**SUPPLEMENTAL METHODS**

*Acute Hemorrhagic Shock Model (Short-term Follow-up)*

Rats were anesthetized with sodium thiopentone (120 mg/kg i.p. initially and 10 mg/kg i.v. for maintenance as needed). Cannulation with polyethylene catheters (Smiths Medical International Ltd., Kent, UK) of the trachea for facilitation of spontaneous breathing (internal diameter [ID] 1.67 mm), left femoral artery for recording of the mean arterial pressure (MAP) (ID 0.40 mm), left carotid artery for blood withdrawal (ID 0.58 mm) and right jugular vein for fluid and drug administration (ID 0.40 mm) was performed. To prevent tissue desiccation, swabs moistened with saline were placed over the surgery incision sites. Body temperature was monitored by a rectal probe thermometer and maintained at 37°C ± 0.3°C by means of a homoeothermic blanket system (Harvard Apparatus). Upon the completion of surgery, the MAP was allowed to stabilize for 15 min. Blood was then withdrawn (up to 1 mL/min into heparinized syringes containing 100 IU/mL heparin mixed with normal saline) through the cannula inserted in the carotid artery in order to achieve a fall in MAP to 35 ± 5 mmHg, which was recorded with a pressure transducer (attached to the femoral artery cannula, 844-31 Memscap, Durham, USA) and coupled to a PowerLab 8/30 data acquisition system (AD Instruments Pty Ltd., Castle Hill, Australia). Thereafter, MAP was maintained at 35 ± 5 mmHg for a period of 90 min either by further withdrawal of blood during the compensation phase or administration of the shed blood during the decompensation phase. At 90 min after initiation of hemorrhage (or when 25% of the shed blood had to be re-injected to sustain MAP at 35 ± 5 mmHg), resuscitation through the jugular vein was performed with the remaining shed blood (mixed with 100 IU/mL heparinized saline) over a period of 5 min plus a volume of Ringer’s lactate identical to the volume of shed blood. Treatment or vehicle was also administered intravenously. One hour after resuscitation, an infusion of Ringer’s lactate (1.5 mL/kg/h) was started as fluid replacement and it was maintained throughout the experiment for a total of 3 h. Under deep anesthesia, the heart was removed to terminate the experiment 4 h after resuscitation. Sham-operated rats were used as control and underwent identical surgical procedures, but without hemorrhage or resuscitation.

Rats were treated with either acalabrutinib (3 mg/kg), fenebrutinib (3 mg/kg) or its vehicle (5% DMSO + 95% Ringer’s lactate) intravenously as a bolus treatment immediately after resuscitation. This dose of acalabrutinib was based on the dose used in studies conducted by the Thiemermann group9. The dose of fenebrutinib was chosen to match that of acalabrutinib.

*Sample Collection - Acute Hemorrhagic Shock Model (Short-term Follow-up)*

Rats remained anesthetized with sodium thiopentone (120 mg/kg i.p.) before sacrifice. Up to 5 mL of blood was taken from the carotid artery via the inserted cannula into a non-heparinized 5 mL syringe and immediately decanted into 1.1 mL serum gel tubes (Sarstedt, Germany). The blood was centrifuged (10,000 g for 5 min) to obtain the serum, which was subsequently stored at -80 °C until analysis. Organs (heart, lungs, liver, spleen and kidneys) were excised of which one section was snap frozen in liquid nitrogen and stored at -80 °C, and another section was placed in 10 % formalin for 24-48 h; followed by transfer to 70 % ethanol until further analysis. All organ injury/dysfunction parameters (urea, creatinine, alanine aminotransferase [ALT], aspartate aminotransferase [AST], creatine kinase [CK], amylase and lactate dehydrogenase [LDH]) in the serum were measured in a blinded fashion by a clinical pathology diagnostic laboratory (MRC Harwell Institute, Oxfordshire, UK).

*Acute Hemorrhagic Shock Model (Long-term Follow-up)*

At 15 min prior to anesthesia, analgesia with tramadol (10 mg/kg i.p.) was administered. Rats were then anesthetized with ketamine-xylazine (ketamine, 100 mg/kg; xylazine, 10 mg/kg i.m. initially and 100 µL ketamine i.p. for maintenance as needed). Cannulation with polyethylene catheters of the left femoral artery and left femoral vein was performed. Body temperature was monitored by a digital ear thermometer and maintained at 36.5 °C ± 0.5 °C by means of a homoeothermic blanket system (Harvard Apparatus). Upon completion of surgery, the MAP was allowed to stabilize for 15 min. Blood was then withdrawn (up to 1 mL/min into heparinized syringes containing 100 IU/mL heparin mixed with normal saline) through the cannula inserted in the femoral artery in order to achieve a fall in MAP to 40 ± 2 mmHg, which was recorded with a pressure transducer (attached to the femoral artery cannula) and coupled to a PowerLab 8/30 (AD Instruments Pty Ltd., Castle Hill, Australia). Thereafter, MAP was maintained at 40 ± 2 mmHg for a period of 90 min either by further withdrawal of blood or administration of the shed blood. At 90 min after initiation of hemorrhage (or when 25 % of the shed blood had to be re-injected to sustain MAP at 40 ± 2 mmHg), resuscitation through the femoral vein was performed with the remaining shed blood over a period of 5 min plus 1.5 mL/kg Ringer’s lactate. An initial bolus of acalabrutinib treatment (1.5 mg/kg) or vehicle was then administered intraperitoneally. Sham-operated rats were used as control and underwent identical surgical procedures, but without hemorrhage or resuscitation. At 20 min after resuscitation was completed, the catheters were removed, the femoral vessels were ligated, and the incision was closed with sutures. Rats were allowed to recover from the anesthesia and given tramadol (5 mg/kg i.p.) 12 h later in addition to a second dose of acalabrutinib (1.5 mg/kg) or vehicle (i.p.). At 24 h post-resuscitation, rats were anesthetized with ketamine-xylazine (100 mg/kg ketamine, 10 mg/kg xylazine i.m.) and samples were collected. Cannulation of the left carotid artery with a polyethylene catheter was performed to measure the mean arterial pressure (MAP) and heart rate (HR); after which up to 5 mL blood was taken into non-heparinized blood collection tubes. The blood was centrifuged (10,000 g for 5 min) to obtain the serum and stored at -80 C until analysis. Organ collection and measurement of organ injury/dysfunction parameters (Hospital Universitário Professor Polydoro Ernani de São Thiago, Brazil) was performed as described in the short-term follow-up acute model.

*Western Blot Analysis*

Semi-quantitative immunoblot analysis was carried out in kidney tissue samples as previously described21. Briefly, kidney samples from the short-term follow-up acute HS model were homogenized in buffer and centrifuged (1320 g, 5 mins, 4 °C). To obtain the cytosolic fraction, supernatants were centrifuged (16,125 g, 4 °C, 40 mins). The pelleted nucleoli were resuspended in extraction buffer and centrifuged (16,125 g, 20 mins, 4 °C). Protein content was determined on both nuclear and cytosolic extracts using bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific Inc, Rockford, IL). Proteins were separated by 8% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) membrane. After blocking (1 hr in 10% dry milk solution), membranes were incubated with primary antibodies in 5% blocking solution overnight [1:1000 rabbit anti-total BTK, 1:1000 rabbit anti-NF-κB, 1:1000 rabbit anti-IKKβ, 1:1000 rabbit anti-Ser176/180 IKKα/β, 1:1000 mouse anti-Ser32/36 IκBα, 1:1000 mouse anti-total IκBα (from Cell Signaling), 1:1000 rabbit anti-Tyr223-BTK, 1:1000 rabbit anti-NLRP3 inflammasome (from Abcam), 1:1000 mouse anti-caspase 1 (p20) (from Adipogen)] followed by incubation with appropriate HRP-conjugated secondary antibodies. Proteins were detected with an ECL detection system and quantified by densitometry using analytic software (Quantity-One; Bio-Rad, Hercules, CA). Results were normalized with respect to densitometric values of tubulin for cytosolic proteins or histone H3 for nuclear proteins.

*Quantification of myeloperoxidase activity*

Lung and liver tissue samples from the long-term follow-up acute HS model were homogenized in liquid nitrogen with a pestle and mortar. The homogenate was then centrifuged at 13,000 x g at 4 °C for 10 min and the supernatant was assayed for myeloperoxidase (MPO) activity by measuring the H2O2-dependent oxidation of 3,3,5,5-tetramethylbenzidine (TMB). MPO activity was determined colorimetrically using an ultra-microplate reader (EL 808, BioTek Instruments, INC, USA) set to measure absorbance at 650 nm. Total protein content in the homogenate was estimated using the BCA assay (Thermo Fisher Scientific, Rockford, IL), according to the manufacturer's instructions. MPO activity was expressed as optical density at 650 nm per mg of protein.

**SUPPLEMENTAL INFORMATION**

*Acalabrutinib*

The half-life of acalabrutinib in rats is 2.74 h1. The half-life of acalabrutinib in humans is ~1-2 h whilst the half-life of the active metabolite (ACP-5862) is ~7 h1.

With regards to the cardiovascular effects of acalabrutinib, as part of a single dose telemetry pilot study in beagle dogs, drug administration (doses: 10 mg/kg and 30 mg/kg) did not result in changes in MAP and HR up to 24 h post-administration in radiotelemetry-implanted male beagle dogs1. In a follow-up study, single doses of acalabrutinib (3 mg/kg, 10 mg/kg and 30 mg/kg) to dogs again did not significantly affect the cardiovascular system1. Furthermore, four studies which included patients with chronic lymphocytic leukemia treated with acalabrutinib clearly demonstrated that acalabrutinib does not increase the risk of cardiovascular abnormalities2 In contrast, treatment of patients with B-cell malignancies with ibrutinib resulted in hypertension in 23.2% of patients compared to only 9.4% of patients treated with acalabrutinib3.

With regards to the gender differences following acalabrutinib treatment (humans), population covariate analysis investigating the effect of gender on the pharmacokinetic parameters of acalabrutinib and its active metabolite (ACP-5862) have not reported a significant effect1.

*Fenebrutinib*

The half-life of fenebrutinib in rats is 2.2 h4. The half-life of fenebrutinib in humans is 6.1-11 h5.

With regards to the cardiovascular effects of fenebrutinib, at therapeutic exposures drug administration is considered to have a low potential to directly affect other cardiovascular parameters6.

To the best of our knowledge, no information relating to gender differences in the response to fenebrutinib has been published.

**SUPPLEMENTAL FIGURES**

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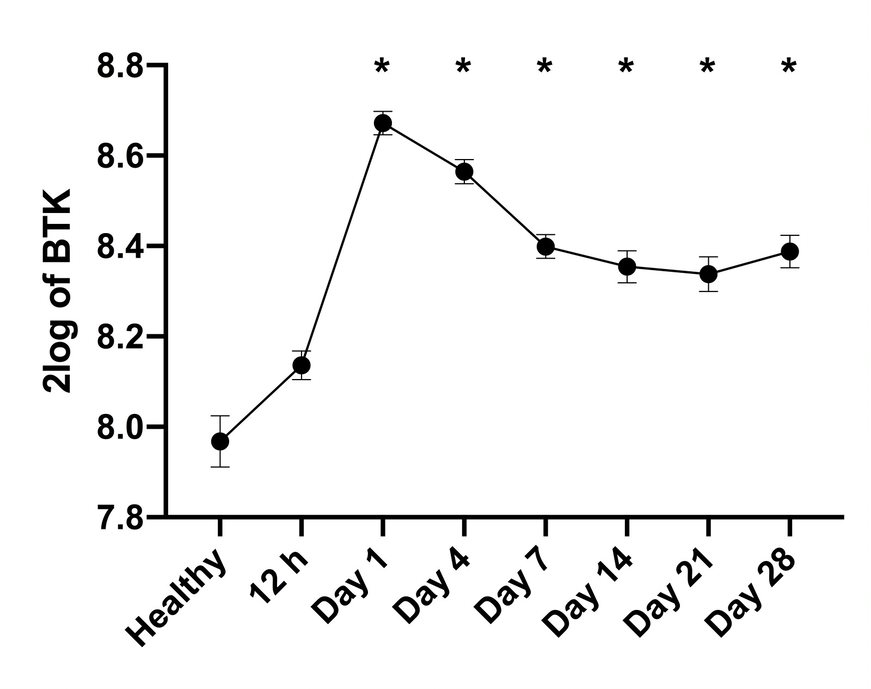


**Supplemental Figure 1: Schematic representations of the acute HS models.** The experimental procedures at each stage of the (**A**) short-term follow-up and (**B**) long-term follow-up acute HS models are shown.

*BTK gene expression is elevated in trauma patients*

It should be noted that repeated sampling between 12 h and 24 h was not conducted to determine the exact point at which BTK gene expression increased in leukocytes or whether the true peak occurs between Day 1 and 4.

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**Supplemental Figure 2: BTK gene expression is elevated in trauma patients.** Original data were obtained from the Gene Expression Omnibus under dataset accession number GSE36809 which was published by Xiao and colleagues7. RNA was extracted from whole blood leukocytes over a 28-day time course from trauma patients (n = 167) and matched healthy controls (n = 37). Data were reanalyzed for BTK gene expression. Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni's *post-hoc* test. \*p < 0.05 denoted statistical significance.

*BTK gene expression does not differ between uncomplicated and complicated recovery patient groups*

Please note that peak BTK expression occurred on Day 1 in both groups and the increase in BTK expression observed in both groups was not different.

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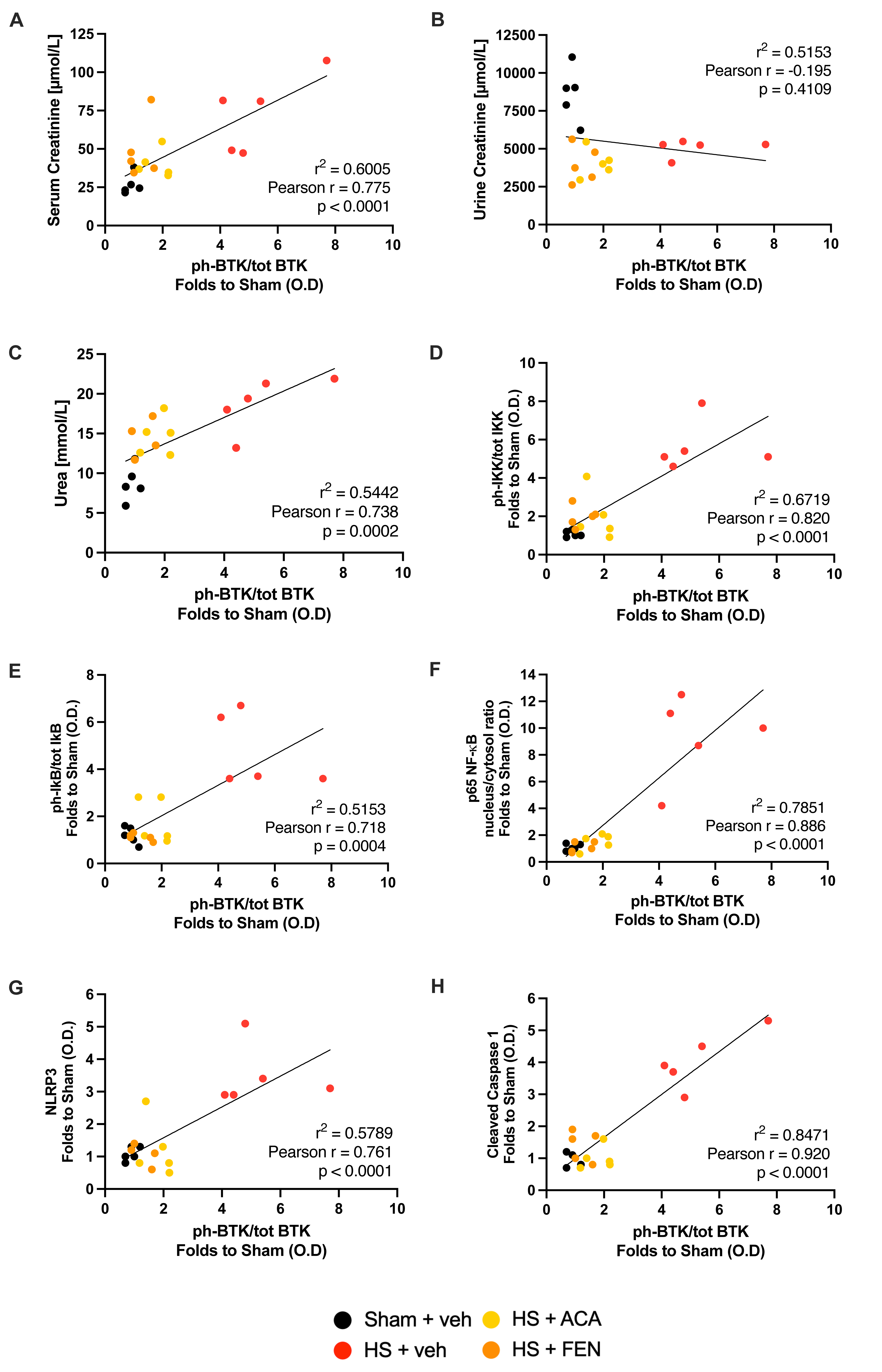


**Supplemental Figure 3: BTK gene expression does not differ between uncomplicated and complicated recovery patient groups.** Original data were obtained from the Gene Expression Omnibus under dataset accession number GSE36809 which was published by Xiao and colleagues7. Data were reanalyzed for BTK gene expression in uncomplicated (n = 55) and complicated (n = 41) recovery patient groups. Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by a Bonferroni's *post-hoc* test. \*p<0.05 denoted statistical significance.

*BTK activation correlates with renal dysfunction, NF-κB and NLRP3 activation in a short-term follow-up acute HS model*

Firstly, to address the question whether the degree of activation of BTK correlates with changes in renal function, we correlated the degree of phosphorylation of BTK at Tyr223 with serum creatinine (Supplemental Figure 4A), urine creatinine (Supplemental Figure 4B) and serum urea (Supplemental Figure 4C). We found a highly significant positive correlation between the degree of BTK activation and the increases in serum creatinine (Supplemental Figure 4A) and urea (Supplemental Figure 4C), suggesting that BTK activation drives or precedes the renal dysfunction associated with HS. No significant correlation was observed between BTK activation and the decrease in urine creatinine (Supplemental Figure 4B). Secondly, the potential relationship between the degree of BTK activation and alterations in the activation of NF-κB was also addressed by correlating the degree of phosphorylation of BTK with the phosphorylation of IKKα/β at Ser176/180 (Supplemental Figure 4D), the phosphorylation of IκBα at Ser32/36 (Supplemental Figure 4E) and the translocation of p65 (Supplemental Figure 4F). We found a highly significant positive correlation between the degree of BTK activation and NF-κB activation when measured as IKKα/β phosphorylation (Supplemental Figure 4D), IκBα phosphorylation (Supplemental Figure 4E) and p65 translocation (Supplemental Figure 4F). Thirdly, whether the degree of BTK activation correlates with changes in the assembly and activation of the NLRP3 inflammasome was investigated by correlating the degree of phosphorylation of BTK with the expression of NLRP3 (Supplemental Figure 4G) and cleaved caspase 1 (Supplemental Figure 4H). We found a highly significant positive correlation between the degree of BTK activation and the NLRP3 inflammasome expression (Supplemental Figure 4G) and activation of caspase 1 (Supplemental Figure 4H).

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**Supplemental Figure 4: BTK activation correlates with renal dysfunction, NF-κB and NLRP3 activation in a short-term follow-up acute HS model.** Linear regression analysis of (**A**) phosphorylation of BTK at Tyr223 vs. serum creatinine, (**B**) phosphorylation of BTK at Tyr223 vs. urine creatinine, (**C**) phosphorylation of BTK at Tyr223 vs. serum urea, (**D**) phosphorylation of BTK at Tyr223 vs. phosphorylation of IKKα/β at Ser176/180, (**E**) phosphorylation of BTK at Tyr223 vs. phosphorylation of IκBα at Ser32/36, (**F**) phosphorylation of BTK at Tyr223 vs. translocation of p65, (**G**) phosphorylation of BTK at Tyr223 vs. expression of NLRP3 and (**H**) phosphorylation of BTK at Tyr223 vs. expression of the cleaved form of caspase 1. Data are expressed as raw individual values of five animals per group. Statistical analysis was performed using simple linear regression to calculate the r2 value, Pearson correlation coefficient test to calculate the r value and a two-tailed t-test to calculate the p-value. \*p<0.05 denoted statistical significance.

**SUPPLEMENTAL DISCUSSION**

**NEUTROPHIL RECRUITMENT**

Following ICAM-1 binding on endothelial cells and subsequent tissue recruitment, neutrophils undergo degranulation and release pro-inflammatory mediators8,9. These mediators (e.g. cytokines, reactive oxygen species and MPO) can contribute to inflammation by exerting direct cytotoxic cellular effects at the local site; subsequently leading to organ dysfunction and potential mortality following HS and resuscitation10,11. As we have demonstrated in the short-term follow-up model that treatment with BTKi attenuates both NF-κB and NLRP3 activation, it can be implied that the decreased recruitment of neutrophils and lowered inflammation is secondary to this reduced activation of NF-κB and NLRP3.

**LIMITATIONS OF THE STUDY**

Although acalabrutinib and fenebrutinib displayed some striking, beneficial effects in the HS models, there are study limitations which should be considered. In the HS models, only male rats were used to prevent any sex-dependent confounding effects (fluctuations in female reproductive hormones and X chromosome) and to represent the population typically most affected by trauma (young males). It should be noted that the majority of trauma patients in our recent clinical trial TOP-ART were male and under 30 years, so the use of male rats is clinically relevant12. Further long-term survival experiments are needed to verify that the observed early reduction in MODS does, indeed, translate to improved outcome and ultimately reduced mortality. In our study, organ injury and dysfunction were used as surrogate markers for mortality (as the determination of mortality is not allowed by our respective ethics and/or Home Office licenses). Therefore, care must be taken when interpreting our pre-clinical results and extrapolating them to the clinical scenario. Additionally, future studies in larger animals and/or higher species may be useful to confirm efficacy and to further investigate the mechanism of action (e.g. blood gas analysis and microcirculatory effects) of BTKi in HS. As BTK is primarily expressed in cells of hematopoietic lineage (except T-lymphocytes, plasma cells and natural killer cells), it is likely that the increase in BTK activity measured in the kidneys was secondary to the infiltration of these organs by invading immune cells rather than an increase in parenchymal renal tissue (where no expression of BTK has been reported). Therefore, it is possible that BTK inhibition leads to decreased leukocyte recruitment into the kidney (as a result of reduced NF-κB and NLRP3 activation) and subsequently to lower levels of BTK activation in the kidney. Furthermore, studies with large cohorts of trauma patients are needed to robustly examine the relationship between BTK activity and clinical outcomes in humans.

**REFERENCES**

1. CHMP. Committee for Medicinal Products for Human Use (CHMP) Assessment report. Published online 2020. Accessed November 15, 2021. www.ema.europa.eu/contact

2. Brown J. Pooled Analysis of Cardiovascular Events from Clinical Trials Evaluating Acalabrutinib Monotherapy in Patients with Chronic Lymphocytic Leukemia (CLL). Published online December 7, 2020.

3. Byrd JC, Hillmen P, Ghia P, et al. Acalabrutinib Versus Ibrutinib in Previously Treated Chronic Lymphocytic Leukemia: Results of the First Randomized Phase III Trial. *Journal of Clinical Oncology*. 2021;39(31):3441-3452. doi:10.1200/jco.21.01210

4. Crawford JJ, Johnson AR, Misner DL, et al. Discovery of GDC-0853: A Potent, Selective, and Noncovalent Bruton’s Tyrosine Kinase Inhibitor in Early Clinical Development. *Journal of Medicinal Chemistry*. 2018;61(6):2227-2245. doi:10.1021/ACS.JMEDCHEM.7B01712/SUPPL\_FILE/JM7B01712\_SI\_001.CSV

5. Herman AE, Chinn LW, Kotwal SG, et al. Safety, Pharmacokinetics, and Pharmacodynamics in Healthy Volunteers Treated With GDC-0853, a Selective Reversible Bruton’s Tyrosine Kinase Inhibitor. *Clinical Pharmacology & Therapeutics*. 2018;103(6):1020-1028. doi:10.1002/CPT.1056

6. Official Title: A Phase II, Multicenter, Randomized, Double-Blind, Placebo-Controlled Pilot and Dose-Ranging Study of GDC-0853 in Patients With Refractory Chronic Spontaneous Urticaria (CSU).

7. Xiao W, Mindrinos MN, Seok J, et al. A genomic storm in critically injured humans. *The Journal of Experimental Medicine*. 2011;208(13):2581. doi:10.1084/JEM.20111354

8. Sauaia A, Moore FA, Moore EE. Postinjury Inflammation and Organ Dysfunction. *Critical Care Clinics*. 2017;33(1):167-191. doi:10.1016/j.ccc.2016.08.006

9. Olanders K, Sun Z, Börjesson A, et al. The effect of intestinal ischemia and reperfusion injury on ICAM-1 expression, endothelial barrier function, neutrophil tissue influx, and protease inhibitor levels in rats. *Shock*. 2002;18(1):86-92. doi:10.1097/00024382-200207000-00016

10. Dewar D, Moore FA, Moore EE, Balogh Z. Postinjury multiple organ failure. *Injury*. 2009;40(9):912-918. doi:10.1016/j.injury.2009.05.024

11. Zhang Y, Zhang J, Korff S, Ayoob F, Vodovotz Y, Billiar TR. Delayed neutralization of interleukin 6 reduces organ injury, selectively suppresses inflammatory mediator, and partially normalizes immune dysfunction following trauma and hemorrhagic shock. *Shock (Augusta, Ga)*. 2014;42(3):218-227. doi:10.1097/SHK.0000000000000211

12. ISRCTN - ISRCTN15731357: The TOP-ART Study: Trauma Organ Protection - Artesunate. Accessed November 15, 2021. https://www.isrctn.com/ISRCTN15731357