Cell Reports, Volume *27*

Supplemental Information

Uncovering the Role of N-Acetyl-Aspartyl-Glutamate as a Glutamate Reservoir in Cancer

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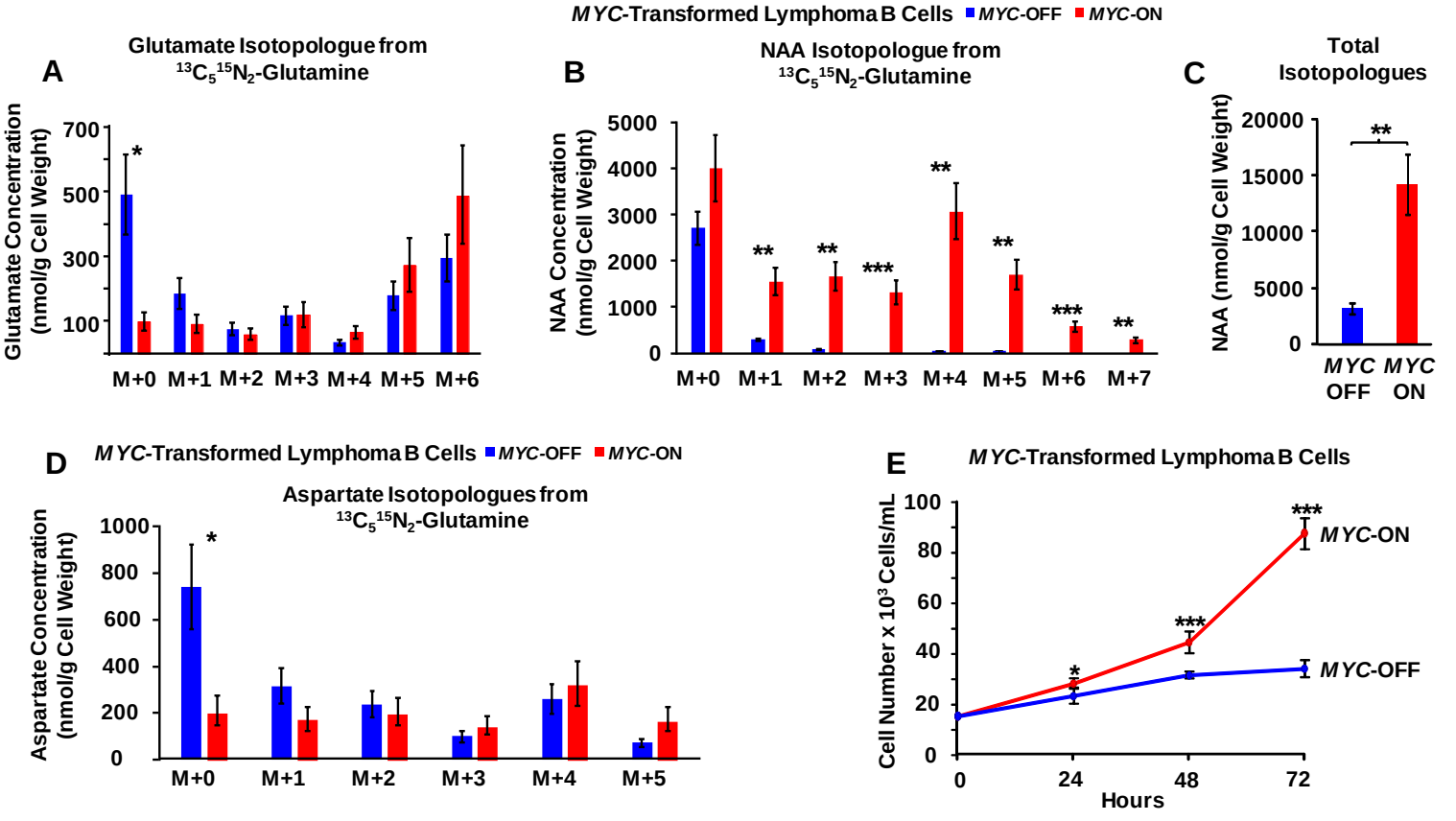

Figure S1. Related to **Figure 1 A-C**. **Incorporation of ^{13}C and ^{15}N in Glutamate (A), NAA (B) and Aspartate (D) from $^{13}C_5{}^{15}N_2$-Glutamine in *MYC*-Transformed Human Lymphoma B cells (*MYC*-ON and *MYC*-OFF P493 cells).** The *MYC*-ON and *MYC*-OFF P493 cells were grown at 37ºC in a 5% CO_2 and 95%(vol/vol) air incubator in RPMI containing 10% FBS and 2 mM $^{13}C_5{}^{15}N_2$-glutamine instead of $^{12}C_5{}^{14}N_2$-glutamine**.** After 24 hours, the cells were harvested and subjected to metabolic extraction for metabolomics analysis. Glutamate, NAA and aspartate intensities were converted into concentrations as described in the method section. The isotopologue concentrations for *MYC*-ON are shown as red bars, and *MYC*-OFF as blue bars, n = 4 per group. **(C) The Total NAA Concentration for all Isotopologues (NAA-Labeled from Glutamine-Labeled).** The experiment was repeated three times with similar results, n = 4 per group. **(E)** Related to **Figure 1A-B**. **Cell Numbers of *MYC*-ON and *MYC*-OFF P493 cells over 3 Days.** Cell numbers were evaluated at three different time points: 24, 48, and 72 hours. The numbers of cells are shown as mean ± SEM (n = 5 per group per time point). *p < 0.05, **p < 0.01, ***p < 0.001 (Student's *t*-test) where indicated.

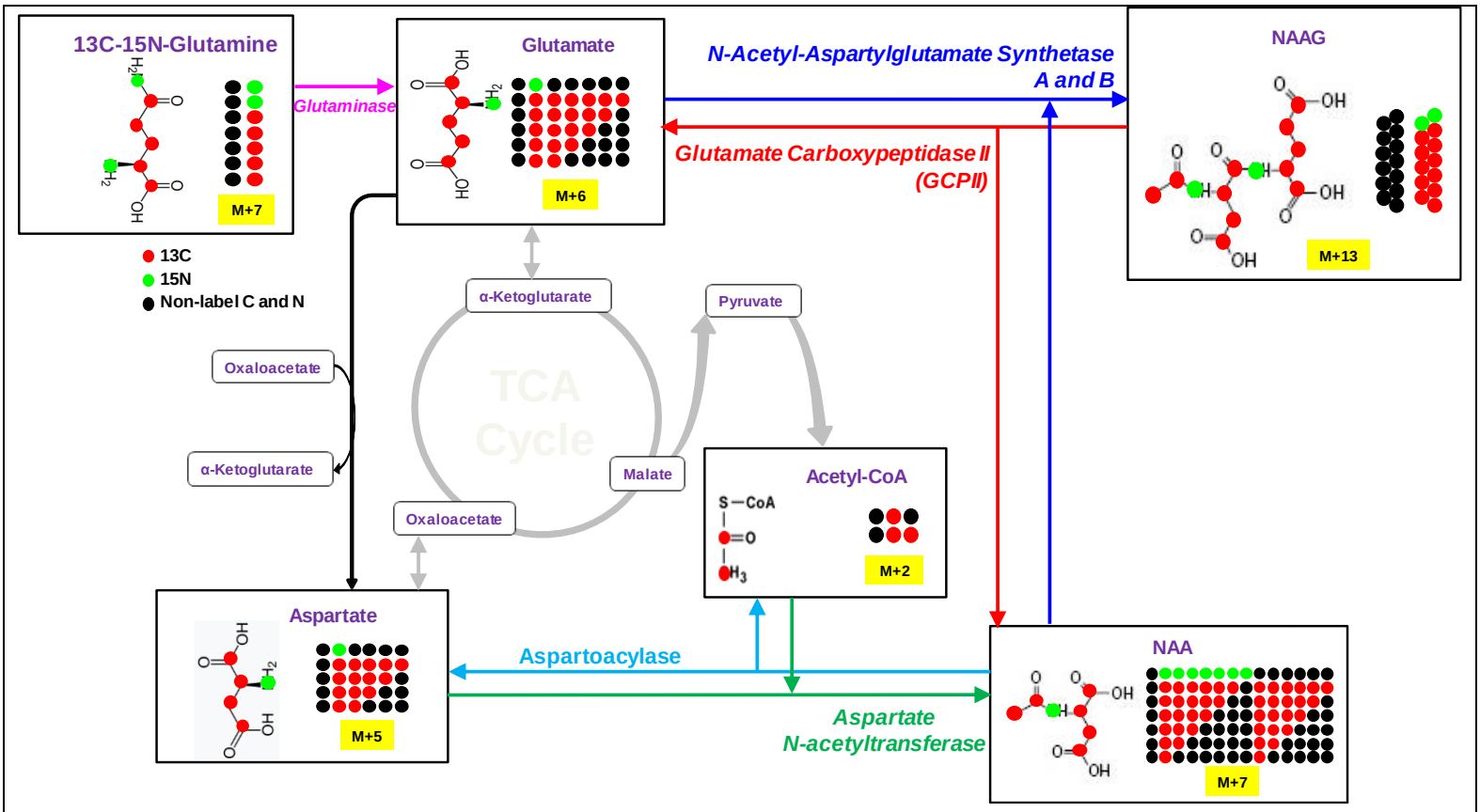

Figure S2. Related to **Figure 1A-C**. Illustration of the production of isotopologues of NAAG, NAA, glutamate, and aspartate from labeled $^{13}C_5{}^{15}N_2$-glutamine. The illustration is presented as following: metabolites in purple, red dots for ^{13}C, green dots for ^{15}N and black dots for non-labeled ^{12}C and ^{14}N. Catalytic enzymes were color-coded the same as reaction arrows.

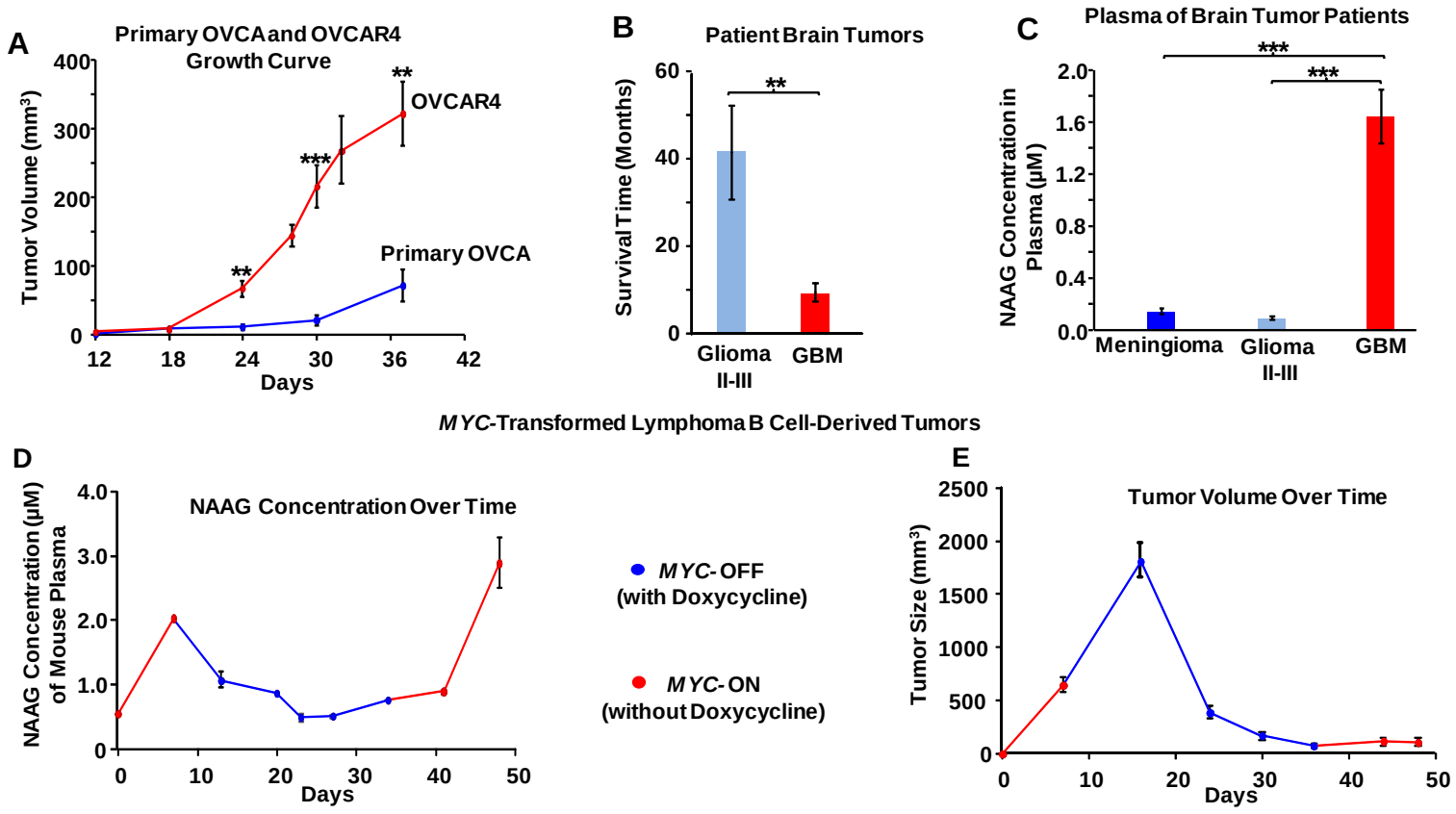

Figure S3. (A) Related to **Figure 1C**. **(A) Primary Ovarian Cancer (OVCA) and Human High-Grade Ovarian Serous Adenocarcinoma OVCAR4 Tumor Growth *in vivo*.** Mice bearing primary OVCA and OVCAR4 tumors were monitored and measured over time. Tumor volumes were calculated based on the tumor width and length and presented as primary OVCA group in blue and OVCAR4 group in red, n = 7 for primary OVCA, and n = 6 for OVCAR4. **(B-C)** Related to **Figure 1D**. **(B) The Survival Time of the Patients with Each Grade of Glioma. (C)** Plasmas of patients with meningioma (n = 51), grade II-III (n = 9) and IV (GBM) (n = 38) gliomas were subjected to metabolic extraction and metabolic assessment. The plasma NAAG concentrations of meningioma are shown as blue bars, grade II-III as light blue bars, and GBM as red bars. **(D-E)** Related to **Figure 1A-B. Relationship Between NAAG Concentration in Plasma of Mice Bearing *MYC*-Transformed Lymphoma B Tumors and Tumors Sizes.** NAAG concentrations in plasma **(D)** and tumor sizes **(E)** of mice bearing *MYC*-transformed lymphoma B tumors were assessed before, during and after doxycycline treatment *in vivo*. Red data points indicate data obtained before *MYC* suppression by doxycycline and after removal of doxycycline to reactivate *MYC*; blue data points during doxycycline treatment to suppress *MYC*. Data are shown as mean ± SEM.

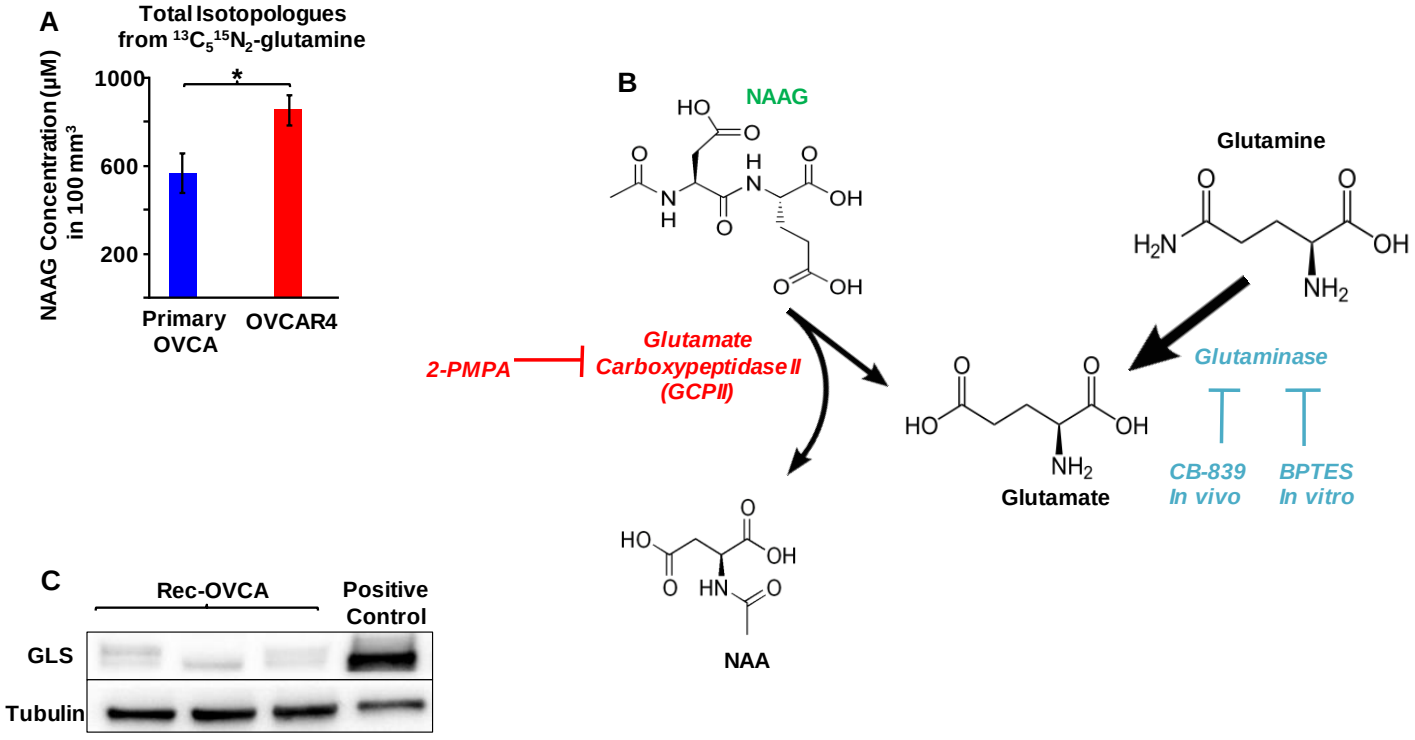

Figure S4. (A) Related to **Figure 1C. Total NAAG Concentration in µM for all Isotopologues (NAAG-Labeled from Glutamine-Labeled) in Primary OVCA and OVCAR4 Tumors.** Mice bearing OVCA and OVCAR4 tumors were injected with 100 µL of 100 mM sterile-filtered $^{13}C_5{}^{15}N_2$-glutamine in PBS at three time points, 15 minutes apart via intra-peritoneal administration. The tumors were then harvested at 1.5 hours after the last injection. The total concentration of NAAG-labeled from glutamine-labeled for OVCAR4 are shown as red bars, and primary OVCA as blue bars. Data are shown as mean ± SEM (n = 4 for the OVCA group and n = 5 for the OVCAR4 group). The experiment was repeated twice with similar results. *p < 0.05, **p < 0.01, ***p < 0.001 (Student's *t*-test) where indicated. **(B)** Related to **Figure 4D-E** and **Figure 5**. Illustration of two sources of glutamate from glutamine via glutaminase and from NAAG via GCPII. **(C)** Related to **Figure 4A**. Western Blot on protein extracted from pancreatic cancer P8 (positive control) and patient-derived rec-OVCA for expression level of glutaminase. Tubulin served as a loading control.

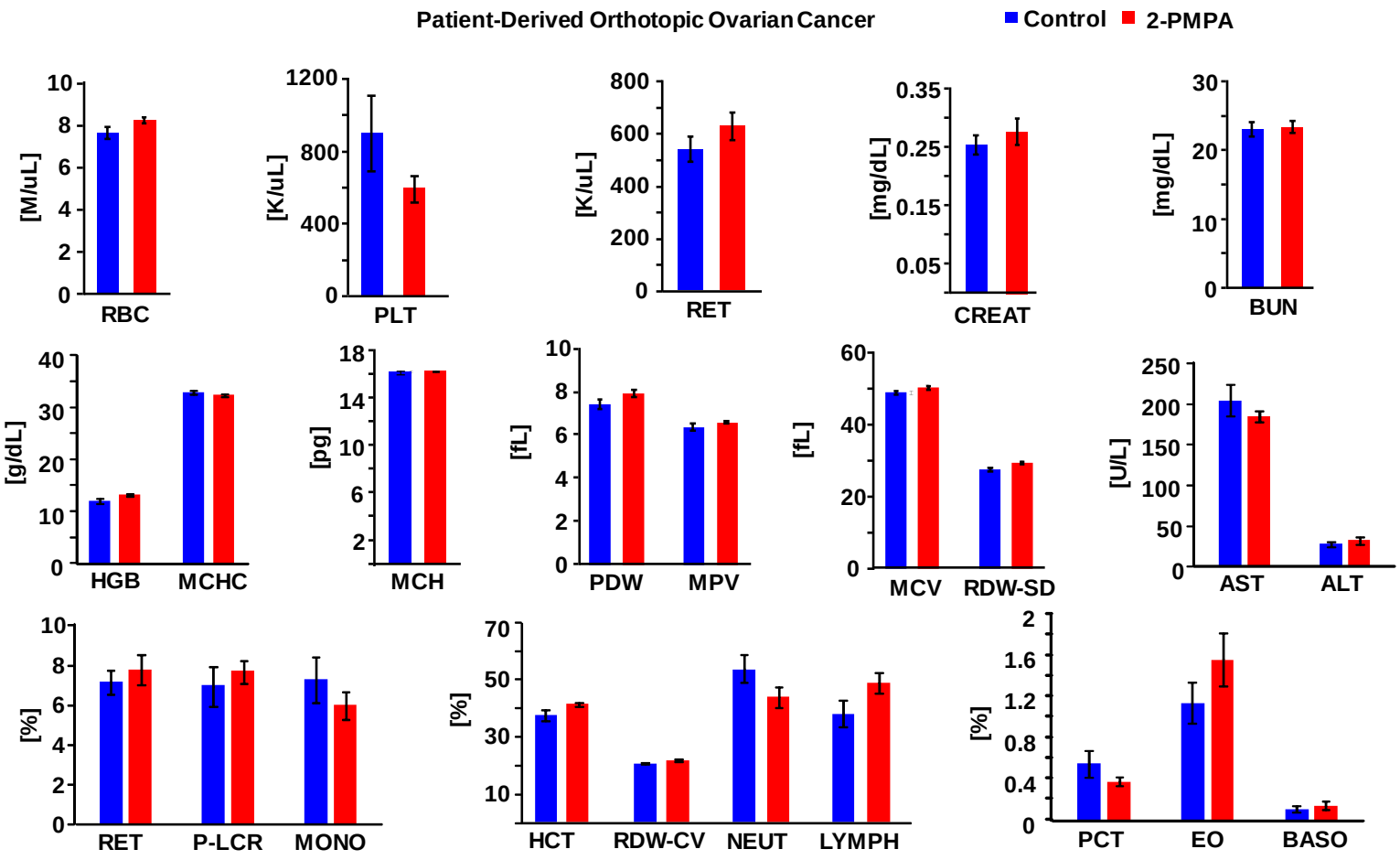

Figure S5. Related to **Figure 4A**. **Effect of GCPII Inhibitor, 2-PMPA Treatment (50 mg/kg 2-PMPA every day for by IP administration) on Blood Chemistries and Hematology of Mice Bearing Patient-Derived Orthotopic Recurrent Ovarian Cancer (Rec-OVCA) Orthotopic Tumors**. U/L, units per liter; ALT, alanine aminotransferase; AST, aspartate aminotransferase; K/µL, thousands per microliter; BASO, basophils; BUN, blood urea nitrogen; CREAT, creatinine; EO, eosinophils; HGB, hemoglobin; HCT, hematocrit; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; MPV, mean platelet volume ; NEUT, neutrophils; PCT, procalcitonin; PLT, platelets; fl, femtoliter; PDW, platelet distribution width; P-LCR, platelet large cell ratio; M/µL, millions per microliter; RBC, red blood cells; RET, reticulocyte count; RDW-CV, red cell distribution width-coefficient of variation; RDW-SD, red cell distribution width- standard deviation**.** The values are shown as mean ± SEM (n = 9 for each group).

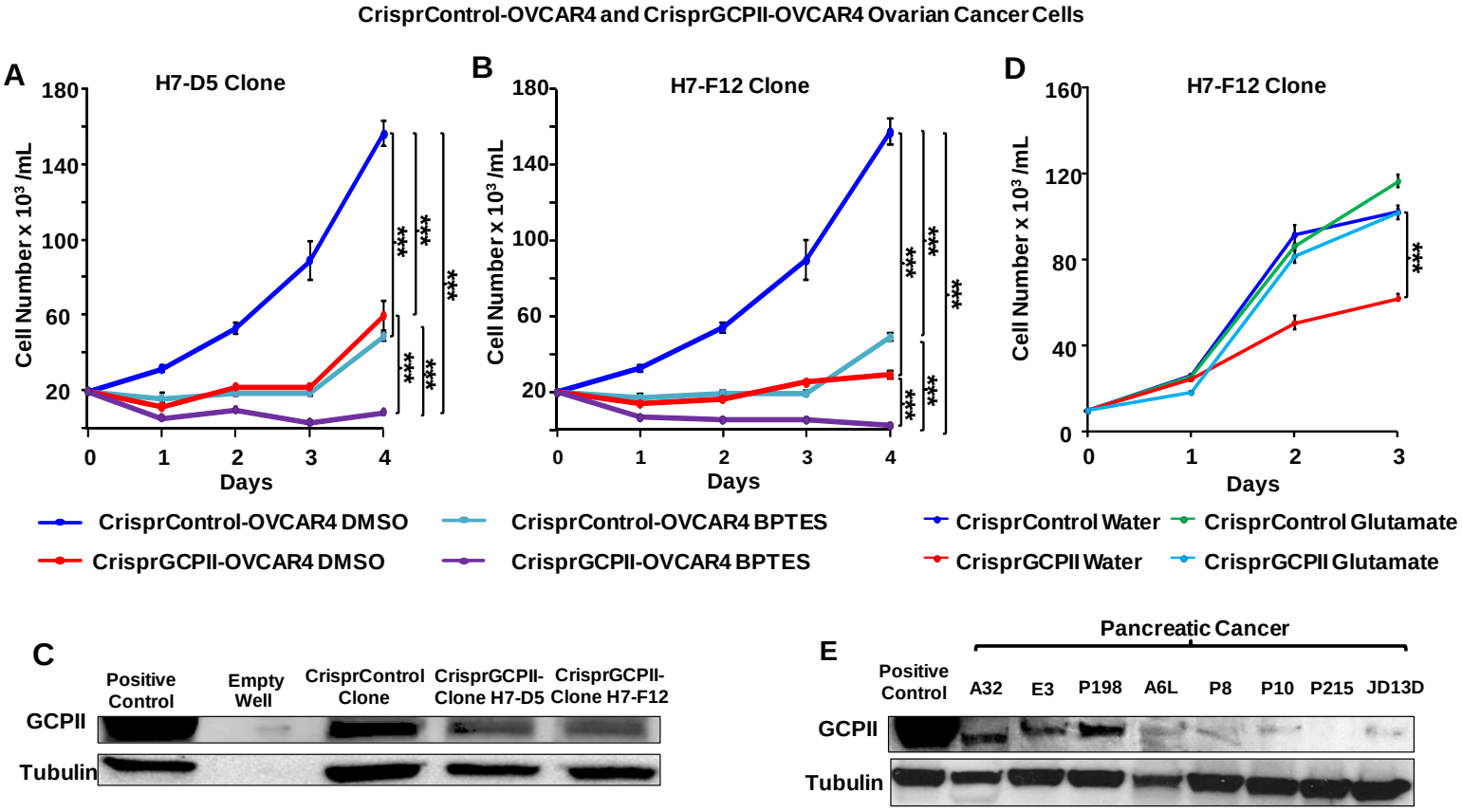

Figure S6. (A-C) Related to **Figure 4D-E. Effect of Glutaminase Inhibitor, BPTES, on Cell Numbers of CrisprControl and CrisprGCPII-OVCAR4 Cells *in vitro*. (A-B)** CrisprControl and CrisprGCPII-OVCAR4 cells were grown at 37ºC in a 5% CO_2 and 95% (vol/vol) air incubator in RPMI containing 10% FBS. After 24 hours, cells were treated with vehicle control (DMSO) or 10µM BPTES as shown. Cell numbers were assessed daily using a hemocytometer for a period of 4 days, n = 4 per group. **(C)** Western Blot on protein extracted from prostate cancer LNCAP cells (positive control) and CrisprGCPII-OVCAR4 clones for expression level of GCPII. Tubulin served as a loading control. (**D**) **Effect of Glutamate on CrisprControl and CrisprGCPII-OVCAR4 Cells *in vitro***. CrisprControl and CrisprGCPII-OVCAR4 cells were grown at 37ºC in a 5% CO_2 and 95% (vol/vol) air incubator in RPMI containing 10% FBS. After 24 hours, cells were supplemented with vehicle control (DMSO) or 4 mM glutamate. Cell numbers were assessed daily using a hemocytometer. The numbers of cells are shown as mean ± SEM (n = 4 per group). *p < 0.05, **p < 0.01, ***p < 0.001 (Student's *t*-test) where indicated. **(E)** Related to **Figure 5A-B**. Western Blot on protein extracted from prostate cancer LNCAP cells (positive control) and pancreatic cell lines: A32, E3, P198, A6L, P8, P10, P215, JD13D. Tubulin served as a loading control.

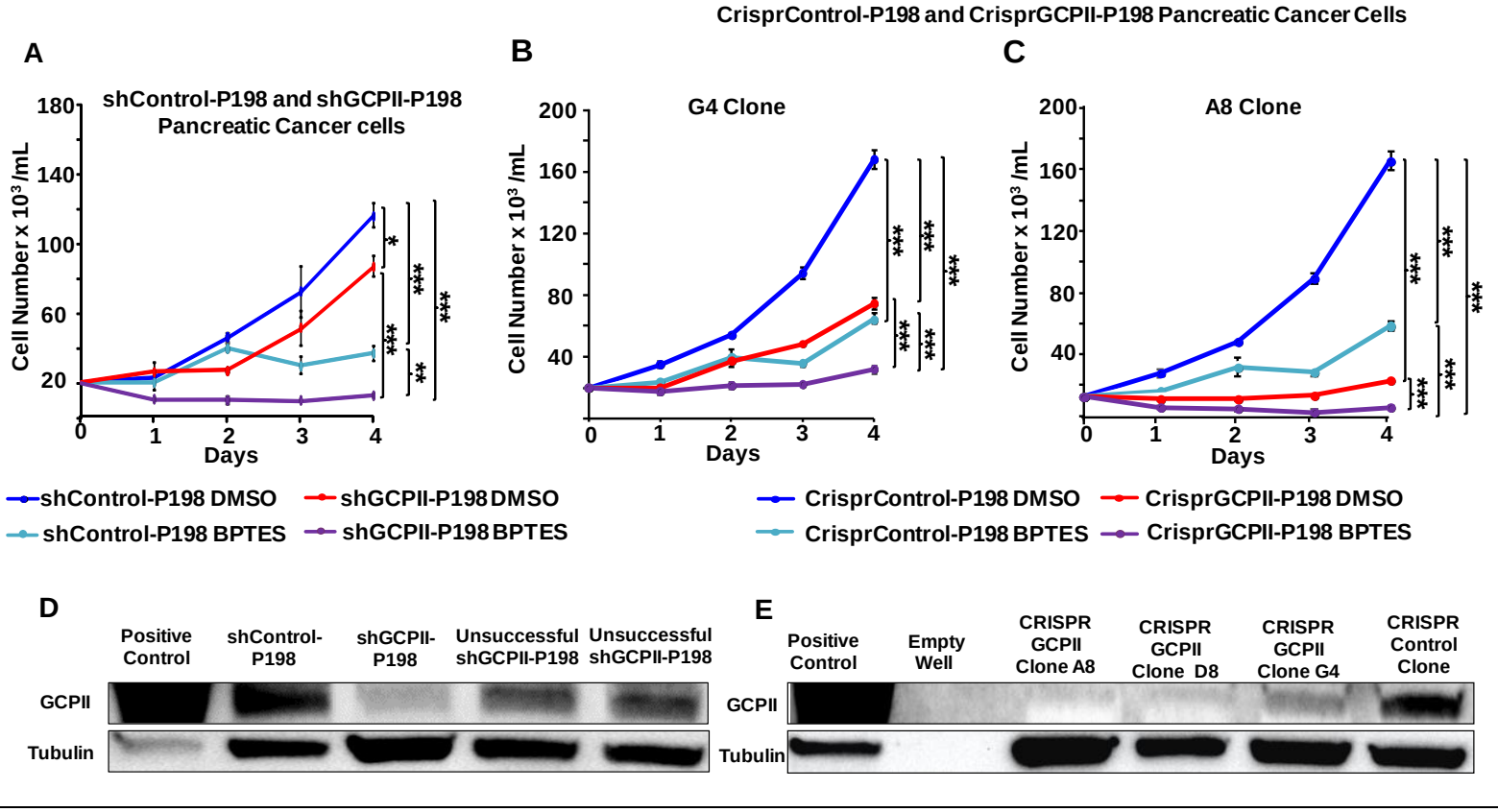

Figure S7. Related to **Figure 5A-B**. **Effect of Glutaminase Inhibitor, BPTES, on Cell Numbers of shControl-P198 and shGCPII-P198 Cells, CrisprControl-P198 and CrisprGCPII-P198 Cells *in vitro*. (A-C)** Cells were grown at 37ºC in a 5% CO_2 and 95% (vol/vol) air incubator in DMEM containing 10% FBS and 1 µg/mL puromycin (for shControl and shGCPII-P198 cells only). After 24 hours, cells were treated with vehicle control (DMSO) or 10µM BPTES as shown. Cell numbers were assessed daily using a hemocytometer for a period of 4 days. The numbers of cells are shown as mean ± SEM (n = 4 per group). ***p < 0.001 (Student's *t*-test) where indicated. **(D-E)** Western Blot on protein extracted from prostate cancer LNCAP cells (positive control), shControl-P198 and shGCPII-P198 cells, CrisprControl and CrisprGCPII-P198 cells for expression level of GCPII. Tubulin served as a loading control.

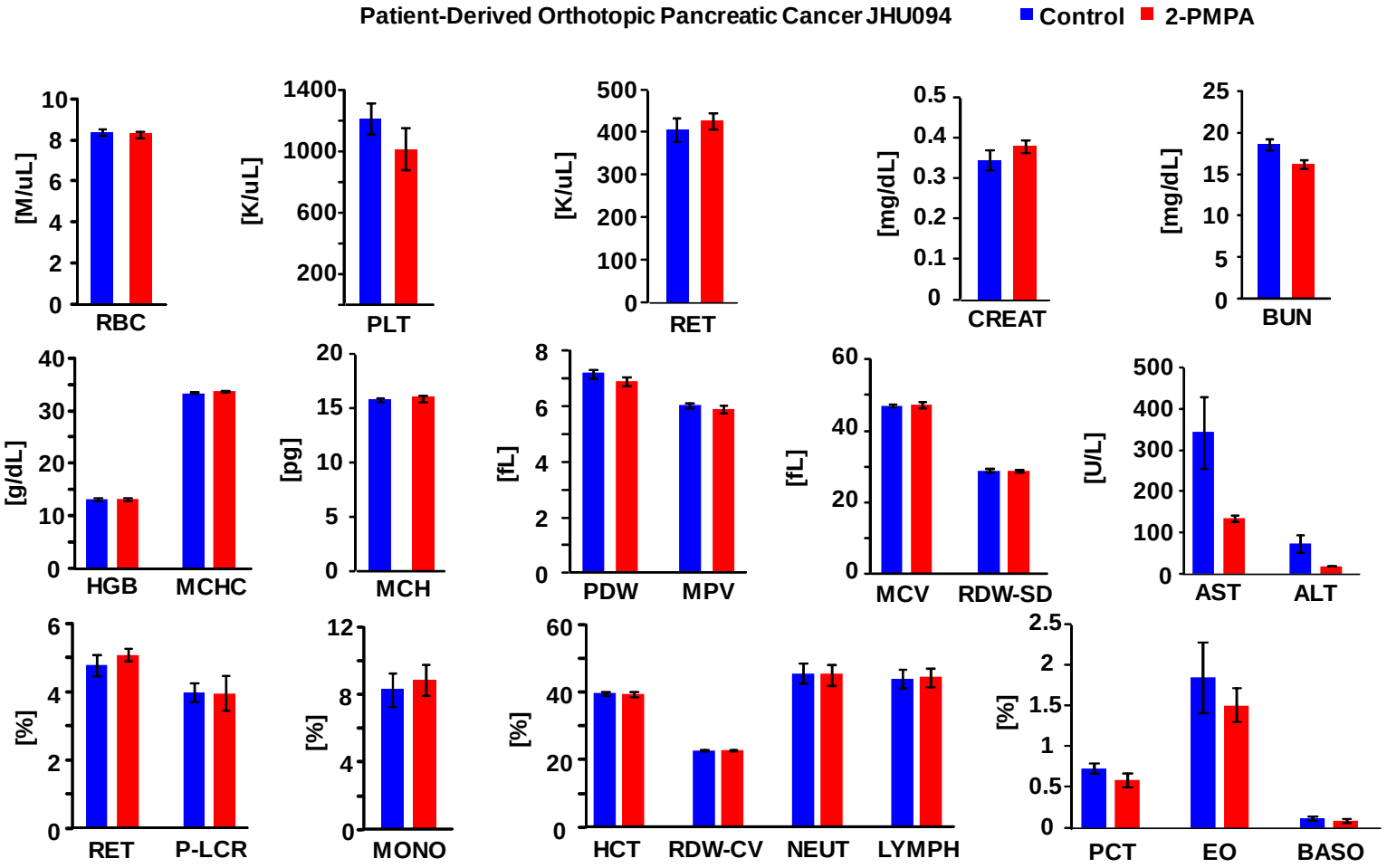

Figure S8. Related to **Figure 5D**. **Effect of GCPII Inhibitor, 2-PMPA Treatment (50 mg/kg 2-PMPA every day for by IP administration) on Blood Chemistries and Hematology of Mice Bearing Patient-Derived Pancreatic Cancer (JHU094) Orthotopic Tumors**. U/L, units per liter; ALT, alanine aminotransferase; AST, aspartate aminotransferase; K/µL, thousands per microliter; BASO, basophils; BUN, blood urea nitrogen; CREAT, creatinine; EO, eosinophils; HGB, hemoglobin; HCT, hematocrit; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; MPV, mean platelet volume ; NEUT, neutrophils; PCT, procalcitonin; PLT, platelets; fl, femtoliter; PDW, platelet distribution width; P-LCR, platelet large cell ratio; M/µL, millions per microliter; RBC, red blood cells; RET, reticulocyte count; RDW-CV, red cell distribution width-coefficient of variation; RDW-SD, red cell distribution width- standard deviation**.** ALT and AST were in normal ranges for both groups. The values are shown as mean ± SEM (n = 10 for the control group and n = 8 for 2-PMPA treated group).

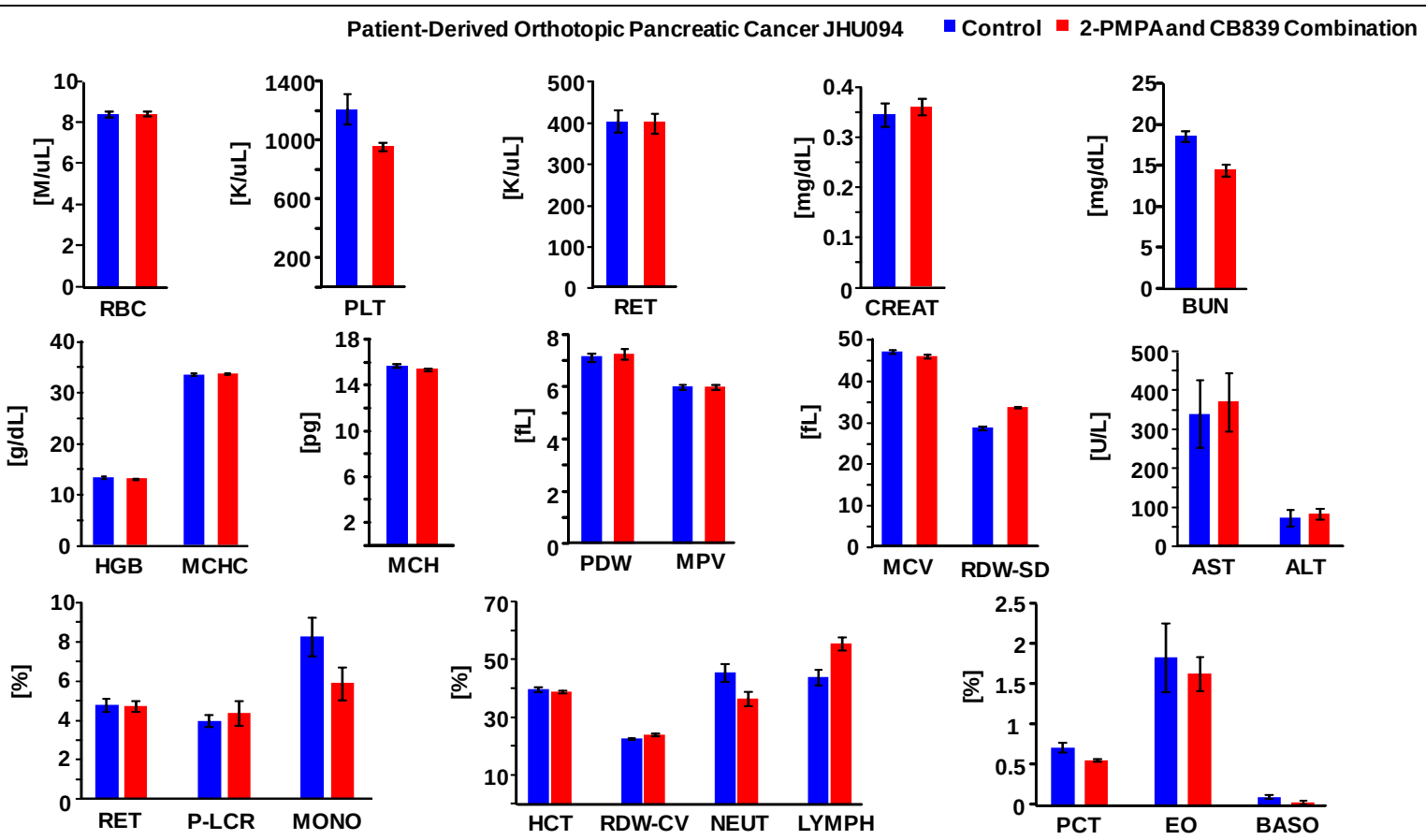

Figure S9. Related to **Figure 5D**. **Effect of GCPII Inhibitor, 2-PMPA Treatment (50 mg/kg 2-PMPA every day for by IP administration) and Glutaminase Inhibitor, CB-839 Treatment (200 mg/kg twice per day via oral gavage) on Blood Chemistries and Hematology of Mice Bearing in the Patient-Derived Pancreatic Cancer (JHU094) Orthotopic Tumors** U/L, units per liter; ALT, alanine aminotransferase; AST, aspartate aminotransferase; K/µL, thousands per microliter; BASO, basophils; BUN, blood urea nitrogen; CREAT, creatinine; EO, eosinophils; HGB, hemoglobin; HCT, hematocrit; LYMPH, lymphocytes; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MONO, monocytes; MPV, mean platelet volume; NEUT, neutrophils; PCT, procalcitonin; PLT, platelets; fl, femtoliter; PDW, platelet distribution width; P-LCR, platelet large cell ratio; M/µL, millions per microliter; RBC, red blood cells; RET, reticulocyte count; RDW-CV, red cell distribution width-coefficient of variation; RDW-SD, red cell distribution width-standard deviation**.** The values are shown as mean ± SEM (n = 10 for each group).