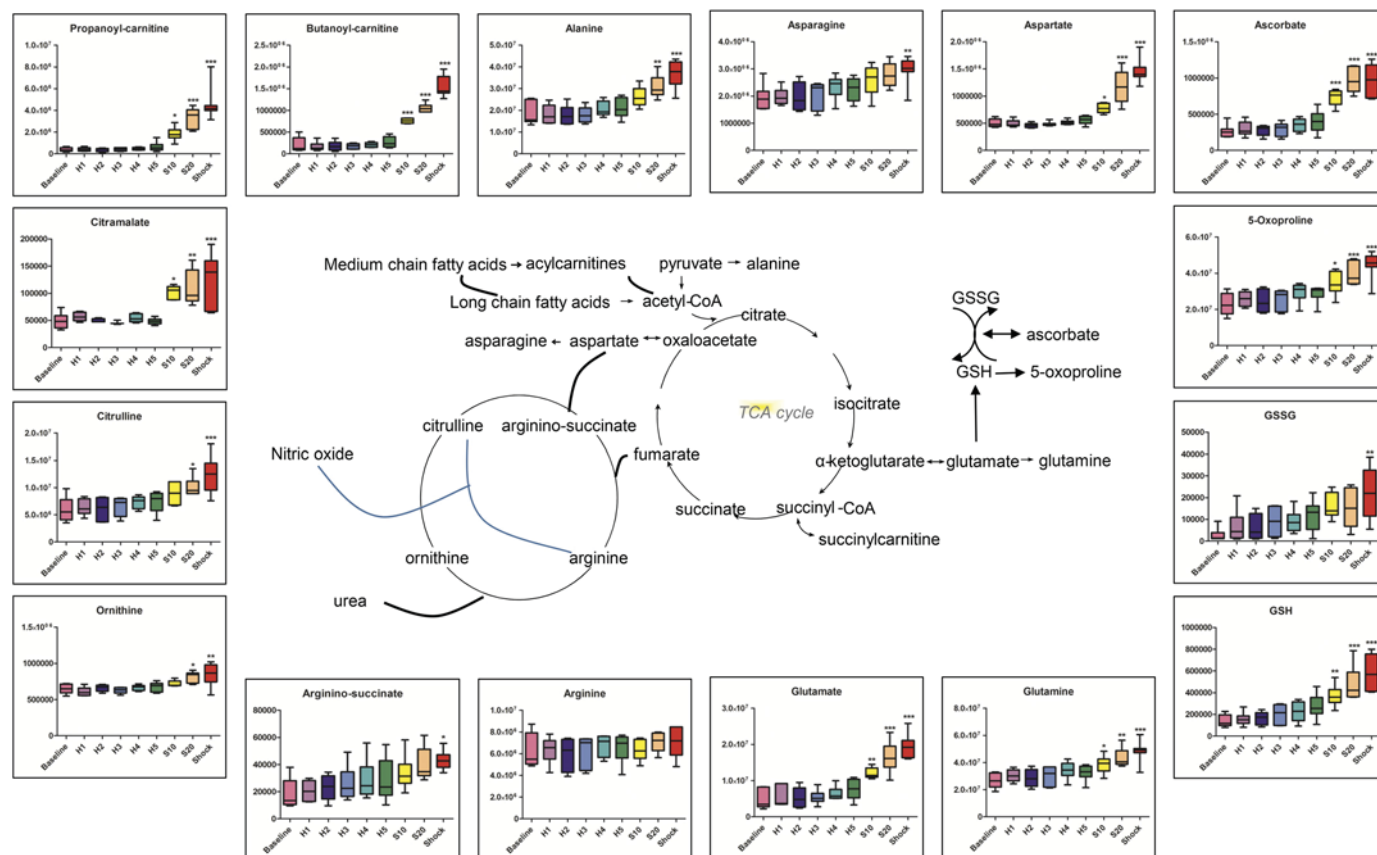


Fig. 6. An overview of protein (GSH homeostasis, transamination, and urea cycle) and lipid metabolism in plasma samples from rats undergoing progressive hemorrhage and hemorrhagic shock. Results are graphed as box plots indicating median values (line), mean values (+), and upper/lower quartile distributions for each group. Asterisks indicate significance upon repeated-measures ANOVA test (* $P \leq 0.1$; ** $P \leq 0.01$; *** $P \leq 0.001$).

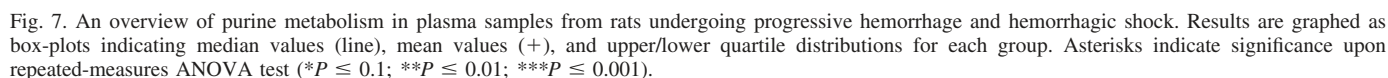
(LY30 >3%) and poor prognosis. The model was designed to test the hypothesis that metabolic changes in response to traumatic injury could ensue during progressive hemorrhage or could, instead, be triggered by acute hemorrhagic shock. As a result, in the shock group, but not in the sham one (a control group to determine the effect of anesthesia and supine positioning), the dynamic alterations of the metabolome involved almost all the metabolic pathways tested in this study, including energy metabolism (glycolysis, β -oxidation of fatty acid, TCA cycle), amino acid metabolism (amino acid levels, urea cycle, polyamines, and nitrogen balance), nucleotide catabolism, one-carbon metabolism, and glutathione homeostasis. No toxic effect by anesthesia alone (e.g., lactate accumulation) was observed in the sham group.

Early increases in plasma glucose is suggestive of ongoing hepatic gluconeogenesis or ongoing glycogenolysis, as to mobilize fast energy sources in response to hypoxia/local anoxia, even before actual hemorrhage shock has enough time to ensue, consistent with the concept of traumatic diabetes or trauma-induced insulin resistance. On the other hand, accumulation of glycolytic byproducts is in line with the literature describing lactate accumulation as the key contributor to acidosis in response to poly-trauma (24), critical illness, and isolated injury, such as in the case of concussive brain injury (30). Twofold increases in lactate observed in some rats at earliest hemorrhage time points might be representative of the existence of a differential metabolic phenotype across individ-

ual rats, with hypercatabolism ensuing immediately during hemorrhage or later upon a transient fast hypometabolic state (conceptually mimicking the “ebb” phase). Such early responses were not observed in the sham group either at early or late time points (baseline through 30 min).

Shock-induced lipolysis was observed, a phenomenon that is under tight hormonal regulation (17, 19). While response to injury may begin even before the injury itself, with awareness of approaching danger activating the hypothalamic defense area, the stability in the levels of ketone bodies in the sham group suggests that the observed phenomena are rather tied to hemorrhage rather than anesthesia and supine positioning alone.

Accumulation of TCA cycle intermediates after shock might result from local cellular breakdown (cell lysis) or mitochondrial uncoupling. These results are consistent with recent findings on ischemia/reperfusion injury in rat organs (6) and human plasma from trauma activation and emergency department thoracotomies subjects (38). These results are consistent with incommensurate oxygen demands by trauma patients to sustain mitochondrial metabolism (35). Accumulation of TCA cycle intermediates, especially succinate, has been reported to occur in response to hypoxia following ischemia through reverse TCA fluxing of carbon backbones from aspartate (6). Consistently, in our model, hypoxxygenation should ensue in response to hemorrhage. On the other hand, reperfusion has been reported to promote reverse electron transport chain



Citramalate is a carboxylic acid isobar to 2-hydroxyglutarate that unexpectedly increased in a shock-dependent fashion. This metabolite, produced by bacteria, has been recently found to be uptaken/pynocytosed by red blood cells (5). Hemolysis secondary to hemorrhagic shock might trigger release of this metabolite in the plasma and indicate as of yet uninvestigated ties between bacterial metabolism and bacterial metabolite-triggered responses to injury, even in the absence of sepsis.

Muscle proteolysis in response to trauma/hemorrhagic shock is known to promote the release of free amino acids for catabolic purposes (27, 37), as we here observe in response to shock, but not in shams. If plasma amino acid accumulation in response to hemorrhagic shock would be dependent in proteolysis alone, fold-change increases in amino acid levels should be proportional to their abundance in proteins. However, this did not seem to be the case upon a rough comparison of the shock/baseline amino acid ratios and the expected abundance of each amino acid in the proteome (as indirectly gleaned by the frequency of the codons for each amino acid in the vertebrate genomes). Amino acids like tyrosine and glutamate were extremely enriched, suggesting ongoing anabolic reactions toward their specific enrichment. Glutamate, a key amine group donor and excitatory neurotransmitter, can, indeed, be generated through glutamine deamination and, thereby, provide a carbon substrate for transamination reactions. In parallel, glutamate can be used as an amine group donor in transamination reactions to generate ketoglutarate, so as to fuel the TCA cycle. Thus, it is worth noting that glutamine levels increased to a lesser extent compared with other amino acids only upon hemorrhagic shock, albeit not in sham animals, suggesting ongoing glutaminolysis in response to hemorrhage/hemorrhagic shock. Enteral supplementation of glutamine is

one nutritional concept for severely injured patients in recent years (27, 37). Additionally, glutamine exerts an important nutritional effect serving as a principle fuel source for enterocytes and intestinal mucosa (24). However, recent evidence from labeling experiments on ischemia/reperfusion injury suggests that glutamine might serve a key role as a nitrogen donor, rather than as a carbon substrate (6).

Glutamine-derived glutamate might serve transamination purposes, thereby, compensating hypoxia-induced accumulation of pyruvate and oxaloacetate by promoting their conversion to alanine and aspartate, respectively. Anabolism of glutamate is further supported by the hemorrhage-dependent accumulation of synthesis intermediates aminobutanoate and succinate semialdehyde.

Hemorrhage-dependent accumulation of glutamate and cysteine was not accompanied by similar trends for glycine. While glycine can also fuel late glycolysis at the trioses stage, these three amino acids are the substrates for the biosynthesis of glutathione (GSH), hinting at alterations of the glutathione pool in response to hemorrhagic shock. Both GSH and GSSG levels increased in response to hemorrhage, suggesting an increased necessity to cope with oxidative stress. Of note, accumulation of 5-oxoproline was observed in shock rats (but not in sham animals), which is relevant in that oxoproline is a marker of glutathione turnover that has been associated with impaired GSH homeostasis in stored blood products (11). At the same time, 5-oxoproline can be converted back to glutamate by the enzyme oxoprolinase in an ATP-dependent fashion, contributing to the observed increase in glutamate. In the context of trauma, oxoproline might play a potential role as osmoprotectant and contribute to unexplained acidosis (15).

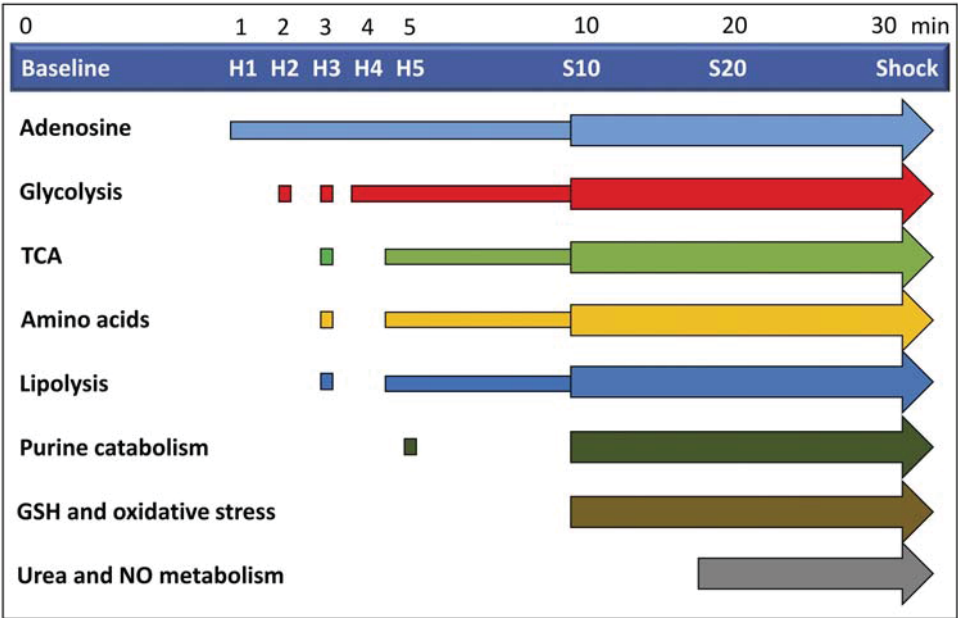
An arginine increase only after late shock is indicative of early arginine consumption, either through the urea cycle or as a substrate to promote nitric oxide generation by endothelial nitric oxide synthase (eNOS). Late citrulline increase supports the hypothesis of nitric oxide (NO) production by eNOS activity only after prolonged shock. NO generation would be relevant in that it would result in the promotion of vasodilation

(to meet higher energy and oxygen demands in response to hemorrhage-induced hypercatabolism). NO would also further strengthen hypoxic responses by stabilizing HIF-1 α (1), and promising beneficial effects have been documented for enteral arginine supplementation in rats undergoing trauma/hemorrhagic shock (43).

At the same time, aspartate accumulation is consistent with proteolysis, or partial impairment of the purine salvage reactions (deaminated IMP conversion back to AMP fueling the TCA cycle at the level of fumarate) (6). This is relevant in the light of the observed upregulation of purine catabolism in response to hemorrhage/hemorrhagic shock, resulting in the accumulation of IMP breakdown products inosine, hypoxanthine, and xanthine, and downstream metabolites urate, hydroxyisourate, and allantoin. Of note, urate conversion to hydroxyurate and allantoin is mediated by the rat enzyme uricase, which is nonfunctional in greater apes and humans, where no significant plasma accumulation of urate was observed in trauma patients (38). On the other hand, urate-to-allantoin conversion in humans might be driven by reacting oxygen species-scavenging reactions (28). In the light of these data, here, we hypothesize that accumulation of urate, a potent antioxidant, might represent an adaptive response against hemorrhage-triggered ischemia.

Hemorrhagic shock also apparently fueled pathways branching from the urea cycle, namely polyamine synthesis pathway. These highly basic compounds might play a role as pH buffers, other than contribute to osmoprotection resulting from hemorrhage-induced hypovolemia, suggesting hitherto under investigated ties between conserved stress-response mechanisms in plants and mammals (20). At the same time, polyamine metabolism has been previously related to edema formation and necrotic formation after traumatic brain injury (13) and increased urinary polyamine excretion has been observed in patients with surgical and accidental trauma undergoing total parenteral nutrition (39). As the hemorrhaged rats tested in this study developed hyperfibrinolysis, it is relevant to note how polyamines have been previously associated with the modula-

Fig. 8. A schematic representation of time course metabolic changes during early hemorrhage and prolonged hemorrhagic shock. Color codes for pathway arrows are consistent with pathway legends in Supplemental Table S2.



tion of blood coagulation and fibrinolysis (25). Further studies will address the specific hypothesis, as the hereby observed fold-change increase in polyamine levels in hemorrhaged rats is also observable in humans and affects coagulation cascades and clot stability.

Early shock increases in the levels of adenosine in response to hemorrhage document the insurgence of a key defensive protective auto/paracrine signaling cascade aimed at limiting cellular damage in response to adverse conditions, including hypoxia or ischemia (22). Indeed, adenosine release promotes vasodilation, stimulation of glycogen breakdown, and reduced neuronal excitability, other than neurotransmitter release to reduce neuronal energy requirements (22). Adenosine signaling via adenosine receptor A2B [A(2B)R] has been implicated in protection during acute kidney injury through the inhibition of neutrophil-dependent TNF- α release (23). While beneficial under control conditions, plasma adenosine increase in response to hypoxia has been observed to promote sickling of red cells in rats and humans through signaling via adenosine receptor A2B [A(2B)R] (50).

Late nicotinamide accumulation might result from the activity of poly-ADP-ribose polymerase, which can use nicotinamide adenine dinucleotides as substrates to trigger DNA damage and apoptosis/necrosis, and, in turn, partially deplete NAD/NADP reservoirs, thereby negatively influencing energy and antioxidant potential.

Perspectives and Significance

Dynamic changes in the rat plasma metabolome in response to progressive hemorrhagic shock were observed during early hemorrhage, before sustained hemorrhagic shock. Conversely, significant changes in early and late time points (30 min through shock) were not observed in the sham group. Specific phenotypes were observed, with half of the biological replicates showing trends toward early metabolic depression during hemorrhage, followed by hypercatabolism, while the other half showing immediate hypercatabolic reactions. While complementing and expanding recent evidence in humans (38), the appreciation of such metabolic phenotypes will pave the way for tailored resuscitative strategies. Testable hypotheses were generated, indicating a likely correlation of metabolic adaptation to hemorrhage (e.g., plasma elevation in the levels of succinate, urate, and polyamines), but not anesthesia and supine positioning, and the hyperfibrinolytic phenotype observed in the tested rats.

Conclusion. Almost immediate increases in the levels of adenosine were observed, suggesting a signaling role for this metabolite in early hemorrhage responses. In keeping with the literature, hypermetabolism corresponded to increased glycemia and glycolysis, accumulation of TCA cycle intermediates, and amino acid during hemorrhage, before prolonged hemorrhagic shock. Fatty acid mobilization and oxidation were observed during prolonged shock. There also appear to be a role for metabolic factors potentially associated with strengthening of hypoxic responses (stabilization of HIF-1 α , induction of vasodilation) upon prolonged shock, such as purine catabolism/salvage and urea cycle by-products (urate and polyamines), TCA cycle intermediates, nitric oxide generation, and adenosine (Fig. 8). In the light of these results, arginine and adenosine, other than glutamine, emerge as

likely candidates for nutrition resuscitative strategies in the intensive care setting.

Most of the observed metabolites, including carboxylic acids, oxoproline and other acids (e.g., urates), increasing up to 36-fold in response to hemorrhage/hemorrhagic shock, albeit not in shams, might justify nonlactate metabolic acidosis in the clinical setting. Future studies will be necessary on a larger animal study/human cohort through absolute quantitation-based metabolomics. These studies will improve our understanding of the correlation between the levels of these metabolites and the severity of shock and investigate whether functional ties exist between hemostatic function (e.g., platelet activation, fibrinolysis, as suggested by the presented correlative evidence herein) or immune responses (e.g., neutrophil priming) and the levels of the metabolites affected by hemorrhage/hemorrhagic shock.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A.D., H.B.M., E.E.M., E.G., A.S., K.C.H., C.C.S., and A.B. conception and design of research; A.D., H.B.M., M.J.W., T.N., E.G., A.S., and M.F. performed experiments; A.D., H.B.M., E.E.M., M.J.W., T.N., K.C.H., and A.B. analyzed data; A.D., H.B.M., E.E.M., K.C.H., C.C.S., and A.B. interpreted results of experiments; A.D. prepared figures; A.D., H.B.M., C.C.S., and A.B. drafted manuscript; A.D., H.B.M., E.E.M., M.J.W., E.G., K.C.H., C.C.S., and A.B. edited and revised manuscript; A.D., H.B.M., E.E.M., M.J.W., T.N., E.G., A.S., M.F., K.C.H., C.C.S., and A.B. approved final version of manuscript.

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BLOOD COMPONENTS

Routine storage of red blood cell (RBC) units in additive solution-3: a comprehensive investigation of the RBC metabolome

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BACKGROUND: In most countries, red blood cells (RBCs) can be stored up to 42 days before transfusion. However, observational studies have suggested that storage duration might be associated with increased morbidity and mortality. While clinical trials are under way, impaired metabolism has been documented in RBCs stored in several additive solutions (ASs). Here we hypothesize that, despite reported beneficial effects, storage in AS-3 results in metabolic impairment weeks before the end of the unit shelf life.

STUDY DESIGN AND METHODS: Five leukofiltered AS-3 RBC units were sampled before, during, and after leukoreduction Day 0 and then assayed on a weekly basis from storage Day 1 through Day 42. RBC extracts and supernatants were assayed using a ultra-high-performance liquid chromatography separations coupled online with mass spectrometry detection metabolomics workflow.

RESULTS: Blood bank storage significantly affects metabolic profiles of RBC extracts and supernatants by Day 14. In addition to energy and redox metabolism impairment, intra- and extracellular accumulation of amino acids was observed proportionally to storage duration, suggesting a role for glutamine and serine metabolism in aging RBCs.

CONCLUSION: Metabolomics of stored RBCs could drive the introduction of alternative ASs to address some of the storage-dependent metabolic lesions herein reported, thereby increasing the quality of transfused RBCs and minimizing potential links to patient morbidity.

Transfusion of red blood cells (RBCs) is a lifesaving therapy and allows for complex surgeries, organ and bone marrow transplantation, the administration of myelotoxic chemotherapy, and survival from serious blunt and penetrating injuries. In most countries, the shelf life of RBCs is limited to 42 days, as to ensure 24-hour recoveries higher than 75% and hemolysis below 0.8% thresholds. During storage, RBCs

ABBREVIATIONS: GSA = glutathione; HCA = hierarchical clustering analysis; LR = leukoreduction; PLS-DA = partial least square discriminant analysis; PPP = pentose phosphate pathway; TCA(s) = tricarboxylic acid(s); UPLC-MS = ultra-high-performance liquid chromatography separations coupled online with mass spectrometry detection.

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are characterized by the progressive accumulation of biochemical products commonly known as the “storage lesion.”¹¹ The storage lesion results in the impairment of cation homeostasis, the accumulation of reactive oxygen species, the alteration of proteomic profiles, and the progressive vesiculation of membrane portions that accompanies the loss of the discocytic morphology in exchange for a spherocytic RBC morphology.¹⁻⁶ Metabolomics studies have been completed on RBC units stored in a number of additive solutions (ASs), including SAGM,^{3,7,8} AS-1,⁹ MAP,¹⁰ PAGGGM,^{11,12} and AS-5.¹³ These studies paved the way for preliminary investigations aimed at determining the influence of alternative storage solutions (e.g., supplementation of antioxidants, vitamin C, *N*-acetylcysteine, or serotonin¹⁴⁻¹⁶) or strategies (such as anaerobic storage¹⁷) on stored RBC metabolic profiles. Recent innovations in metabolomics include the introduction of ultra-high-performance liquid chromatography separations coupled online with mass spectrometry detection (UPLC-MS), the assembly of publicly accessible metabolome databases, and the implementation of software dedicated to the mining and interpretation of extraordinarily large data sets.¹⁸

Despite encouraging results, none of the published studies provided comprehensive metabolome coverage of stored RBCs comparable to proteomics investigations, especially in the light of recent articles suggesting that the RBC proteome might be more complex (2289 distinct gene products) than previously thought.¹⁹ The metabolomics studies published so far have focused on either the intra- or the extracellular fraction without a comprehensive assessment of relative metabolite quantities in both fractions. Thus, data regarding the uptake or efflux and anabolism- or catabolism-dependent fluctuations were precluded by the experimental design. In addition, most of the reported studies use control samples removed from the RBC units on Day 1 after processing, which includes plasma removal, leukoreduction (LR), and resuspension in ASs. Finally, despite recent data that found that AS-3 ameliorated a number of important proteomic changes versus SAGM,²⁰ no complementary metabolomic analysis of AS-3–stored RBCs has been reported.

Therefore, we aimed to test the hypothesis that AS-3 preserves RBC metabolic homeostasis longer than other ASs, yet this preservation is limited to a shorter time window than the actual shelf life of the unit (42 days). Within this time window, we expect the RBC metabolome to undergo significant biochemical changes, leading to the depletion of high-energy phosphates, the impairment of glutathione (GSH) homeostasis, and the accumulation of a number of biochemical intermediates (especially amino acids), due to either the interruption of RBC anabolism or the increased proteolysis. We performed a comprehensive metabolomics investigation of AS-3 RBC extracts and supernatants and assayed both matrices before,

during, and minutes after processing, as well as at early storage time points (3 hr and Day 1) and weekly time points until the expiration of the unit (Day 42). Samples were analyzed via UPLC-MS, a strategy that allowed for significantly improved metabolome coverage and relative quantitation in comparison to previous studies.^{7,17}

MATERIALS AND METHODS

Blood processing and sample collection

One unit of whole blood (500 ± 50 mL) was collected from five healthy donors per AABB and FDA guidelines, using CP2D in the collection bag system (Nutricel, Pall Medical, Braintree, MA). Plasma was separated from RBCs by centrifugation followed by manual expression, and AS-3 (Nutricel) was added to a final hematocrit of approximately 60%. The estimated amount of residual plasma was 5 to 10 mL/unit. RBC units were leukoreduced before storage via filtration using the in-line LR filter (RC2D, Haemonetics, Braintree, MA) and stored at 1 to 6°C. Samples (0.5 mL) were obtained through sterile couplers before (PRE), during (POST), or after LR, at 3 hours from LR, or on Days 1, 7, 14, 21, 28, 35, and 42 (the last day a unit can be transfused). The supernatant was separated from RBCs via centrifugation ($5000 \times g$ for 7 min at 4°C) followed by a second centrifugation at $12,500 \times g$ for 6 minutes at 4°C to sediment residual cellular material and contaminating platelets. Although snap-freezing the samples would have resulted in immediate arrest of cell metabolism, the conditions adopted in this study were optimized as to allow separation of RBCs from supernatants while minimizing the technical bias on the metabolic readout by performing cold centrifugation steps (4°C).

Metabolomics extraction

RBCs and supernatants were immediately extracted in ice-cold lysis and extraction buffer (methanol : acetonitrile : water 5:3:2) at 1:3 and 1:25 dilutions. Samples were then agitated at 4°C for 30 minutes and then centrifuged at $10,000 \times g$ for 15 minutes at 4°C. Protein and lipid pellets were discarded, while supernatants were stored at -80°C before metabolomics analyses.

Metabolomics analysis

Five microliters of samples for both RBC and supernatant extracts were injected into a UPLC system (Ultimate 3000, Thermo, San Jose, CA) and run on an HPLC column (150×2.1 mm i.d., $1.7 \mu\text{m}$ particle size; XB-C18, Kinetex, Phenomenex, Torrance, CA) using a 3-minute isocratic gradient at $250 \mu\text{L}/\text{min}$ (mobile phase—5% acetonitrile, 95% $18 \text{ m}\Omega \text{ H}_2\text{O}$, 0.1% formic acid). The UPLC system was coupled online with a mass spectrometer (Q Exactive

system, Thermo), scanning in full MS mode (2 μ scans) at 70,000 resolution in the 60 to 900 m/z range, 4 kV spray voltage, 15 sheath gas, and 5 auxiliary gas, operated in negative and then positive ion mode (separate runs). Calibration was performed before each analysis against positive or negative ion mode calibration mixes (Piercenet, Thermo Fisher, Rockford, IL) to ensure sub ppm error of the intact mass. Metabolite assignments were performed using computer software (Maven,¹⁸ Princeton, NJ), upon conversion of .raw files into .mzXML format through MassMatrix (Cleveland, OH). The software allows for peak picking, feature detection, and metabolite assignment against the KEGG pathway database. Assignments were further confirmed against chemical formula determination (as gleaned from isotopic patterns and accurate intact mass) and retention times against a subset of standards including commercially available glycolytic and Krebs cycle intermediates, amino acids, GSH homeostasis, and nucleoside phosphates (Sigma Aldrich, St Louis, MO).

Relative quantitation was performed by exporting integrated peak areas values into a computer spreadsheet (Excel, Microsoft, Redmond, WA) for statistical analysis including t test and analysis of variance (significance threshold for p values < 0.05) and partial least square discriminant analysis (PLS-DA), calculated through the macro MultiBase (freely available at <http://www.NumericalDynamics.com>).

Hierarchical clustering analysis (HCA) was performed through computer software (GENE-E, Broad Institute, Cambridge, MA). XY graphs were plotted through statistical software (GraphPad Prism 5.0, GraphPad Software, Inc., La Jolla, CA), dot plots were generated with an in-house R script (<http://www.r-project.org>), and figure panels were assembled through digital image editing software (Photoshop CS5, Adobe, Mountain View, CA).

RESULTS

The complete metabolomic analyses of both RBC extracts and supernatants, which include compound names, KEGG pathway IDs, pathway assignments (color code explained in the legend at the end of each table), mass-to-charge ratios (m/z = parent), median retention times, median values for each time point, sparkline graphs indicating trends at a glance, the polarity mode (either positive or negative) in which the metabolite has been detected, and t test between each independent time point and Storage Day 1 were comprehensively reported (Tables S1 and S2, available as supporting information in the online version of this paper). Unambiguous assignment and relative quantitation was achieved for 229 metabolites in RBC extracts and 200 in supernatants. Clustering of metabolic profiles for RBC extracts and supernatants through PLS-DA (Figs. 1A and 1B, respec-

tively), indicated that pre-LR (PRE) samples were comparable to Day 1 RBCs or supernatants.

A trend was evident when analyzing samples from the LR stages (POST, LR), throughout storage duration on a weekly basis, indicating Storage Day 14 as a recurring inflection point both in RBC extracts and in supernatants (Figs. 1A and 1B). HCA through heat maps further confirmed the PLS-DA output and highlight trends toward a progressive decrease or increase in both RBC extracts and supernatants (Figs. 1C and 1D). These results illustrate evident quantitative (color code) changes from Storage Day 7 to Storage Day 14 (heat maps are also provided in Figs. S1 and S2 [available as supporting information in the online version of this paper] in a vectorial format, also including clustering layouts and metabolite names).

In Fig. 2 we provide an overview of the top 10 increasing (including lactate, alanine, sarcosine, asparagine, poly-gamma-glutamate, uracil, nicotinamide, adenosyl homocysteine, homocysteine, oxoglutarate, 5-oxoproline) and the top five decreasing (including phosphoglycerate, NAD⁺, cyclic adenosine monophosphate (AMP), oxalomalate, hexose phosphate, orthophosphate) metabolites in RBC extracts and supernatants (Figs. 2A and 2B, respectively) when comparing results from analyses of Storage Day 42 samples against Day 1 controls. Roughly, increasing metabolites can be classified as oxidative stress and transamination/one-carbon metabolism products, while decreasing metabolites are mostly related to energy metabolism. Box and whisker plots are provided for a subset of metabolites at the very same time points in either the cellular compartment (Fig. 2C) or the supernatants (Fig. 2D). We highlight the extreme (Day 1 vs. Day 42) trends for two metabolites involved in redox poise and GSH or purine turnover, 5-oxoproline, and hypoxanthine, respectively (Fig. 2C). In addition, NADH/NAD⁺ and NADPH/NADP⁺ were progressively depleted during storage (Supporting Information Table and Fig. 1), while nicotinamide accumulated in both RBCs and supernatants (Fig. 2).

Metabolites detected in this study could be deemed representative of the RBC metabolome (Fig. 3A). A glance at key metabolic intermediates involved in rate-limiting reactions for each pathway is depicted in Fig. 3B. Metabolite abbreviations are consistent with previous reports.¹⁷

The heat map in Fig. 3C summarizes the quantitative trends for metabolites of glycolysis, pentose phosphate pathway (PPP), and tricarboxylic acids (TCAs) generated from the cytosolic version of TCA cycle enzymes, all of which have been enlisted in the most recent RBC proteome lists.^{19,21} Glycolytic precursors (hexose phosphates, phosphogluconate, erythrose phosphate—Fig. 3C; Supporting Information Tables/Figs. 1 and 2) and PPP intermediates increased early during storage and subsequently decreased after Storage Day 7. Conversely,

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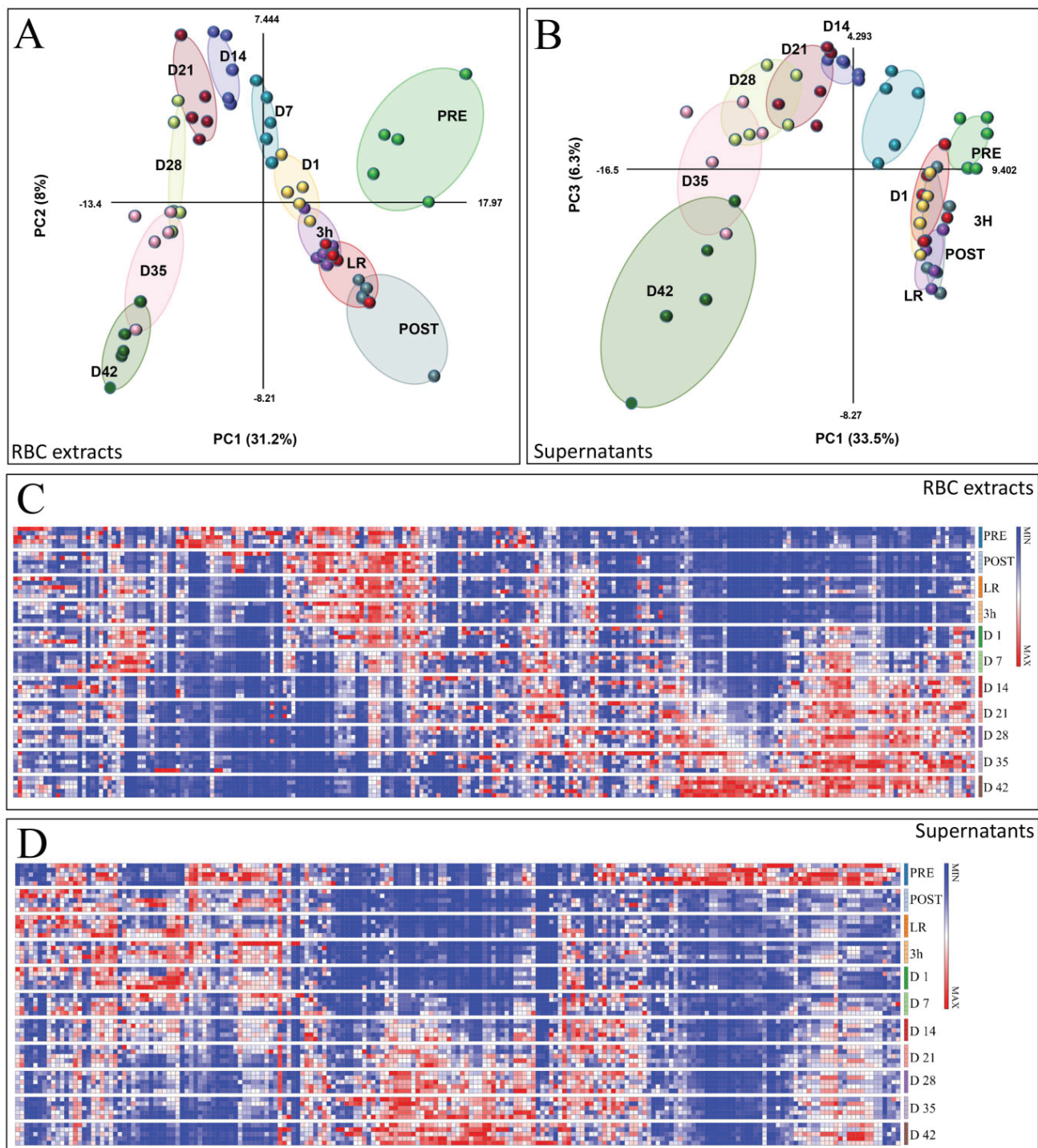


Fig. 1. RBC storage metabolomics analyses: PLS-DA and HCA. (A and B) Results from PLS-DA based on time course metabolomics analyses of RBC extracts and supernatants, respectively. (C and D) Heat maps from hierarchical clustering analyses of time course metabolomics changes in RBC extracts and supernatants, respectively. A vectorial format version of the HCA maps from C and D, also including metabolite names and clusters, is provided in Figs. S1 and S2, respectively.

acidic compounds including lactate and TCAs (malate) increased with storage duration in RBC extracts (Fig. 3C).

RBC membranes are endowed with a complex amino acid transport system, which includes the most abundant membrane protein, the anion exchanger 1 Band 3 protein²² (Fig. 4A), one of the main targets of the storage lesion to the membrane proteome,¹ especially after Storage Day 14.³ Amino acid levels in RBC extracts and supernatants (Figs. 4B and 4C) were monitored throughout the processing stages and storage period. Observed trends included progressive increases in both RBCs and supernatants for the majority of amino acids, including threonine, tyrosine, cysteine (cysteine dimer generated by oxidative stress), histidine, and neutral amino acids (leucine or isoleucine, valine, phenylalanine); transient increases (glutamine, intracellular); or progressive decreases (glutamine, arginine, methionine, and tryptophan in the supernatants). Pre-LR supernatants were characterized by higher levels of amino acids, consistently with the presence of residual plasma or contaminating cells other than RBCs (e.g., white blood cells [WBCs]).

Glutaminolysis, transamination reactions, GSH homeostasis, and serine biosynthesis or metabolism are deeply intertwined pathways (Fig. 5). Relative quantitative trends for RBC or supernatant metabolites involved in these pathways indicate that storage depletes glutamine, GSH, and ketoglutarate reservoirs, while generating transamination and serine catabolism byproducts such as alanine, aspartate, serine, glycine, cysteine, and sarcosine (Fig. 5). Accumulation in both RBC extracts and supernatants of the GSH turnover biomarker,²³ 5-oxoproline (Fig. 5), reached significant differences by the end of the storage period (Fig. 2C). Storage-dependent accumulation of glutathionyl-cysteine was observed as well (Fig. S3, available as supporting information in the online version of this paper).

Purine deamination and catabolism reactions are interconnected with the cytosolic version of TCA and urea cycle intermediate enzymes (Fig. 6). Increases of nicotinamide, AMP, and hypoxanthine were observed in RBC extracts and/or supernatants, resulting in adenine consumption (Fig. 6). Arginine uptake and catabolism likely contribute to the accumulation of urea cycle intermediates (citrulline argininosuccinate and ornithine) in the cytosol, but not in supernatants (Fig. 6). In addition, malate accumulated in RBC extracts and supernatants, while urate did not (Fig. 6 and Tables S1 and S2).

DISCUSSION

Evidence from biochemistry-based approaches suggest that lesions affecting RBC physiology and capacity to transport and deliver oxygen are evident as early as hours after blood withdrawal.²⁴ Despite this evidence, logistic issues have hampered the determination of metabolic

profiles during the early stages after blood collection. In this study, we tackled this issue and report for the first time a metabolomics overview of RBC extracts and supernatants soon after withdrawal and cell separation (PRE), during, and immediately after LR (3.5 log—POST, LR), and at 3 hours post-LR. These early time points represent a baseline for the determination of metabolic fluctuations until the expiration date of the units (42 days).

Previous metabolomics studies have focused on the impact of metabolic lesions on RBC viability during storage in the presence of different ASs, including SAGM,^{7,8} MAP,¹⁰ PAGGGM,^{11,12} and AS-5.¹³ Accordingly, we focus here on RBC storage in AS-3, an AS that is characterized by half the level of saline in comparison to SAGM, AS-1, and AS-5 (70 mmol/L vs. 150, 154, and 150 mmol/L, respectively), high phosphate loading (23 mmol/L NaH₂PO₄, absent in other ASs, and only 5 mmol/L in MAP), almost a double dose of adenine in comparison to SAGM (2 mmol/L vs. 1.2 mmol/L) and half the dose of dextrose in comparison to AS-1 (55 mmol/L vs. 111 mmol/L).²⁵ This composition is expected to exploit a lower level of chloride loading to promote band 3-dependent chloride shift toward partial alkalization or slower acidification of the intracellular milieu, thereby preventing early pH-dependent inhibitory feedback on glycolytic enzymes such as phosphofructokinase. At the same time, adenine and phosphate loading would theoretically boost energy metabolism by providing the building blocks for adenosine triphosphate (ATP) biosynthesis through salvage reactions. While AS-1 and AS-5 are characterized by high doses of mannitol (750 and 525 mg/100 mL), AS-3 is mannitol free, which makes it the eligible choice for RBC transfusion of pediatric patients.

Beneficial effects of RBC storage in AS-3 in comparison to SAGM have already been reported from a proteomics standpoint,²⁰ suggesting a reduced likelihood of protein fragmentation or vesiculation during RBC storage in the former AS. Storage in the presence of SAGM has been previously associated with the progressive accumulation of biochemical (metabolic and proteomics) and morphologic lesions, reaching significance after 14 days of storage.^{2,3,7} Besides, proinflammatory lipids accumulate in RBC supernatants in a significant fashion by Storage Day 14 irrespective of the ASs^{26,27} and trigger neutrophil priming and transfusion-related acute lung injury,²⁸ a phenomenon that is in part abrogated by poststorage washing.²⁷ This result was consistent with highly debated retrospective studies suggesting that RBCs stored longer than 14 days might no longer be viable for transfusion to certain categories of recipients, such as traumatized, critically ill, or perioperative patients.^{1,4,29,30} In this study, RBCs stored in AS-3 displayed significant metabolic alterations starting from Storage Day 14 onward.

Overall, storage resulted in energy impairment (ATP and diphosphoglycerate depletion), progressive lactate

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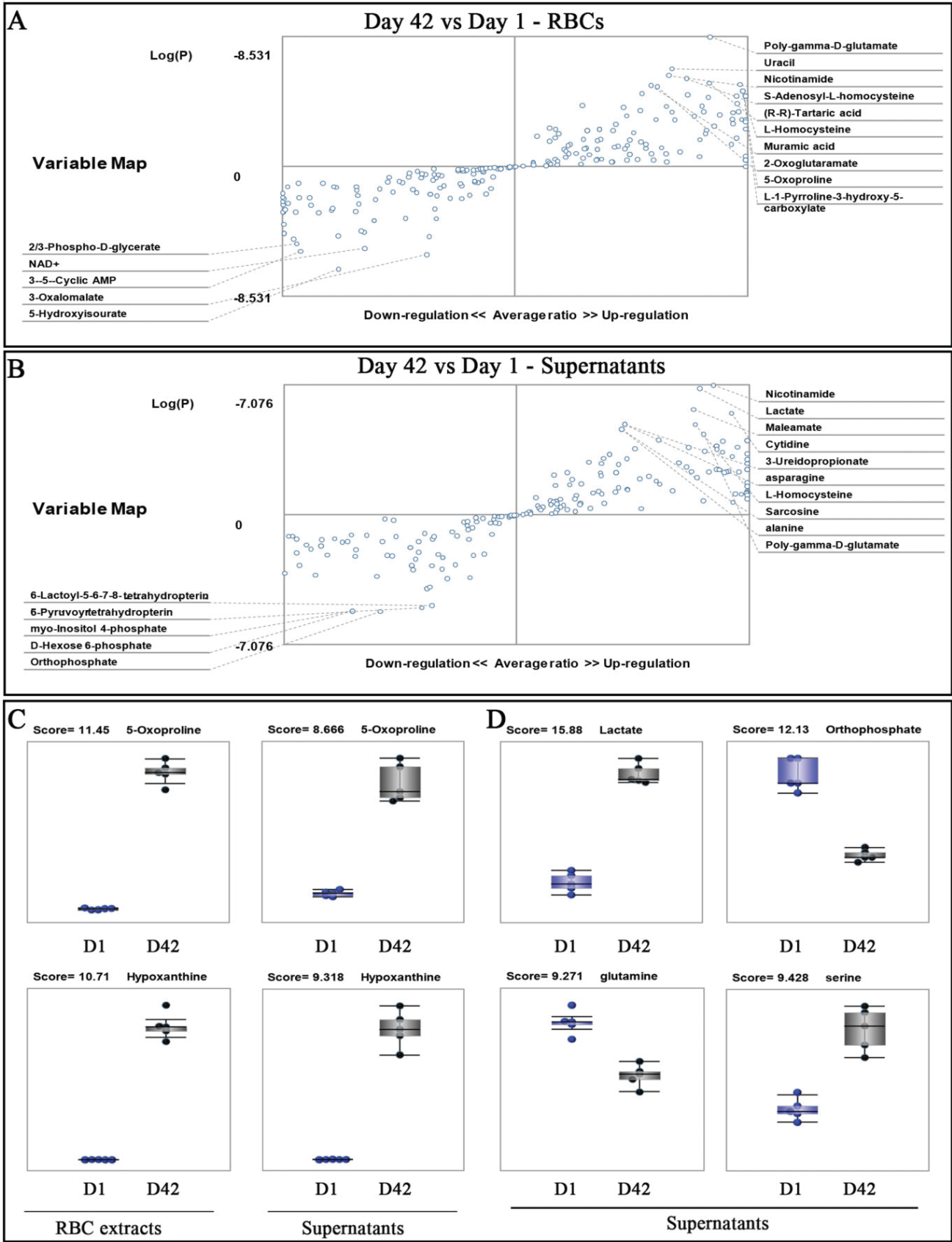


Fig. 2. Metabolomics changes in Day 42 samples against Day 1 controls. The graphs in A and B show logarithmic scale fold changes in metabolite concentrations upon comparison of Day 42 versus Day 1 RBC extracts or supernatants, respectively. Top 10 metabolites increasing and top 5 decreasing in Day 42 samples in comparison to Day 1 controls are indicated in the right and left side of the panels, respectively. (C) Box and whisker plots of oxoproline and hypoxanthine levels in Storage Day 1 and Storage Day 42 RBC extracts and supernatants. (D) Box and whisker plots of a subset of metabolites showing significant fold change variations in Day 42 supernatant samples in comparison to Day 1 controls.

accumulation in the cytosol and supernatant, and incomplete depletion of intracellular and supernatant glucose. A transient activation of PPP during early storage weeks was observed, together with the accumulation of glycolytic precursors, consistently with previous reports for SAGM RBCs.^{7,31} These consistencies are relevant in that PPP is deemed to be a key contributor to NADPH generation and thus GSH homeostasis in RBCs, a mechanism that is oxygen and storage dependent in *in vitro* aging RBCs and results in lesions targeting the cytosolic domain of band 3 (reviewed in D'Alessandro et al.¹). In this study, the entire NADH/NAD⁺ and NADPH/NADP⁺ compartment was progressively depleted during storage with concomitant accumulation of nicotinamide, a NAD/NADP breakdown product in both RBCs and supernatants. This is consistent with the hypothesis that nicotinamide accumulation results in glucose-6-phosphate dehydrogenase inhibition³² and thus PPP depression.

A key role for one-carbon metabolism downstream of serine synthesis in NADPH generation has been highlighted in the metabolome of several cancer cell lines, including colorectal cancer HCT116 cells.³³ By promoting a metabolic diversion from late glycolysis toward serine biosynthesis, cells might exploit serine catabolism and one-carbon metabolism to boost NADPH generation via folate metabolism³⁴ and GSH biosynthesis by providing glycine and cysteine precursors.³³ Of note, cysteine efflux has been previously associated with the process of RBC senescence *in vivo* in concordance with an increase in RBC and supernatant levels of glycine and cysteine during routine storage.^{35,36} Of note, their transport is dependent on both the G and the ASC systems,²² but also on band 3,²² which is one of the well-established targets of oxidative and protease-dependent storage lesions in the blood bank.^{1,37} It is worthwhile to recall that most of the amino acid transport systems in RBC membranes are either ATP or cation (e.g., Na⁺) dependent,²² which is relevant since both energy and ion homeostasis are impaired in stored RBCs.¹ Therefore, together with the observed increase in the intracellular levels of sarcosine and methylenetetrahydrofolate (one-carbon metabolism), these results are better explained by *de novo* synthesis, rather than intake, of glycine, cysteine, and serine.

Glutamate, the last precursor to the tripeptide GSH, also increased in both RBCs and supernatants, at the expense of its precursor, glutamine. By comparing the relative abundance of glutamine in both fractions, it can

be hypothesized that storage promotes intracellular intake of glutamine until it is almost completely removed from the supernatant. This in turn might fuel glutamine deamination to glutamate and, indirectly, the promotion of transamination reactions that are dependent on glutamate or ketoglutarate as the amine group donor or acceptor, respectively. These metabolites act as substrates of alanine aminotransferase and aspartate aminotransferase, resulting in the production of alanine and aspartate, hereby accumulating in both extracts and supernatants. Such reactions might contribute to preserve the NADH/NAD⁺ balance, the former being relevant for detoxification reactions mediated by cytochrome b5 reductase and the latter to sustain oxidative glycolytic reactions. In this study NADH/NAD⁺ ratios remained relatively stable until Storage Day 21.

Despite these observations, it should be noted that *de novo* GSH biosynthesis is an ATP-dependent process, and cytosolic ATP depletion and AMP accumulation is observed early by Storage Day 7. ATP and GSH metabolite concentrations are also donor dependent and their levels in RBCs are characterized by storage and heritability dependency.³⁸⁻⁴⁰ Furthermore, GSH and its oxidized form GSH disulfide both decrease during storage duration as previously reported.^{38,41} This decrease may be due to increased GSH turnover, rather than impaired *de novo* biosynthesis as previously proposed.⁴¹ As expected, the byproducts of GSH metabolism increased, including glutathionylated amino acids and 5-oxoproline, as previously postulated²³ and reported.^{8,13}

During routine storage of RBCs, protease activities increase (caspases and calpains), as is proteasome-mediated proteolysis, as long as the functional proteasome is not extruded to the supernatant.^{1,5} The presented data demonstrated that there was both intra- and extracellular accumulation of amino acids, which is suggestive of efflux of amino acids derived from ongoing biosynthesis or proteolysis. However, levels of arginine decreased both in RBCs and in supernatants during storage duration, which is suggestive of arginine catabolism in RBCs, possibly through an intact intracellular urea cycle,⁴² as confirmed by accumulation of the specific intermediates. Alternatively, arginine can be metabolized by a functional arginase and/or endothelial nitric oxide synthase,⁴³ to generate nitric oxide and thus promote vasodilation. No urate accumulation, despite the elevation in the levels of arginine and citrulline, supports

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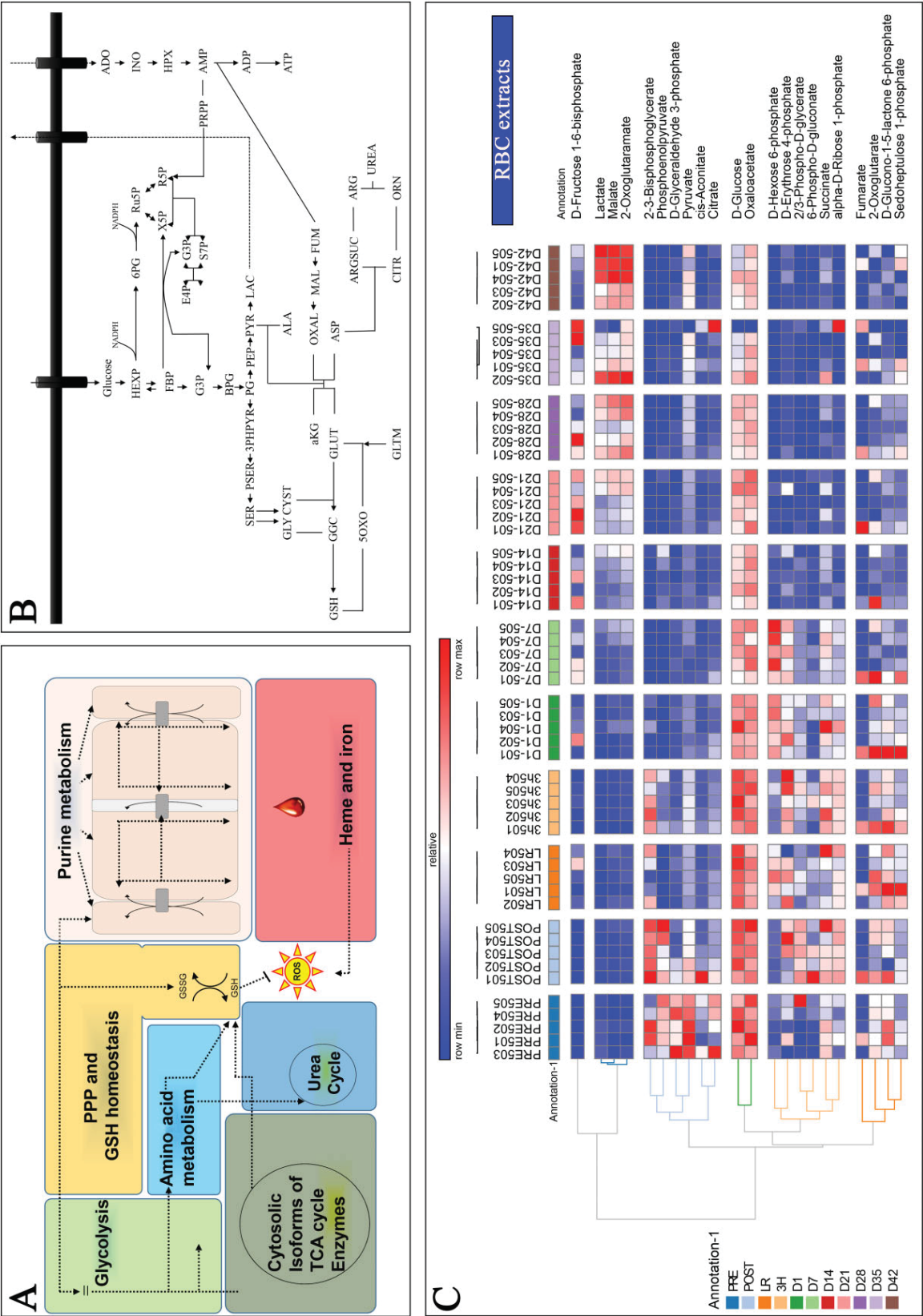


Fig. 3. Central metabolic pathways and quantitative changes during storage in AS-3. (A) A simplified overview of the interconnectedness of metabolic pathways investigated in this study, including glycolysis (Embden-Meyerhof pathway), PPP and GSH homeostasis, cytosolic TCA, amino acid metabolism, urea cycle, purine metabolism, and heme metabolism. (B) The same pathways are dissected to highlight a subset of key metabolites that represent key nodes in metabolic cascades in RBCs. (C) Heat maps show the outcome of hierarchical clustering analyses for metabolites enlisted in B. Relative quantities are indicated through a color code (blue to red = low to high levels), while storage time points are displayed on the basis of the color code explained in the left-side legend of the panel.

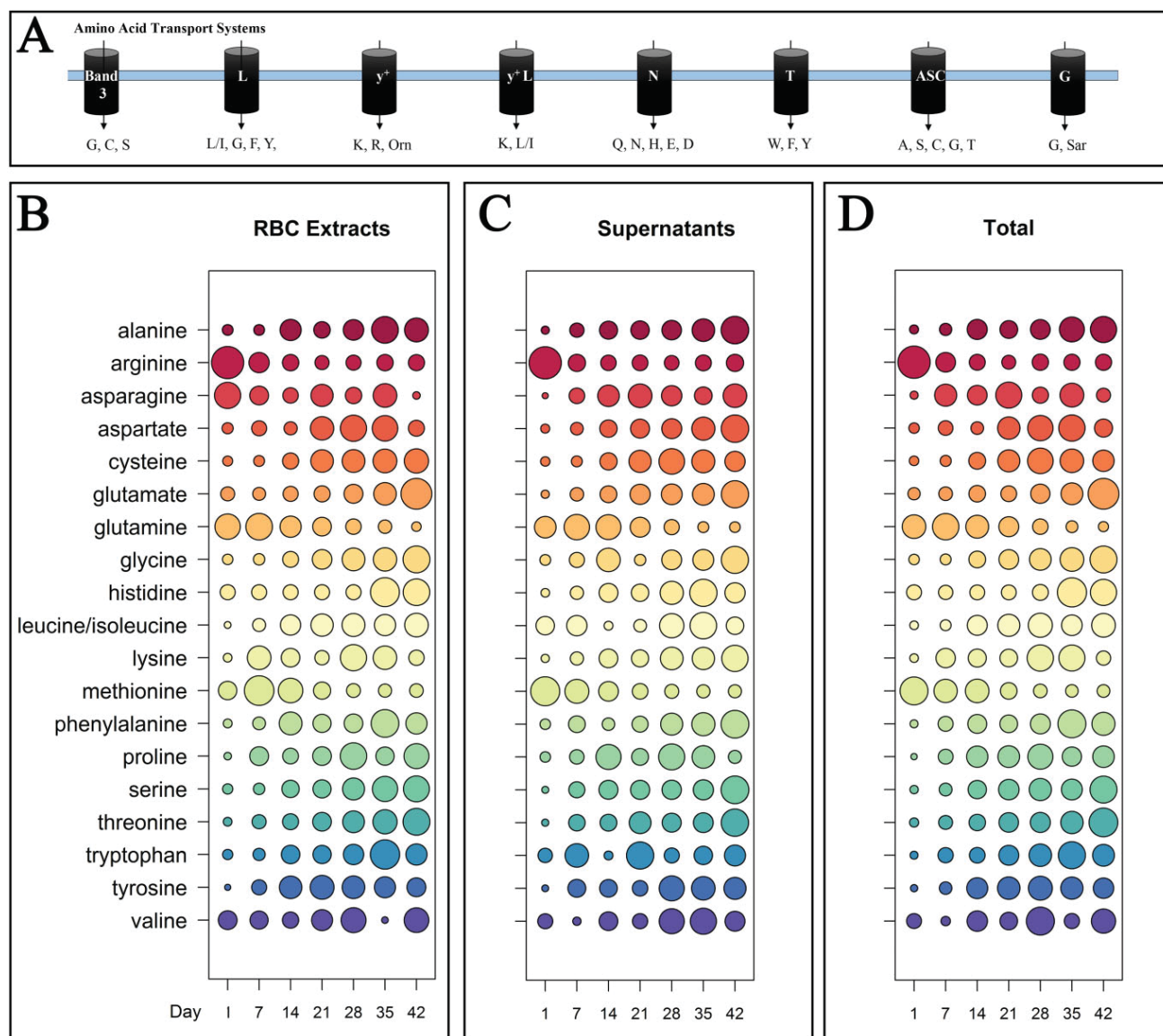


Fig. 4. Amino acid metabolism during storage in AS-3. (A) A simplified overview of amino acid transport systems in RBC membranes. (B-D) Dot plots represent the quantitative temporal changes of amino acid levels in RBC extracts, supernatants, and the total amino acid levels (both matrices). The area of each node is proportional to the relative abundance, which is representative of the Z-score of median values for extract ion chromatogram peak areas for each biologic replicate per time point per each amino acid. Each amino acid has been assigned a different color for visualization.

the hypothesis of arginine depletion through arginase activity.

Nitrogen imbalance might also derive from purine catabolism, as cells are fueled with adenine from the AS. In the presence of oxidative stress, adenine can be deaminated to hypoxanthine^{7,17} and then be fluxed to the supernatants as demonstrated in the presented data, similar to previous observations.^{8,13} In RBCs, purine catabolism would also result in the accumulation of fumarate,⁴⁴ which in turn can be converted into malate and oxaloacetate by cytosolic fumarate hydratase⁴⁵

and malate dehydrogenase, both enzymes in the RBC proteome.¹⁹ Such purine catabolism was present as evidenced by the accumulation of malate during storage in both RBCs and in the supernatants. Finally, there was storage-dependent accumulation of oxoproline⁴⁶ or TCAs (e.g., malate). Organic acids might impair prolyl-hydroxylase activity and thus prevent degradation of hypoxia-inducible factor 1 α .⁴⁷ Additionally, accumulation of these anions in RBC supernatants might contribute to strong anion gap, pH acidification, and base deficit in transfused trauma patients.⁴⁸

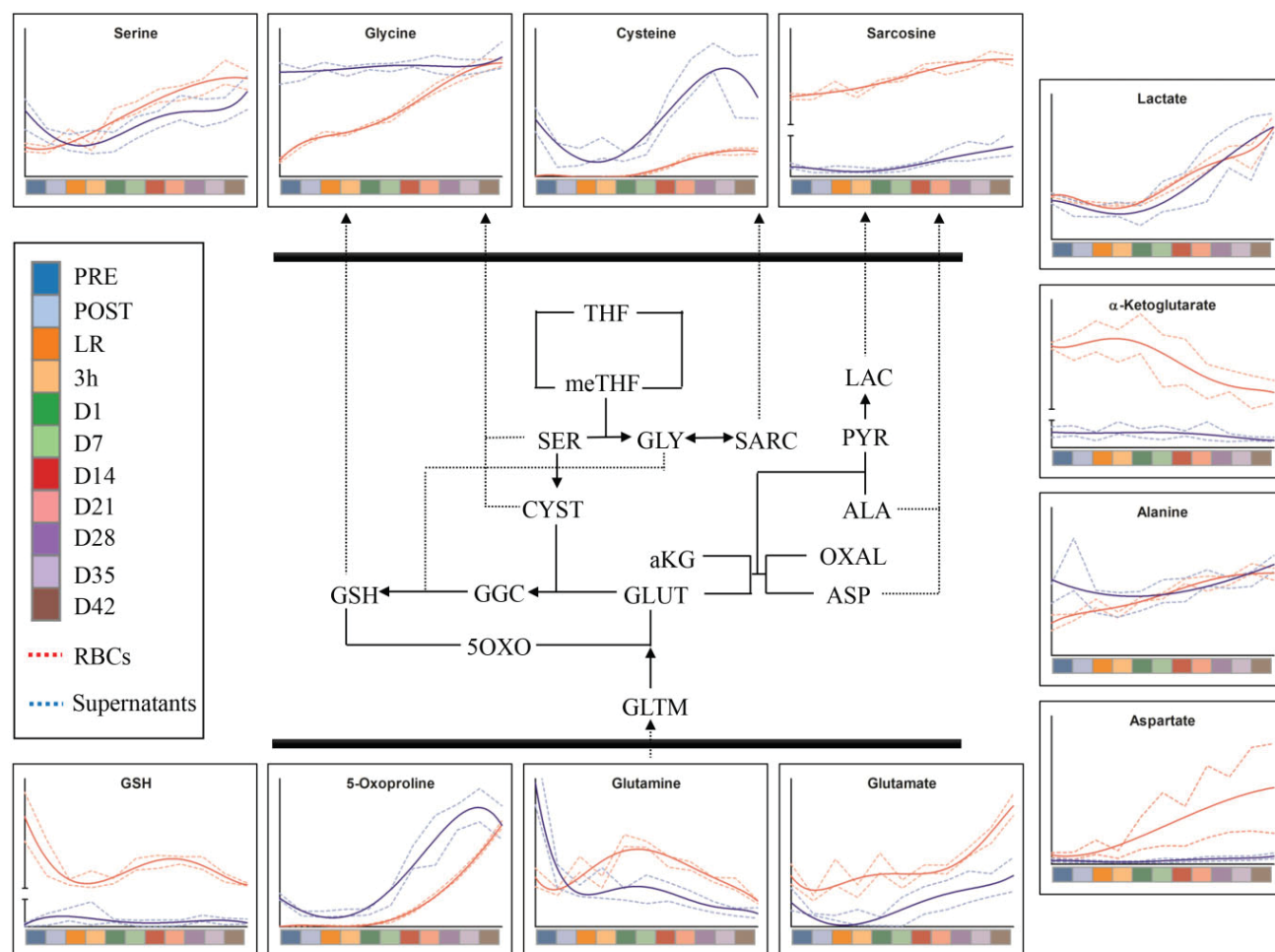


Fig. 5. Glutamine and serine metabolism during RBC storage in AS-3. An overview of the metabolites directly or indirectly involved in glutaminolysis or serinolysis and catabolic or anabolic reactions related to glutamine, serine consumption, or biosynthesis. Color codes under each graph represent a distinct time point, as indicated in the legend on the left side of the panel. Red and blue lines represent quantitative changes (y axes indicate MS signal intensities in arbitrary ion counts) for RBC and supernatant levels for each metabolite, respectively. Fifth-degree polynomial interpolation solid curves are provided for each metabolite, together with standard errors of the mean (SEM—dashed red and blue lines for RBC extracts or supernatants, respectively).

RBC storage in the blood bank offers an invaluable model for cell senescence *in vitro*, one that holds immediate translational applications in many areas of medicine including transfusion medicine¹ and resuscitation strategies in the intensive care setting. Despite intrinsic differences in comparison to aging *in vivo*,^{5,49} biochemical analysis of RBC aging *in vitro* provides relevant clues about this process in the bloodstream. Likewise, metabolomics profiles of freshly withdrawn blood from healthy controls might serve as a baseline for future translational studies on hematologic infections (e.g., malaria⁵⁰) or diseases (e.g., sickle cell anemia or glucose-6-phosphate dehydrogenase deficiency^{51,52}). On the other hand, changes unique to RBC storage *in vitro* might inform biopreservation experts as to alternative strategies for extending the shelf life and improving the quality of

RBCs or blood components, such as in the case of pathogen reduction technologies.⁵³

In this study employing RBC stored in AS-3, a baseline of metabolic profiles for RBCs cytosol and supernatants was provided by assaying very early time points, during and upon processing of RBCs for WBC removal through leukofiltration. In comparison to previous studies,^{3,7-13} this analysis provides a comprehensive coverage of the hydrophilic component of the metabolome, as recently reconstructed by proteomics-based systems biology models of RBC metabolic pathways.⁵⁴ Limitations of this analysis include the quantitation of the compounds (relative, instead of absolute) and the lack of coverage of hydrophobic compounds, a portion of the metabolome that has been recently addressed through orthogonal approaches.^{26,55}

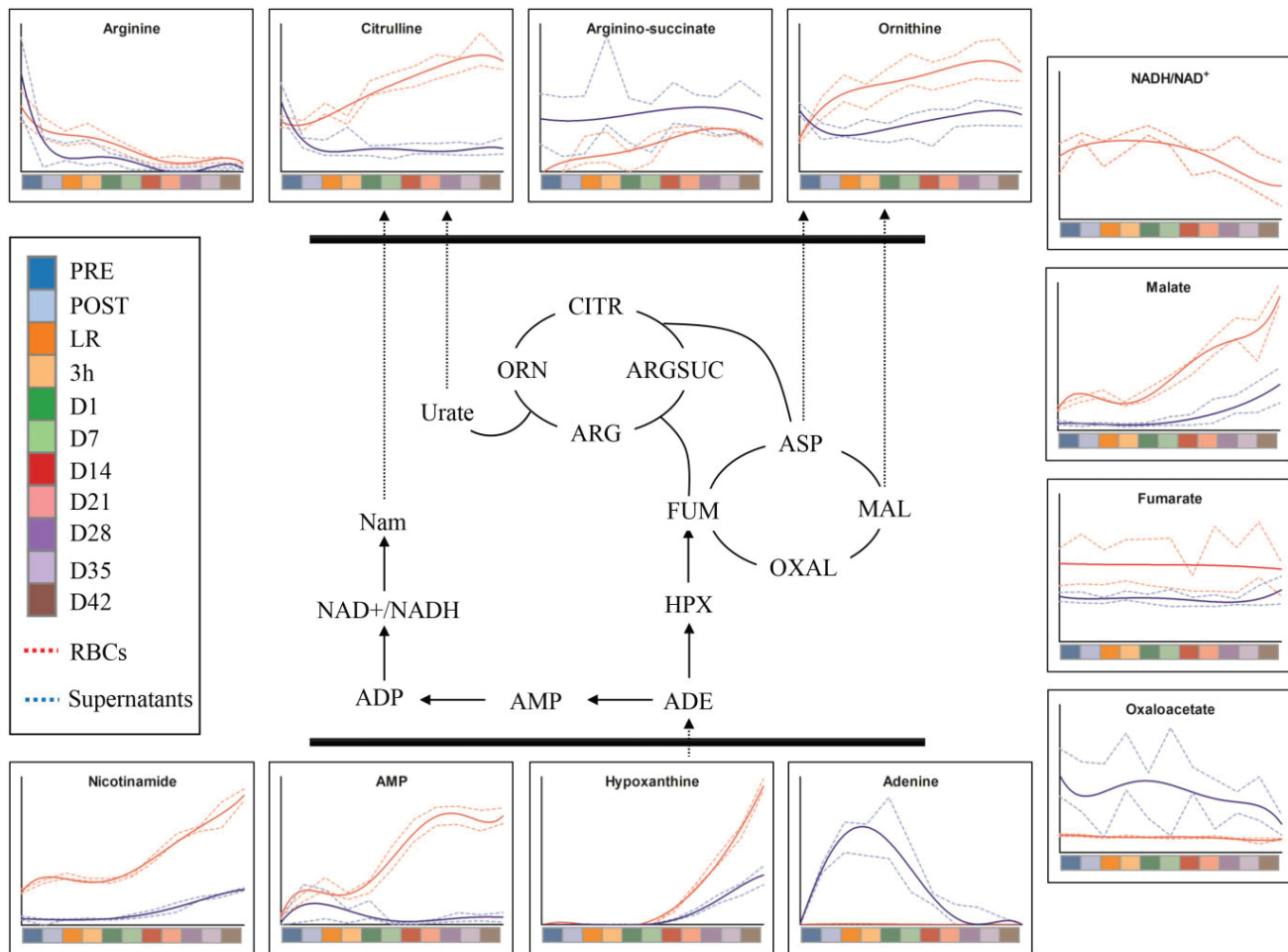


Fig. 6. Urea cycle and TCAs during RBC storage in AS-3. An overview of the metabolites from urea cycle and TCAs. Color codes under each graph represent a distinct time point, as indicated in the legend on the left side of the panel. Red and blue lines represent quantitative changes (y axes indicate MS signal intensities in arbitrary ion counts) for RBC and supernatant levels for each metabolite, respectively. Fifth-degree polynomial interpolation solid curves are provided for each metabolite, together with standard errors of the mean (SEM—dashed red and blue lines for RBC extracts or supernatants, respectively).

Significant relative metabolomics changes were detected starting from Storage Day 14 onward. Such changes include impairment in energy homeostasis, as demonstrated previously, and metabolic changes related to previously unreported amino acid homeostasis; glutaminolysis, serine metabolism, or transamination; urea cycle; and purine catabolism byproducts both in RBC extracts and in supernatants. The reported data will likely impact RBC biopreservation strategies by driving the development of novel ASs. These results might also be useful for translational studies in diverse fields, including hematology, nutrition, neurobiology, and clinical biochemistry and might pave the way for metabolomics-oriented clinical trials in the fields of trauma, transplant, cardiac surgery, neurosurgery, or intensive care in general, to evaluate the potential risks associated with the transfusion of RBC units stored longer than 14 days.

CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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AB, ADA, CCS, EEM, and KCH designed the experimental workflow; MK and FBW collected and prepared the samples for metabolomics analyses; ADA, KCH, and TN set up the metabolomics platform and performed metabolomics extraction and analyses; ADA and TN analyzed the data and prepared figures and tables; RKS contributed to figure preparation; ADA and CCS wrote the paper, while all the authors contributed to the finalization of the manuscript through critical comments and revisions of the first draft; and AB, EEM, CCS, and KCH provided economic support, biologic material, instrumentation, and hematology/transfusion medicine or mass spectrometry expertise to carry out the experiments.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Hierarchical clustering analysis of metabolite levels in red blood cell extracts during storage in AS-3. Heat maps display intra-row normalized quantitative fluctuations for each metabolite (blue to red = low to high levels), as detected through UPLC-MS metabolomics analyses of red blood cell extracts during storage in AS-3. Metabolite names are indicated on the right side of the figure, while storage time points are indicated on top of the map and are annotated through the color code explicated in the top left corner of the figure.

Fig. S2. Hierarchical clustering analysis of metabolite levels in red blood cell supernatants during storage in AS-3. Heat maps display intra-row normalized quantitative fluctuations for each metabolite (blue to

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red = low to high levels), as detected through UPLC-MS metabolomics analyses of red blood cell supernatants during storage in AS-3. Metabolite names are indicated on the right side of the figure, while storage time points are indicated on top of the map and are annotated through the color code explicated in the top left corner of the figure.

Fig. S3. Glutathionyl cysteine. Glutathionyl cysteine levels in AS-3 RBC extracts (red line) and supernatants (blue line) during storage in the blood bank. Storage days are represented through a color code, as indicated in the legend on the left hand panel.

Table S1. Metabolomics report of AS-3 RBC extracts.

Table S2. Metabolomics report of AS-3 RBC supernatants.

REVIEW ARTICLE

Postinjury fibrinolysis shutdown: Rationale for selective tranexamic acid

Ernest E. Moore, MD, Hunter B. Moore, MD, Eduardo Gonzalez, MD, Michael P. Chapman, MD, Kirk C. Hansen, PhD, Angela Sauaia, MD, PhD, Christopher C. Silliman, MD, PhD, and Anirban Banerjee, PhD, Denver, Colorado

Postinjury systemic fibrinolysis has been recognized as a biologic process for more than 200 years, but the mechanisms of regulation and their clinical implications remain unclear. In 1794, John Hunter from Edinburgh observed that the last blood exiting from fatal gunshot wounds did not clot.¹ Albert Dastre from Paris proposed the term *fibrinolysis* in 1893 (*Archives de Physiologie*) based on experimental work demonstrating digestion of fibrin. In 1927, interest in fibrinolysis was piqued by a Russian report that victims of sudden death were preferred as blood donors because their blood “reliquified” within a few hours, permitting transfusion without an anticoagulant. Scientific knowledge of physiologic fibrinolysis improved rapidly during the ensuing two decades and, by the 1950s, the plasminogen (PLG)-plasmin-antiplasmin system was established as critical in preserving microvascular patency during clotting to maintain hemostasis.^{1,2} Thus, in parallel to the highly regulated clot formation system, clot stabilization and physiologic degradation by the fibrinolytic system was also appreciated to be highly regulated.

THE CHALLENGES OF MODIFYING FIBRINOLYSIS

In 1963, Starzl et al.³ identified systemic fibrinolysis by thrombelastography (TEG) during the anhepatic phase of liver transplantation and advocated routine antifibrinolytics (aminocaproic acid). Three years later,⁴ however, this Colorado transplant team reversed their recommendation when three of their four transplant survivors given aminocaproic acid developed multiple pulmonary emboli. Interestingly, during the same period, Hardaway et al.⁵ demonstrated the benefits of fibrinolytic administration to prevent irreversible experimental hemorrhagic shock. During the ensuing three decades, fibrinolytic therapy became the standard for arterial thromboemboli in the coronary, cerebral, mesenteric, and peripheral vasculature, with selective use in the venous system. By the late 1980s, recombinant tissue

PLG activator (tPA) became the fibrinolytic of choice. On the other side, with the widespread availability of TEG, excessive fibrinolysis was incriminated in post-coronary artery bypass grafting mediastinal bleeding presumably because of contact activation. But the enthusiasm for antifibrinolytics was dampened after the BART (Blood Conservation using Antifibrinolytics in a Randomized Trial) indicated increased renal failure, myocardial infarction, and mortality after coronary artery bypass grafting when a plasmin inhibitor (aprotinin) was given.⁶

ENTHUSIASM FOR TRANEXAMIC ACID IN TRAUMA MANAGEMENT

Acknowledging the potential role of the PLG-plasmin system in trauma is a relatively recent event and largely caused by the implementation of TEG⁷ and ROTEM (rotational thromboelastometry).⁸ The stage was set by Hoffman and Monroe⁹ in 2001 who proposed the cell-based model of hemostasis. Based on this construct, Brohi et al.¹⁰ introduced the provocative concept that trauma-induced coagulopathy was mediated via the activation of protein C (aPC), resulting in the degradation of clotting factors V and VIII. Embedded within this novel proposal was the consumption of PLG activator inhibitor-1 (PAI-1) by aPC, thus, indirectly enhancing fibrinolysis.¹¹ Within a year, our group in Denver documented systemic hyperfibrinolysis by TEG in 18% of acutely injured patients requiring a massive transfusion.¹² These data were further supported by contemporary reports from the United States¹³ and Europe.¹⁴ The CRASH-2 (Clinical Randomization of an Antifibrinolytic in Significant Hemorrhage) trial, reported in 2010,¹⁵ provided the ultimate impetus for the widespread adoption of an antifibrinolytic (tranexamic acid [TXA]) for trauma management.

However, the significant limitations of this prospective randomized trial were soon emphasized by a number of groups.^{16,17} Although 20,211 patients were enrolled in this study designed to reduce mortality caused by coagulopathy, only half of the patients required a red blood cell (RBC) transfusion. Furthermore, there was no reduction in transfused blood products; each group received six units of RBCs. Finally, an additional analysis of the data indicated a 1.44 increased risk for mortality when TXA was given more than 3 hours after injury.¹⁸ While the MATTERS (Military Application of Tranexamic Acid in Trauma Emergency Resuscitation Study) suggested a benefit of TXA in combat casualty care, this was a retrospective analysis confounded by the administration of fibrinogen.¹⁹ Recently, Valle et al.²⁰ in a retrospective analysis

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of civilian data, using propensity score matching, found an increased mortality in severely injured patients administered TXA, and Harvin et al.²¹ in a retrospective study of civilian patients confirmed to have fibrinolysis by TEG observed no benefit from TXA.

POSTINJURY HYPERFIBRINOLYSIS, PHYSIOLOGIC FIBRINOLYSIS, AND FIBRINOLYSIS SHUTDOWN

Principal component analyses of patients with trauma-induced coagulopathy performed by the Denver group²² and the San Francisco General group²³ indicated that clotting factor deficiencies and systemic hyperfibrinolysis are mechanistically distinct. These analyses stimulated us to investigate the mechanistic regulation, and consequent manifestation, of fibrinolysis in our animal shock/trauma models. Although systemic fibrinolysis is difficult to replicate in animal models, we developed a tPA challenge assay to unmask latent hyperfibrinolysis versus fibrinolysis shutdown.²⁴ We define *shutdown* as a relative resistance to tPA caused by a physiologic dysregulation of the PLG-plasmin system. Interestingly, in both our rodent and swine models, shock (ischemia/reperfusion) produced consistent systemic hyperfibrinolysis, whereas tissue injury (thoracotomy/laparotomy/femur fracture) provoked physiologic fibrinolysis shutdown.²⁵

Stimulated by these experimental findings, we then interrogated our prospectively collected TEG database from 2010 to 2013. Patients were eliminated if the first TEG was obtained longer than 12 hours after injury or the patient was taking preinjury anticoagulants. A citrated kaolin TEG was conducted by standard TEG 5000 methods (Haemonetics) and used because of its greater accuracy for identifying lysis compared with the rapid TEG. Systemic fibrinolysis was quantified as the percent clot lysis at 30 minutes after maximum strength was achieved (LY30). Patients were then stratified into three groups by LY30 criteria: hyperfibrinolysis ($\geq 3\%$); physiologic fibrinolysis (0.81–2.9%); and fibrinolysis shutdown ($\leq 0.08\%$). The hyperfibrinolysis cutoff was based on previous clinical studies indicating increased blood product consumption and mortality in acutely injured patients.^{13,26} Because there were no previous reports addressing fibrinolysis shutdown, we used a receiver operating characteristic curve for mortality in the remaining patients with an LY30 less than 3%. The point of greatest specificity and sensitivity was 0.8% based on a Youden Index. Our study population consisted of 180 severely injured patients who were 43 years old (interquartile range [IQR], 28–55 years), 70% male, and 79% sustained blunt trauma. The median Injury Severity Score (ISS) was 29 (IQR, 22–36), median initial base deficit (BD) was 9 (IQR, 6–3), and the mortality was 20%, with two thirds occurring within 24 hours. Fibrinolysis shutdown (as previously defined) was the most common phenotype, accounting for 64% ($n = 115$) with physiologic fibrinolysis ($n = 32$) and hyperfibrinolysis ($n = 33$) representing 18% each. Interestingly, the three phenotypes could not be distinguished by age, sex, ISS, or BD. When considering patients who required RBCs, the systemic hyperfibrinolysis patients required more RBCs and fresh-frozen

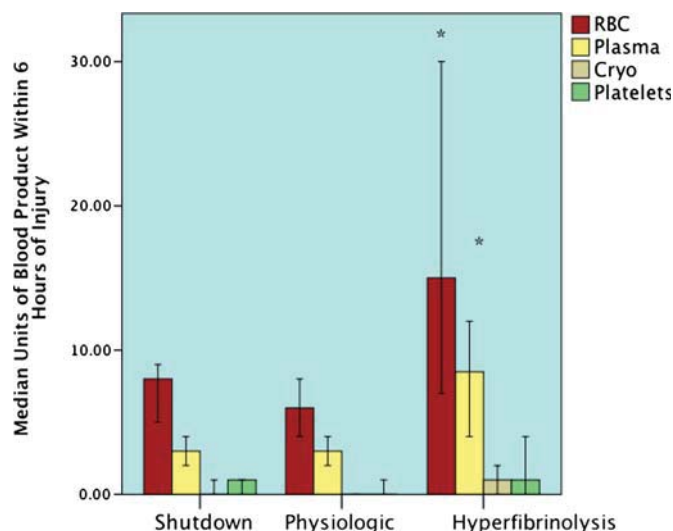


Figure 1. Blood product transfusions between systemic fibrinolysis phenotypes. The blood component transfusion among the systemic fibrinolysis phenotypes summarized; y axis represents the number of units within the first 6 hours after injury. RBC and plasma transfusions were higher in the systemic hyperfibrinolysis phenotype. Cryo indicates cryoprecipitate. * $p < 0.05$ after pairwise adjustment. Reproduced from Moore et al.²⁴ with permission from Lippincott Williams & Wilkins.

plasma, and this correlated with an increased need for massive transfusion (Fig. 1).

Of note, mortality among the fibrinolysis phenotypes had a U-shaped distribution (Fig. 2), with the nadir in the physiologic group (3%) compared with systemic hyperfibrinolysis (44%) and fibrinolysis shutdown (17%). The cause of death was also substantially different between the phenotypes (Fig. 3). Acute blood loss accounted for 66% of the mortality in the hyperfibrinolysis group compared with 15% in the fibrinolysis shutdown patients. Conversely, death because of multiple organ failure occurred in 7% of the hyperfibrinolysis group compared with 40% in the shutdown patients.

In sum, our clinical study identified three distinct phenotypes of fibrinolysis after severe injury. Unfortunately, it was not possible to predict which phenotype would be manifest based on injury severity (ISS) and magnitude of shock (BD). The majority of these patients (64%) had fibrinolysis shutdown and typically had delayed mortality from multiple organ failure. These patients should not improve with further blockade of the fibrinolytic system and may even be harmed by the untimely administration of TXA. Conversely, the 18% with documented systemic hyperfibrinolysis will presumably benefit from timely TXA delivery, as mortality in this group was early and caused by acute blood loss. We believe that these findings argue against the empiric use of TXA in acutely injured patients and support the routine monitoring for lysis with TEG or ROTEM in high-risk patients. There are limitations to our initial clinical study identifying fibrinolysis phenotype caused by the delay in acquiring the TEG analysis up to 12 hours.²⁴ Consequently, we have an ongoing prospective study to define the timing of fibrinolysis shutdown using our graded tPA challenge assay. The experience to date

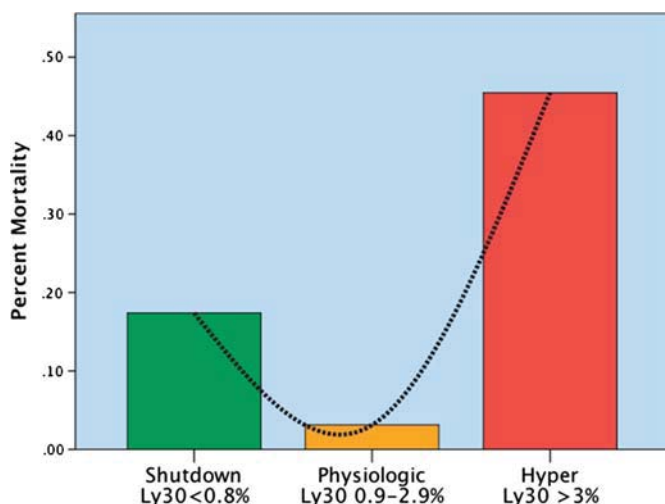


Figure 2. U-shaped distribution of mortality related to systemic fibrinolysis phenotype. The mortality among the systemic fibrinolysis phenotypes was U-shaped; y axis represents mortality by phenotype. *Ly30* indicates percent fibrinolysis 30 minutes after reaching maximum amplitude measured by thrombelastography (y axis represents the percent mortality per phenotype); *shutdown*, fibrinolysis shutdown; *physiologic*, physiologic fibrinolysis; *hyper*, hyperfibrinolysis. There is a U-shaped distribution of mortality with a nadir in mortality identified in the physiologic group (*Ly30* between 0.9 and 2.8%). *Ly30* above and below this range had statistical increases in mortality. Reproduced from Moore et al.²⁴ with permission from Lippincott Williams & Wilkins.

indicates that the majority of severely injured patients manifest tPA resistance/fibrinolysis shutdown within 3 hours of injury. Considering the half-life of 2 hours for TXA, we believe that our data support our recommendation for the selective administration of TXA.¹⁶

REGULATORY MECHANISMS OF POSTINJURY FIBRINOLYSIS

At a simplistic level, the regulation of postinjury fibrinolysis can be viewed as a set of activators and inhibitors of the PLG-plasmin system (Fig. 4), but the molecular events are more complex and yet to be fully elucidated.²⁷ A perusal of the molecular structure of fibrinogen underscores the complexities of this regulation.^{28,29} Our experimental work and clinical studies^{11,24–26,30} indicate that circulatory arrest provokes systemic hyperfibrinolysis. Our experimental work further confirms that shock (ischemia) stimulates systemic fibrinolysis.^{24,25} Collectively, these studies suggest that a primary mechanism for systemic hyperfibrinolysis is the release of tPA that overwhelms the counter-regulatory mechanisms. At this point, we believe a major component is that tPA release exceeds the capacity of its cognate inhibitor PAI-1 (Serpin E1). Tissue PLG activator and PAI-1 form a mutually inhibitory covalent complex with 1:1 stoichiometry and are cleared by the liver. PAI-1 activity may be further impaired because of the actions of other proteolytic enzymes, including activated protein C (aPC) and neutrophil elastase, which are known to be upregulated by acute injury and

inflammation. While the endothelium is a major source of tPA, the precise mediators in trauma are unclear and production is organ specific (unpublished data). Furthermore, there may be other direct contributors such as neuronal tissue.²⁷ Regulation of the PLG-plasmin system involves a myriad of molecular events at multiple steps, including overt and covert protein domains that modulate the binding of tPA and PLG to fibrinogen.^{27,31} For example, our experimental work, using proteomics, indicates that partners bearing accessible lysine residues enhance plasmin fibrinolysis independent of tPA and PAI-1 levels.^{30,31} Furthermore, our ongoing investigation with metabolomics indicates a number of potential modifiers of the plasmin system (unpublished data).

The pathogenesis of postinjury systemic fibrinolysis shutdown remains even further mysterious. There are a number of potential regulatory events after severe trauma. Plasminogen activation on fibrin is initiated when tPA binds to fibrin followed by the binding of PLG. Once this trimolecular complex is formed, plasmin cleaves fibrin and exposes carboxy terminal lysine residues. Kringle 2 of tPA and kringles 1 and 4 of PLG contain lysine-binding sites. Their counterbinding sites in fibrin (Aa 148–160 and c 312–324) are cryptogenic in fibrinogen

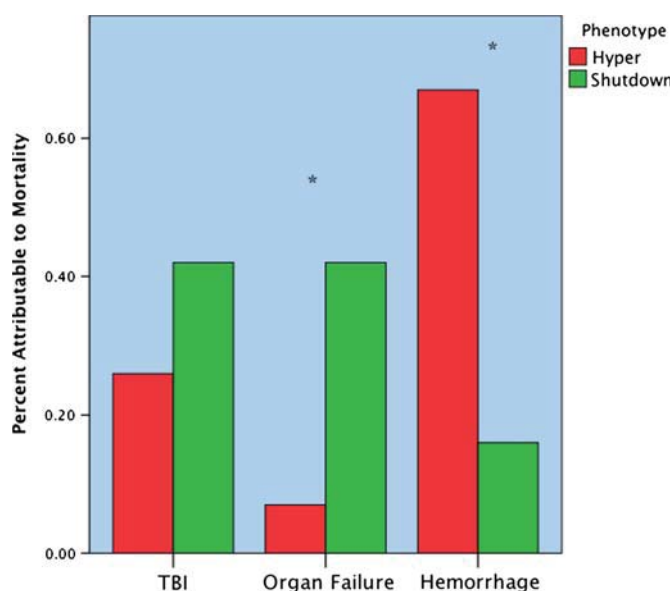


Figure 3. Distribution of mortality according to systemic fibrinolysis phenotype. The distribution of mortality among the systemic fibrinolysis phenotypes was substantially different; y axis represents percentage of total mortality per systemic fibrinolysis phenotype. The systemic hyperfibrinolysis phenotype died primarily because of hemorrhage, whereas the systemic fibrinolysis shutdown phenotype succumbed to multiple organ failure. *TBI* indicates traumatic brain injury; *hyper*, hyperfibrinolysis; *shutdown*, fibrinolysis shutdown. * $p < 0.05$. The y axis represents the percent of total mortality per phenotype. The hyperfibrinolytic phenotype had a high frequency of mortality associated with hemorrhage. The shutdown phenotype has a high frequency of organ failure-related death. *TBI* did not reach statistical difference between phenotypes but was more common in the shutdown cohort. Reproduced from Moore et al.²⁴ with permission from Lippincott Williams & Wilkins.

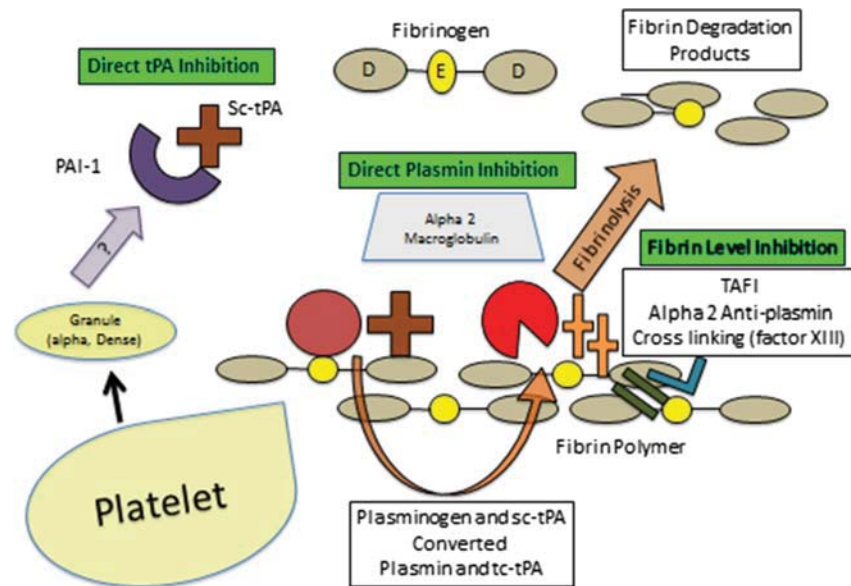


Figure 4. Regulation of postinjury systemic fibrinolysis. The control of systemic fibrinolysis driven by tPA is controlled at multiple levels, including the direct inhibition of tPA, the direct inhibition of plasmin, and the inhibition at the fibrin cross-linking stage.

but become exposed during fibrin cross-linking because of intermolecular D:E interactions that result in conformational changes in the D region. The Aa 148–160 site binds tPA and PLG with similar affinity, whereas the c 312–324 site binds tPA exclusively. Fibrin polymerization occurs with considerable diversity, and the resulting viscoelastic properties are generally referred to as clot stability. Fibrin structure affects the rate of fibrinolysis; thinner fibrin strands with frequent branch points are more resistant to plasmin disassembly. Disaggregation of the fibrin fibers proceeds by lateral transection rather than surface erosion. When plasmin is generated, it converts single-chain tPA to a double-chain form that has much greater activity. In degrading cross-linked fibrin, plasmin initially cleaves the C termini of the α and β chains within the D region, resulting in a variety of fibrin degradation products. Degradation of fibrin cross-linked by factor XIII releases the fibrin degradation product known as D-dimer, which consists of fragments containing two D regions and one E region.

Once plasmin is generated, there are a number of inhibitors that can attenuate its activity. The most active is α 2-antiplasmin (α 2-AP), a 70-kD single-chain glycoprotein that is a serpin. The α 2-AP is made in the liver and has a circulating half-life exceeding 2 days. The α 2-AP forms a lysine-binding site dependent α 2-PI plasmin complex, which is cleared by the liver. The α 2-AP can be cross-linked to fibrin α chains, and this further enhances resistance to fibrinolysis. Factor XIII is also capable of incorporating α 2-AP into fibrinogen. The α 2-macroglobulin is a 72-kD dimeric protein synthesized by endothelial cells and macrophages. The α 2 macroglobulin, like the α 2-AP, is also in the platelet granule. Thrombin-activatable fibrinolysis inhibitor (TAFI), also known as procarboxypeptidase B, is a 60-kD polypeptide that is generated in the liver and present in platelets. Thrombin activation of TAFI is enhanced 1,250-fold in the presence of thrombomodulin. TAFI inhibits fibrinolysis by cleaving lysine

residues on fibrin that bind tPA and PLG. There are additional proteins released after platelet activation that can influence fibrinolysis. Polyphosphate, a negatively charged polymer of inorganic phosphate, is secreted from the dense granules of platelets and promotes tighter fibrin aggregates with reduced sites for tPA and PLG binding. Enhanced interactions with the extracellular matrix by binding fibrin to fibronectin also impair fibrinolysis.

Our clinical studies indicate that the majority of severely injured patients manifest fibrinolysis shutdown within 3 hours of injury, but predicting that response based on injury pattern has been challenging. Our experimental work indicates that tissue injury provokes fibrinolysis shutdown, but the precise mechanisms remain unclear.²⁵ While the release of PAI-1 exceeding tPA activity is an intuitive mechanism,³² the process seems to involve other molecular interactions. Plasmin activity on fibrin might be obstructed by differential expression of cellularly produced regulators or incur interactions with novel inhibitors released by tissue injury. Structurally, it seems that modulators of the kringle domains in plasmin and tPA can both be activators and inactivators.²⁷ With tissue injury as an emerging unifying stimulus, danger signaling could be another partial explanation. Recently, we have shown experimentally that lysed platelets strongly inhibit systemic fibrinolysis. Although the mechanism is unknown, data suggest that platelet activation and the release of granule contents may have a role.³⁰ Finally, we have now documented that fibrinolysis shutdown is prevalent in the postinjury recovery period, a dominant phenotype in the surgical intensive care unit and may explain sequelae ranging from acute lung injury to venous thromboembolism.³³

DISCLOSURE

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Pathologic metabolism: An exploratory study of the plasma metabolome of critical injury

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BACKGROUND: Severe trauma is associated with massive alterations in metabolism. Thus far, investigations have relied on traditional bioanalytic approaches including calorimetry or nuclear magnetic resonance. However, recent strides in mass spectrometry (MS)-based metabolomics present enhanced analytic opportunities to characterize a wide range of metabolites in the critical care setting.

METHODS: MS-based metabolomics analyses were performed on plasma samples from severely injured patients' trauma activation field blood and plasma samples obtained during emergency department thoracotomy. These were compared against the metabolic profiles of healthy controls.

RESULTS: Few significant alterations were observed between trauma activation field blood and emergency department thoracotomy patients. In contrast, we identified trauma-dependent metabolic signatures, which support a state of hypercatabolism, driven by sugar consumption, lipolysis and fatty acid use, accumulation of ketone bodies, proteolysis and nucleoside breakdown, which provides carbon and nitrogen sources to compensate for trauma-induced energy consumption and negative nitrogen balance. Unexpectedly, metabolites of bacterial origin (including tricarballoylate and citramalate) were detected in plasma from trauma patients.

CONCLUSION: In the future, the correlation between metabolomics adaptation and recovery outcomes could be studied by MS-based approaches, and this work can provide a method for assessing the efficacy of alternative resuscitation strategies. (*J Trauma Acute Care Surg.* 2015;78: 742-751. Copyright © 2015 Wolters Kluwer Health, Inc. All rights reserved.)

KEY WORDS: Metabolomics; trauma; energy metabolism; proteolysis; lipolysis; ketoacidosis.

Despite significant advances in prehospital care, resuscitation strategies, operative techniques, and surgical critical care, trauma remains the leading cause of death for individuals younger than 40 years.¹ Uncontrolled hemorrhage and traumatic brain injury are paramount as early causes of posttraumatic death (<24 hours), while multiple-organ failure (MOF) remains the leading cause of death for patients surviving the initial 24 hours following injury.²

Severe metabolic changes are a hallmark of major traumatic injury. A complex interplay of altered systems biology contributes to profound inflammatory and immunologic dysfunction, activation of the complement system, induction of the hepatic acute-phase response, altered acid/base metabolism, and

coagulopathy.³ Concurrent with this proinflammatory state, a disequilibrium exists between catabolic and anabolic pathways characterized by hypermetabolism with increased energy expenditure,⁴ enhanced protein catabolism, lipolysis, insulin resistance with associated hyperglycemia, failure to tolerate a high glucose load, high plasma insulin levels ("traumatic diabetes"), and hyperlactatemia predisposing to metabolic acidosis.⁵⁻¹⁴ The predominance of a catabolic state in polytrauma patients leads to increased oxygen demands and corresponds to an enhanced mitochondrial oxygen use.¹⁵

It is likely that no single element is independently responsible for the development of posttraumatic systemic inflammatory response syndrome and that alternatively, elaboration of MOF results from a culmination and interplay of a multiple-component, systems biology-level pathology. Advances in genomics,¹⁶ proteomics,¹⁷ and pioneering metabolomics investigations¹⁸ provide insight into this deranged pathophysiology. However, the complex biochemistry and metabolism responsible remain largely unknown.

Previous investigations of posttraumatic metabolic changes have been conducted with classic bioanalytic approaches that directly assessed metabolite levels by means of nuclear magnetic resonance (NMR).^{7,10-13} These investigations were limited by their ability to resolve the complex metabolites of blood using ¹H-NMR. The paucity of unique hydrogen environments (e.g., CH₃, CH₂) and the overwhelming abundance of H₂O in the samples hinder a broad analytic coverage of the metabolome via NMR. Mass spectrometry (MS)-based metabolomics is a powerful complementary tool in terms of sensitivity and specificity for the unambiguous assignment of a broader range of metabolic species in biologic fluids in comparison with NMR.¹⁸

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Advantages of the MS-based metabolomics workflow include increased sensitivity and specificity, as it has been extensively reviewed.^{19–22} MS techniques enable detection of thousands of small molecules from key metabolic pathways (e.g., glycolysis, Krebs cycle, ATP/purine catabolism), which are intertwined to physiologic modulations in response to trauma (e.g., coagulation cascades, neutrophil priming, inflammatory cascades, gut microbiome translocation).¹⁸

In this study, we used a mass spectrometric analysis of the plasma metabolome of trauma activation patients’ field blood samples (TAFB) and blood samples obtained during emergency department thoracotomy (EDT) in comparison with fresh frozen plasma (FFP). The EDT population was chosen since the most profound plasma metabolic changes would be anticipated in this severely injured group, which will serve as a reference for extreme metabolic shock in future studies. Specifically, we hypothesize that metabolic signatures will mirror injury-triggered alterations in fuel source use to preserve cellular energy production, help identify potential mediators in postshock pathology (such as metabolites mediating nonlactate metabolic acidosis or those which may impact postinjury coagulopathy), and will further the investigation of early nitrogen imbalance following injury. The precise metabolome following trauma remains incompletely investigated, and this exploratory MS study is designed to provide a foundation that will be hypothesis generating to stimulate further direct investigations of early metabolic shock associated with severe traumatic injury and hypoperfusion.

MATERIALS AND METHODS

Blood samples were collected from 13 critically injured adult trauma patients (mean [SD] age, 43 [13] years; 62% male; 85% blunt injury; median Injury Severity Score [ISS], 26) presenting to Denver Health Medical Center (Level I trauma center, University of Colorado) in accordance with a Colorado Multiple Institutional Review Board–approved protocol. Patients identified for inclusion were 18 years or older with an ISS greater than 15, who experienced blunt or penetrating injury less than 6 hours before admission, and who were likely to require transfusion of red blood cells (RBCs) within 6 hours of presentation as indicated by clinical assessment. Patients younger than 18 years, those with known inherited coagulopathy, prisoners, and pregnant females were excluded.

To compare phenotypic changes in the plasma metabolome immediately following injury and for patients with the most profound injury and shock, we evaluated two subsets of patients meeting the inclusion criteria in this study: (i) seven patients presenting as TAFB obtained by paramedics shortly after injury and before crystalloid resuscitation or blood product administration and (ii) six patients who required EDT for refractory hypotension secondary to hemorrhage and tissue injury. Patient blood samples were drawn in citrated blood tubes and compared with control plasma from five healthy volunteers (mean [SD] age, 50 [15.8] years; 40% male).

Metabolomics Analyses

Extended details about the protocols adopted for metabolomics analyses are reported in Supplementary Materials

and Methods extended (see Supplemental Digital Content 1, <http://links.lww.com/TA/A527>. Briefly, samples were assayed by gas chromatography/MS and liquid chromatography/MS/MS platforms (Thermo-Finnigan Trace DSQ or Waters ACQUITY UPLC/Thermo-Finnigan LTQ-FT mass spectrometer). Compounds were identified by comparison with library entries of purified standards within a 5-ppm window range. Statistical significance was determined by calculating Welch’s two-sample *t* test (*p* < 0.05).

RESULTS

TAFB and EDT plasma samples differed greatly from control plasma (90 and 106 significantly different features, respectively) but were similar to each other (only 19 significantly different metabolites) (Table 1). Metabolomic analyses highlighted a 97- and 166-fold increase in plasma heme from EDT and TAFB patients, respectively (Fig. 1). Consistently, all trauma patients had large (750-fold in EDT, 150-fold in TAFB) increases in mannitol and oxidative stress markers (Fig. 1).

Global changes in energy metabolism involved glucose/sugar use at the glycolytic and mitochondrial level (Fig. 2). Severely injured patients manifest a strong signature of altered central energy metabolism (Fig. 2). While pyruvate levels did not increase in response to trauma, lactate levels did, with the greatest elevations observed in the most severely injured EDT group (Fig. 2). Despite lactate accumulation, fueling of the TCA cycle was also observed through the increase in levels of all Krebs cycle intermediates with the exception of citrate (Fig. 2).

Second, there was a significant increase in fatty acid mobilization and ongoing lipolysis as demonstrated by trauma-dependent decrease in the levels of several lipid classes (Supplemental Digital Content 2, <http://links.lww.com/TA/A528>). In parallel, there were increased plasma levels of ketone bodies (3-hydroxybutyrate, acetoacetate and 1,2-propanediol; Fig. 3) and the glycogen breakdown metabolite maltotriose (Fig. 2), together with the accumulation of citramalate and tricarballylate (Fig. 3). In addition, there were significant increases in carnosinase products (1-methylhistidine and β-alanine; Fig. 3) and 4-hydroxybutyrate (GHB) for both TAFB and EDT patient plasma samples.

Injured patients also demonstrated significant increases in proteolysis as shown by the accumulation of amino acids: alanine, aspartate, cysteine, glutamate, glutamine, histidine, lysine, and phenylalanine (Fig. 3, Supplemental Digital Content 3,

TABLE 1. Statistical Summary

Welch’s Two Sample <i>t</i> Test	EDT/Control	TAFB/Control	TAFB/EDT
Total no. biochemical with <i>p</i> < 0.05	106	90	19
Biochemicals decreasing ↓ or increasing ↑	60 ↓/46↑	44↓/44↑	6↓/13↑
Total no. biochemical with 0.05 < <i>p</i> < 0.10	40	43	19
Biochemicals decreasing ↓ or increasing ↑	14 ↓/26↑	22↓/21↑	6↓/13↑

Heme metabolism and oxidative stress

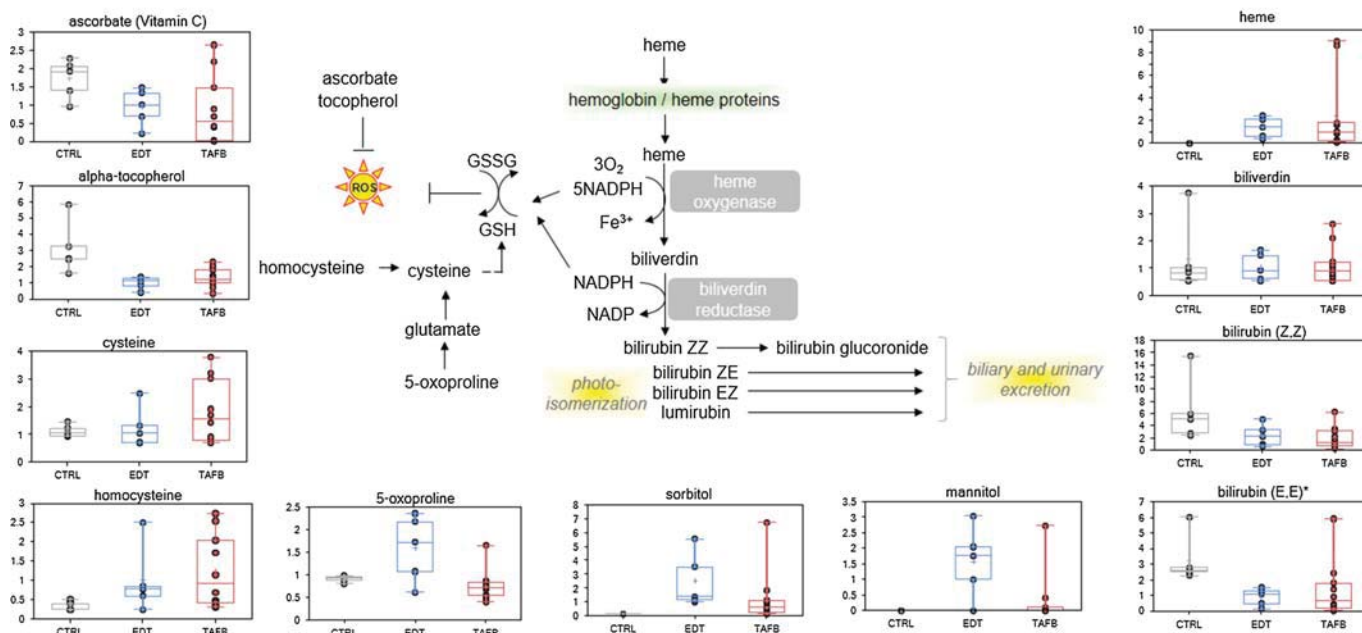


Figure 1. An overview of heme metabolism and antioxidant compounds in plasma samples from controls, EDT, and TAFB patients. Results are graphed as box plots, indicating median values (line), mean values (+), and upper/lower quartile distributions for each group.

<http://links.lww.com/TA/A529>) and cyclic dipeptide cyclo (glu-glu) (Supplemental Digital Content 3, <http://links.lww.com/TA/A529>).

In addition, there was significant nucleoside breakdown for metabolic purposes as demonstrated by trauma patients' increased levels of purine (xanthine, hypoxanthine and inosine) and pyrimidine catabolites (uracil, 5,6-dihydrocuracil and 3-aminobutyrate) (Figs. 3 and 4).

Lastly, there was metabolic evidence for the activation of coagulation in these injured subjects. Three fibrinogen cleavage peptides accumulated following injury (ADSGEGDFA-EGGGVR, corresponding to fibrinopeptide A, and two other cleavage products, namely, DSGEGDFAEGGGVR, and ADp-SGEGDFAEGGGVR) (Supplemental Digital Content 3, <http://links.lww.com/TA/A529>).

DISCUSSION

Metabolomics describes the complement of metabolites present in a biologic matrix and is reflective of the host's pathologic state or response to stimuli.¹⁹ This pilot study represents the first MS-metabolomic description of profound postinjury metabolic aberration in a critically injured group of trauma patients and serves as the essential foundation for future investigation in postinjury metabolic stress (Fig. 5).

Technical issues might affect the metabolomics readouts. In this study, extraction efficiency was tested by the addition of recovery standards and normalizing extraction volumes to protein concentrations and total ion currents. Single platform-associated limitations, such as retention time

and chromatographic issues, were controlled by running both liquid chromatography/MS and gas chromatography/MS analyses.²³ MS resolution issues were addressed by running samples both in low- and high-resolution instruments (LTQ-FT and FT-ICR) in both polarity modes, either full MS or MS/MS so as to monitor transition fingerprints against validated standards. Biologic bias should be considered because the metabolome is influenced by the patients' genetic makeup and is readily responsive to environmental stimuli (e.g., diet, habitudes), including the nature and severity of injury. In this view, it is worth noting that the present study addresses a small heterogeneous group of trauma patients, sharing a comparable elapse time from trauma as a result of different injury mechanisms. Nonetheless, plasma metabolomics provides the opportunity to elucidate pathway-specific responses to injury and develop hypotheses for investigating mechanistic pathology. Future investigations on larger cohorts of injury patients may benefit from incorporating principal component analysis or other variable reduction techniques (e.g., partial-least square discriminant analysis), to evaluate metabolomic associations to a patient's clinical presentation and hospital course, such as injury severity, transfusion requirement, responsiveness to therapy, risk for morbidity and mortality outcomes.

Critical injury may provoke both complementary and competing physiologic responses in an attempt to maintain homeostasis. Clinical responses include the adrenergic "fight or flight" response, which maintains blood pressure, heart rate, and critical perfusion, which also mobilizes substrates for energy production (proteolysis, lipolysis, glycogenolysis, and gluconeogenesis) to preserve cellular and tissue metabolic

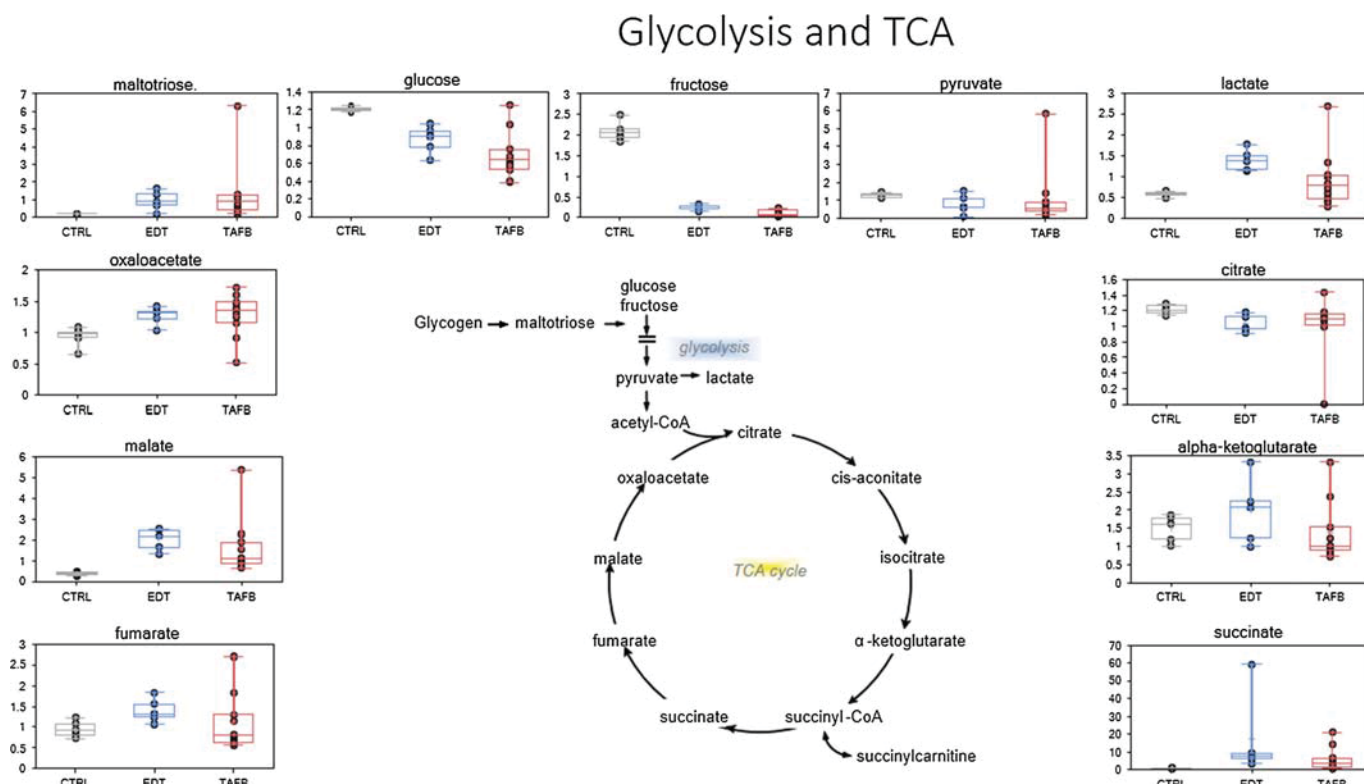


Figure 2. An overview of glycolysis and the TCA cycle in plasma samples from controls, EDT, and TAFB patients. Results are graphed as box plots, indicating median values (line), mean values (+), and upper/lower quartile distributions for each group.

functions.^{4,5,15} Adrenergic mobilization of fuel substrates overlaps with hormonal regulation including cortisol release and insulin resistance, which may preserve glucose for sensitive tissues (i.e., brain, heart), thereby potentiating stress hyperglycemia. Other critical response mechanisms include physiologic modulation of the coagulation cascades, endothelium, and platelets to achieve hemostasis at points of bleeding (thrombogenesis and fibrinolysis shut down) while seemingly competing mechanisms exist to prevent systemic clot formation and maintain perfusion to critical tissue beds (fibrinolysis).²⁴ In addition, the complex interplay of proinflammatory and anti-inflammatory cytokines, growth factors, as well as the complement system and acute hepatic phase response all combine to create the relative anabolic or catabolic state of the system as a whole.

Central to these physiologic adaptations are the multiple interrelated metabolic pathways. Postinjury systemic response mechanisms (i.e., adrenergic response, innate immune response/cytokines/complement) may influence multiple subcellular pathways, whose extended coverage is warranted by the sensitivity of the MS-based analyses.

Hemolysis and Hemostasis

Hemorrhagic shock is one of the main features of trauma.³ Plasma from trauma patients was characterized by the accumulation of mannitol, heme, and oxidative stress markers. Mannitol increase secondary to trauma might stem from its use

as an osmotherapeutic agent or rather accumulate upon the administration of blood-derived therapeutics such as packed RBCs (containing mannitol in the storage solutions). In line with this, EDT patients, receiving blood product resuscitation in the emergency department, demonstrated higher levels of plasma mannitol (Fig. 1). This finding may convey a potential confounding variable with blood product administration influencing the observed trauma metabolome. However, our data suggest limited transfusion effect, aside from mannitol increase, in this study population. We have recently published several reports and meta-analyses of biochemical and metabolic lesions to stored packed RBCs.^{25–28} Specifically, alteration in glutathione and NADPH homeostasis and oxidized proinflammatory lipid accumulation by Day 42 of storage were identified. This corresponds to a consumption of storage nutritive components (glucose and adenine) and the accumulation of catabolic byproducts: lactate, carboxylic acids (fumarate, succinate, α-ketoglutarate) and nucleotide metabolism byproducts (i.e., hypoxanthine). However, although no TAFB patient (0/6) received transfusion before sampling by paramedics, there is no difference in the levels of the metabolites mentioned earlier between EDT patients who received limited transfusion and TAFB patients, in whom blood draws preceded intravenous fluid or blood products. Furthermore, no patients in this study received platelets, and there was limited FFP administration (2 of 7 EDT patients), which would not be expected to grossly affect the observed metabolic signatures because FFP was used as the baseline control metabolome. Transfusion likely had minimal effect on the global metabolic changes

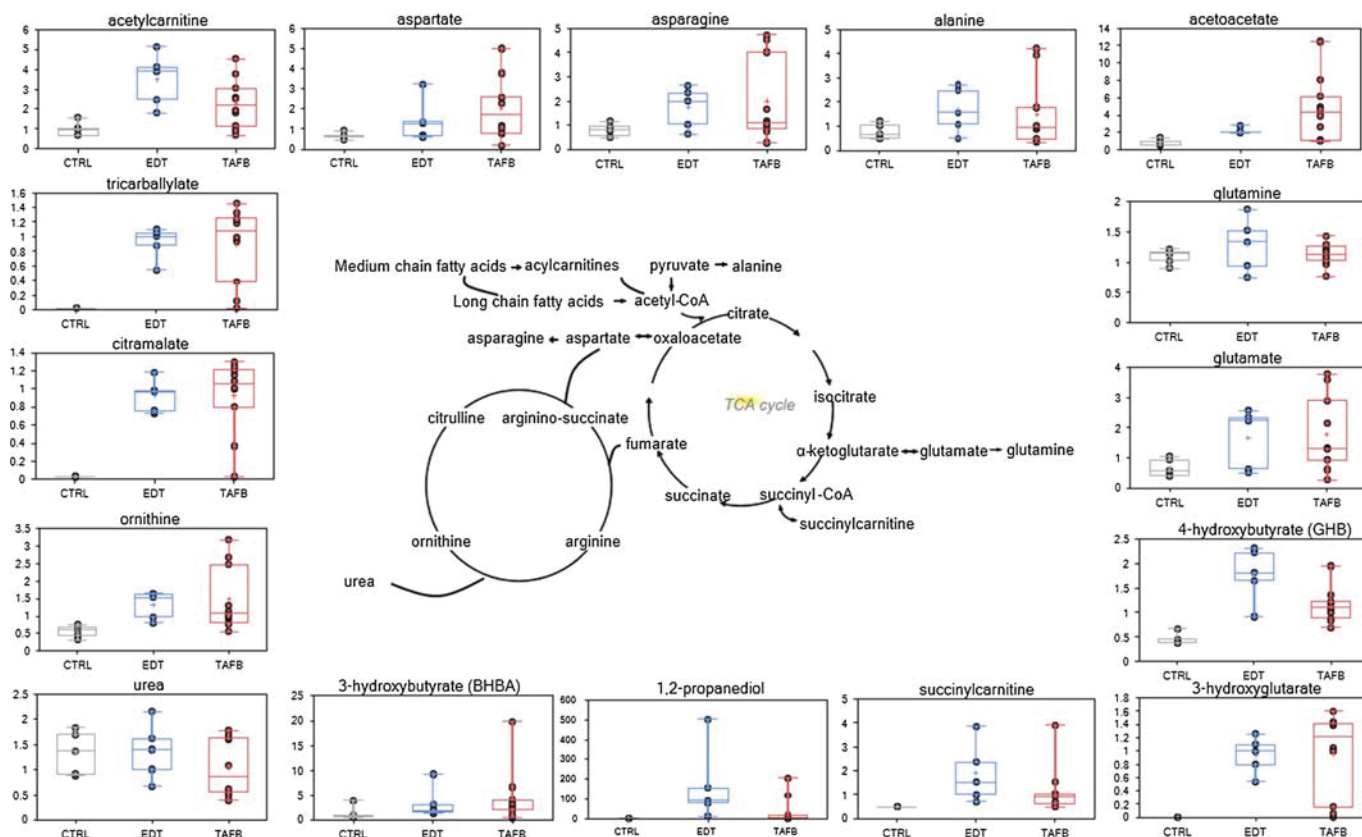


Figure 3. An overview of protein and lipid metabolism in plasma samples from controls, EDT, and TAFB patients. Results are graphed as box plots, indicating median values (line), mean values (+), and upper/lower quartile distributions for each group.

seen in these patients. Moreover, the elevated mannitol in the EDT population also confirms the sensitivity of this technique to detect potential treatment effect. A possible dose-response effect of blood product transfusion on the plasma metabolome could be considered for future investigation in populations receiving massive transfusion.

Hemolysis might promote oxidative stress in the form of heme-iron-generated reactive oxygen species. 5-oxoproline significantly increased after injury, with a concomitant decrease in the levels of antioxidant vitamins including ascorbate and tocopherol, especially in EDT patients.

Balance of hemostasis and physiologic modulation of coagulation are prominent trauma research interests. Competing mechanisms seem to exist to halt hemorrhage, while preventing systemic thrombosis and preserving microcirculatory flow. Distinct coagulopathy phenotypes in the trauma population have been proposed, including hypercoagulable (fibrinolysis shutdown), physiologic fibrinolysis, and coagulopathic (hyperfibrinolysis).²⁴ While the mechanistic genesis of each remains to be fully elucidated, these coagulation patterns are suggestive of both altered thrombin generation and circulating metabolites, which may contribute to coagulopathy in the injured patient. Fibrinogen cleavage peptides were detected in 60% to 80% of samples from trauma patients but not in controls, suggesting fibrin cleavage by thrombin to promote clot formation. However, sequelae of this response may exist as evidenced by the identification of a specific fibrin

cleavage peptide phosphorylated at Ser3 (Ser22 of fibrinogen α chain). This posttranslational modification is known to be elevated in acute injury and may play a role in the onset of trauma-induced coagulopathy.²⁹ These important metabolic implications on the coagulation phenotype of the patient could further be explored in controlled animal models of hemorrhagic shock and resuscitation or longitudinal investigation of massive transfusion with correlation to functional coagulation evaluations (TEG, platelet mapping, LY-30, etc.)

Glycolysis and TCA

Previous investigations of the metabolic response to trauma have highlighted a trauma-dependent impairment in energy metabolism, resulting in increased rates of glycolysis, through the modulation of hepatic gluconeogenesis and glycogenolysis.^{30,31} Accumulation of the glycogen catabolite maltotriose was observed in trauma patients likely because of increased glycogen breakdown and increased consumption of fructose and glucose. Accumulation of lactate in plasma from trauma patients is consistent with metabolic adaptations to polytrauma, critical illness, and isolated injury, such as in the case of concussive brain injury.³⁰⁻³²

Plasma accumulation of TCA cycle intermediates was observed in trauma samples, a phenomenon that can be associated with metabolic acidosis.³³ Succinate accumulation

Nucleic acid metabolism

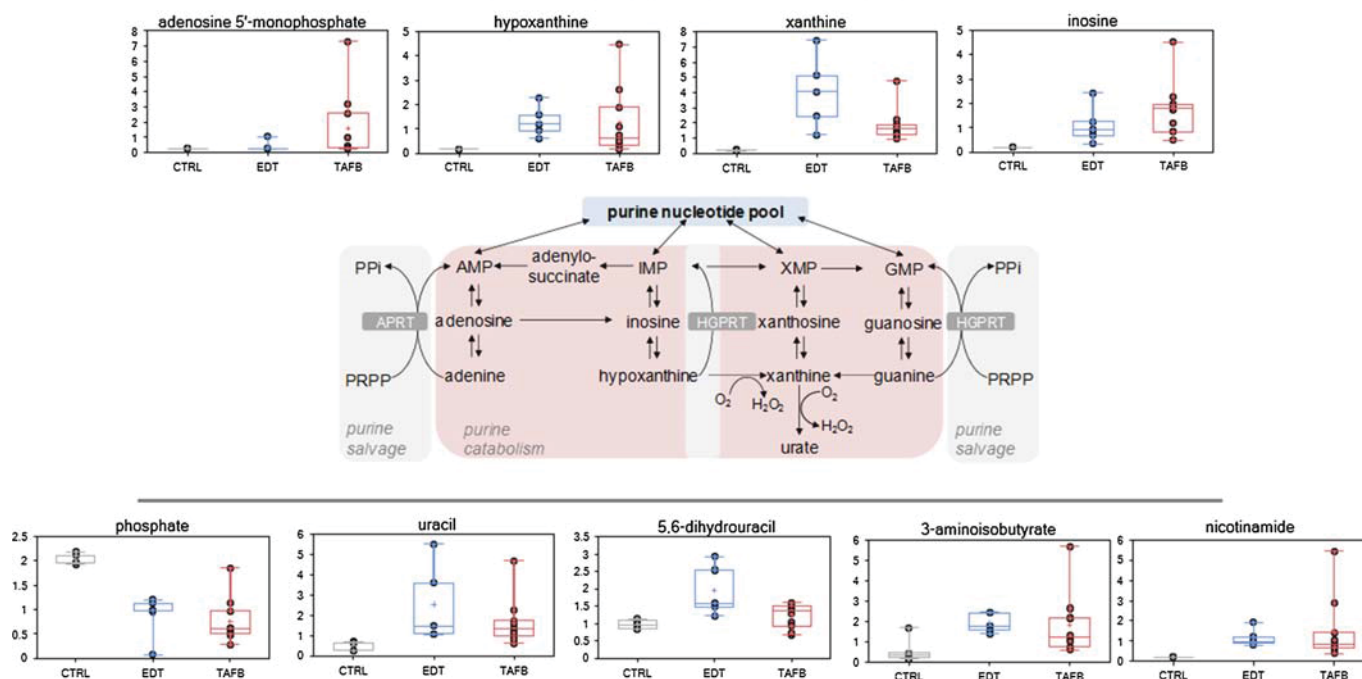


Figure 4. An overview of purine and pyrimidine metabolism in plasma samples from controls, EDT, and TAFB patients. Results are graphed as box plots, indicating median values (line), mean values (+), and upper/lower quartile distributions for each group.

might mediate platelet overactivation and thus potentiate untoward hypercoagulation in trauma patients.³⁴ Concomitant accumulation of lactate and TCA intermediates is suggestive that early metabolic alterations following trauma promote channeling to the TCA cycle of additional carbon substrates other than sugars, such as lipids and proteins.

Lipolysis and Ketoacidosis

In healthy subjects, lipolysis is under tight hormonal regulation, and altered lipidomic profiles are a hallmark of trauma-induced metabolic adaptation.³⁵ In response to trauma, lipolysis is activated, and lipogenesis is blocked, facilitating the transition to lipids as a primary fuel substrate for energy production. Consistently, metabolomic analyses hereby highlighted fatty acid mobilization (accumulation of acylcarnitines) and lipid breakdown (buildup of ketone bodies and breakdown products of fatty acids—e.g., choline, glycerol, ethanolamine, glycerol phosphate, and glycerophosphocholine moieties). In addition, elevations in postinjury proinflammatory arachidonate metabolites such as prostaglandin PGE2 and leukotriene LTB4, observed here, support the proposed beneficial effect of Ω -3 fatty acid supplementation diets as a measure to inhibit arachidonate-mediated leukocyte activation and chemotaxis and thus attenuate proinflammatory gene expression levels.³⁶ Moreover, hyperactivation of lipid metabolism in response to trauma results in the accumulation of anionic compounds such as ketone bodies, which in turn promotes the development of a base deficit and ketoacidosis.³⁷ Organic acidurias, such as ketoacidosis, stem from the accumulation

and subsequent excretion of acid metabolites that are typically only present in low abundance, such as ketone bodies.³⁷

Organic Acids

The trauma-dependent accumulation of certain organic acids might underpin both alterations to hemostatic functions and partially explain cross-talk between metabolic adaptations and the neuroendocrine system. Accumulation of 2-hydroxyglutarate in trauma patient samples could be related to platelet activation because this metabolite has been reported to impair platelet aggregation *in vitro*.³⁸ Elevation of citramalate (methylmalonate) was also observed (Fig. 3). This metabolite can accumulate rare pathologies such as methylmalonic aciduria, a metabolic disorder caused by methylmalonyl-CoA mutase deficiency (genetic or B12 deficiency), and might be related to alterations in the gut microbiome.³⁹ *In vivo*, bacterial citramalate is taken up by RBCs and can be released in trauma plasma samples upon hemolysis.⁴⁰ Analogously, tricarballic acid (Fig. 3), a magnesium-chelating tricarboxylic acid thought to be of bacterial origin, was greatly elevated in trauma patients and may be associated with acidurias.⁴¹

These observations are relevant in that they link the previously reported phenomenon of trauma-dependent gut microbiome effects on the plasma metabolic phenotypes. In addition, they foster translational considerations in the light of the posited role for certain metabolites of microbial origin, rather than bacteria themselves, in tethering neutrophil activation, a known precursor to systemic inflammatory response syndrome/MOF.

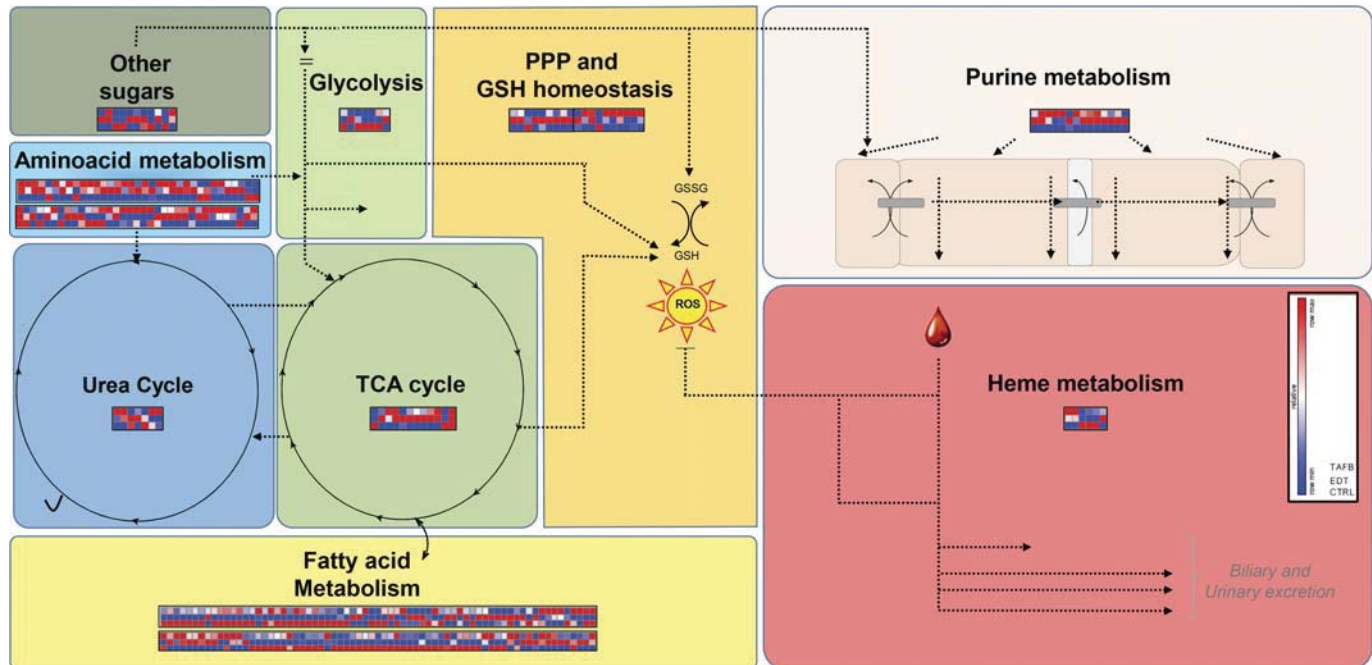


Figure 5. A summary of the main metabolic pathways assayed in this study. Mean metabolite quantitative fluctuations were Z score normalized and graphed as heat maps in GENE-E. For each metabolite, three values are given, indicating values detected, from top to bottom, in TAFB, EDT, or control samples, respectively. Quantitative values progressively increase from blue (minimum) to red (maximum), while white indicates normalized values close to 1.

Accumulation of organic acids often has a significant negative impact on central nervous system function.⁴² Indeed, one of the largest and statistically significant fold-changes (>500-fold increase, $p < 0.05$) for a posttrauma metabolite was observed in the organic acid 3-hydroxyglutarate (3-HG). This organic acid is a potent neurotoxin contributing to neuropathology in both ketosis and glutaryl-CoA dehydrogenase deficiency. Altered carnosinase activity in trauma patients could change levels of carnosine, a brain antioxidant and neuroprotectant. The organic acid 4-hydroxybutyrate (GHB) is a central nervous system neuromodulator that accumulates with impaired activity of succinate dehydrogenase, an enzyme involved in catabolism of the neurotransmitter GABA. In contrast, the observed stability of classic neuroendocrine mediators (cortisol, cortisone, and epinephrine levels; see Supplemental Digital Content 3, <http://links.lww.com/TA/A529>) is suggestive of the presence of a yet unexplored neuroendocrine stress response to trauma. Together, these results suggest previously unrecognized potential etiologies for acute mental status change following severe trauma at the metabolic level.

Proteolysis and Amino Acid Accumulation

Accumulation of amino acids, as observed here, can occur from three principal mechanisms: (i) enhanced proteolysis, (ii) de novo synthesis and altered anabolic reactions using amino acids as building blocks for larger biomolecules, or (iii) arrest of catabolic processes using amino acids for energy production purposes.

Proteolysis

Trauma recovery is known to involve an initial catabolic phase, often with negative nitrogen balance, followed by an anabolic phase. Proteolysis ensues in response to trauma in an effort to provide carbon backbones for gluconeogenesis in the liver.^{43,44} In trauma patients, accumulating cyclic dipeptides (diketopiperazines) can be generated as byproducts of protein metabolism and have been reported to have biologic activity including an immunomodulatory role in the stimulation of T lymphocytes.

De Novo Synthesis and Anabolic Purposes

Amino acid accumulation (as observed with plasma increases in alanine, aspartate, and glutamate) could be secondary to de novo synthesis from activation of transaminases for detoxification purposes and redox balance. Glutamate and cysteine accumulation could fuel new reduced glutathione (GSH) synthesis (Fig. 2), thereby serving as physiologic protection from the increase in trauma-dependent oxidative stress (see previous paragraphs).

Arrest of Catabolic Reactions

Alternatively, amino acid accumulation may result from arrest of metabolic processes during low tissue oxygen conditions or with insufficient phosphate availability to sustain oxidative phosphorylation. These essential criteria are fulfilled in this study population as it is well recognized that trauma

patients are characterized by incommensurate oxygen consumption in relation to maximal oxygen availability. This might be associated with mitochondrial uncoupling or an inefficient electron transport chain. In addition, we found that circulating phosphate levels, representative of high-energy phosphate groups, were significantly decreased following injury in both trauma patient populations (Fig. 4).

Notably, trauma did not result in the accumulation of glutamine. A potential explanation is that trauma enhanced consumption of this specific amino acid for direct cellular energy production or for fueling transamination reactions. However, although glutamine supplementation in intensive care unit patients has been a long-sought after-issue, no definitive evidence has been produced to date about the association between patient survival and glutamine supplementation.⁴⁵ Glutamine has been shown to promote neutrophil phagocytic activity and oxidative burst. In addition, glutamine exerts an important nutritional effect serving as a principal fuel source for enterocytes and intestinal mucosa.

While the levels of most amino acids were increased in response to trauma, tryptophan and its associated metabolites (tryptophan betaine, N-acetyltryptophan, C-glycosyltryptophan, 3-indoxyl sulfate, indolepropionate) decreased in trauma samples (Supplemental Digital Content 3, <http://links.lww.com/TA/A529>). Altered tryptophan metabolism may be related to oxidative stress in the central nervous system and brain injury.⁴⁶

Nucleoside Metabolism as an Additional Resource to Sustain Energy and Nitrogen Metabolism

Nucleosides may also provide additional carbon backbones and act as nitrogen donors to compensate for the negative nitrogen balance in response to trauma. However, increased generation of xanthine and hypoxanthine could also reflect increased xanthine oxidase activity, which would generate reactive oxygen species and potentiate oxidative stress as has been reported in a rat model of traumatic brain injury.⁴⁷

Altered purinergic metabolism may underlie cellular responses to glucagon stimulation in response to trauma-associated fasting/starvation-like catabolic response. In line with this, the accumulation of nicotinamide (Fig. 4), a breakdown product of the purine metabolite NAD as generated by the activity of poly-ADP ribose polymerase, may have significant clinical impact by contributing to posttraumatic motor, cognitive, and histologic sequelae.⁴⁸ Indeed, exhaustion of NAD⁺/NADH reservoirs would compromise the activity of many energy and redox metabolism enzymes that are dependent on these cofactors.

Unexpected Nonmammalian Metabolites

We have previously highlighted citramalate (methylmalonate) and tricarballic acid as organic acids contributing to an acidotic milieu and acidurias. The significant elevation in these metabolites shortly after trauma is a unique, unexpected finding as these metabolites have rarely been described in human physiology and are likely of bacterial origin. Investigations of remote organ dysfunction following trauma have implicated postshock mesenteric

lymph as the conduit by which etiologic agents are conveyed from the stressed splanchnic beds to the systemic circulation.⁴⁹ Previous investigations have excluded bacterial translocation and endotoxin within postshock mesenteric lymph or portal venous blood as mediators, and although a number of alternative substances have been investigated (cytokines, lipids, proteins, DAMPS), the culprit mediators remain elusive.⁵⁰

Our findings suggest an alternate hypothesis where bacterial metabolites, and not bacteria themselves, may translocate during reperfusion of ischemic splanchnic beds to manifest systemic pathology. Alternatively, bacterial metabolites such as citramalate may elaborate systemically during RBC lysis associated with trauma and hemorrhagic shock.⁴⁰ The precise source of these nonmammalian metabolites and their role in postinjury pathophysiology deserve further investigation, which could include MS-metabolomic analysis for bacterial metabolites in postshock mesenteric lymph and blood product cell lysates.

CONCLUSION

Results from this study expand on existing NMR-based metabolomics investigation for trauma-induced hypercatabolism, driven by sugar consumption, lipolysis and fatty acid use, ketone body accumulation, proteolysis and nucleoside breakdown providing substrates, which compensate for increased energy demands and contribute to a negative nitrogen balance. While TAFB and EDT patients were characterized by massive plasma metabolic changes in comparison with controls, minimal alterations were observed between these two critically injured populations. These similarities suggest that the profound metabolic changes, which ensue immediately after severe traumatic injury (TAFB), persist and may develop into progressive pathologic deterioration as the patient becomes terminal (EDT).

This MS-metabolomic description provides an essential foundation for the development of multiple investigational hypotheses. Cellular energy balance and acute resuscitation are likely to be tied to metabolic end points. Resuscitative efforts with crystalloid, colloid, and blood products or the inclusion of alternative therapeutics with acute resuscitation (i.e., antioxidants or nutritive support to balance metabolic deficiencies or meet essential substrate requirements) may be evaluated with respect to normalization of clinical physiology in conjunction with metabolomic end points. Specific metabolites identified, such as succinate or modified fibrinogen cleavage products (Ser22 fibrinogen- α), may be evaluated in conjunction with functional coagulation assays to investigate potential mechanistic impact on coagulation phenotypes. In addition, the unique finding of early elaboration of nonmammalian metabolites in plasma may suggest an etiologic link between the ischemic gut, mesenteric lymph, and remote organ injury, which could be further elucidated in established animal models with the incorporation of MS-metabolomic analyses.

From this study, it emerges that, while initial measures of resuscitation by hemorrhage control and optimizing oxygen balance are aimed at correcting ongoing posttraumatic physiologic aberration, an optimal therapeutic regimen may include the concept of a "metabolic resuscitation" to specific end points to maintain or to reinstitute physiologic homeostasis.

AUTHORSHIP

E.D.P., E.E.M., C.C.S., A.S., K.C.H., and A.B. planned the experimental design and the patient enrollment criteria as well as supervised metabolomics analyses. E.D.P. and T.C. collected the samples. E.D.P., A.D., E.E.M., C.C.S., K.C.H., and A.B. analyzed the results, wrote the paper, and critically commented on the paper. E.D.P. and A.D. prepared the figures and tables.

DISCLOSURE

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