SUPPLEMENTAL MATERIALS

Long-term ET_A receptor antagonism provides robust renal protection in humanized sickle cell disease mice

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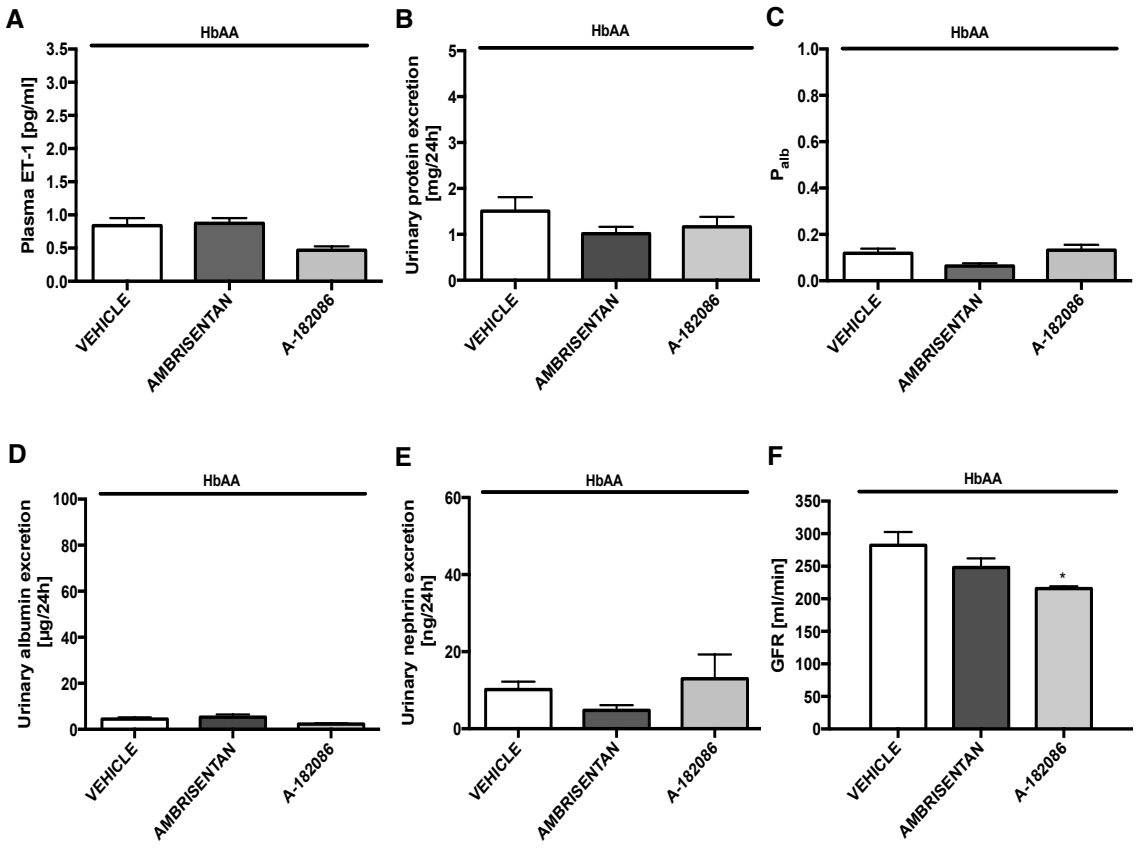

Figure 1. Plasma ET-1, proteinuria, glomerular permeability to albumin (P_{alb}), urinary excretion of markers of glomerular injury and glomerular filtration rate (GFR) in vehicle-treated or treated with 10-week treatment protocol (beginning at 4 weeks of age) with selective ET_A antagonist, ambrisentan or combined $ET_{A/B}$ antagonist, A-182086, HbAA mice. Panel A depicts the average ET-1 in plasma after 10-week treatment protocol. Panel B depicts the average proteinuria after 10-week treatment protocol. Panel C depicts the average P_{alb} after 10-week treatment protocol. Panel D depicts the average urinary albumin excretion after 10-week treatment protocol. Panel E depicts the average urinary nephrin excretion after 10-week treatment protocol. Panel F depicts the average GFR after 10-week treatment protocol. Data are mean ± S.E.M; n=6-9 in HbAA groups; $^{*}p<0.05$ versus vehicle-treated HbAA.

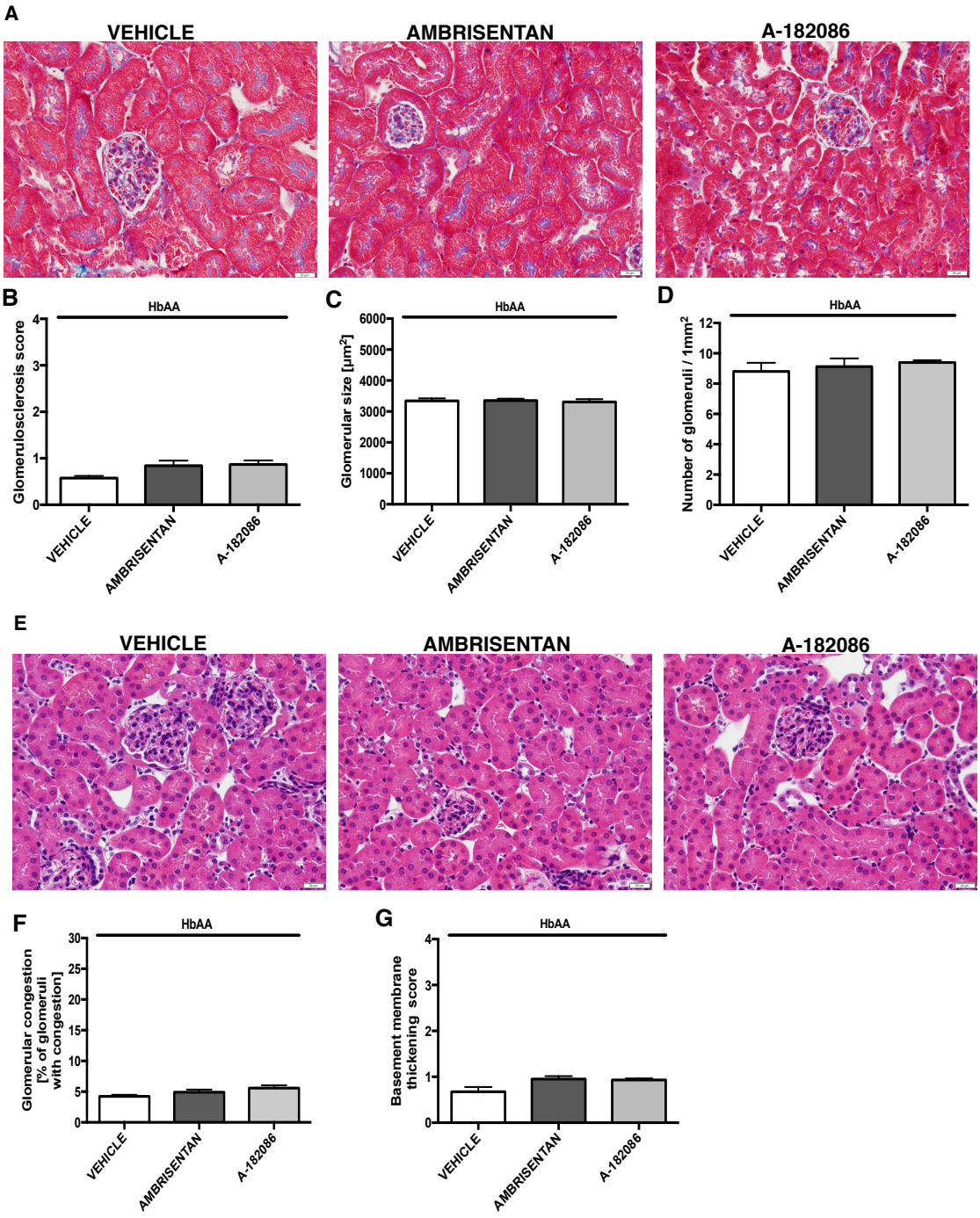

Figure 2. Histological examination of the renal cortex in vehicle-treated or treated with 10-week treatment protocol (beginning at 4 weeks of age) with selective ET_A antagonist, ambrisentan or combined $ET_{A/B}$ antagonist, A-182086, HbAA mice. Panel A depicts representative Masson's trichrome stained sections of glomeruli. Original magnification, x40 (scale bar=50 µm). Panel B depicts quantification of panel A represented as sclerosis index score. Panel C depicts glomerular size represented as mean area of glomeruli [μm^2]. Panel D depicts number of glomeruli per mm^2. Panel E depicts representative hematoxylin and eosin (H&E) stained sections of glomeruli. Original magnification, x40 (scale bar=50 µm). Panel F depicts glomerular vascular congestion represented as percentage of glomeruli with congestion. Panel G depicts basement membrane of Bowman's capsule thickening score. All the glomerular characteristics were counted in 10 sections per slide (minimum 20 glomeruli). Data are mean ± S.E.M; n=5 in HbAA groups.

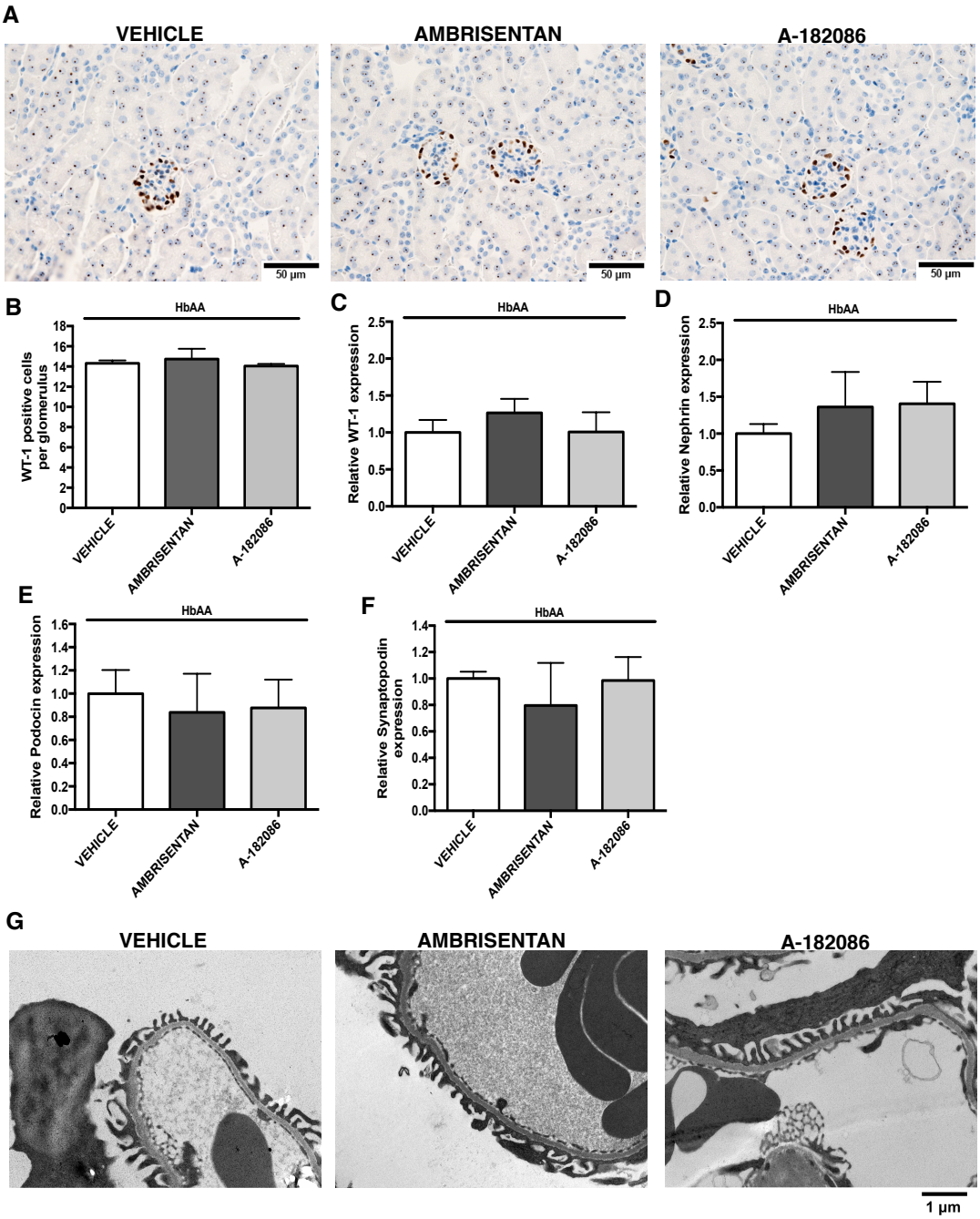

Figure 3. Immunohistochemical examination and mRNA expression of markers of podocyte injury in vehicle-treated or treated with 10-week treatment protocol (beginning at 4 weeks of age) with selective ET_A antagonist, ambrisentan or combined $ET_{A/B}$ antagonist, A-182086, HbAA mice. Panel A depicts representative Wilm's tumor antigen 1 (WT-1) positive stained sections of glomeruli. Original magnification, x40 (scale bar=50 µm). Panel B depicts the quantification of panel A represented as the average number of WT-1 positive cells per glomerulus. Podocytes were counted in minimum 20 glomeruli in 10 sections per slide. Data are mean ± S.E.M; n=5 in HbAA groups. Panel C depicts the relative WT-1 mRNA expression in glomeruli after 10-week treatment protocol. Panel D depicts the relative nephrin mRNA expression in glomeruli after 10-week treatment protocol. Panel E depicts the relative podocin mRNA expression in glomeruli after 10-week treatment protocol. Panel F depicts the relative synaptopodin mRNA expression in glomeruli after 10-week treatment protocol. Panel G depicts representative photomicrographs of transmission electron microscopy sections of glomeruli. Data are mean ± S.E.M; n=5-6 in HbAA groups.

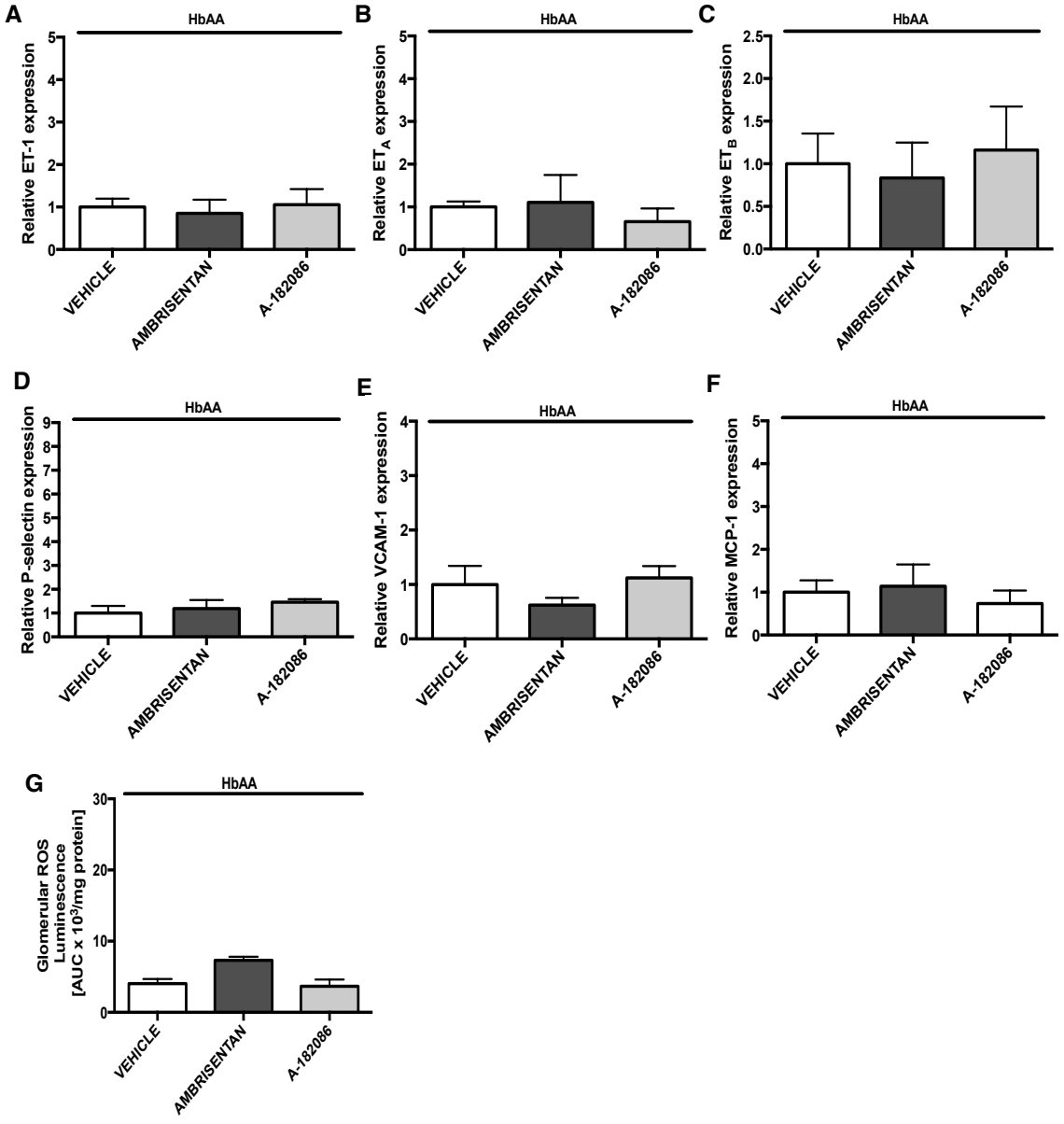

Figure 4. Examination of ET signaling, endothelial dysfunction, inflammation and oxidative stress in glomeruli of vehicle-treated or treated with 10-week treatment protocol (beginning at 4 weeks of age) with selective ET_A antagonist, ambrisentan or combined $ET_{A/B}$ antagonist, A-182086, HbAA mice. Panel A depicts the relative ET-1 mRNA expression in glomeruli after 10-week treatment protocol. Panel B depicts the relative ET_A receptor mRNA expression in glomeruli after 10-week treatment protocol. Panel C depicts the relative ET_B receptor mRNA expression in glomeruli after 10-week treatment protocol. Panel D depicts the relative P-selectin mRNA expression in glomeruli after 10-week treatment protocol. Panel E depicts the relative vascular cell adhesion molecule 1 (VCAM-1) mRNA expression in glomeruli after 10-week treatment protocol. Panel F depicts the relative monocyte chemoattractant protein-1 (MCP-1) mRNA expression in glomeruli after 10-week treatment protocol. Panel G depicts the glomerular reactive oxygen species (ROS) production after 10-week treatment protocol. Data are mean ± S.E.M; n=5-6 in HbAA groups.

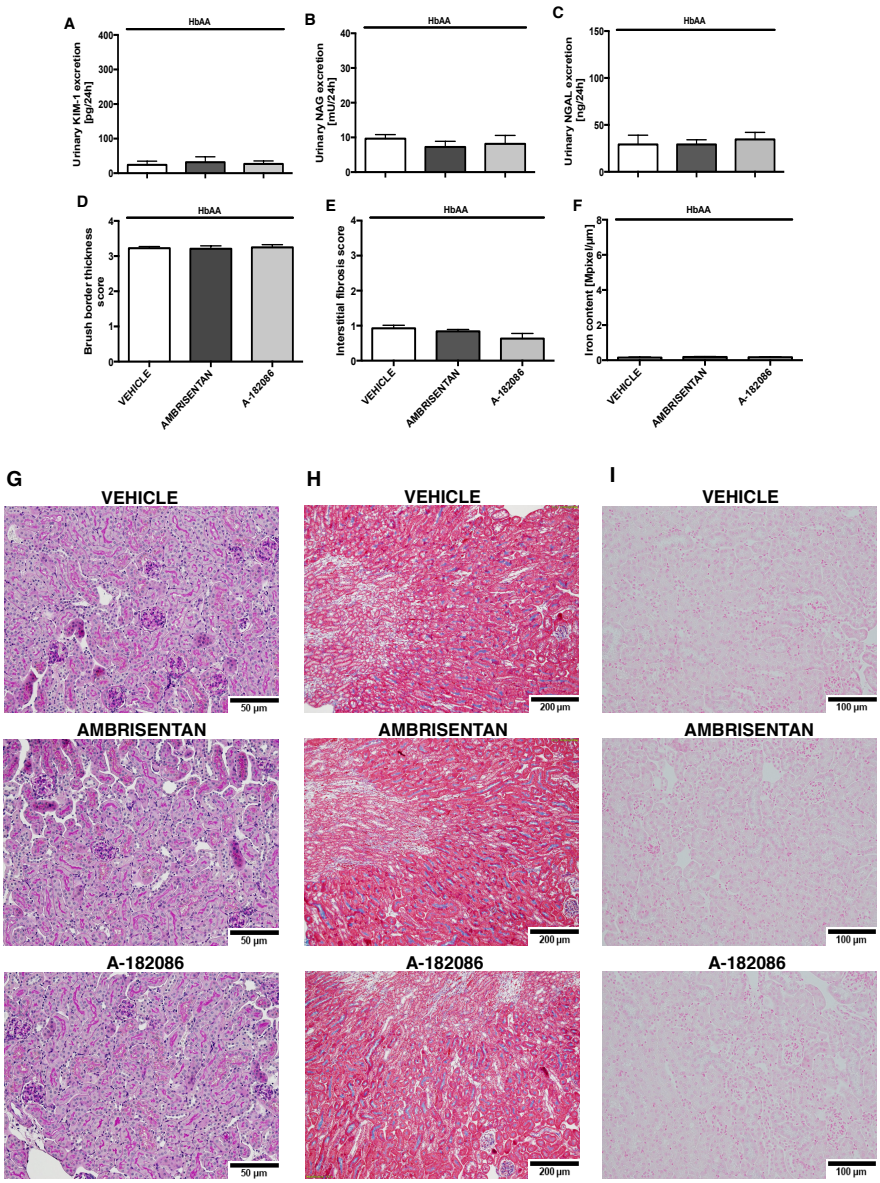

Figure 5. Histological examination of the renal cortex and urinary excretion of markers of tubular injury in vehicle-treated or treated with 10-week treatment protocol (beginning at 4 weeks of age) with selective ET_A antagonist, ambrisentan or combined $ET_{A/B}$ antagonist, A-182086, HbAA mice. Panel A depicts the average urinary kidney injury marker 1 (KIM-1) excretion after 10-week treatment protocol. Panel B depicts the average urinary N-acetyl-β-D-glucosaminidase (NAG) after 10-week treatment protocol. Panel C depicts the average urinary neutrophil gelatinase-associated lipocalin (NGAL) excretion, iron-binding protein, after 10-week treatment protocol. Data are mean ± S.E.M; n=6-9 in HbAA groups. Panel D depicts brush border thickness index score. Panel E depicts interstitial fibrosis index score. Panel F depicts iron deposition in the whole kidney sections (Mpixel/µm). Panel G depicts representative periodic acid Schiff-hematoxylin (PASH) stained sections of tubules. Original magnification, x20 (scale bar=50 µm). Panel H representative Masson's trichrome stained sections of renal cortex and medulla. Original magnification, x10 (scale bar=200 µm, respectively). Panel I depicts Prussian blue iron stained sections of renal cortex. Original magnification, x20 (scele bar=100 µm). Fibrosis and brush border thickness were assessed in 10 sections per slide and calculated. Data are mean ± S.E.M; n=5 in HbAA groups.

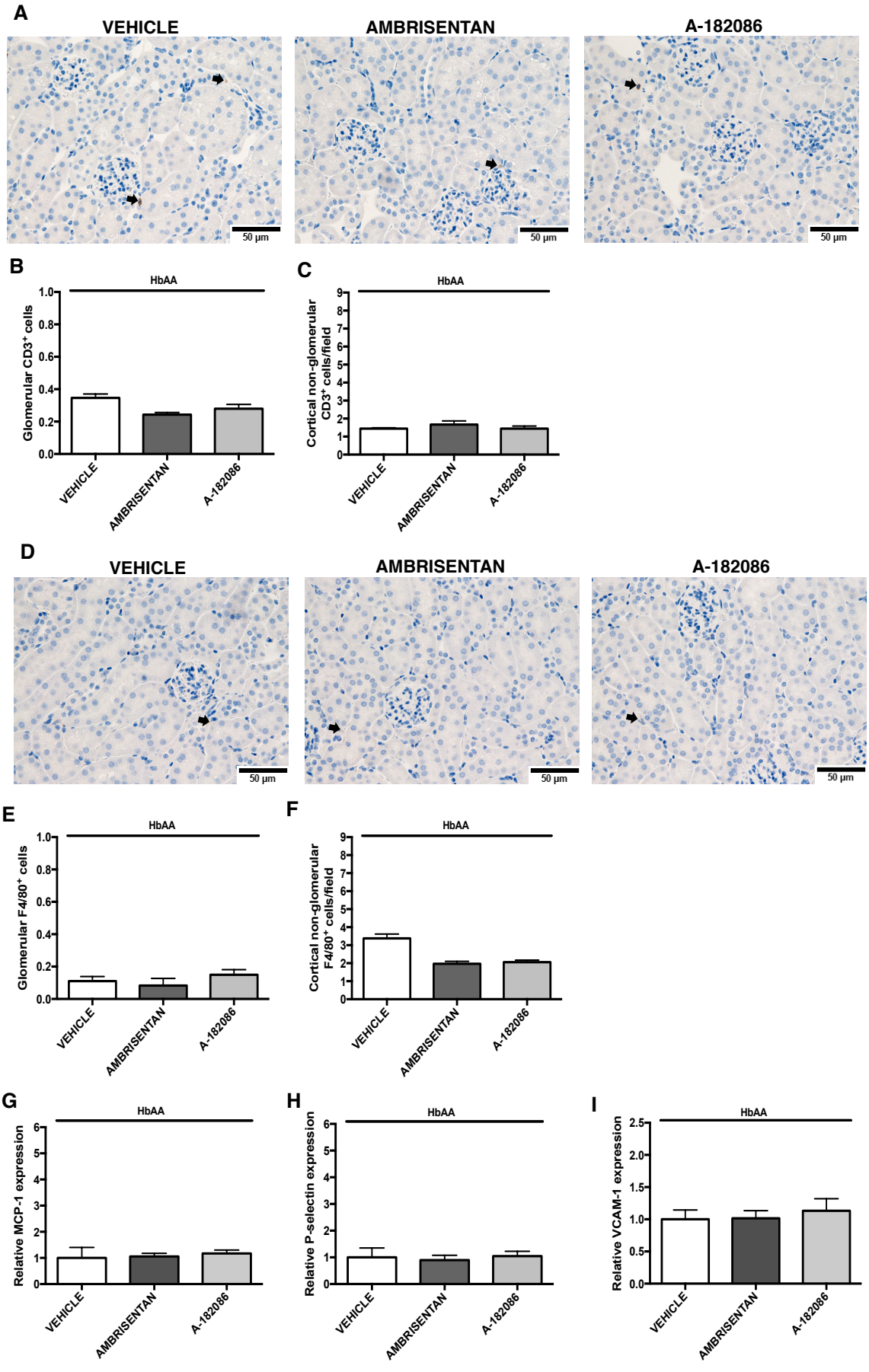
A
VEHICLE
AMBRISENTAN
A-182086
50 µm
B
HbAA
Glomerular CD3+ cells
C
Cortical non-glomerular CD3+ cells/field
D
E
Glomerular F4/80+ cells
F
Cortical non-glomerular F4/80+ cells/field
G
Relative MCP-1 expression
H
Relative P-selectin expression
I
Relative VCAM-1 expression

Figure 6. Immunohistochemical examination and mRNA expression of proinflammatory and profibrotic markers in renal cortex in vehicle-treated or treated with 10-week treatment protocol (beginning at 4 weeks of age) with selective ET_A antagonist, ambrisentan or combined $ET_{A/B}$ antagonist, A-182086, HbAA mice. Panel A depicts representative $CD3^+$ stained cortical sections. Original magnification, x40 (scale bar=50 μm). Arrows indicate $CD3^+$ cells. Panel B depicts the quantification of glomerular $CD3^+$ represented as the average number of $CD3^+$ cells per glomerulus (minimum 20 glomeruli counted). Panel C depicts the quantification of cortical non-glomerular $CD3^+$ represented as the average number of non-glomerular $CD3^+$ cells per field. Panel D depicts representative $F4/80^+$ stained cortical sections. Original magnification, x40 (scale bar=50 μm). Arrows indicate $F4/80^+$ cells. Panel E depicts the quantification of glomerular $F4/80^+$ cells represented as the average number of glomerular $F4/80^+$ cells per glomerulus (minimum 20 glomeruli counted). Panel F depicts the quantification of cortical non-glomerular $F4/80^+$ cells represented as the average number of non-glomerular $F4/80^+$ cells per field. $CD3^+$ and $F4/80^+$ cells were assessed in 10 sections per slide. Data are mean ± S.E.M; n=5 in HbAA groups. Panel G depicts the relative MCP-1 mRNA expression in cortex after 10-week treatment protocol. Panel H depicts the relative P-selectin mRNA expression in cortex after 10-week treatment protocol. Panel I depicts the relative VCAM-1 mRNA expression in cortex after 10-week treatment protocol. Data are mean ± S.E.M; n=5-6 in HbAA groups; $^*p<0.05$ versus vehicle-treated HbAA.

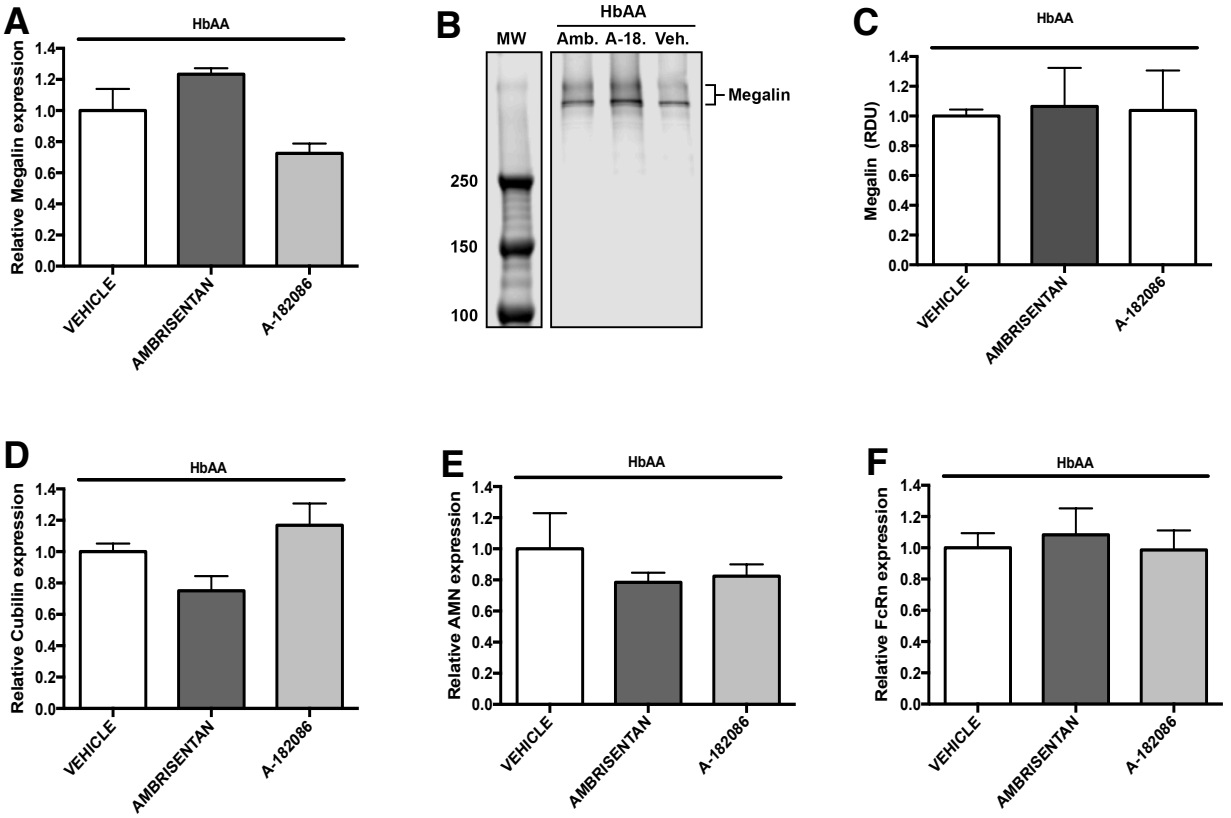

Figure 7. Examination of mRNA expression of proximal tubule albumin handling receptors in cortex of vehicle-treated or treated with 10-week treatment protocol (beginning at 4 weeks of age) with selective ET_A antagonist, ambrisentan or combined $ET_{A/B}$ antagonist, A-182086, HbAA mice. Panel A depicts the relative megalin mRNA expression in cortex after 10-week treatment protocol. Panel B depicts Western blot analysis of megalin expression in cortical extracts. Panel C depicts quantification of Western blot bands for megalin. Panel D depicts the relative cubilin mRNA expression in cortex after 10-week treatment protocol. Panel E depicts the relative amnionless (AMN) receptor mRNA expression in cortex after 10-week treatment protocol. Panel F depicts the relative neonatal Fc receptor (FcRn) mRNA expression in cortex after 10-week treatment protocol. Data are mean ± S.E.M; n=5-6 in HbAA groups.