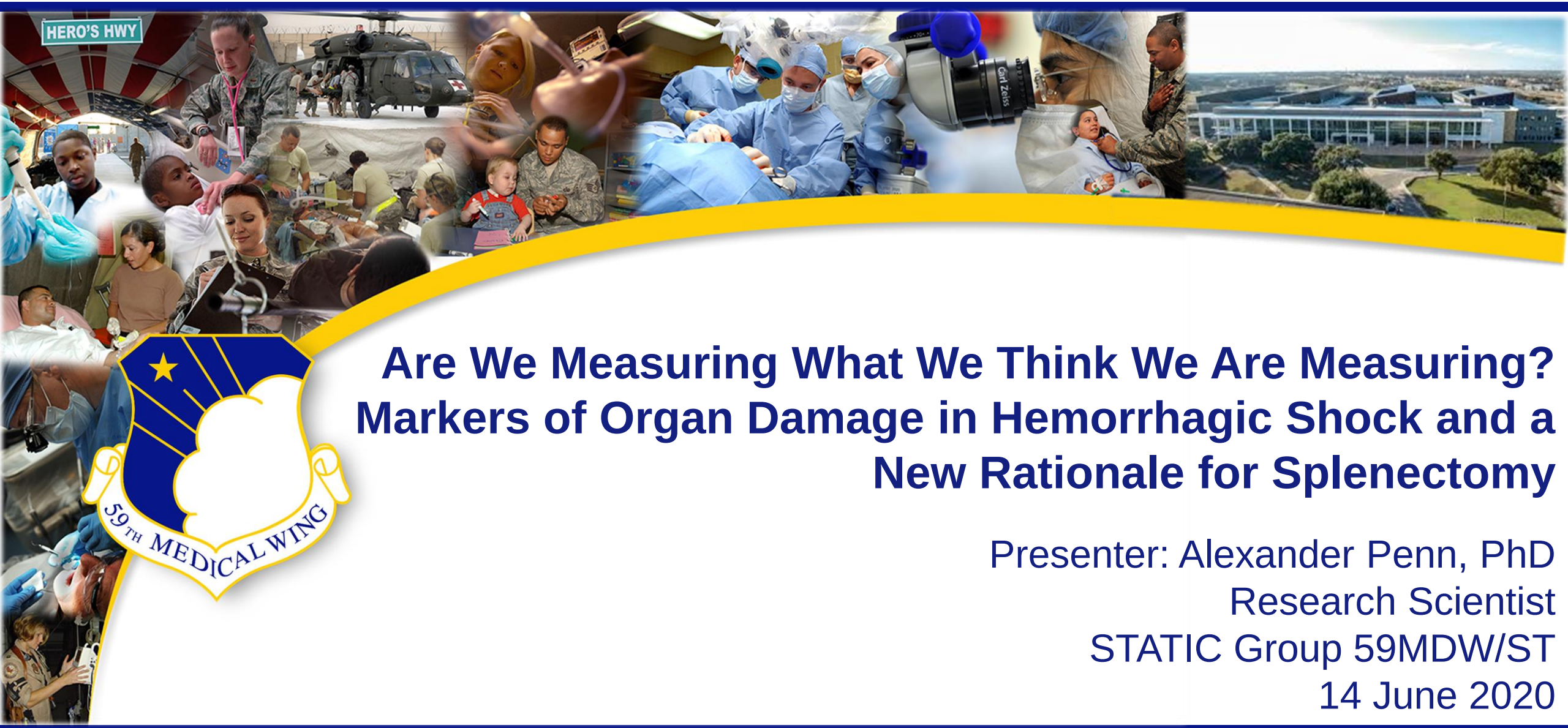
59th Medical Wing



Are We Measuring What We Think We Are Measuring? Markers of Organ Damage in Hemorrhagic Shock and a New Rationale for Splenectomy

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Disclaimers

- The views expressed are those of the presenter and do not reflect the official views or policy of the Department of Defense or its Components.
- The experiments reported herein were conducted according to the principles set forth in the National Institute of Health Publication No. 80-23, Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966, as amended.
- The authors have no conflicts of interest to report.

Background

- Hemorrhage and hemorrhagic shock (HS) are major concerns for military medicine.
- The three main hurdles to surviving severe hemorrhage are:

	Time scale of onset
1) Stopping the bleed (initially by pressure, tourniquets, and hemostatic dressings; later by surgery)	(< 1 h)
2) Compensating for hypovolemia until surgical control of hemorrhage and full resuscitation with blood products	(< 1 d)
3) Avoiding, treating, or outlasting organ dysfunction resulting from ischemia/reperfusion injury and other damage mechanisms	(<1 wk)

- The mechanisms leading to death from decompensation versus organ failure are interrelated but separate (e.g. the earliest decompensation deaths have the least organ injury).
- Treatments to aid in overcoming earlier hurdles can make the challenge of later hurdles greater

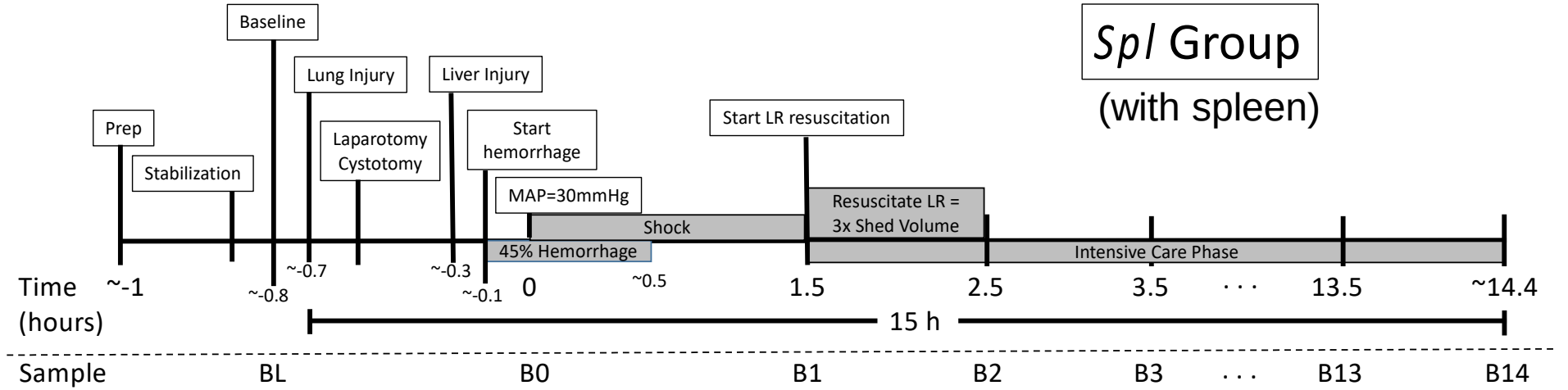
  Ex 1: Tourniquets and permissive hypotensive resuscitation may increase the odds of fatal decompensation

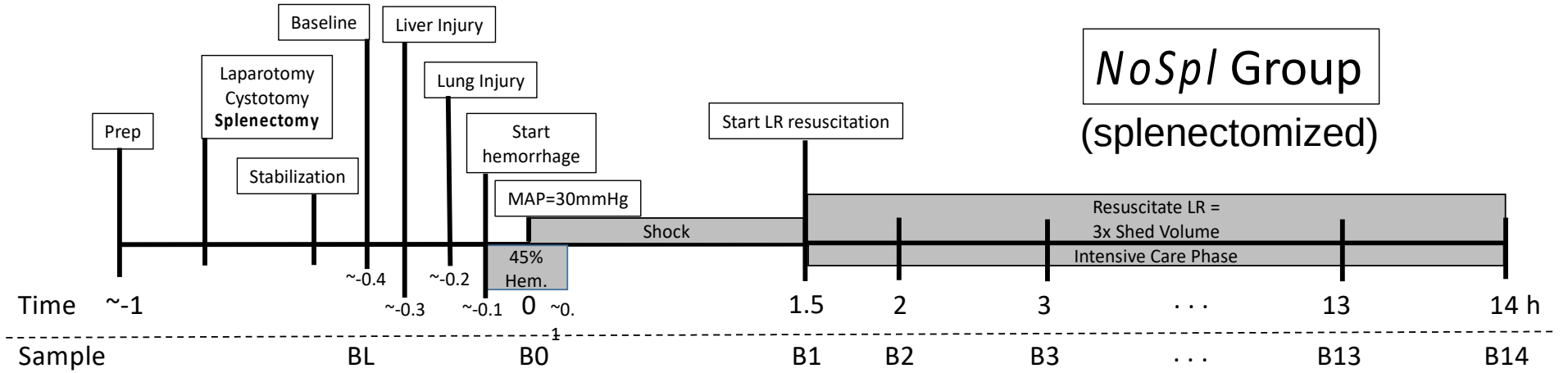
  Ex 2: Vasopressors may be able to aid compensation, but can lower perfusion of blood in organs increasing subsequent organ dysfunction
- Similarly, creating animal models to study the later hurdles is complicated by the earlier hurdles
  - In our swine prolonged field care (14 h) model of trauma and HS, we attempted treatment for organ dysfunction, but found little histological organ damage despite 65% mortality. Increasing severity would likely further reduce the number of animals surviving to develop organ damage.
  - We need a model that is severe enough to cause organ damage but somehow avoids death from decompensation.

Background

- One of the main functions of the spleen is to trap older red blood cells (RBCs), removing them from circulation and allowing their hemoglobin and other components to be safely processed.
- However, if a need for greater oxygen carrying capacity in the circulation arises (e.g. after hemorrhage), the spleens of many large animals (humans included) can contract, pushing blood volume with high hematocrit into circulation.
- Splenectomy prior to hemorrhagic shock (HS) in large animal models remains controversial.
- Previous studies of splenectomy in HS found minimal effect but were of short duration and non-fatal severity.
- We hypothesized that splenic contraction may not be sustainable for long periods of time. Therefore, the spleen may transition from being a volume source to a volume sink, contributing mortality in HS models of sufficient severity to display decompensation.
- As part of a model development protocol, we examined the effects of splenectomy (NoSpl group) prior to trauma and HS in 9 swine compared to a historical set of 14 swine subjected to a near identical trauma and HS protocol without splenectomy (Spl group).

Timelines

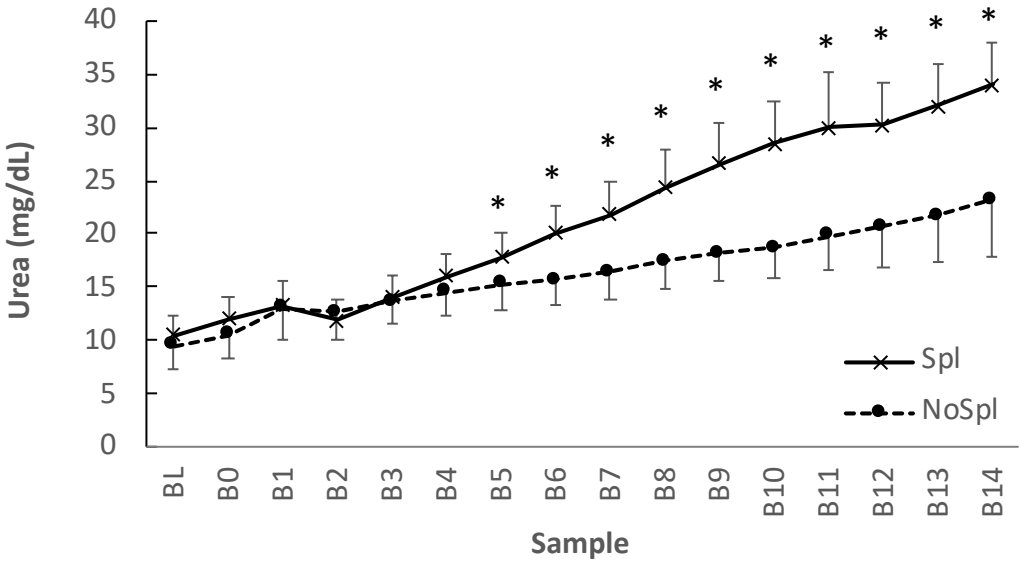

Baseline
Lung Injury
Liver Injury
Prep
Stabilization
Laparotomy
Cystotomy
Start
hemorrhage
Start LR resuscitation
MAP=30mmHg
Resuscitate LR =
3x Shed Volume
Shock
45% Hemorrhage
Intensive Care Phase
Time
(hours)
~-1
~-0.8
~-0.7
~-0.3
~-0.1
0
~0.5
1.5
2.5
3.5
· · ·
13.5
~14.4
15 h
Sample
BL
B0
B1
B2
B3
· · ·
B13
B14
Spl Group
(with spleen)

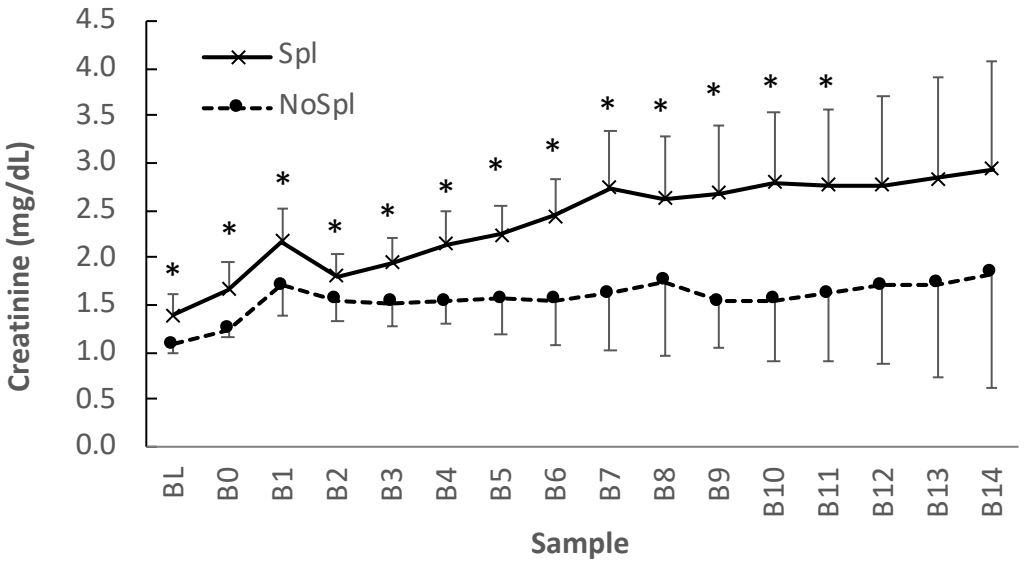

Baseline
Liver Injury
Laparotomy
Cystotomy
Splenectomy
Lung Injury
Prep
Start
hemorrhage
Start LR resuscitation
Stabilization
MAP=30mmHg
Resuscitate LR =
3x Shed Volume
Shock
45%
Hem.
Intensive Care Phase
Time
~-1
~-0.4
~-0.3
~-0.2
~-0.1
0
~0.
1
1.5
2
3
· · ·
13
14 h
Sample
BL
B0
B1
B2
B3
· · ·
B13
B14
NoSpl Group
(splenectomized)

Results

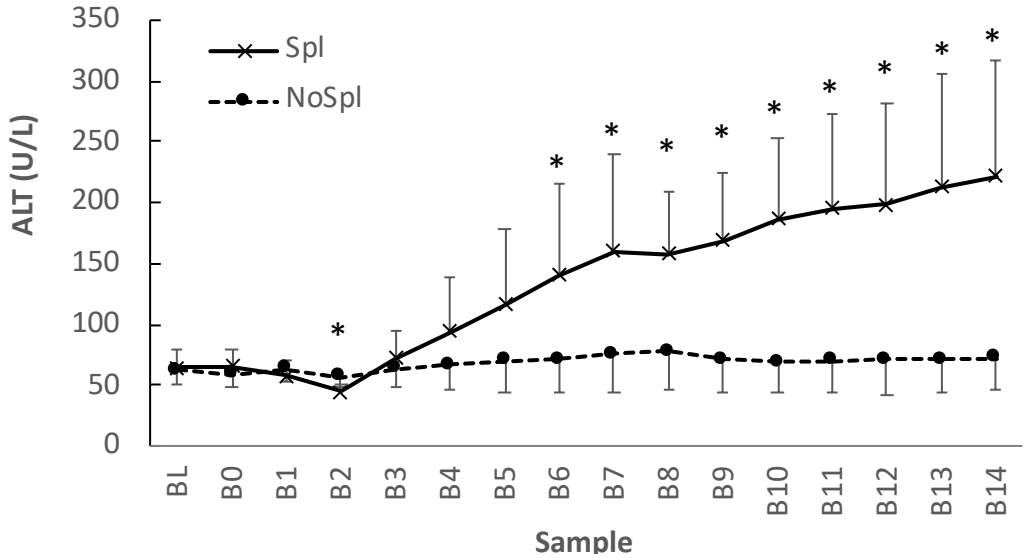
- Previously Reported at MHSRS 2019:
  - Survival was significantly greater in the NoSpl group (89% vs 36%, p=0.02).
  - The resuscitation fluid was used up much faster in the Spl group (100% vs. 35% used in 1st hour). Multiple animals in NoSpl group still had fluid left to give at 9 h.
  - We concluded that if the goal is to study decompensation, the spleen is critical and should be left in.
  - If the goal, however, is to study multi-organ failure, which occurs by separate mechanisms, splenectomy may help balance the high severity needed to achieve sufficient organ injury with the need to not lose most of the animals to decompensation before the organ injury can develop.
- But wait… there's more!
  - Analysis of our routine chemistry and electrolyte measures revealed another important event occurring unbeknownst to us.

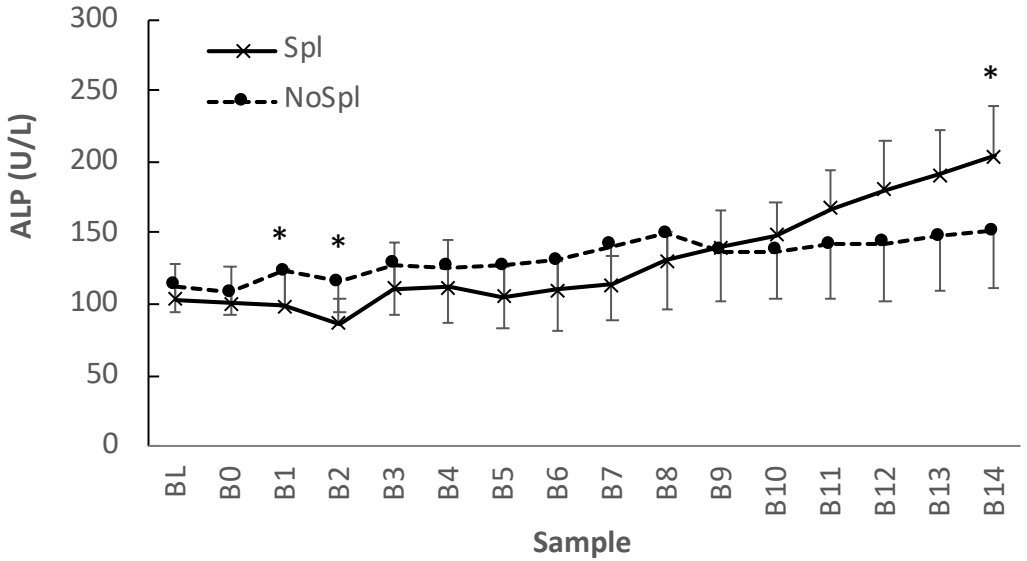
“Renal” Damage Markers Higher in Spl Group

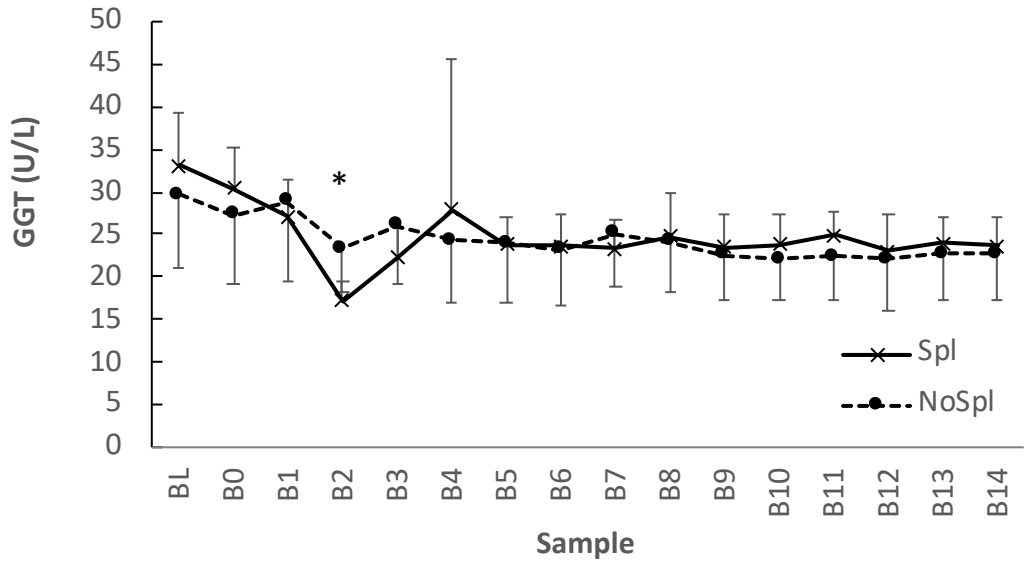




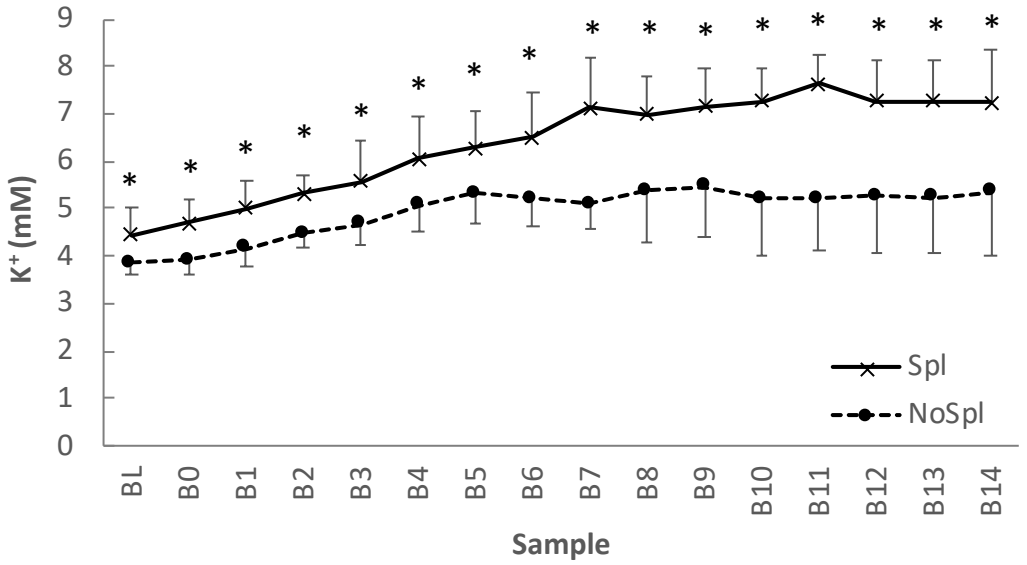
Some “Liver” Damage Markers Higher in Spl Group

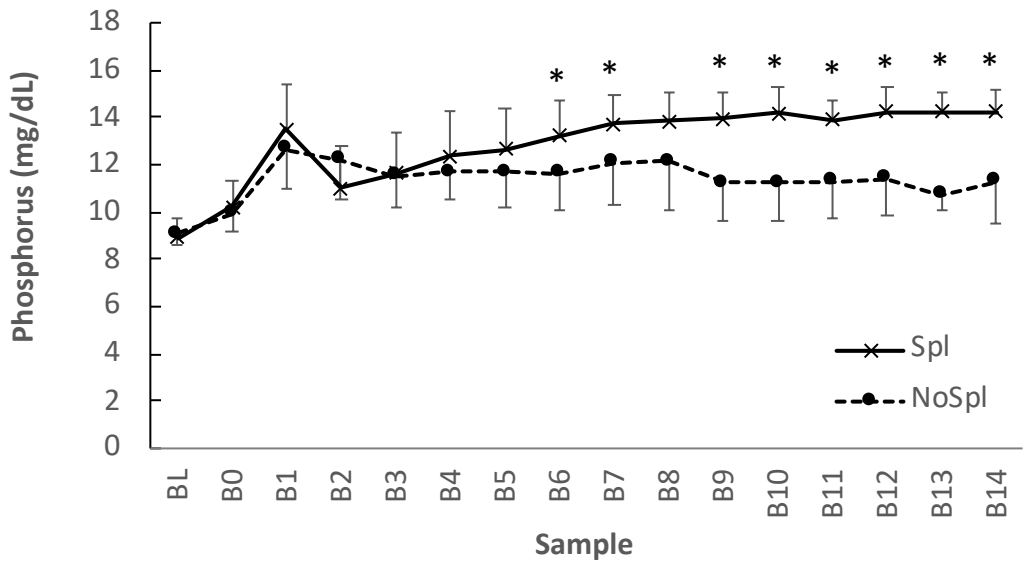




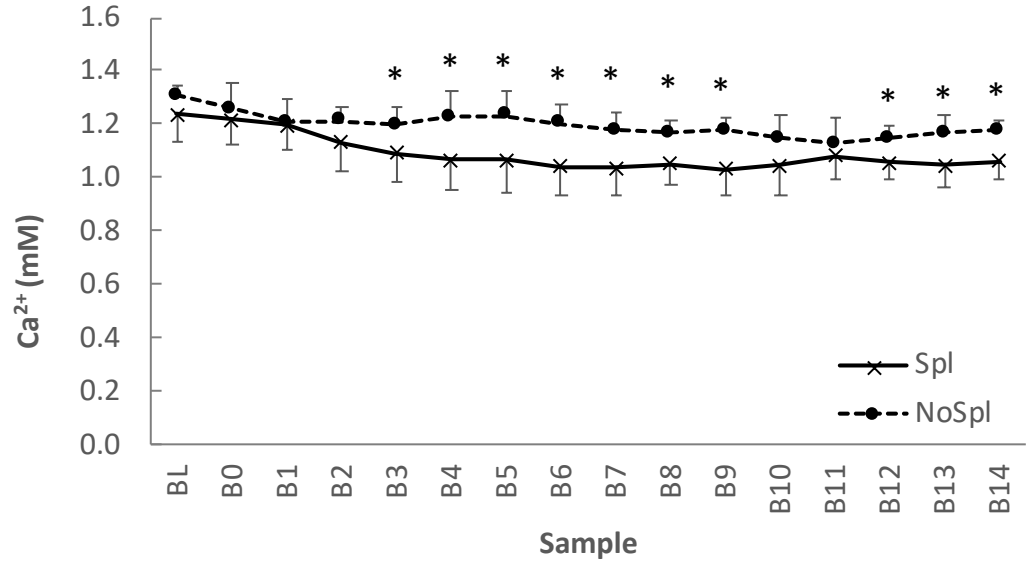


Elements and Ions Suggest Cell Lysis



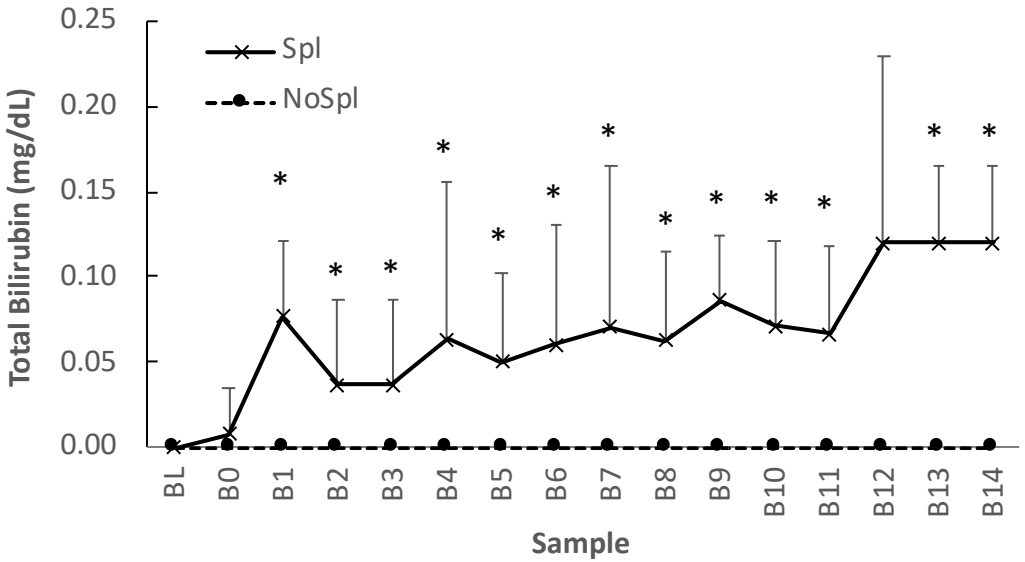


- Spl group had higher K^+ & phosphorus, but lower Ca^{2+}.
- K^+ and Phosphate are found in much higher concentrations intracellularly than in plasma.
- Phosphate ions that encounters calcium ions form an insoluble salt.



Bilirubin suggests hemolysis

- If cell lysis is occurring, which cells are being lysed?
- There was far less kidney damage in the NoSpl group according to the biomarkers
- Likewise, ALT only appeared in the Spl group and there was little to no increase in the other measured markers of liver damage in either group.
- There is no obvious connection between splenectomy and a reduction in liver and kidney damage.
- **What if those biomarkers are indicating something other than liver or kidney damage?**



- Bilirubin is the downstream product of hemoglobin degradation and it only appeared in the Spl group.
- Since the spleen (when present) is ejecting a bunch of older/frailer cells into circulation in HS, maybe what we are really detecting is **hemolysis**.

In Vitro Hemolysis Causes Similar Changes

Lippi et al. Influence of hemolysis on routine clinical chemistry testing. Clin Chem Lab Med 2006;44(3):311–316.

They added serial dilutions of hemolyzed blood to whole blood in vitro and measured a variety of chemistry values.

Analyte	Change per g/L of Hb released by hemolysis	If multiply by 270 g/L Hb **	Change in value in Spl group
Blood Urea Nitrogen (BUN)	0.03 mM	7.0	8.4
Creatinine	0.38 uM	102	136
Alanine Aminotransferase (ALT)	0.58 U/L	157	157
Alkaline Phoshpatase (ALP)	-1.42 U/L *	<0	101
Γ-glutamyltransferase (GGT)	-0.27 U/L *	<0	<0
Potassium	0.22 mM	60.3 (wow!)	2.8
Phosphorus	0.01 mM	3.2	1.7
Bilirubin	-0.04 uM	<0	2.05 ***
Aspartate Aminotransferase (AST)	7.13 U/L	1921	Not Tested
Creatine Kinase (CK)	12.44 U/L	3353	Not Tested
Lactate Dehydrogenase (LDH)	165.12 U/L	44501	Not Tested

Highlight = significantly elevated by hemolysis in their study

* Likely Hb interfered with measurement

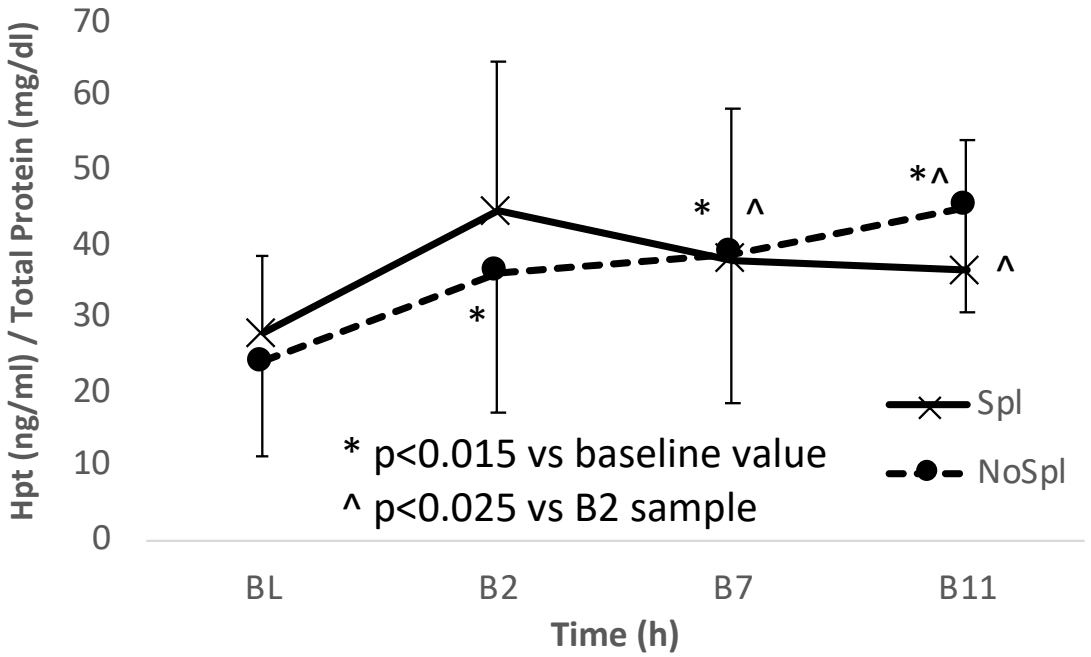
** Amount theoretically released in swine study based on increase in ALT in Spl group.

*** Bilirubin should only increase from in vivo hemolysis, not in vitro hemolysis.

Measuring Intravascular Hemolysis

- *In vitro* hemolysis is easily spotted by the appearance of cell-free hemoglobin (cfHb) in plasma.
- *Intravascular* hemolysis cannot easily be detected because cfHb binds immediately to haptoglobin (Hpt) and the complex is removed from the circulation in minutes. Plasma cfHb will only increase after Hpt has been depleted.
- <u>The gold standard for measuring intravascular hemolysis is to measure Hpt</u>.
- Hpt is an acute phase protein (i.e. production is increased in response to inflammation or stress).
- Haptoglobin is therefore typically only used to detect <u>chronic</u> intravascular hemolysis.
- It was shown to detect intravascular hemolysis during cardiopulmonary bypass, so in theory it may detect acute hemolysis in shock. Warkentin et al. *Clin Chem* 33(7):1265-1266, 1987

Haptoglobin Supports Hemolysis



- Hpt increased sharply in response to hemorrhage and resuscitation in both groups by B2 and continued to rise in the NoSpl group.
- It is likely that production also continued to increase in the Spl group, yet Hpt levels dropped significantly after the B2 sample, supporting the idea that hemolysis was occurring in the Spl group.
- However, there were no significant differences between groups at any timepoint and Hpt did not drop below baseline in the Spl group suggesting Hpt will not be useful as a clinical indicator of hemolysis in shock.

Discussion and Future Efforts

- Our data suggests the majority of the increase in markers of liver and kidney damage is not due to actual liver or kidney damage, but to release of the same markers by hemolysis.
- This agrees with histology data from the Spl group which placed the liver necrosis as minimal and kidney tubular necrosis between mild and moderate.
- We never observed an increase in plasma cfHb (Hpt never depleted).
- We would have liked to show correlation between a drop in Hpt and increase of markers like ALT, but this has been complicated by the acute phase response.
- Our next step will be to measure some of the markers of in vitro hemolysis identified by Lippi et al. In particular, we are resurrecting an old assay that measures the activity of the LDH-1 isoform which is found predominantly in RBCs and cardiac tissue. We will determine if increases in LDH-1 activity correlate to the increase in other markers.

Conclusions

- Hemolysis appears to be occurring early in hemorrhagic shock and is amplified by the presence of the spleen (presumably due to the influx of older RBCs).
- Since hemolysis has been proposed to cause ARDS, this finding may have a major impact on our understanding of the pathogenesis of shock and may lead to new treatments.
- Markers of liver and kidney damage may also detect hemolysis.
- Therefore much of the liver and kidney damage reported in shock based on these markers (both in animal studies and clinically) may be false.
- This finding may apply to other disorders beyond shock.
- If the goal of a study is to determine the effect of a treatment on organ injury after HS, splenectomy may be necessary to reduce the confounding effect of hemolysis.

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