UNIVERSITY OF GHANA

COLLEGE OF BASIC AND APPLIED SCIENCES

INVESTIGATING THE ROLE OF NEUREGULIN-1 IN MITIGATING HAEMOLYSIS-MEDIATED KIDNEY INJURY IN HUMANIZED SICKLE CELL MICE.

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF PHD IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASE DEGREE.

DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

NOVEMBER 2024

Declaration

Presented in this thesis are studies conducted by me, William Agbozo, at the West African Center for Cell Biology of Infectious Pathogens, University of Ghana and the Department of Microbiology, Biochemistry and Immunology, Morehouse School of Medicine, Atlanta, GA, USA. Prof. Solomon Ofori-Acquah (University of Ghana), Prof. Jonathan Stiles (Morehouse School of Medicine), Dr. Lily Paemka (University of Ghana) and Dr. Samuel Adjei (University of Ghana) supervised this thesis.

I assert that neither the whole work nor any part of it has been or is being submitted for another degree in this or any other university and that this thesis is my original work (except where acknowledgments indicate otherwise).

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Acknowledgment

I am grateful to God for his grace to complete this project. My sincere gratitude goes to Professors Solomon Ofori-Acquah, Jonathan Stiles and Doctors Lily Paemka, Samuel Adjei, for their mentorship, guidance, and support. I am extremely grateful to Dr. Wesley Solomon and the members of Stiles lab for their support.

I want to thank the following people, laboratories, and facilities for their impactful contributions.

i. Dr. Oguljahan Babayewa (Center for Laboratory Animal Resources, Morehouse School of Medicine, Atlanta, USA)
ii. Dr. Isaac Joe Erskine (Department of Pathology, Korle Bu Teaching Hospital, Accra, Ghana)
iii. Staff and Technicians (Center for Laboratory Animal Resources, Morehouse School of Medicine, Atlanta, USA)
iv. Department of Pathology (Center for Neurodegenerative Disease, Emory University, Atlanta, USA)
v. Prof. David Archer (Emory University, Atlanta, USA)
vi. Dr. Larry Boyle (Cytek Biosciences, Inc, California, USA)
vii. Histopathology Department (Parker H. Petit Institute for Bioengineering and Biosciences, Georgia Institute of Technology, Atlanta, USA)
viii. Department of Animal Experimentation (NMIMR, University of Ghana, Legon, Ghana)
ix. Paemka Genomics/Genetics Research Lab (Biochemistry, Cell and Molecular Biology Dept/WACCBIP, University of Ghana, Legon, Ghana)
x. Staff of West African Genetic Medicine Center (University of Ghana, Legon, Ghana)
xi. Classmates, faculty, and staff (WACCBIP, University of Ghana, Legon, Ghana)

Funding

This PhD project received funding support from.

1. National Institutes of Health Grant U54HL141011; Sickle Cell Disease Genomics Network of Africa, University of Ghana.
2. National Institutes of Health Grant R01NS125775; Morehouse School of Medicine, GA, USA.
3. World Bank African Centre's of Excellence (ACE) Impact project - The West African Centre for Cell Biology of Infectious Pathogens (WACCBIP) PhD fellowships; University of Ghana.

Dedication

"And the things you have, entrust to dependable people who will also be qualified to teach others".

I dedicate this work to Professors Solomon Ofori-Acquah and Jonathan Stiles, whose interest in my career growth and support with invaluable opportunities have been key to achieving this milestone.

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List of abbreviations

A1M	Alpha-1-microglobulin
ACE	Angiotensin converting enzyme
ACS	Acute chest syndrome
ADAM17	A disintegrin and metalloproteinase domain 17
AKI	Acute kidney injury
Ang II	Angiotensin II
APOL1	Apolipoprotein L1
ARE	Antioxidant response elements
Aβ	Amyloid Beta Peptide
Bax	Bcl-2-associated X protein
BBB	Blood-brain barrier
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BMECs	Human brain microvascular endothelial cells
CCL-2	C-C motif chemokine ligand 2
CD86	Cluster of Differentiation 86
CKD	Chronic kidney disease
CLAR	Center for Laboratory Animal Resources
CXCL-1	C-X-C motif chemokine ligand 1
CXCL-10	C-X-C motif chemokine ligand 10
DFP	Diisopropylfluorophosphate
ED	Endothelial dysfunction
eDAMPs	Erythrocyte damage-associated molecular patterns
EGFR	Epidermal growth factor family of receptor tyrosine kinases
ErbB2	Erb-b2 receptor tyrosine kinase 2
ErbB3	Erb-b2 receptor tyrosine kinase 3
ErbB4	Erb-b2 receptor tyrosine kinase 4

ESRD	End stage renal diseases
ET-1	Endothelin-1
ETA	Endothelin A
FFPE	Fresh frozen paraffin embedded
GFR	Glomerular filtration rate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPx-1	Glutathione Peroxidase-1
Hb	Haemoglobin
HbAA	Haemoglobin AA
HbF	Fetal haemoglobin
HMOX-1	Hemeoxygenase-1 gene
HO-1	Hemeoxygenase-1
Hp	Haptoglobin
HSP70	Heat shock protein 70
HU	Hydroxyurea
Hx	Hemopexin
ICAM-1	Intercellular adhesion molecule-1
ICAM-1	Intercellular adhesion molecule 1
IFNγ	Interferon-gamma
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1 beta
IL-33	Interleukin 33
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
KIM-1	Kidney injury molecule-1
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
M1	Classically activated macrophages (pro-inflammatory)

M2	Alternatively activated macrophages (anti-inflammatory)
MAP	Mitogen-Activated Protein Kinases
MCP-1	Monocyte chemoattractant protein 1 (another name for CCL-2)
MIF	Macrophage Migration Inhibitory Factor
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B
NGAL	Neutrophil gelatinase–associated lipocalin
NO	Nitric oxide
NOX4	NADPH Oxidase 4
NQO1	NAD(P)H quinone oxidoreductase 1
Nrf2	Nuclear translocation of nuclear factor erythroid 2 related factor 2
NRG-1	Neuregulin-1
OGD	Oxygen-glucose deprivation
OPN	Osteopontin
PI3K	Phosphoinositide 3-kinases
PI3K/AKT	Phosphoinositide 3-kinase / Protein kinase B
PKC	Protein kinase C
RAF	Rapidly Accelerated Fibrosarcoma
RBC	Red blood cell
rhNRG-1	Recombinant human Neuregulin-1
RTECs	Renal tubular epithelial cells
SCA	Sickle cell anaemia
SCD	Sickle cell disease
sFLT-1	soluble fms-like tyrosine kinase 1
SOD	Superoxide Dismutase
TACE	Tumor necrosis factor-alpha converting enzyme
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion protein 1

VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor A
VEGFR1	Vascular endothelial growth factor receptor 1
VOC	Vaso-occlusive crisis
vWF	von Willebrand factor
ZO-1	Zonula occludens-1

ABSTRACT

Background: Chronic kidney disease (CKD), defined as the persistent and gradual decline in kidney function, is a common and severe complication of sickle cell disease (SCD). CKD in SCD is reported in childhood and worsens with age. More than half of adults with SCD over the age of forty (40) develop CKD, leading to end-stage renal disease. This contributes significantly to 16-18% of SCD global deaths each year. Acute kidney injury (AKI), the sudden loss of kidney function, is a major risk factor for developing chronic kidney disease (CKD). In sickle cell disease, the incidence of AKI is three times higher than in non-sickle cell patients, affecting over 30% of hospitalized individuals. Again, AKI is a leading cause of early death in critically ill SCD patients. Despite its significant role in kidney disease, treatment options for AKI in SCD remain limited and uncertain, posing a significant challenge in its clinical management.

A major driver of kidney injury in SCD is intravascular haemolysis, which releases excess protein-free heme into circulation, triggering kidney injury through vascular inflammation, endothelial dysfunction, oxidative stress, and cytotoxicity. Hydroxyurea (HU), the approved SCD-modifying therapy, partly improves renal function, but its impact on heme-driven kidney injury remains uncertain and underexplored. There is an urgent need for targeted interventions against heme driven kidney injury in SCD. Neuregulin-1 (NRG-1), an endothelium-derived peptide with anti-inflammatory, antioxidant, and cytoprotective properties, holds therapeutic potential in SCD. *It is hypothesized that Neuregulin-1 reduces kidney injury in sickle cell mice by modulating haemolysis, inflammation and inducing anti-heme cytoprotective factors*. This PhD work assessed the effects of NRG-1 and HU on haemolysis, inflammation, kidney injury, and renal histopathologic changes in a humanized sickle cell mouse model that mimics clinical features of SCD.

Methods: The HbSS-Townes mouse model, developed by Dr. Tim Townes' laboratory (University of Alabama, Birmingham), which carries human haemoglobin knock-in genes and develops kidney injury resembling human disease, was used. Control mice (HbAA), which do not develop the disease, were also included. Cohorts of twelve-week-old Townes sickle cell (HbSS) and non-sickle (HbAA) mice (n = 8 per group, sex-matched) were intraperitoneally administered with recombinant human neuregulin-1 (NRG-1) at doses of 5 µg/kg/day and 25 µg/kg/day; 50 mg/kg of hydroxyurea (HU) or sterile phosphate-buffered saline (vehicle) for two weeks. Following treatment with NRG-1, HU or vehicle, blood and urine samples were collected for analysis of complete blood count, haemolysis markers, and kidney injury biomarkers. Kidney tissue sections were examined for histopathologic renal changes, and immunohistochemistry was used to assess the effect of neuregulin-1 on the expression and tissue distribution of heme oxygenase-1, a heme-degrading enzyme.

Results: HbSS mice exhibited anaemia, leucocytosis, elevated haemolysis (total plasma heme, lactate dehydrogenase [LDH]), increased urinary kidney injury biomarkers (neutrophil gelatinase-associated lipocalin [NGAL] and cystatin C), and reduced renal repair biomarkers (clusterin, epidermal growth factor [EGF]) prior treatment. Urinary levels of NGAL and Cystatin C positively correlated with total plasma heme ($\rho = 0.98$, $p < 0.05$) and LDH ($\rho = 0.97$, $p < 0.05$) respectively. Both NRG-1 and HU significantly reduced white blood cell counts, total plasma heme, lactate dehydrogenase, and pro-inflammatory kidney injury mediators (NRG-1: FN-γ, IL-6, CXCL10, VEGF-A, CCL19, MCP-5; HU: CXCL10, VEGF-A, IFN-γ) in HbSS mice. Notably, NRG-1 (25 µg/kg/day), like HU, significantly increased circulating fetal haemoglobin containing red blood cells (F-cells). HU reduced urinary cystatin C and NGAL levels more than NRG-1, whereas NRG-1 significantly increased urinary clusterin and EGF. HU significantly reduced medullary

congestion, while NRG-1 markedly reduced iron deposition in cortical tubular epithelial cells. Both treatments reduced glomerular congestion, Bowman's membrane thickening, tubular brush border loss, and glomerulosclerosis. NRG-1 enhanced HO-1 expression in HbSS kidneys, whereas HU had no significant effect on HO-1 expression. ErbB4 was highly expressed in HbSS kidneys compared to HbAA, suggesting its involvement in kidney protection pathways.

Conclusion: This work provides new insight supporting the exploration of NRG-1 as a targeted therapeutic agent for kidney injury in SCD. The complementary renoprotective effects observed with NRG-1 and hydroxyurea support further evaluation of their combined use to improve kidney outcomes in SCD.

1.0 CHAPTER ONE: INTRODUCTION

1.1 Background

Sickle cell disease (SCD) is a lifelong genetic disorder affecting approximately eight million people worldwide, predominantly those of African ancestry (Hegemann et al., 2023; Thomson et al., 2023). SCD is caused by a single point mutation in the beta-globin gene of haemoglobin (Hb) on chromosome 11, leading to the formation of sickled red blood cells (Sundd et al., 2019). Two key characteristics of these sickled cells are their tendency to adhere to one another and/or undergo haemolysis (Gladwin & Ofori-Acquah, 2014). These cellular abnormalities drive the numerous clinical complications of SCD, which can affect nearly every organ. While survival rates among pediatric SCD patients have improved in recent years, multiorgan dysfunction and failure have become increasingly prevalent in adult patients (Thein & Howard, 2018; Thomson et al., 2023). This progressive organ damage is a leading cause of early mortality and contributes to the declining survival rates among adults with SCD (Gardner et al., 2016; Lanzkron et al., 2013; Thomson et al., 2023). As a result, SCD is now considered a chronic adult illness marked by progressive multiorgan failure (Vichinsky, 2017).

Chronic kidney disease (CKD), defined as the persistent and gradual decline of kidney function, is one of the most common and serious complications among SCD patients. More than half of SCD patients over 40 years old develop CKD, with 10 to 12% progressing to end-stage renal disease (ESRD) (Roy et al., 2023; Valle et al., 2024). In the United States, a national cohort study of African Americans with ESRD reported that 25% of these individuals were SCD patients (Olaniran et al., 2020). This study also reported that mortality rates among SCD patients were twice as high as those of non-SCD African Americans with ESRD (Olaniran et al., 2020).

Acute kidney injury (AKI), a condition characterized by a sudden decline in kidney function, is a major risk factor for the development of CKD. Individuals with sickle cell disease (SCD) are two to three times more likely to develop severe acute kidney injury (AKI) than non-SCD patients with other medical conditions (Olaniran et al., 2021). Clinical reports show that more than 30% of SCD patients admitted with vaso-occlusive crises or acute chest syndrome develop AKI (Batte et al., 2022). SCD-AKI patients experience higher rates of morbidity, hospitalization, and mortality (Cecchini et al., 2014; McCormick et al., 2020; Nath & Hebbel, 2015). Together, AKI-driven CKD and ESRD account for 16-18% of deaths among adult SCD patients (Roy et al., 2023). Despite its critical role in the progression of kidney disease, treatment options for AKI in SCD remain limited and uncertain, posing a significant challenge in clinical management and underscoring the need for improved therapeutic strategies. Targeting modifiable risk factors and modulating their effects may offer opportunities to improve clinical outcomes of AKI in SCD.

Intravascular haemolysis, a life-threatening event where red blood cells break apart within blood vessels, is a major risk factor for developing kidney injury (Aban et al., 2017; Lebensburger et al., 2016). Haemolysis releases toxic erythrocyte damage-associated molecular patterns (eDAMPs), particularly cell-free haemoglobin (Hb) and its breakdown product, protein-free heme, into the bloodstream (Gladwin & Ofori-Acquah, 2014). When cell-free Hb and protein-free heme are exposed to the kidneys, they trigger inflammatory and cytotoxic pathways that result in renal injury (Aban et al., 2017; Avondt et al., 2019; Baddam et al., 2017; Lebensburger et al., 2016; Qian et al., 2010). Glomerular endothelial cells are particularly vulnerable to heme-mediated damage and demonstrate an insufficient counteractive response (May et al., 2018). Similarly, podocytes, specialized cells involved in kidney filtration, suffer oxidative injury and cell death when exposed to cell-free Hb or protein-free heme (Rubio-Navarro et al., 2018). Podocyte injury or loss disrupts

the renal filtration function and is linked to proteinuria observed in both patients and animal models (Raij et al., 2016). Renal epithelial tubular cells are the primary targets of Hb and heme nephrotoxicity. Excessive heme exposure leads to tubular injury, characterized by elevated kidney injury biomarkers, histopathological changes, and cell death (Ofori-Acquah et al., 2020a). Preclinical studies have confirmed that circulating haemolytic biomarkers, including cell-free Hb and heme, drive kidney injury in SCD (Avondt et al., 2019), and clinical data from SCD patients have established a link between haemolysis and AKI (Olaniran et al., 2020).

SCD patients are especially vulnerable to haemolysis-mediated kidney injury during episodes of acute haemolysis (Ofori-Acquah et al., 2020a). This vulnerability arises from a dysregulated heme clearance process, where an unusually high amount of circulating cell-free haemoglobin and heme is directed to the kidneys instead of the liver (Ofori-Acquah et al., 2020a). This PhD thesis focuses on modulating the effects of haemolysis and heme in mediating kidney injury in a sickle cell mice model.

1.2 Problem statement

Acute kidney injury (AKI) leads to a sudden decline in kidney function and increases the risk of developing long-term loss of kidney function (Leung et al., 2013; Nath & Hebbel, 2015). In sickle cell disease (SCD), the incidence of AKI is three times higher compared to non-SCD individuals and is linked to increased mortality in patients admitted to intensive care (Cecchini et al., 2014; Olaniran et al., 2021). The high burden of poor clinical outcomes and early deaths among SCD-AKI patients underscores the urgent need for effective therapies and improved clinical management strategies. (Nath & Hebbel, 2015).

Current treatments for SCD-AKI focus on sickle cell-approved drugs and interventions aimed at reducing sickling, alleviating vaso-occlusion, improving hydration, and enhancing blood flow to mitigate symptoms. While these interventions have shown some improvement in kidney function, their effectiveness against kidney injury remains unclear. Hydroxyurea, an approved SCD drug, has been shown to improve glomerular filtration rate (GFR), reduce proteinuria, and decrease renal tubular injury (Bartolucci et al., 2016; Han et al., 2020; Park et al., 2019; Taylor et al., 2019). Also, prolonged hydroxyurea therapy is associated with adverse effects, including bone marrow suppression and neutropenia (López Rubio & Argüello Marina, 2024). Blood transfusion therapy, another treatment option for acute or chronic SCD complications, has conflicting efficacy reports and notable adverse effects, limiting its routine use (Alvarez et al., 2006; Lebensburger et al., 2011). Additionally, angiotensin-converting enzyme (ACE) inhibitors are used to treat albuminuria/proteinuria in SCD, but they have not demonstrated long-term improvement in renal outcomes (Quinn et al., 2017; Xie et al., 2016; Yee et al., 2018). Taken together, the optimal treatment for SCD- kidney injury remains elusive, with current therapies being suboptimal and associated with adverse effects, underscoring the need for improved treatment options.

Clinical studies have linked AKI in SCD to acute haemolysis which releases cell-free haemoglobin and excess protein-free heme into circulation (Aban et al., 2017; Baddam et al., 2017; Lebensburger et al., 2016). There is currently no specific drug available to mitigate haemolysis-induced kidney injury in SCD (Avondt et al., 2019). Identifying a drug that can effectively attenuate haemolysis-mediated mechanisms may prove beneficial in managing AKI in SCD. Cell-free haemoglobin and protein-free heme cause kidney injury through pro-inflammatory effects, oxidative stress, and cytotoxic pathways (Avondt et al., 2019). Therapeutic strategies targeting these pathways including inflammation, complement activation, oxidative stress, immune cell trafficking, and cytotoxicity, have shown potential in protecting the kidney (Boutaud & Roberts, 2011; Legendre et al., 2013; Scindia et al., 2015; Ware et al., 2013; Young et al., 2014).

This PhD research repurposed a therapeutic agent that has been shown to mitigate organ damage in diseases with pathophysiological similarities to SCD's haemolytic environment. Neuregulin-1, an anti-inflammatory and cytoprotective peptide known to counteract haemolysis and heme-mediated processes, is investigated as a potential renal protective agent against kidney injury in sickle cell disease.

1.3 Rationale and Justification

Sickle cell patients are particularly vulnerable to kidney injury due to an acquired deficiency of the primary heme-scavenging plasma protein, hemopexin (Hx) (Santiago et al., 2018). As a result, a compensatory mechanism occurs, where the secondary heme scavenging protein, alpha-1-microglobulin (A1M), preferentially transports heme to the kidneys (Ofori-Acquah et al., 2020a). Hence, the sickle cell kidney is exposed to excessive levels of cell-free haemoglobin and heme proteins (Ofori-Acquah et al., 2020a).

Excess protein-free heme induces glomerular endothelial injury by activating proinflammatory pathways. Heme binds to toll-like receptor 4 (TLR4) on renal endothelial cells, initiating an inflammatory cascade that increases the production of proinflammatory cytokines, the expression of adhesion molecules, and the breakdown of the endothelial barrier, ultimately leading to immune cell infiltration (Belcher et al., 2014; Wagener et al., 2001). Heme-mediated inflammation and immune cell infiltration are major contributors to kidney injury (Chen et al., 2020; Daniels et al., 2021; Imig & Ryan, 2013; Merle et al., 2018; Nath, Vercellotti, et al., 2001; Wagener et al., 2001). Activation of the renal endothelium is evident in the glomeruli of SCD kidneys, contributing to glomerular injury (Heimlich et al., 2016; Kasztan et al., 2017; Taylor et al., 2019). Also, increased levels of proinflammatory cytokines found in the urine of SCD patients are linked to haemolysis (Cerqueira et al., 2011). Evidence from animal studies shows that targeting heme-induced pathways protect against glomerular endothelial injury (Avondt et al., 2019), with strategies such as reducing endothelial activation, oxidative stress, and inflammatory signaling providing potential renal protection (Belisário et al., 2020; Fujihara et al., 2007; Lin et al., 2021; Nath et al., 2018; Shi et al., 2017; Taylor et al., 2019).

Heme also induces direct injury within renal tubular epithelial cells and podocytes (Gonzalez-Michaca et al., 2004; Rubio-Navarro et al., 2018). Cell-free haemoglobin and heme in circulation are endocytosed by renal epithelial cells via megalin and cubilin receptors. In SCD patients, excessive uptake is evidenced by iron accumulation in kidney biopsy specimens (Zahr, et al., 2019). Within tubular epithelial cells, heme activates the inflammasome (NLRP3), oxidative stress pathways, and caspase-3-mediated cell death (Belcher et al., 2014).

Neutralizing intracellular haemoglobin and A1M-bound heme is crucial for preventing tubular cell injury and death (Grunenwald et al., 2021). Heme oxygenase-1 (HO-1), an intracellular heme-degrading enzyme, plays a key role in defending against kidney injury (Grunenwald et al., 2021). Clinical and animal studies demonstrate that upregulation of HO-1 is protective against heme-mediated kidney injury in SCD and other haemolytic conditions (Lever et al., 2016; Li et al., 2021; Nath, 2014; Nath et al., 1992, 2000; Ohta et al., 2000; Shi et al., 2016). Additionally, specific *HMOX-1* promoter alleles that increase HO-1 expression are linked to reduced risk of AKI and CKD in patients (Saraf et al., 2015a, 2018). Therefore, effective therapeutic induction of HO-1 could protect renal cells from heme-induced toxicity in SCD (Grunenwald et al., 2021).

Neuregulin-1 (NRG-1), an endothelium-derived peptide, is a versatile endogenous signalling molecule that induces anti-inflammatory and cytoprotective pathways (Cespedes et al., 2018). Studies have demonstrated the systemic and tissue-protective effects of NRG-1 in various cell types, tissues, organs, and disease models. NRG-1 treatment reduces proinflammatory responses, increases anti-inflammatory activity, maintains vascular barrier integrity, reduces endothelial cell activation, and promotes immune homeostasis (Alizadeh et al., 2018; Dreymueller et al., 2014; Kang et al., 2019; Liu et al., 2020; Lok et al., 2012; Mencel et al., 2013; Pascual-Gil et al., 2019; Shahriary et al., 2019; Simmons et al., 2016; Wu et al., 2015; Zhou et al., 2016).

NRG-1 also induces the expression of cytoprotective factors that have antioxidant, anti-apoptotic, restorative, and regenerative effects (Di Segni et al., 2005; Gauthier et al., 2013; Ji et al., 2017; Jiang et al., 2016; Kim et al., 2020; Li et al., 2007; Ma et al., 2022; Shahsavani et al., 2021; Xu et al., 2004; Yoo et al., 2021). NRG-1 acts by activating two key homeostatic pathways, PI3K/AKT and RAF/MAPK (Cespedes et al., 2018).

Studies administering NRG-1 in mouse models of kidney disease have shown promising results, with reductions in serum creatinine, proteinuria, urinary tubular injury markers, renal fibrosis, and glomerulosclerosis (Sárközy et al., 2023; Vandekerckhove et al., 2016). Furthermore, elevated serum NRG-1 levels in humans are strongly associated with kidney recovery and improved survival in patients with dialysis-dependent AKI (Daniels et al., 2021). In experimental models of heme exposure, NRG-1 mitigates inflammation and prevents cell death. NRG-1 administration reduces the production of endothelial proinflammatory chemokines (CXCL-1 and CXCL-10), downregulates expression of adhesion molecules (ICAM-1, E-selectin, and VCAM-1), and protects against endothelial barrier disruption and immune cell infiltration (Chambliss et al., 2023; Liu et al., 2018). Harbuzariu *et al.* (2019) showed that NRG-1 protects brain organoids from heme-induced cell death and structural disorganization (Harbuzariu et al., 2019). Given its systemic anti-inflammatory and cytoprotective properties, NRG-1 could protect the kidneys in SCD (Cespedes et al., 2018).

The relevance of investigating Neuregulin-1 (NRG-1) lies in its translational potential for sickle cell disease (SCD). NRG-1 therapy is already undergoing clinical trials, where it has been shown to be safe and effective in reducing myocardial injury following infarction. (Gao et al., 2010; Jabbour et al., 2011; Xu et al., 2023).

1.4 Aims and Hypotheses

1.4.1 Specific Aim 1

Evaluate the effect of Neuregulin-1 treatment on haemolysis, inflammation, and kidney injury biomarkers in humanized sickle cell mice.

Hypothesis 1: Neuregulin-1 mitigates kidney injury in Townes sickle cell mice by reducing haemolysis and inflammation.

1.4.2 Specific Aim 2

Determine the effect of Neuregulin-1 treatment on renal histopathological changes associated with kidney injury in humanized sickle cell mice.

Hypothesis 2: Neuregulin-1 improves renal histopathologic changes in Townes sickle cell mice by modulating heme toxicity.

1.4.3 Specific Aim 3

Determine the effect of Neuregulin-1 treatment on renal heme oxygenase-1 expression in humanized sickle cell mice.

Hypothesis 3: Neuregulin-1 treatment increases the expression and distribution of heme oxygenase-1 in the kidneys of Townes sickle cell mice.

2.0 CHAPTER TWO: LITERATURE REVIEW

2.1 Sickle cell disease (SCD).

2.1.1 Disease epidemiology and burden.

Sickle cell disease (SCD) is a lifelong genetic disease that was first described in a patient with symptoms linked to having abnormal red blood cells with a crescent appearance in his blood (Herrick, 2001, 2014). Following its first report by Dr. James Herrick in 1910, the disease burden remains a global health problem with high prevalence in sub-Saharan Africa, the Mediterranean, the Middle East, and India (Thomson et al., 2023). Globally, the total number of sickle cell births has increased by approximately 13.7% between 2000 and 2021, with an estimated 425,000–614,000 babies born each year with the disease (Thomson et al., 2023). Africa is home to about three-quarters of global sickle cell disease new births (Oron et al., 2020). A survey in Africa reported about 150,000–300,000 sickle cell child deaths before the age of five years (Piel et al., 2013). Sickle cell disease alone contributes to about one-tenth of Africa's annual total child mortality (Kato et al., 2018). The surviving adolescents and adults with sickle cell disease experience significant disease complications, morbidity, and a high risk of organ failure compared to non-sickle individuals (Macharia et al., 2018). The number of people living with sickle cell disease globally is estimated to be 8 million people (Thomson et al., 2023). Mortality burden is about 400,000 deaths per year, with the highest burden in western and central sub-Saharan Africa (Thomson et al., 2023). SCD is also present in well-resourced regions, among people of African Ancestry. The United States has about 100,000 persons affected by the disease, with 1 in every 365 Black or African American births presenting with sickle cell disease (Thomson et al., 2023).

2.1.2 Genetics of SCD.

SCD is characterized by a person carrying a genetic defect in the gene that makes haemoglobin, an oxygen-carrying protein in red blood cells (Kato et al., 2018). The haemoglobin protein comprises a tetrameric combination of two subunits, α-globin, and β-globin subunits. The tetramer of the native α-globin and β-globin subunits form the normal haemoglobin A (Azar & Wong, 2017). Individuals with sickle cell disease inherit a copy of the mutant β-globin subunit from each parent. (Azar & Wong, 2017; Kato et al., 2018). The β^S mutation is the most common of the β-globin gene with an amino acid substitution at codon six [Glu6Val, rs334], giving rise to the sickle haemoglobin (HbS). Other common mutations of the β-globin gene are β^C [Glu6Lys], β^0 thalassemia [no protein translation], and β^+ thalassemia [decreased protein translation] (Henderson et al., 2016; Indrák et al., 2018). SCD genotypes are defined by the combination of at least one HbS mutant and any other β-globin mutation; thus, it can be genotype HbSS, HbSC, HbSβ^0 thalassemia, Hbβ^+ thalassemia, and other reported combinations such as HbSD, HbSE, HbSOArab, etc. Sickle cell anaemia (SCA) or HbSS is the most common genotype, which accounts for the severe forms of the disease. HbSC is the second most common genotype with a high incidence rate in West Africa. HbSC is less severe compared to SCA. Among patients of African descent, HbSS constitutes 65-70% of SCD cases, followed by HbSC (30-35%), and the rest HbSβ thalassemia. Individuals carrying the β^S allele and the normal β allele are sickle cell trait carriers and not with SCD (Kato et al., 2018; Piel et al., 2017; Thein et al 2017).

2.1.3 Clinical complications of SCD.

Clinical outcomes of sickle cell disease are multisystem and can affect every organ in the body. The basis for disease complication is the altered physical and biochemical state of sickle red blood cells (Piel et al., 2017). Mutant haemoglobins (HbS, HbC, etc.) undergo polymerization within the red blood cell under hypoxic conditions. This abnormal biophysical change gives rise to red blood cell membrane distortions characterized by a sticky surface or fragility to break up (Sundd et al., 2019). Sticky sickle red blood cells tend to adhere to one another, vascular endothelium, or other blood cells and get stuck, blocking blood flow to tissues (Moerdler & Manwani, 2018; Puri et al., 2018). Again, the tendency for these fragile sickle red cells to hemolyze is high, releasing cell-bound intracellular toxic molecules into vascular space (Kato et al., 2017). A cascade of pathogenic processes is initiated by haemolysis and vaso-occlusion, including disruption of vascular endothelial homeostasis, inflammation, nitric oxide deficiency, reperfusion injury, increased neutrophil adhesiveness, platelet activation, hypercoagulability, and oxidative stress (Moerdler & Manwani, 2018; Puri et al., 2018; Tran et al., 2017; Zhang et al., 2016). Haemolysis and vaso-occlusion drive the development of acute and chronic complications. Acute complications are the common cause of frequent hospitalizations in SCD individuals. Pain in the extremities, bones, joints, chest, and back are examples of common acute complications (Azar & Wong, 2017). Chronic complications caused by vessel vasculopathy or prolonged tissue ischemia result in organ damage and dysfunction. Acute chest syndrome, enlarged spleen, eye problems, gallstones, heart problems, kidney problems, leg ulcers, priapism, liver problems, stroke, and silent brain injury are complications that patients experience throughout their life. (Khargekar et al., 2024; Usmani & Machado, 2018; Vichinsky, 2017). There is however heterogeneity in the clinical presentations of SCD complications among individuals and between genotypes (Thein, 2017).

2.1.4 Chronic haemolysis and hyperinflammation in SCD mediates disease complications.

Sickle red blood cells have an unstable cell membrane and are susceptible to breaking up in hypoxic environments (Vallelian et al., 2022). This releases significant amounts of toxic molecules into the vascular space (Kato et al., 2017). Haemolytic products, which include cell-free haemoglobin and its derivative heme, arginase, are cytotoxic molecules within vascular space and hence referred to as erythrocyte damage-associated molecular patterns (eDAMPs) molecules (Gladwin & Ofori-Acquah, 2014; Roumenina et al., 2016). eDAMPs are the primary initiators of pathogenesis in SCD (Gladwin & Ofori-Acquah, 2014) causing deleterious effects in patients when not sufficiently neutralized (Santiago et al., 2018). The extent of eDAMPs release into vascular space is associated with SCD severity and organ damage (Shankar et al., 2021; Vallelian et al., 2022). Cell-free Hb reacts with and depletes nitric oxide (NO), an important vasodilator and endothelial homeostatic factor (Thomson et al., 2023). Also, arginase reduces plasma arginine levels, the substrate for NO production (Nader et al., 2021; Ofori-Acquah, 2020). During haemolysis, cell-free haemoglobin and arginase within the vascular space cause NO depletion, impaired vasodilation, and endothelial dysfunction (ED). SCD is a vascular disorder where vasculopathy found in SCD promotes complications and organ damage (Ofori-Acquah, 2020; Pavan et al., 2024)

Furthermore, heme, a by-product of haemoglobin breakdown, mediates pro-inflammation and endothelial injury (Roumenina et al., 2016). Protein-free heme acts by binding with the receptor, Toll-like receptor 4 (TLR4) on endothelial cells (Belcher et al., 2014). Heme-TLR4 activation mediates downstream signaling via the Nuclear Factor kappa-light-chain-enhancer of activated B (NF-kB) and NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome pathways (Belcher et al., 2014; Erdei et al., 2018; Ofori-Acquah, 2020; Salgar et al., 2023). NF-kB activation increases pro-inflammatory cytokine production, intensifying the inflammatory

environment within the vascular space (Owusu-Ansah et al., 2016). Endothelial cell activation, mediated by vascular inflammatory factors, leads to the surface expression of adhesive molecules E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1). This causes the recruitment and subsequent abnormal vascular endothelium adhesive interactions with erythrocytes, platelets, and leukocytes (Mendonça, et al., 2016; Owusu-Ansah et al., 2016). This gives rise to vaso-occlusion, particularly in microvessels (Guarda et al., 2017). Vascular dysfunction and endothelial activation are key adverse effects of haemolysis due to inflammatory pathogenesis (Guarda et al., 2017; Owusu-Ansah et al., 2016).

The generation of reactive oxygen species (ROS) by cell-free haemoglobin and heme also drives the cytotoxicity of haemolytic products. The buildup of ROS in and outside cells outweighs antioxidant clearance and leads to oxidative stress. ROS can cause direct vascular endothelial damage through lipid peroxidation and DNA fragmentation. Hence, oxidative stress further promotes vascular endothelial activation, inflammation, and injury (Balla & Zarjou, 2021; Martins & Knapp, 2018).

Several studies have demonstrated the link between excess-free heme and SCD clinical outcomes such as vaso-occlusive crises, acute chest syndrome, kidney damage, liver damage, brain damage, and stroke (Adisa et al., 2013; Cardoso et al., 2023; Chonat et al., 2022; Ghosh et al., 2019; Ghosh & Ofori-Acquah, 2010; Ofori-Acquah et al., 2020; Panda et al., 2019; Runge et al., 2022; Schmidt et al., 2018; Shankar et al., 2021). Altogether, intravascular haemolysis and eDAMPs trigger interconnected pathogenic processes which are drivers of SCD clinical outcomes (Ofori-Acquah, 2020).

2.2 Sickle cell disease kidney injury.

2.2.1 SCD kidney disease burden.

Progressive organ damage and early deaths are common complications among SCD individuals (Cançado et al., 2023; Njoku et al., 2024). SCD pathophysiology results in organ damage, and the kidneys are one of the most affected organs (Abbasi et al., 2024). Kidney disease in SCD is common in both adults and children, with renal manifestations showing up as early as age four (4) (Zahr et al., 2019). The spectrum of SCD renal abnormalities is progressive, with microalbuminuria being an early sign of dysfunction. Microalbuminuria progresses to reduced urine concentration ability, impaired tubular acidification, and electrolyte imbalance (Nath & Hebbel, 2015). Severe proteinuria, hematuria, increased plasma markers of tubular injury, and changes to the glomerular filtration rate are seen in early adulthood (Ataga et al., 2022; Zahr et al., 2019). Recurrent episodes of AKI, marked by impaired renal excretory function, is a key risk factor for the development of CKD and progression to ESRD. CKD is particularly prominent in adulthood (Roy et al., 2023).

Kidney disease is a significant burden for SCD and is associated with frequent visits and longer stays at the hospital, expensive cost of care, and eventually death (Kauf et al., 2009; Yeruva et al., 2016). The incidence of AKI and CKD is 2 to 3 times more common among sickle cell patients compared to non-sickle individuals. (Olaniran et al., 2021; Yeruva et al., 2016). Altogether, AKI, CKD, and ESRD contribute to 16–18% of causes of death in SCD (Roy et al., 2023). About two-thirds (more than 60%) of sickle cell patients with abnormal renal manifestations progress to developing CKD, and up to 10 – 12% end up with ESRD (Zahr & Saraf, 2023). Although newborn screening and early interventions have improved the survival rates of SCD children (Galadanci et al., 2024), surviving adolescents and adults are faced with age-related complications. Increasing cases of kidney diseases are now a significant health issue in this population.

In Ghana, kidney disease burden among the SCD population shows up in children through to adult patients (Osei-Yeboah & Rodrigues, 2011). Pediatric cohorts have reported early urinary abnormalities and the prevalence rises with age, with proteinuria around 26% and CKD near 40%, highest in HbSS cohorts (Anto et al., 2019; Osei-Yeboah & Rodrigues, 2011). Adult steady-state cohorts commonly reports microalbuminuria (~33%) and increased early kidney injury biomarkers (e.g., serum NGAL, fibrinogen-to-albumin ratio)(Twumasi et al., 2025). Clinic-based studies estimated CKD by reduced eGFR at ~39% overall (~ 68% in adults; ~32% in children). The burden of SCD kidney diseases in Ghana prompts the need for routine screening and longitudinal renal function monitoring in Ghanaian SCD care (Ephraim et al., 2015).

2.2.2 Susceptibility to kidney injury in SCD.

The sickling phenotype of red blood cells leads to two adverse outcomes, namely vaso-occlusion and haemolysis (Kato et al., 2017). These two are cardinal pathophysiological events that make patients susceptible to kidney injury compared to non-SCD individuals. Anaemia, an outcome of intense haemolysis, a common haematological abnormality in SCD, is an independent risk factor for AKI and predisposes patients to CKD and ERSD (Aban et al., 2017; Baddam et al., 2017; Lebensburger et al., 2016; McClellan et al., 2012) . Low Hb levels are associated with a high incidence of AKI. Patients with significantly low Hb during hospital stays develop AKI compared to those with relatively steady Hb levels (Chaturvedi et al., 2016; Saraf et al., 2018). Severe anaemia (Hb: 7-9 g/dl) was observed 71% of SCD patients with ERSD compared to 25% of non-SCD patients with ERSD (McClellan et al., 2012).

Furthermore, anaemia and loss of systemic vascular resistance are common features in SCD patients at an early age. This leads to increased renal blood flow and a high glomerular filtration

rate (GFR) (Afangbedji & Jerebtsova, 2022). A high glomerular filtration rate (hyperfiltration) is associated with the loss of plasma proteins in the urine, a common clinical phenomenon among SCD children (Audard et al., 2017). Early-stage proteinuria in the form of microalbuminuria (urine albumin 30–300 mg/g creatinine) increases with age as patients develop macroalbuminuria (urine albumin >300 mg/g creatinine) as they grow older (Audard et al., 2017; Vazquez et al., 2014). The prolonged hyperfiltration phenomenon in young patients causes damage to glomerular structures and predisposes them to develop focal segmental glomerular sclerosis (FSGS). This is a common histopathological change in kidney biopsies of SCD children (Zahr et al., 2019).

At later stages of disease progression, reduced blood flow caused by vaso-occlusion is evident within renal blood vessels. Vaso-occlusion reduces blood flow and leads to ischemia. Re-occurring episodes of ischemia-reperfusion trigger painful crises, a major cause of hospitalization among patients. Vaso-occlusion also develops fatal cardiopulmonary events like pulmonary hypertension and acute chest syndrome (ACS) (Sundd et al., 2019). The SCD kidney is vulnerable and has a substantial risk of developing AKI in situations of underlying lung and heart conditions. Patients hospitalized for vaso-occlusive crisis and acute chest syndrome have an increased incidence of suffering AKI. Reports indicate about 14% of adults (Audard et al., 2010), 8% of children with ACS (Lebensburger et al., 2016), and 49% of children in crisis (Batte et al., 2022) develop AKI. Moreover, occlusion in renal blood vessels generates reactive oxygen species, increased oxidative stress, a disruption to the endothelial barrier, mesangial proliferation, and thickening of the glomerular basement membrane (Afangbedji & Jerebtsova, 2022; Nath & Hebbel, 2015).

Predisposing factors such as infections, rhabdomyolysis, dehydration, and the use of non-steroidal analgesics have also been implicated in SCD-AKI development (Ataga et al., 2022).

2.2.3 Haemolysis: a cardinal pathophysiological event that mediates AKI.

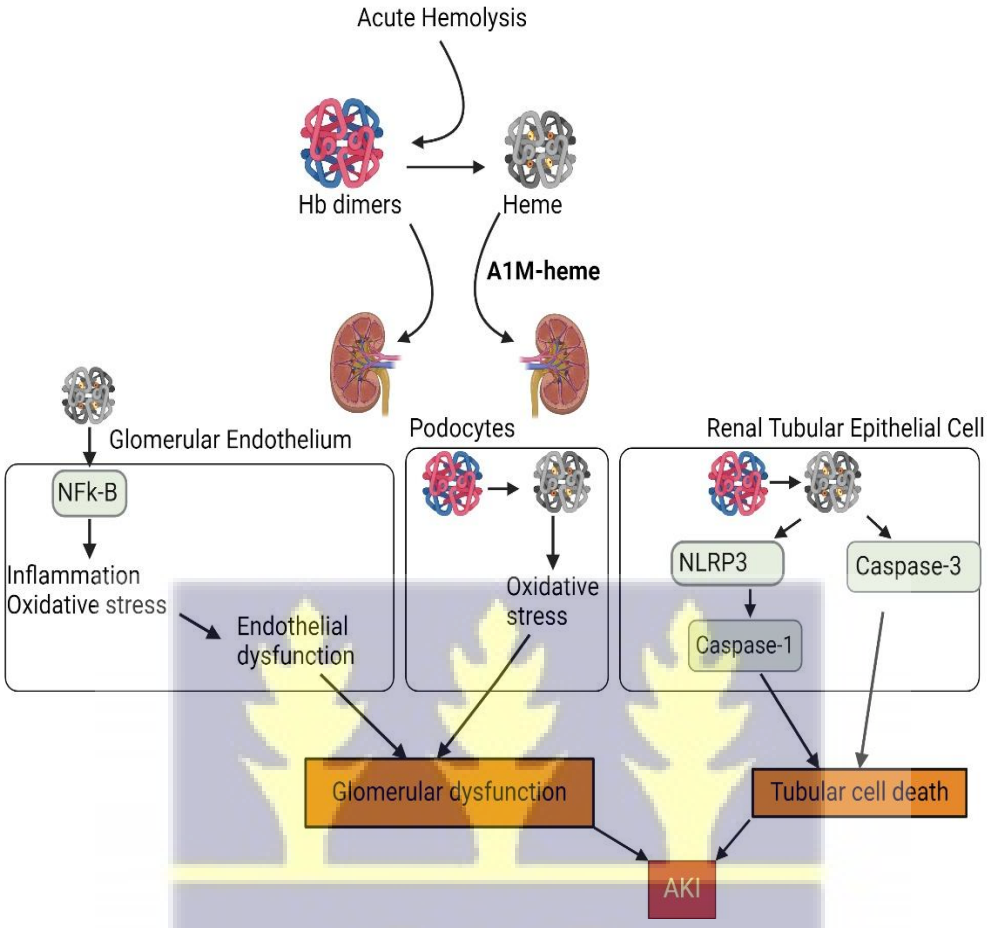

Diagram modified from Ghosh, Samit. (2022). Kidney Injuries in Sickle Cell Disease. IntechOpen. doi: 10.5772/intechopen.102839.

Figure 1 Haemolysis is a risk factor for acute kidney injury.

Haemolysis causes the release of excess cell-free Hb and its derivative heme into the bloodstream. Cell-free Hb and heme bound to A1M mediate kidney injury when exposed to the major functional cells of the kidney. Heme triggers inflammatory cascades that cause glomerular endothelial dysfunction. Endocytosis of Hb dimers and A1M-heme by podocytes and tubular epithelial cells induce cytotoxic pathways leading to podocyte and tubular cell death. Tubular cell death and/or glomerular damage develop AKI.

2.2.4 SCD kidneys are disproportionately exposed to haemolytic products.

Intravascular haemolysis, a pathophysiological event in SCD, increases plasma cell-free Hb and arginase levels. Haptoglobin (Hp) is an endogenous plasma protein that clears cell-free Hb from circulation for degradation (di Masi et al., 2020). SCD individuals have an acquired deficiency of haptoglobin (Santiago et al., 2018). Excess cell-free Hb accumulates in the blood due to insufficient clearance by haptoglobin. Circulating in an oxidative environment, cell-free Hb is oxidized to methaemoglobin (metHb) and the release of ferric heme (Mandal et al., 2020). Hemopexin (Hx), a plasma heme scavenger, binds heme to form a Hx-heme complex, which is transported to the liver for enzymatic degradation by heme oxygenase-1 (HO-1) (Montecinos et al., 2019). Hemopexin is also deficient in SCD (Santiago et al., 2018). Therefore, the SCD vascular milieu is characterized by increased circulating excess cell-free Hb and heme levels. A compensatory secondary heme scavenger, alpha-1 microglobulin (A1M), is elevated when hemopexin activity is overwhelmed in SCD (Saraf, 2020). Studies have shown the preferential transport of the A1M-heme complex to the kidney rather than the liver, as in the case of hemopexin (Larsson et al., 2004; Ofori-Acquah et al., 2020). A high hemopexin-A1M ratio is seen in SCD patients compared to individuals without haemolytic diseases. This is identified as a risk factor for AKI. Within the glomerulus, circulating cell-free Hb dimers and A1M-heme complexes are filtered through the glomerulus into the renal tubular lumen. An abnormally high level of haemoglobin in urine is a common clinical manifestation in AKI patients with underlying anaemia or haemolysis (Deuel et al., 2016).

2.2.5 Heme-mediated renal tubular injury and cell death.

Acute tubular injury is one primary pathological manifestation of AKI. Within the renal tubular lumen, filtered Hb dimers and A1M-heme complexes are taken up by renal tubular epithelial cells (RTECs) using megalin and cubulin receptors (Gonzalez-Michaca et al., 2004). The internalized Hb dissociates into heme. The degradation of heme within RTECs is critical in mitigating tubular cell death and subsequent AKI development (Saraf et al., 2018).

The heme-degrading enzyme HO-1 is key in neutralizing the harmful effects of heme in RTECs (Grunenwald et al., 2021). HO-1 breaks down heme into iron (Fe), carbon monoxide (CO), and biliverdin, countering heme-mediated pathological outcomes (Lever et al., 2016). Previous studies have shown that rapid induction and increased activity of HO-1 under haemolytic stress is protective against kidney injury (Lever et al., 2016; Li et al., 2021; Nath et al., 2000; Nath, 2014). However, in SCD, the excess heme protein presence within the RTECs can overwhelm activity in HO-1. Also, carriers of specific polymorphisms in the HO-1 gene (*HMOX1*) promoter with low HO-1 expression have a high risk of AKI. (Saraf et al., 2018).

In situations of insufficient heme degradation, as in the case of SCD, excess heme within the RTECs induces multiple lethal pathways that lead to tubular cell death. Heme induces caspase-3-mediated apoptosis in RTECs (Gonzalez-Michaca et al., 2004). Also, heme induces the NLRP3 inflammasome pathway, which releases pro-apoptotic factor caspase-1 with subsequent secretion of pyroptotic pro-inflammatory signals IL-1 beta and IL-18 (Xiong & Zhao, 2024).

Besides damage to RTECs, podocytes, highly differentiated visceral epithelial renal cells, suffer a similar fate. Podocytes are critical components of the glomerular structure. Podocyte injury is accompanied by proteinuria and GFR decline. A reduction in GFR clinically defines AKI (Kellum et al., 2021). One study has shown that podocytes, when exposed to Hb, have increased oxidative

stress and undergo apoptosis, leading to cell dysfunction (Rubio-Navarro et al., 2018). Focal segmental glomerulosclerosis (FSGS), a histopathological lesion typical in SCD, arises from multiple AKI events (Nath & Hebbel, 2015).

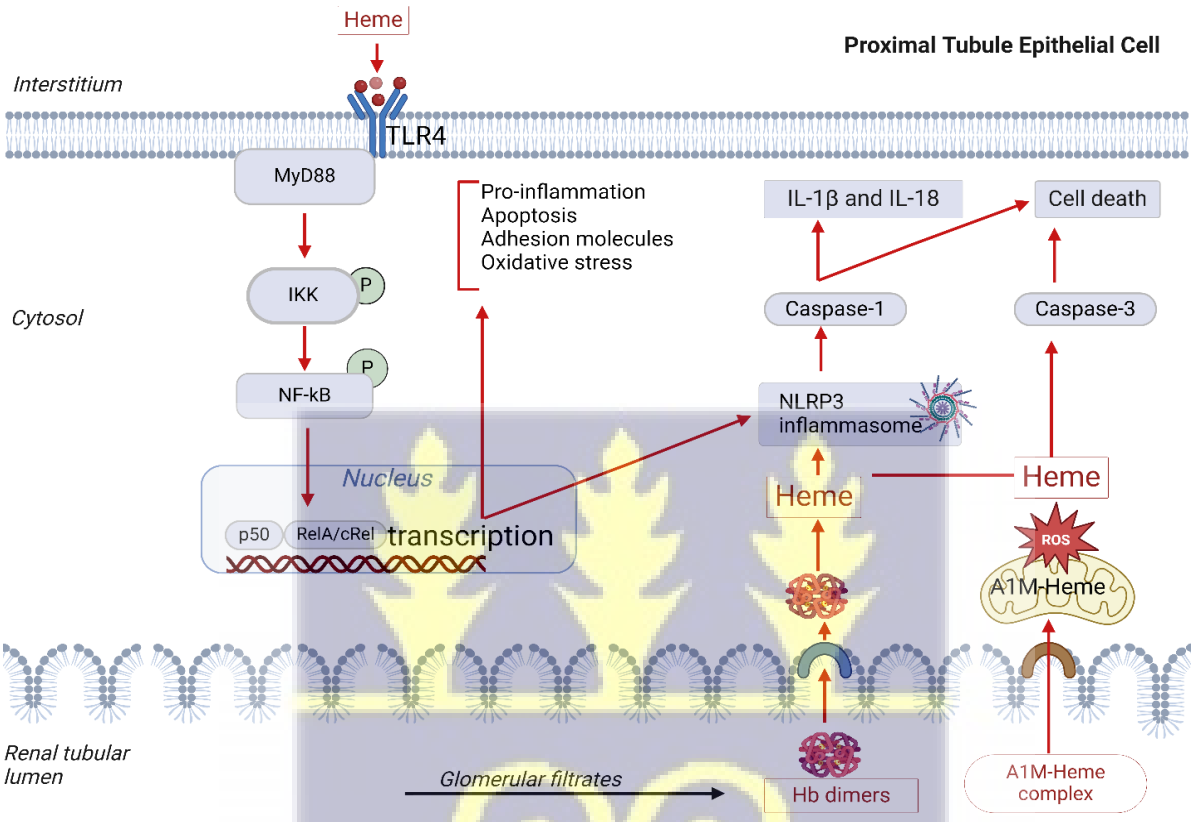

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Figure 2 Heme-mediated renal tubular epithelial cell injury.

Haemolysis causes the release of cell-free Hb and heme derivatives. Excess cell-free Hb and heme-bound alpha-1 microglobulin (A1M) pass through glomerular filtration and are internalized into renal tubular epithelial cells. Undegraded heme mediates direct or indirect reactive oxygen species (ROS) activation of apoptotic NLRP3 inflammasome/caspase-1 and caspase-3 pathways. This leads to tubular cell death and release of .pro-inflammatory IL-1β and IL-18. On the other hand, heme in the interstitial space binds to its receptor toll-like receptor 4 (TLR4). Heme binding triggers the NF-kB pathways of pro-inflammatory, oxidative stress, and cell death.

2.2.6 Heme-mediated renal endothelium injury and cell death.

In SCD, inherent chronic haemolysis, heightened inflammatory milieu, and hypercoagulative state contribute to endothelial dysfunction (Ofori-Acquah, 2020). Endothelial activation, interaction with blood components including red blood cells, leucocytes, platelets, cytokines, and reactive species, injures the endothelium. A breakdown of endothelial barrier and obstruction to blood flow affects internal organs (Zhang et al., 2016). The etiology of AKI and CKD involves the glomerulus and intrarenal blood vessels, which are relevant functional structures of the kidney.

Glomerular endothelial dysfunction affects podocytes, which maintain the integrity of the glomerular molecular sieve barrier. Proteinuria occurs when podocytes are damaged and this is associated with levels of plasma haemolytic markers and haemoglobinuria in SCD patients (Day et al., 2012; Hamideh et al., 2014).

Extensive peritubular microvascular congestion and chronic thrombotic microangiopathy are observed in kidney biopsies of SCD patients. CKD develops by peritubular microvascular rarefaction, interstitial fibrosis, and tubular atrophy (Bissonnette et al., 2016; Kida et al., 2014; Lusco et al., 2016). Histopathological changes like rarefaction and interstitial fibrosis are closely related to endothelial injury in the renal peritubular capillaries (Bábíčková et al., 2017). Endothelial injury to renal peritubular capillaries disruption may also cause a split, leading to hematuria through extravasation of red blood cells (Ataga et al., 2022).

Heme initiates disruption to the endothelial barrier in various organs, including the kidney (Belcher et al., 2014; Chen et al., 2024; Ghosh et al., 2012, 2013; James et al., 2020; Meegan et al., 2021; Santaterra et al., 2020). Heme activates toll-like receptor 4 (TLR4) on endothelial cell surfaces (Belcher et al., 2014). Heme-TLR4 activation triggers an inflammatory milieu involving increased production of pro-inflammatory cytokines, vascular permeability, increased expression of

adhesion molecules, and increased leucocyte infiltration into the interstitial space. (Habib, 2021; Ma et al., 2019). Endothelin-1(ET-1), generated by endothelial cells under heme or inflammatory challenge, causes endothelial injury via reactive oxygen species or by reducing nitric oxide bioavailability (Heimlich et al., 2016). ET-1 mediates podocyte injury in sickle mice by binding the endothelin A (ETA) receptor. Antagonists to ETA receptor have protective effects on the glomerulus in SCD mice (Kasztan et al., 2017). A study also observed that damage to the glomerular endothelium in SCD is associated with increased expression of soluble fms-like tyrosine kinase 1 (sFLT-1), a splice variant of vascular endothelial growth factor receptor-1 (VEGFR1) (Youssry et al., 2015). sFLT-1 promotes endothelial activation and dysfunction by blocking vascular endothelial growth factor (VEGF). VEGF maintains endothelial health by interacting with glomerular endothelium (Ataga et al., 2010; Youssry et al., 2015).

2.2.7 Current management and therapies for sickle cell kidney disease.

Protecting the SCD kidney is critical in facilitating the poor outcomes and early deaths suffered by patients (Obadina et al., 2023). The optimal treatment for sickle cell nephropathy is still lacking. Generally, current strategies for improving renal manifestations in SCD include targeting the underlying disease to reduce haemolytic events, release of Hb into plasma, or attenuating the damage induced by downstream effects of haemolysis and vaso-occlusion (Avondt et al., 2019).

Red blood cell (RBC) transfusions are administered for the acute or chronic management of SCD complications, including kidney injury. RBC transfusions, a whole cell replacement therapy, reduce the percentage of sickle red blood cells in circulation, improving haemoglobin concentration and oxygen delivery (Howard, 2016). Chronic RBC transfusions have been shown to protect against endothelial injury (Hyacinth et al., 2014). In the past, transfusion therapy has

been used to enhance osmolality and improve urine concentrating ability for SCD children (Audard et al., 2017). These reports have been supported by recent observations where having chronic transfusion therapy at early ages is associated with low incidence of microalbuminuria (Alvarez et al., 2006). However, another study assessing the risk of CKD in children did not find a significant relationship between blood transfusion and low prevalence of microalbuminuria (Lebensburger et al., 2011).

Hydroxyurea is currently the standard disease-modifying therapy for sickle cell patients (Thein & Howard, 2018). Hydroxyurea therapy reduces the frequency of vaso-occlusive crises and pain in SCD (López Rubio & Argüello Marina, 2024). The mechanism of action of hydroxyurea includes induction of fetal haemoglobin (HbF) expression, reduction of leucocyte and platelet counts, and maintenance of nitric oxide bioavailability (McGann & Ware, 2015). Hydroxyurea therapy in a prospective study with SCA children normalized 44% of patients microalbuminuria excretion (McKie et al., 2007). Multivariate analyses found a low prevalence of albuminuria among patients on hydroxyurea therapy (Laurin et al., 2014). Children before the age of ten who are on hydroxyurea therapy have a low incidence of developing microalbuminuria compared to children who start therapy after the age of ten (Zahr et al., 2019). Hydroxyurea's effect on glomerular filtration rate has been conflicting; however, it has improved urine concentration (Alvarez et al., 2012; Aygun et al., 2013). Hydroxyurea's renoprotective effects are still not clear. More studies are needed to establish its role in protecting SCD kidney health. There are ongoing trials evaluating the protective effects of recently approved SCD drugs like crizanlizumab on kidney function among patients (Obadina et al., 2023).

Angiotensin converting enzyme inhibitors (ACE-inhibitors) have been used conventionally in treating albuminuria/proteinuria conditions and CKD (Xie et al., 2016). ACE inhibitors vasodilate

efferent arterioles to help decrease glomerular pressure. ACE inhibitors are widely used in treating sickle cell nephropathy to control albuminuria. For example, ACE-inhibitors lisinopril treatment among cohorts of SCD patients was found to decrease proteinuria significantly (Aleem et al., 2023). Also, the use of ACE-inhibitor losartan in SCD patients with albuminuria had positive outcomes of reducing albuminuria and stabilizing GRF (Quinn et al., 2017; Yee et al., 2018). Testing for the synergistic protective effect of ACE inhibitors and SCD-related therapies has been proposed (Obadina et al., 2023).

Emerging therapies like Endothelin (ET)-1 antagonists have been investigated in sickle cell mice models (Kasztan et al., 2017) and are currently being evaluated in a phase I trial (NCT02712346) in SCD. ET-1 is a peptide that binds its receptor Endothelin A (ETA) to trigger pro-inflammatory, vasoconstriction, and pain in SCD. Increased serum ET-1 expression and urinary excretion are seen in SCD patients, positively correlating with the urinary albumin-creatinine ratio (Ataga et al., 2016). Overall, current approaches to managing sickle cell nephropathy and potential drugs in trials have yet to be shown to be fully effective.

2.2.8 Future research perspectives in mitigating haemolysis-mediated kidney injury and disease.

Clinical data and animal studies emphasize the link between haemolysis, the damaging role of heme and cell-free Hb, and kidney injury. However, the precise mechanisms of this association have not been established. Mechanistic studies are encouraged to help develop targeted therapies in the future.

Infiltration and accumulation of leucocytes and platelets are common in SCD kidneys. Understanding the mechanisms that interactions of blood cells with renal endothelium lead to AKI and CKD is of importance to research (Ataga et al., 2016; Sarakpi et al., 2023).

Again, podocytes maintain glomerular endothelial integrity via intra- and intercellular signaling. Attenuating podocyte injury as a strategy to maintain glomerular integrity is yet to be fully explored (Jiang et al., 2024; Rubio-Navarro et al., 2018).

Heme mediates multiple lethal pathways by activating TLR4, of which their role in SCD kidney injury is unknown. The role of heme-mediated activation of the inflammasome and subsequent induction of AKI still lacks clarity. Again, future studies should delineate relevant biomarkers involved in heme signaling that are critical to the development of AKI (Jana et al., 2022). Identifying specific biomarkers involved in the transition of AKI to CKD is warranted for developing therapeutics to protect patients from progressive renal complications.

Heme oxygenase-1 (HO-1) role in neutralizing heme is clearly defined (Lever et al., 2016). However, the mechanism of cellular induction and regulation of HO-1 during AKI events in SCD is limited (Nath & Agarwal, 2020). To guide future therapeutic development, further studies are needed to establish the role of HO-1in SCD kidneys.

2.3 Neuregulin-1; a signaling molecule that mediates anti-inflammatory and cytoprotective pathways.

2.3.1 Neuregulin-1.

Neuregulin-1 (NRG-1), a signaling molecule, belongs to the family of epidermal growth factors (EGF), including Neuregulin-2, Neuregulin-3, and Neuregulin-4 (Cespedes et al., 2018). The human *NRG1* gene, located on chromosome 8, makes multiple isoforms through alternative splicing (Shi & Bergson, 2020). NRG-1 α and β isoforms are the two main isoforms containing common amino-terminal segments, but distinct α or β variant sequences in their EGF-like domains (Cespedes et al., 2018).

NRG-1 is primarily expressed by endothelial cells in response to several stimuli, such as inflammatory insult, oxidative stress, tissue injury, and cell damage (Noll et al., 2024). Other cell types including neurons, Schwann cells, cardiomyocytes, skeletal muscle cells, and epithelial cells have all been reported to express NRG-1. The physiological role of NRG-1 is diverse, including vascular homeostasis, nerve development, cardiac repair, muscle function, and breast development, among others. The homeostatic role of NRG-1 in both human and animal studies highlights its beneficial role in maintaining homeostasis (Cespedes et al., 2018; Wang et al., 2022).

2.3.2 Neuregulin-1 signaling.

In response to inflammatory insult, oxidative stress, tissue injury, and cell damage, NRG-1 is released by endothelial cells to function as a paracrine or autocrine response factor. Neuregulin-1 acts through binding and activating the epidermal growth factor family of receptor tyrosine kinases (EGFR), specifically Erb-b2 receptor tyrosine kinase 4 (ErbB4) and Erb-b2 receptor tyrosine kinase 3 (ErbB3). NRG-1-receptor interaction triggers homodimerization of two ErbB4 receptors or heterodimerization of ErbB2 with ErbB4 (ErbB4/2) or ErbB3 (ErbB3/2). Activation of its receptor drives downstream signaling pathways that modulate inflammation, oxidative stress, cell

differentiation, proliferation, survival, and cell death (Cespedes et al., 2018; Li et al., 2011; Ou et al., 2021).

ErbB4:ErbB4 homodimers or ErbB2:3, ErbB2:4 heterodimers upon activation, trans or auto phosphorylate tyrosine residues in their cytoplasmic tail, which become docking sites for downstream signaling molecules. NRG-1 activates two important downstream homeostatic pathways, namely Phosphoinositide 3-kinases (PI3K)/Protein Kinase B (AKT) and Rapidly Accelerated Fibrosarcoma/Mitogen-Activated Protein Kinases (RAF/MAP) pathways (Borg et al., 2000; Citri et al., 2004; Xu et al., 2001, 2005). This triggers downstream anti-inflammatory, antioxidant, and survival mechanisms that define its protective function.

2.3.3 Anti-inflammatory effects of neuregulin-1.

The anti-inflammatory role of NRG-1 has been explored in different organs, specific cell types, and disease models. Table 1 summarizes studies demonstrating its ability to prevent pro-inflammatory responses in haemolytic, ischemic, and other conditions.

2.3.4 Antioxidant and cytoprotective function of NRG-1.

The cytoprotective role of NRG-1 is evident in several studies where NRG-1 promotes cell survival in different *in vitro* and *in vivo* disease models. Table 2 summarizes studies reporting on the pro-survival functions of NRG-1.

Table 1 Neuregulin-1 treatment activates multiple anti-inflammatory pathways.

Disease condition/model	Method	Mechanism	Outcome	Reference
Cerebrovascular				
Blood-brain barrier (BBB) disruption/injury	1. *In vitro:* Cytokine-induced BBB disruption - rat brain microvascular cells pre-treated with NRG-1 and incubated with inflammatory cytokine IL-1β.		↓ Permeability	(Lok et al., 2012)
	2. *In vitro*: Heme and ischemia-induced BBB inflammation: human brain microvascular cells and astrocytes pretreated with NRG-1 under ischemic stress or heme challenge.	Increased ErbB4 expression	↓ Pro-inflammation (CXCL-1, CXCL-10) ↓ Adhesion (ICAM-1, VCAM-1) ↓ Angiogenesis (VEGF-A)	(Chambliss et al., 2023)
	3. *In vitro*: Cytokine-induced endothelial injury - human brain microvascular endothelial cells (BMECs) treated with IL-1β and co-incubated with NRG-1.		↓Adhesion (E-selectin, VCAM-1, neutrophils)	(Wu et al., 2015)
	4. *In vitro*: Oxygen-glucose deprivation (OGD) stress on astrocytes – NRG-1 treatment under OGD.		↓ Pro-inflammation (TNF-α, IL-1β)	(Wang et al., 2012)
Neuroinflammation	1. *In vitro:* Lipopolysaccharide (LPS)-induced microglia inflammation - murine microglial cell line treated NRG-1 and LPS	Increased ErbB4 expression	↓ I Pro-inflammation (TNF-α, IL-6)	(Mencel et al., 2013)

	2. *In vitro:* Cytokine-induced microglia inflammation - Primary cerebral cortex microglial cells from mice were treated with IFN-γ, TNF-α, and NRG-1		↓ Pro-inflammation (TNF-α, IL-6, CD86) ↑ Phagocytosis	(Shahriary et al., 2019)
	3. *In vitro;* LPS-induced microglia inflammation - murine microglial cell line treated NRG-1 and LPS	Inhibits NF-kB signaling pathways	↓ Pro-inflammation (TNF-α, IL-6)	(Simmons et al., 2016a)
	4. *In vitro*; Amyloid Beta Peptide (Aβ)-stimulated microglia inflammation - BV2 microglial cell line treated with Aβ and NRG-1	Inhibits NF-kB signaling pathways	↓ Pro-inflammation (TNF-α, iNOS) Anti-inflammatory phenotype switch (M1 to M2)	(Ma et al., 2022)
	5. *In vivo*: Hippocampal inflammation in rats treated with NRG-1	Increased ErbB4 activation ErbB4/ TACE/ADAM17 activation	↓Pro-inflammation (IL-1α, TNF-α, IL-6, IL-33, GM-CSF, CCL-2, IFN-γ)	(Vega-Torres et al., 2022)
	6. *In vivo*: Neuroinflammation induced in mouse cerebellum via LPS injection with NRG-1	Activates ErbB4/PI3/AKT signaling	↓ Pro-inflammation (Iba-1, GFAP, IL1-β and TNFα.)	(Xu et al., 2017)
	7. *In vivo;* Neurotoxin-mediated inflammation on rat brain – NRG-1 treatment in rats following neurotoxin diisopropylflurophosphate (DFP) intoxication		Suppressed microglial activation ↓ Pro-inflammation (IL-1β, IL-6)	(Li et al., 2015)
Ischemic stroke	*In vivo:* Ischemia-induced inflammatory responses - Injection of rats with NRG-1 before middle cerebral artery occlusion.		Prevents microglial infiltration and activation ↓Pro-inflammation (IL-1β, HSP70, MCP-1)	(Xu et al., 2005)

Cardiovascular				
Cardiac endothelial barrier loss/ dysfunction	*In vivo*: Sepsis-induced loss of endothelial barrier – LPS administered to rats to induce sepsis with NRG-1 treatment.	Inhibits the activation of RhoA/ROCK signaling	↓ Cardiac vascular permeability (vWF) ↓ Endothelial activation (ICAM-1, VEGF) ↓ Pro-inflammation (TNF-α, IL-6)	(Kang et al., 2019)
Septic cardiomyopathy	*In vivo*: Sepsis-induced myocardial injury - cecal ligation and puncture model septic rats treated with NRG-1		↓ Pro-inflammation in myocardium (Interstitial edema, inflammatory cell infiltration, MIF, and Ang II) ↓ Pro-inflammation in serum (TNF-alpha, IL-1β, and IL-6)	(Zhou et al., 2016)
Immunomodulation				
Macrophage anti-inflammatory phenotype polarization	*1. In vitro; Reparative inflammatory response – Macrophage treated with NRG-1 microparticles*		↑ Regenerative phenotype (CD206+ cells) ↓ Inflammatory phenotype (B7-2+ cells)	(Pascual-Gil et al., 2019)
	2. In vitro; NRG-1 immunomodulatory effect - U937 monocytic cells were activated by incubation with PMA and treated with NRG-1		↓ Pro-inflammatory and stress gene expression (COX-2)	(Xu et al., 2004)
	3. Ex vivo; NRG-1 immunomodulatory effect – PBMNCs from subjects with heart	ErbB3/PI3K signaling	↓ Pro-inflammation (TNF-α)	(Ryzhov et al., 2017)

	failure cultured with LPS and NRG-1			
Adaptive immunity regulatory phenotype polarization	*In vivo;* NRG-1 effects on adaptive immunity response following injury in rats - -	NF-κB pathway inhibition	Stimulated a regulatory phenotype in T and B cells (Treg cells, Breg cells) ↑ Anti-inflammatory (CXCL-10)	(Alizadeh et al., 2018)
Others				
Sepsis-induced diaphragm dysfunction	*In vivo;* Sepsis-induced diaphragm dysfunction - caecal ligation and puncture model of sepsis in rats treated with NRG-1	PI3K-Akt Signaling enhanced	↓ Pro-inflammation (CXCL8 and IL-6)	(Liu et al., 2020)
Pulmonary inflammation	*In vivo:* Inflammation-induced lung injury – acid-induced pulmonary inflammation	ErbB4/ADAM17 activation	↓ Pro-inflammation (TNF-α, IL-1β, and IL-6)	(Dreymueller et al., 2014)

Abbreviations: *BBB - Blood-brain barrier; IL-1β - Interleukin-1 beta; BMECs - Human brain microvascular endothelial cells; OGD - Oxygen-glucose deprivation; LPS - Lipopolysaccharide; IFN-γ - Interferon-gamma; Aβ - Amyloid Beta Peptide; DFP - Diisopropylfluorophosphate; ErbB4 - Erb-B2 receptor tyrosine kinase 4; NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells; TACE/ADAM17 - Tumor necrosis factor-alpha converting enzyme / A disintegrin and metalloproteinase domain 17; PI3K/AKT - Phosphoinositide 3-kinase / Protein kinase B; CXCL-1 - C-X-C motif chemokine ligand 1; CXCL-10 - C-X-C motif chemokine ligand 10; ICAM-1 - Intercellular adhesion molecule 1; VCAM-1 - Vascular cell adhesion molecule 1; VEGF-A - Vascular endothelial growth factor A; TNF - Tumor necrosis factor; IL-6 - Interleukin 6; CD86 - Cluster of Differentiation 86; iNOS - Inducible nitric oxide synthase; M1 - Classically activated macrophages (pro-inflammatory); M2 - Alternatively activated macrophages (anti-inflammatory); IL-33 - Interleukin 33; GM-CSF - Granulocyte-macrophage colony-stimulating factor; CCL-2 - C-C motif chemokine ligand 2; MCP-1 - Monocyte chemoattractant protein 1 (another name for CCL-2); HSP70 - Heat shock protein 70; ZO-1 - Zonula occludens-1; Bax - Bcl-2-associated X protein; Bcl-2 - B-cell lymphoma 2; vWF - von Willebrand factor; MIF - Macrophage Migration Inhibitory Factor; Ang II - Angiotensin II; PMA - Phorbol 12-myristate 13-acetate; COX-2: Cyclooxygenase-2; PBMNCs - Peripheral blood mononuclear cells. ↑ - Increase; ↓- Decrease.*

Table 2 Neuregulin-1 treatment activates multiple antioxidant and cell survival pathways.

Disease condition/model	Method	Mechanism	Outcome	Reference
Cerebrovascular				
Blood-brain barrier (BBB) disruption/injury	1. *In vitro*: Heme-induced BBB dysfunction - human brain microvascular endothelial (hCMEC/D3) and human neuroglial cells/astrocytes (M059 K) cells co-culture with NRG-1 and heme or CXCL10		↓ Apoptosis Attenuate tight junction disruption (↑ Claudin 5, Occludin, ZO-1)	(Chambliss et al., 2023; Liu et al., 2018)
	2. *In vivo*; experimental cerebral malaria (ECM) model (Plasmodium berghei ANKA in C57BL/6) treated with NRG-1	ErbB4 phosphorylation AKT activation STAT3 inactivation	↓ Mortality	(Liu et al., 2018)
Neurotoxicity/Oxidative injury	1.*In vitro*; Neuronal damage caused by activated immune cells - SH-SY5Y cells cultured with activated BV2 microglia and NRG-1	Inhibits NF-kB signaling pathways	↓ Apoptosis (Bax)	(Ma et al., 2022)
	2. *In vitro;* Oxidative stress-induced neuronal injury - Primary mouse neurons were treated with H_2O_2 or LPS or $CoCl_2$ or oxygen-glucose deprivation and with NRG-1	Activates ErbB3/PI3/AKT signaling Inhibits NF-kB signaling	↓ Apoptosis (caspase 3) ↑ Anti-apoptosis (ratio of Bcl2/Bax, Bcl-XL) ↓ Lactate dehydrogenase ↑Antioxidants (SOD, GPx-1)	(Jiang et al., 2016; Kim et al., 2020a; Lee et al., 2019; Lu et al., 2016; Xu et al., 2017)
	3. *In vitro*; Neuronal death induced by neurotoxins - PC12 cells treated with Aβ or MPP+ and NRG-1	Activation of PI3K/PKB/Akt and PKC	↑ Antiapoptotic protein BclxL ↓ Proapoptotic protein Bax	(Di Segni et al., 2005)

	4. *In vivo*; Injury mediated neuronal cell death - clip-compression model of spinal cord injured rats were treated with NRG-1	MAP kinase and AKT pathways	↓Apoptosis (cleaved caspases 3,8 and 9) ↓ Neuronal injury (HMGB1, E06, 4-HNE)	(Shahsavani et al., 2021)
	5. *In vitro*; Amyloid precursor protein-related Neurotoxicity - Primary cortical neuronal cells treated with Aβ and NRG-1	ErbB4 signaling.	↓ Lactate dehydrogenase ↓ Apoptosis (caspase-3 activation) ↑ Anti-apoptotic protein (Bcl-2)	(Cui et al., 2013; Woo et al., 2012; Yoo et al., 2021)
	6. *In vitro:* Oxidative stress-induced neuronal injury - N9 microglial cells treated with phorbol 12-myristate 3-acetate (PMA) with NRG-1		↓ Oxidative burst activity and nitrite release Protects against cell death	(Dimayuga et al., 2003)
Haemolytic brain injury/cell death	*In vitro* Heme-induced cortical brain injury - human brain cortical organoids treated with heme combined with NRG-1	ErbB4 activation	Improves structural disorganization Reduced cell death (apoptosis, necrosis)	(Harbuzariu et al., 2019)
Neurodegeneration	1. *In vivo*; Injury induced degeneration of oligodendrocytes - Spinal cord injury rat models treated with NRG-1	ErbB-receptor signaling	↑ New oligodendrocytes and preservation of existing ones ↑ Axonal preservation ↓ Astrogliosis ↓ Tissue degeneration	(Gauthier et al., 2013)

	2. In vivo: Effect of NRG-1 on neuronal proliferation and survival – NRG-1 was delivered into the developing rat brain.		Promote oligodendrocyte survival Reduced apoptosis	(Fernandez et al., 2000)
Ischemia-reperfusion brain injury/cell death	1. *In vivo*: Ischemia-induced neuronal damage, Injection of NRG-1 before middle cerebral artery occlusion models in rats	Decrease in JNK signaling activity.	Reduced abnormal morphological structures Reduced infarct volume Reduced apoptosis	(Ji et al., 2017; Li et al., 2007; Xu et al., 2004)
Cardiovascular				
Myocardial injury/damage	1.*In vivo;* Sepsis-induced myocardial damage – LPS administered to rats to induce sepsis with NRG-1 treatment.	Inhibits the activation of RhoA/ROCK signaling	Reduced apoptosis	(Kang et al., 2019)
	2.*In vitro;* Oxidative injury on cardiomyocytes – Human cardiac myocytes and neonatal rat cardiac myocytes exposed to H_2O_2 with oNRG-1	ErbB-PI3K/Akt signaling	Reduced apoptosis Reduce endoplasmic reticulum stress ↓ Lactate dehydrogenase ↑Antioxidant (SOD)	(Jie et al., 2012; Xu et al., 2014)
	3. *In vivo;* Myocardial infarction - rat model of heart model induced by myocardial infarction treated with NRG-1		↓Reactive oxygen species ↑Antioxidant (GPx-1)	(Guo et al., 2012)
Cardiotoxicity	*In vitro; D*oxorubicin (Dox)-induced cardiotoxicity – rat myocytes treated with doxorubicin and NRG-1	PI3K/Akt pathway	Reduced autophagy (LC3-II and Beclin 1) ↑ Anti-apoptosis (Bcl-2)	(An et al., 2013; Horie et al., 2010; Parry et al., 2017; Timolati et al., 2006)

Ischemic cardiac injury	1.*In vitro*; Hypoxia-reoxygenation (H/R). cardiac injury - myocyte cells cultured under conditions of H/R and NRG-1 expression 2.*In vivo;* Myocardial ischemia-reperfusion injury – rat model of ischemia-reperfusion injury treated with NRG-1	 ERK1/2	Reduced apoptosis Reduced myocardial infarct size ↓Oxidative stress (NOX4, 4-HNE) Inhibit NLRP3/caspase-1	(Hedhli et al., 2012) (Wang et al., 2021)
Microvascular dysfunction	*In vitro*; Homocysteine (Hcy) induced microvascular dysfunction – Rat cardiac microvascular endothelial cells treated with Hcy and NRG-1	Notch signaling pathway	Reduced apoptosis (Caspase-3) ↑Anti-apoptosis (Bcl-2/Bax ratio)	(Qin et al., 2023)
Others				
Ovarian Granulosa Cell Survival	*In vitro:* Protein kinase C inhibitor staurosporine (STS) induced cell death on Granulosa Cell in the presence of NRG-1		Reduced apoptosis (Caspase-3) ↑Anti-apoptosis (Bcl-2/Bax ratio)	(Chowdhury et al., 2017)

Abbreviations: *BBB - Blood-brain barrier; BMECs - Human brain microvascular endothelial cells; ErbB4 - Erb-B2 receptor tyrosine kinase 4; NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells; PI3K/AKT - Phosphoinositide 3-kinase / Protein kinase B; ZO-1 - Zonula occludens-1; Bax - Bcl-2-associated X protein; Bcl-2 - B-cell lymphoma 2; Bcl-XL - B-cell lymphoma-extra large; STAT3 - Signal transducer and activator of transcription 3. Aβ - Amyloid Beta Peptide; PKC - Protein kinase C; MPP+ - methyl-4-phenylpyridinium ion (MPP+); HMGB1 - High mobility group box 1; E06 - Anti-oxidized low-density lipoprotein antibody E06; 4-HNE - 4-Hydroxynonenal; JNK - c-Jun N-terminal kinase; LC3-II - microtubule-associated protein 1A/1B-light chain 3-II; Hcy – Homocysteine; H/R - Hypoxia-reoxygenation; PMA - phorbol 12-myristate 3-acetate; SOD - Superoxide Dismutase; GPx-1 – Glutathione Peroxidase-1; NOX4 - NADPH Oxidase 4; NLRP3 - NOD-like Receptor Pyrin Domain-Containing 3. ↑ - Increase; ↓- Decrease.*

2.4 Proposing Neuregulin-1 (NRG-1) as a therapeutic drug to mitigate haemolysis-mediated kidney injury in sickle Cell Disease.

The mechanism of action employed by NRG-1 in attenuating inflammation, oxidative stress, and cell death is through activation of ErbB4/(PI3K-Akt) or ErbB4/(/MAPK-Erk1/2) signaling pathways (Figure 3) (Brero et al., 2010; Chambliss et al., 2023; Di Segni et al., 2005, 2006; Giraud et al., 2005; Goldshmit et al., 2001; Jiang et al., 2016; Li et al., 2001; Liu et al., 2020; Liu et al., 2018; Mòdol-Caballero et al., 2018; Shahsavani et al., 2021;Wang et al., 2021; Xu et al., 2017; Yan et al., 2017; Yang et al., 2016; Yoo et al., 2021). Park (2021) demonstrated that NRG-1 through activation of ErbB4/(PI3K-Akt) or ErbB4/(MAPK-Erk1/2) signaling pathways triggers the nuclear translocation of nuclear factor erythroid 2 related factor 2 (Nrf2) (Figure 2). Nrf2 is a master homeostatic transcription factor whose downstream activity is critical for host cells' defense against oxidative and inflammatory damage (Park et al., 2021). NRG-1 signaling activity has been shown to upregulate relevant host defense factors that are typically downstream of activated Nrf2 functions; and this confirms that NRG-1 is a potent activator of Nrf2. Inhibition of the NF-κB pathway (Ma et al., 2022; Simmons et al., 2016), blocking the NLRP3/caspase-1 pathway (Wang et al., 2021), upregulating antioxidant enzymes (SOD, GPx) (Guo et al., 2012; Kim et al., 2020), promoting cell survival (Di Segni et al., 2005; Li et al., 2001; Ma et al., 2022; Wang et al., 2015; Woo et al., 2012; Xu et al., 2017), inhibiting caspase 3 cleaving (Kim et al., 2020; Wang et al., 2015; Woo et al., 2012; Yan et al., 2017; Yoo et al., 2021) are all downstream effects of NRG-1 signalling (Figure 3).

Nrf2 binds to the transcriptional regulatory enhancer sequence known as the antioxidant response elements (ARE). The antioxidant response element (ARE) is a DNA sequence responsible for regulating the expression of cytoprotective proteins that are antioxidant, anti-inflammatory, anti-apoptotic and pro-survival including heme oxygenase-1 (*HMOX1*) and NAD(P)H quinone

oxidoreductase 1 (*NQO1*) (Aranda-Rivera et al., 2022; Chen, 2022; Dodson et al., 2022; Farina et al., 2021; Bardallo et al., 2022; Murakami et al., 2023; Nezu & Suzuki, 2020; Piotrowska et al., 2021; Wang & He, 2022; Zgorzynska et al., 2021; Zhou et al., 2022).

The role of nuclear factor erythroid two related factor 2 (Nrf2) in sickle cell disease has been of interest in recent decades. (Belcher et al., 2017; Doss et al., 2016; Ghosh et al., 2011, 2016, 2018; Keleku-Lukwete et al., 2015, 2019; Li et al., 2019; Macari & Lowrey, 2011; Panda et al., 2019; Zhu et al., 2018, 2019). Studies have shown Nfr2 dual protective function in SCD. First, Nrf2 is counteracts the downstream effects of SCD disease mechanisms. Also, Nrf2 can induce fetal haemoglobin (HbF) expression, an important therapeutic target for SCD management (Li et al., 2019; Zhu et al., 2019).

Fetal haemoglobin induction inhibits HbS polymerization in hypoxic conditions, reducing sickling and subsequent vasocclusion and haemolysis (Steinberg, 2020).

Nrf2 activation by various agents in SCD murine models increased haemoglobin F (HbF) in circulating red blood cells (Krishnamoorthy et al., 2017), reduced blood white blood cell count (Doss et al., 2016), oxidative stress (Panda et al., 2019) and vascular stasis (Belcher et al., 2017). At the tissue level, reduced liver inflammation and injury (Keleku-Lukwete et al., 2019), reduced liver iron deposition and damage (Panda et al., 2019), improved lung injury (Ghosh et al., 2018), improved renal function (Doss et al., 2016) were observed when Nrf2 is activated.

2.5 Conclusion.

Chambliss *et al.* (2023) recently quantified NRG-1 levels in SCD children and mouse models. NRG-1 levels were elevated in both SCD children and mice compared to healthy controls (Chambliss et al., 2023). The elevated levels of NRG-1 are suggested to be a cytoprotective response to inflammation or oxidative damage caused by the disease. It is unclear whether the elevated level of NRG-1 is sufficient to mitigate disease-induced effects. However, administration of exogenous NRG-1 in a dose-dependent manner is shown to counteract heme-mediated inflammation, oxidative stress, and cell death (Chambliss et al., 2023; Harbuzariu et al., 2019; Liu et al., 2018; Solomon et al., 2014). This PhD thesis reports on the administration of NRG-1 in SCD mouse models to investigate its potential benefit in modulating kidney injury in SCD.

If NRG-1 therapy demonstrates the ability to mitigate haemolysis-induced kidney injury in sickle cell mice, then its translation to human disease can be facilitated. NRG-1 is already being used in clinical trials to increase cardiac function in patients with heart failure (Gao et al., 2010; Jabbour et al., 2011). Again, recombinant human NRG-1 therapy is reported to be safe and well tolerated in healthy subjects (Xu et al., 2023).

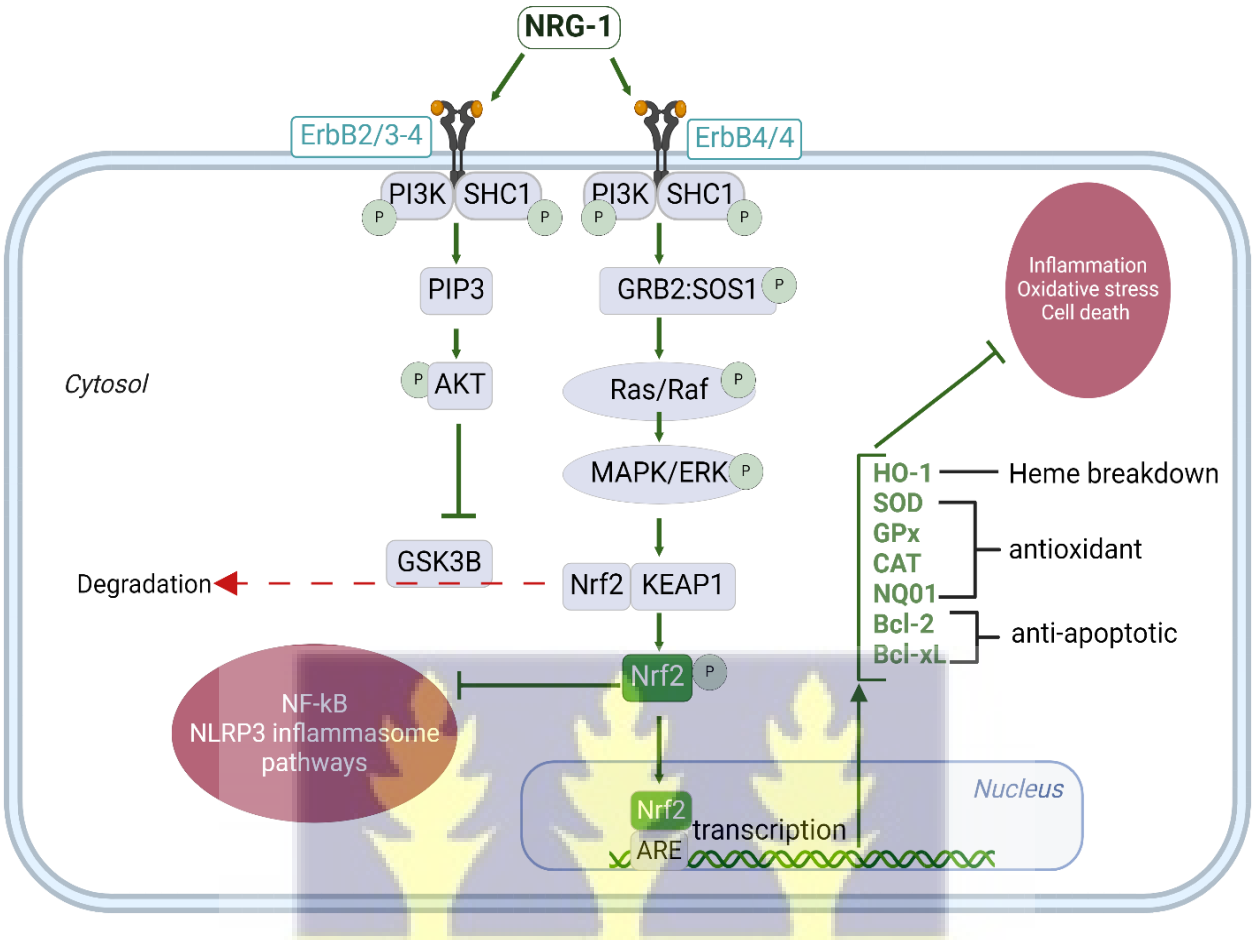

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Figure 3 Neuregulin-1 anti-inflammatory, antioxidant, and pro-survival signaling pathway.

Neuregulin-1 acts as a paracrine or autocrine stress response molecule by binding and activating its receptors Erb-b2 receptor tyrosine kinase 4 (ErbB4) and Erb-b2 receptor tyrosine kinase 3 (ErbB3). Downstream signaling pathways, Phosphoinositide 3-kinases (PI3K)/Protein Kinase B(AKT), and Rapidly Accelerated Fibrosarcoma/Mitogen-Activated Protein Kinases (RAF/MAPK/ERK) are subsequently activated by NRG-1. Activated ErbB/(PI3K-Akt) or ErbB/(MAPK-Erk1/2) pathways trigger the nuclear translocation of nuclear factor erythroid 2 related factor 2 (Nrf2). Nrf2 binds to the transcriptional regulatory enhancer sequence, antioxidant response elements (ARE). ARE transcribed genes are antioxidant, anti-inflammatory, and anti-apoptotic genes, which, are critical in maintaining homeostasis. Nrf2 inhibits the activation of the Nuclear Factor kappa-light-chain-enhancer of activated B (NF-Kb) pathway responsible for pro-inflammatory, oxidative stress and cell death induction.

2.6 Theoretical Framework

The theoretical framework guiding this thesis is based on the axis of haemolysis-mediated kidney injury and the renoprotective potential of NRG-1 in sickle cell disease (SCD). Chronic haemolysis in SCD results in persistent excess protein free heme in circulation. The exhaustion of hemopexin, the primary heme scavenger, is compensated by increased α-1-microglobulin (A1M), a secondary heme scavenger which transports heme to the kidneys instead of the liver. This maladaptive plasma heme clearance in SCD exposes the kidney to heme toxicity.

Heme uptake by renal tubular cells and activation of TLR4–NF-κB signaling on renal endothelium promotes pro-inflammation, oxidative stress and apoptosis that contributes to renal dysfunction, fibrosis, and tubular cell death. In contrast, circulating NRG-1 binding to its ErbB4 receptor activates the AKT and MEK/ERK pathways, which promote nuclear factor erythroid 2-related factor 2 (NRF-2)–mediated induction of cytoprotective, antioxidant and anti-apoptotic factors, while concurrently inhibiting NF-κB signaling. Together, these mechanisms counteract heme-mediated inflammatory, oxidative, and apoptotic pathways, thereby attenuating kidney injury (Figure 4).

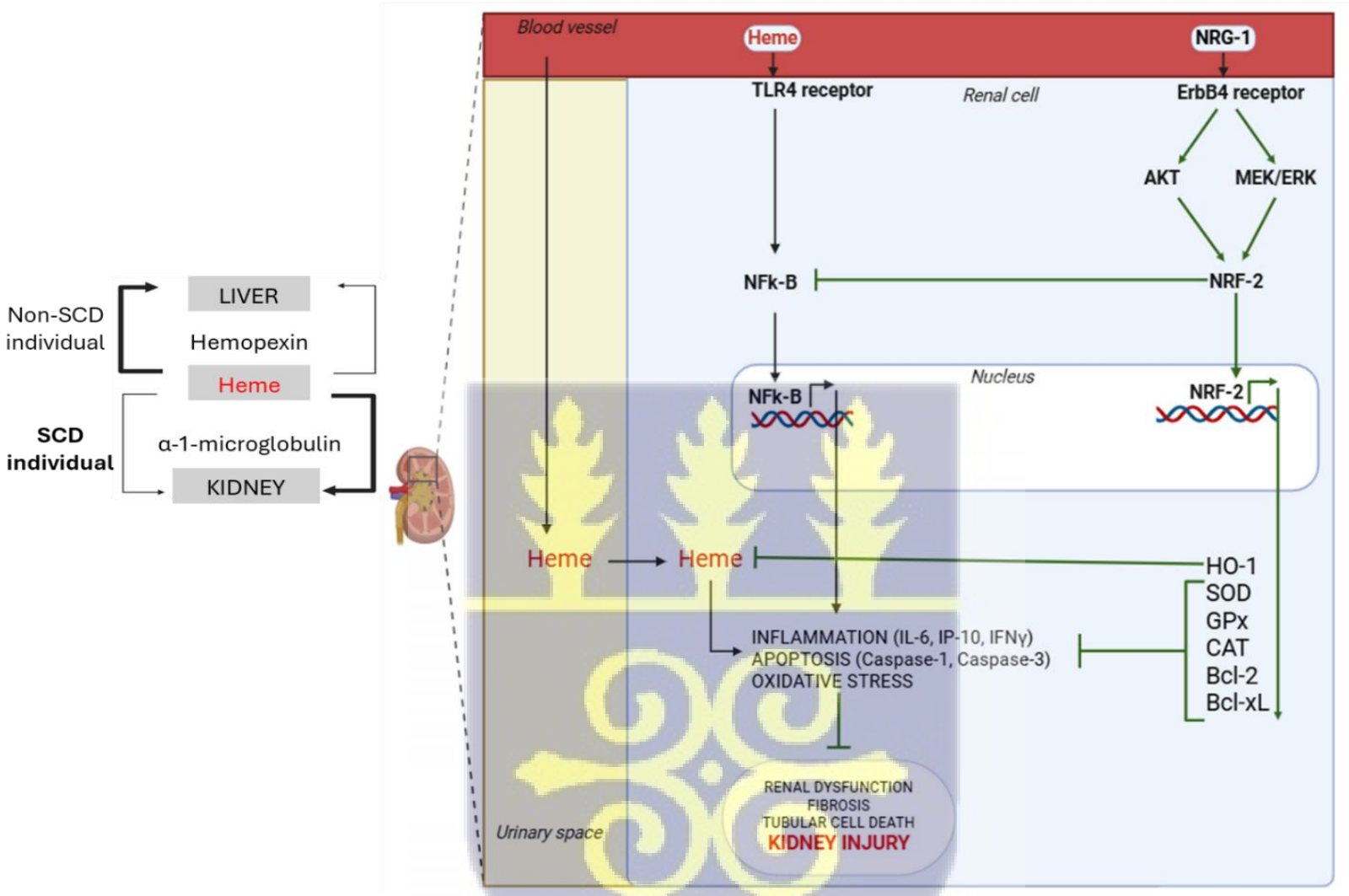

Figure 4. Mechanisms of heme-mediated kidney injury and renoprotective effects of NRG-1.

The diagram illustrates how maladaptive excess circulating heme handling in SCD leads to renal deposition, and activation of TLR4–NFκB signaling that promotes inflammation, apoptosis, and oxidative stress. It also shows the renoprotective role of Neuregulin-1(NRG-

1), which through ErbB4–AKT/MEK/ERK activation enhances NRF-2–dependent antioxidant and cytoprotective responses while inhibiting NFκB. ***Abbreviations:*** *SCD, sickle cell disease; A1M, α-1-microglobulin; TLR4, Toll-like receptor 4; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; IL-6, interleukin-6; IP-10, interferon gamma-induced protein 10 (CXCL10); IFNγ, interferon-gamma; NRG-1, neuregulin-1; ErbB4, Erb-B2 receptor tyrosine kinase 4; AKT, protein kinase B; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; NRF-2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma extra large*

3.0 CHAPTER THREE: METHODOLOGY

3.1 Mice work.

3.1.1 Ethical statement.

This research was conducted at the Center for Laboratory Animal Resources (CLAR), Morehouse School of Medicine, Atlanta, Georgia, USA. CLAR is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Mice handling, housing, and experimental procedures were conducted following the guidelines by the US National Research Council's Guide for the Care and Use of Laboratory Animals and recommendations stated in the book "Guide for the Care and Use of Laboratory Animals" (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). The Institutional Animal Care and Usage Committee (IACUC) at the Morehouse School of Medicine reviewed this project. It granted approval for all procedures involving animal use (IACUC Reference number: 22-14).

3.1.2 Animals.

Transgenic humanized Townes mice developed by Dr. Tim Townes laboratory (University of Alabama, Birmingham, USA) were purchased from the Jackson Laboratories (JAX stock #01307). This mouse model was established by replacing the endogenous adult mouse α-globin and β-globin genes with human α-globin and β-globin genes. The sickle cell Townes mouse model carries the genotype *HbSS* (Hba hα/hα::Hbb hAγβ^S/hAγβ^S) and phenocopies the severe haemolytic disease that is observed in human SCD (Nguyen et al., 2014; Wu et al., 2006). Townes sickle cell mice manifest disease characteristics like anaemia, a shortened half-life of red blood cells, and severe disease phenotype. This mouse model is widely used for studying the pathophysiology of SCD and assessing potential therapeutic interventions. The homozygous non-sickling Townes mice

have the genotype HbAA (Hba hα/hα::Hbb hAγβ^{A}/hAγβ^{A}), are phenotypically normal, and were used as controls for HbSS mice. Mice genotypes were validated by The Jackson Laboratory (JAX), and each mouse was identified with RapID tags based on genotype and sex upon arrival.

Fifty-six (56) Townes mice aged eight weeks were purchased for the study: forty (40) HbSS mice (20 males and 20 females), and sixteen (16) HbAA mice (8 males and 8 females). Mice were randomly allocated in equal numbers into study groups. Mice were housed by keeping four females per cage and two males per cage in microisolator cages (Supermouse Microisolator; Lab Products, Seaford, DE) with filter tops and lined with corncob bedding. The cages were kept in well-ventilated chambers with controlled temperature (23 ± 2 °C) and humidity (50 ± 10%). Mice were on a 12-hour light-dark cycle and given unrestricted access to food (PMI LabDiet 5001, St. Louis, MO) and bottled tap water. Before starting the study, mice were kept acclimatizing and growing to the desired age of 12 weeks.

3.1.3 Animal welfare.

Body weight and body condition assessment were used to monitor and identify signs of pain or distress beyond acceptable, humane endpoints. A digital weight scale was used to measure body weight twice weekly (Mondays and Thursdays). Measurements were taken at the same time in the morning between the hours of 7 am to 10 am. Body condition was assessed weekly as previously described (Burkholder et al., 2012). This approach is validated to evaluate the health status of mice because of its strong correlation with vital indicators of health status, such as white blood cell count and adjusted body weight. Briefly, the mice were positioned on a level surface, and the base of the tail was held with the thumb and index finger of one hand to evaluate body condition. The degree of flesh and fat cover was scored by running the little finger of the same hand over the

sacroiliac region or by palpating the sacroiliac region with the fingers of the opposite hand. An attending veterinarian/technician also independently checked on the animal's health weekly per the center's animal health policy.

3.1.4 Study design.

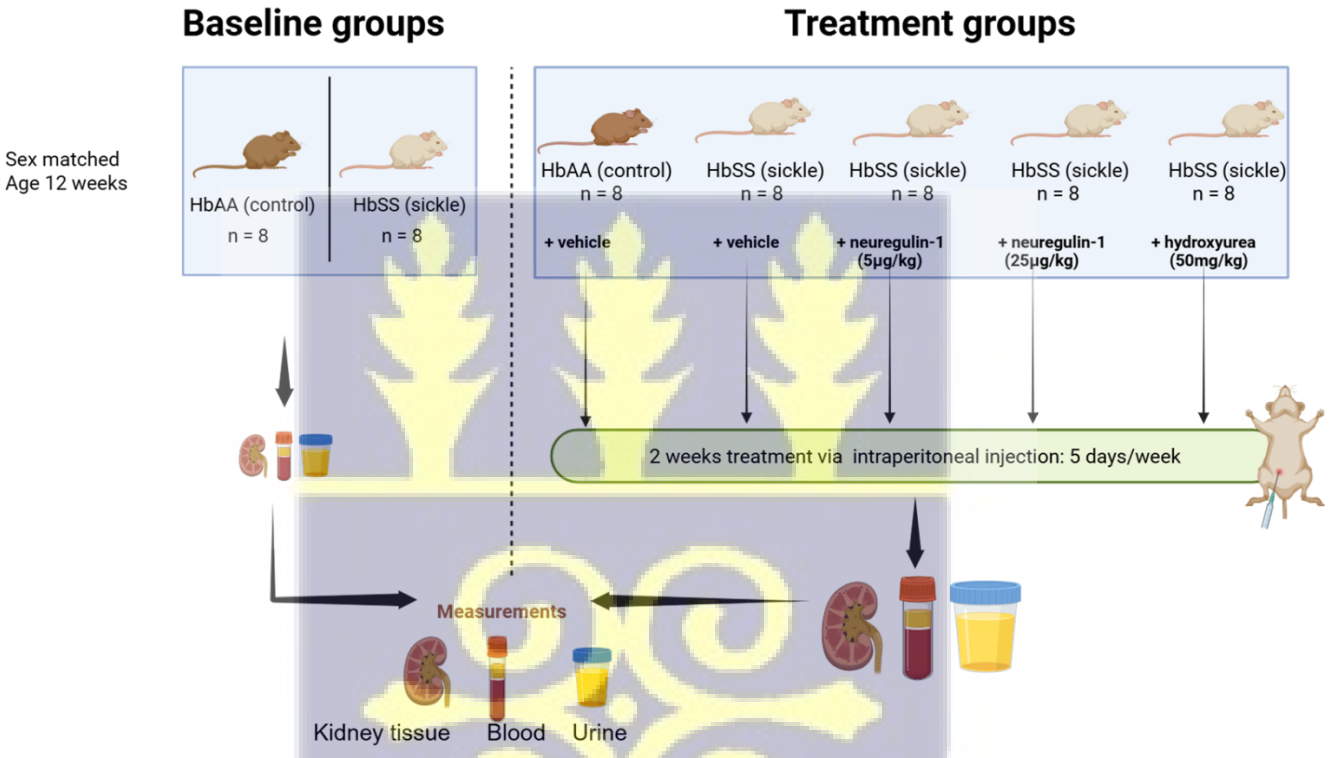

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Figure 5 Study design: Investigating the beneficial effects of neuregulin-1 and hydroxyurea on kidney injury in Townes sickle cell mice.

Twelve-weeks old Townes HbSS humanized sickle cell mice and genetic HbAA controls were randomized into groups (n=8) to evaluate the renal protective effects of neuregulin-1 (NRG-1) and hydroxyurea in SCD. Mice received either vehicle or NRG-1 at low (5 µg/kg) or high (25 µg/kg) and hydroxyurea (50 mg/kg) doses, administered intraperitoneally for two weeks. Urine and blood samples were collected for hematological, haemolytic, inflammatory and kidney injury biomarker

analysis, while renal tissues were processed and examined for histopathological changes and immunohistochemical quantification of heme degrading enzyme, heme oxygenase-1 expression.

3.1.5 Mice groupings

A cohort of 56 Townes mice at age eight weeks was randomly grouped as described:

1. Non-sickle baseline group (HbAA; 4 males, 4 females);

2. Sickle baseline group (HbSS; 4 males, 4 females);

3. Non-sickle vehicle-treated group (HbAA; 4 males, 4 females);

4. Sickle vehicle-treated group (HbSS; 4 males, 4 females);

5. Sickle neuregulin-1 (5µg/kg) treated (HbSS; 4 males, 4 females);

6. Sickle neuregulin-1 (25µg/kg) treated (HbSS; 4 males, 4 females);

7. Sickle hydroxyurea (50mg/kg) treated (HbSS; 4 males, 4 females).

3.1.6 Baseline measurements

Renal manifestations in sickle cell disease patients begin at an early age and progress from acute episodes of injury to irreversible end-stage renal disease. Similarly, humanized Townes sickle cell mice develop spontaneous nephropathy with early signs of kidney injury, which is also seen in young adult mice (Kasztan et al., 2017, 2019).

This study focused on mitigating kidney lesions and dysfunction during early adulthood. To model the renal manifestations observed in adolescent or young adult humans, 12-week-old Townes mice were used to establish baseline levels of kidney injury. From the literature, early signs of significant

elevations in tubular and glomerular markers of injury or malfunction are observed at age 12 weeks old in sickle cell mice. (Kasztan et al., 2017, 2019).

3.1.7 Treatments

Neuregulin-1 treatment: Townes HbSS mice at age 12 weeks were treated for two weeks (five times per week) via intraperitoneal injection with 50 µl doses of recombinant human NRG1-beta1 EGF domain (R&D Biosystems, Minneapolis, MN, USA. CAT # 396-HB-050). This research work is the first study to administer Neuregulin-1 in Townes mice. Two doses, 5 µg/kg/day or 25 µg/kg/day, were adopted based on previous studies. Liu *et al.* (2018) demonstrated that exogenous recombinant human NRG1-β1 attenuates systemic inflammation, neuroinflammation, and blood–brain barrier damage in experimental murine models of cerebral malaria (Liu et al., 2018). Cerebral malaria is characterized by markedly elevated levels of protein free plasma heme, a critical mediator of cerebrovascular injury (Ramos et al., 2024). Notably, plasma heme concentrations in cerebral malaria mouse models are comparable to those observed in Townes sickle cell mice. Given the analogous pathogenic effects of heme (resulting from haemolysis) in sickle cell disease (SCD) and cerebral malaria, the present study adopted the effective NRG-1 dosing regimen reported by Liu et al. (2018). Neuregulin-1 stock solution was prepared by reconstituting lyophilized neuregulin-1 in 0.1% bovine serum albumin in PBS (R&D Biosystems, Minneapolis, MN, USA. CAT # RB02) at 100 µg/mL concentration. On the first day of treatment, the body weights of all mice were determined. Based on body weights, groups were created at intervals of three grams, starting from the least weight. The average weight for each group was then used to calculate the dose for the mice in that group. A final volume of 100µL was prepared. This allowed 50µL to be injected into a mouse, and the remaining 50µL fluid remained in the syringe's dead space.

***Hydroxyurea (HU) treatment**:* Hydroxyurea (HU) is the standard disease-modifying therapy for sickle cell disease (SCD). Hydroxyurea (HU), partly improves renal function, but its impact on heme-driven kidney injury remains uncertain and underexplored (Laurin et al., 2014; McKie et al., 2007; Taylor et al., 2019; Zahr et al., 2019). A cohort of eight sex-matched mice was treated with HU. Using intraperitoneal injection, mice were treated with 50mg/kg hydroxyurea (Sigma-Aldrich, St. Louis, MO, USA. SKU # H8627) for two weeks (five times per week). This dose was selected based on an initial dose-finding study, where 50mg/kg was well tolerated and demonstrated an effect on blood parameters similar to hydroxyurea therapy in SCD patients (Lebensburger et al., 2010). HU was freshly prepared by dissolving it in sterile phosphate-buffered saline (R&D Biosystems, Minneapolis, MN, USA. CAT # RB01) and filtered via sterile syringe filter (pore size 0.2µm) (Millipore, Burlington, MA, USA. CAT # SLFGL25BS). A final volume of 100µL was prepared. This allowed 50µL to be injected into a mouse, and the remaining 50µL fluid remained in the syringe's dead space.

Vehicle-treated HbSS mice (n = 8) and HbAA mice (n = 8) groups were injected with sterile phosphate-buffered saline. All injection procedures were performed carefully to ensure effective delivery and to minimize variability. Intraperitoneal injections were conducted following established protocols. The mice were manually restrained and placed in the Trendelenburg position. The abdomen was cleansed with an alcohol swab, and a 27-gauge needle, 1.0-ml tuberculin syringe (BD Precision Glide, BD, Franklin Lakes, NJ, USA. BD 309623) containing 100 µl of the treatment drug or vehicle was used to puncture the lower left abdominal cavity. All subsequent injections were administered on alternating sides of the abdomen. Injection procedures were conducted daily between 7:00 a.m. and 10:00 a.m. for ten days.

3.1.8 Urine collection.

Urine was collected using a quick and straightforward method, as described by Parasuraman & Raveendran, (2022). The mouse was placed on a disposable plastic tray. One hand's fore and middle fingers were used to tap the mouse's lower back (dorsal posterior), while the other hand resisted the mouse by the tail. Equal pressure was applied, massaging on both sides of the lower back near the tail, with the thumb on one side and the fore and middle fingers on the other side, rubbing up and down. Once urination commenced, the massaging fingers were used to pull the skin at the lower back upward to immobilize the mouse and prevent premature cessation of urination (Parasuraman & Raveendran, 2022). The voided urine was then aspirated using a 100µl micropipette and transferred into a microcentrifuge tube, and it was subsequently stored at −80 °C until assayed. Per protocol, a new disposable plastic container was used for each mouse. Two-time points urine collection was done for each mouse at twelve and fourteen weeks old before being sacrificed.

3.1.9 Blood collection.

Blood was collected at time points: 1. baseline animals (12 weeks old) and 2. Treated animals (14 weeks old). Mice were anesthetized using isoflurane (3% induction and 2% maintenance with 100% O2 carrier) to minimize stress. The blood was collected via cardiac puncture using a 3cc syringe with a 25-gauge 1-inch needle (BD Integra, BD, Franklin Lakes, NJ, USA. BD 305270). Cardiac puncture was used to allow for enough blood to be collected. Although this terminal procedure entails a certain level of stress, a standardized approach was used to avoid any potential variability that may arise between animals or groups. Mice were placed in dorsal recumbency on a dissection board and pinned to the board using clips at the extremities of their four limbs. Fur is dampened with 70% alcohol prior to incision to prevent aerosolization. A needle was inserted into

the thoracic cavity, where the heartbeat was strong, and blood was drawn steadily but quickly to avoid clotting. Whole blood was aliquoted into K_2EDTA tubes (Greiner Bio-One VACUETTE™, Monroe, NC, USA. CAT # 454428) and gently mixed with EDTA anticoagulant. Blood mixed with anticoagulant was centrifuged for 10min at 1500 x g to collect plasma. Aliquots of whole blood in serum-gel microtubes (Sarstedt, Nümbrecht, Germany. CAT # 41.1378.005) were spun for serum. Blood in serum-gel tubes was allowed to clot for 2 hours at room temperature and centrifuged for 5min at 10000 x g for serum separation. Plasma and serum samples were immediately stored at -80°C.

3.1.10 Organ harvesting and tissue processing.

Immediately after blood collection, the mouse was perfused with normal saline solution. Mouse's fur was dampened with 70% alcohol before incision to prevent aerosolization. A stab incision and then an extended ventral incision were made through the skin and abdominal muscle across the caudal edge of the rib cage below the xiphisternum. The abdomen was then cut at either side towards the pubis and then across the caudal abdomen. The abdominal cavity was then opened just behind the ribs, along the midline, and laid back like a flap pinned to the dissection board. The kidneys were carefully removed, longitudinally halved, and fixed in 10% neutral buffered formalin (Thermo Scientific, Waltham, MA, USA. CAT # 23-101-005) for 18 to 20 hours. The tissue was processed using a Sakura tissue processor (Rankin Biomedical Corporation, Davisburg, MI, USA. Model VIP 5). The tissue was dehydrated using two changes of 70%, 95%, and 100% alcohol, cleared with two changes of xylene, and impregnated with two changes of liquified paraffin wax. Tissue blocks generated after embedding the kidneys in paraffin wax were sectioned into 4mm

sections using Leica microtome (Leica Biosystems, Deer Park, IL, USA. Model RM2235) for histopathological analysis.

3.2 Assays/Measurements.

3.2.1 Specific Aim 1: To evaluate the effect of neuregulin-1 treatment on haemolysis, inflammation, and kidney injury in humanized sickle cell mice.

3.2.1.1 Complete blood count

A fully automated veterinary hematology analyzer (Heska Element HT5, Loveland, USA) was used to measure the complete blood count using a whole blood sample (30µL). Samples were carefully collected and handled to minimize clots or small dark red or white translucent particles (fibrin). Absolute values on White Blood Cells (WBC), Neutrophils (NEU), Red Blood Cell Count (RBC), Haemoglobin Concentration (HGB), Hematocrit (HCT), Mean Corpuscular Volume (MCV), and Platelet Count (PLT) were obtained.

3.2.1.2 Reticulocyte count

Reticulocyte (immature red blood cells) count was determined within 3 hours of sample collection using the BD Rectic-Count reagent kit (BD, Franklin Lakes, BD, Franklin Lakes, NJ, USA. CAT # 349204). Two polystyrene tubes were labeled for each mouse sample. BD rectic-count reagent was added to one tube, and Dulbecco's Phosphate-Buffered Saline (DPBS) (Thermo Scientific, Waltham, MA, USA. CAT # 14-190-144) with 0.1% sodium azide added to the second tube. Five (5) µL of well-mixed whole blood from the same mice was added to each tube and vortexed gently. Both samples (stained blood with BD Rectic -count reagent and unstained with DPBS) were incubated for 30 minutes in the dark at room temperature. Reticulocytes as a percentage of total red blood cells were determined by a Cytek Guava flow cytometer (Cytek Biosciences, Fremont,

CA, USA. Model: easyCyte 12HT system). Using size and granularity parameters, forward scatter (FSC) and side scatter (SSC) gating (log mode) was done to exclude noise and debris. Green-B-Hlog laser channel was used to acquire 50,000 events from stained and unstained samples. Data analysis was done using the Guava Incyte software (Cytek Biosciences, Fremont, CA, USA. Version 4.5.50). Unstained samples were used to set markers for background fluorescence. The percentage of positive events (reticulocytes) was determined from the stained sample. Samples were read in duplicates, and the average was used for further analysis.

3.2.1.3 Haemoglobin F measurement.

An anti-haemoglobin F flow cytometry protocol was used to determine the percentage of fetal cells within the sample red blood cell population. This measurement was carried out within 6 hours of sample collection. Ten (20µL) of EDTA whole blood per sample was fixed in 1 ml of 0.05% Glutaraldehyde (Millipore Sigma, St. Louis, MO, USA. CAT # G5882-10X1ML) for 10 minutes at room temperature. The mixture was vortexed after the addition of cells. Following fixation, cells were washed three (3) times with 2mL of PBS-0.1%BSA (Millipore Sigma, St. Louis, MO, USA. CAT # P4474500ML). Cell pellets were resuspended by vortex in 0.5 ml 0.1% Triton X-100 (Millipore Sigma, St. Louis, MO, USA. CAT # X100-5ML) and incubated for 5 minutes at room temperature. Cells were washed one more time with 2mL of PBS-0.1%BSA. Cell pellets were resuspended in 0.5mL PBS-0.1%BSA and gently vortexed. Ten (20µL) of the cell suspension was added to 10µL of human monoclonal fetal haemoglobin antibodies haemoglobin, anti-HbF-APC (Invitrogen, Waltham, MA, USA. CAT# MHFH05), and 140µL of PBS 0.1% BSA. The mixture was incubated at room temperature for 15 minutes. Following incubation, cells were washed twice with 2mL of PBS-0.1%BSA. Cells were finally resuspended in 0.5 ml of 1% Formaldehyde (Polysciences, Inc., Warrington, PA, USA. CAT # 040181). Samples were run in duplicates.

Unstained samples (treated the same as stained sample but with no addition of antibody) from each mouse were included in the assay. Flow cytometric acquisition was done immediately on a Cytek Guava flow cytometer (Cytek Biosciences, Fremont, CA, USA. Model: easyCyte 12HT system). Log FSC, log SSC gating, and log Red-R fluorescence laser channel were used to acquire 50,000 events. Unstained samples were used to set markers for background fluorescence. Data was analyzed using the Guava Incyte software (Cytek Biosience, Fremont, CA, USA. Version 4.5.50). The percentage of positive events (HbF cells) was determined from the stained sample. Samples were read in duplicates, and the average was used for further analysis.

3.2.1.4 Total plasma heme quantification.

Total plasma heme was quantified using the QuantiChrom™ Heme Assay Kit (Bioassay Systems, Hayward, CA, USA. CAT # DIHM-250) per manufacturers instructions. First, the kit's calibrator reagent was serially diluted in distilled water to create working standard concentrations of 62.5µM, 31.25µM, 15.63µM, 7.81µM, 3.91µM and 1.95µM. Samples were diluted 1:4 with distilled water. A volume of 250µL of blank (distilled water) and 250µL of calibrator solution were transferred into a transparent bottom 96-well plate. Aliquots of 50µL plasma samples (in duplicates) were transferred into wells. Heme quantification reagent (200µL) was added to the sample wells. The plate was incubated for 5 minutes at room temperature. Optical density reading at 400nm was taken on a BioTek Synergy H1 multimode microplate reader (Agilent, Santa Clara, CA, USA). Samples were run in duplicates. The final concentration of total plasma heme was calculated by Biotek Gen5 data analysis software (Agilent, Santa Clara, CA, USA. Version: 3.14).

3.2.1.5 Plasma lactate dehydrogenase (LDH) quantification.

Plasma LDH was measured as a marker of haemolysis. LDH was quantified as total LDH activity in plasma. Previous studies have demonstrated that the plasma LDH pattern in SCD is dominated by erythrocyte isoforms LD-1 and LD-2. Because hemolysis in SCD releases these isoforms, an

increase in total LDH activity reliably reflects intravascular hemolysis (Kato et al., 2006). The QuantiChrom™ Lactate Dehydrogenase Kit (Bioassay Systems, Hayward, CA, USA. CAT # D2DH-100) was used to measure plasma lactate dehydrogenase (LDH) activity. A working reagent was prepared from reagents supplied by the kit manufacturer as follows: 14 µL MTT Solution, 8 µL NAD Solution, 1 µL Diaphorase, and 175 µL Substrate Buffer was calculated per sample to make a sufficient solution for all samples. Samples were diluted 1:4 with distilled water. A volume of 200µL of blank (distilled water) and 200µL of calibrator solution were transferred into a transparent bottom 96-well plate. Ten (10) µL of each sample (in duplicates) were transferred into separate wells, and then 190µL working solution was added to each sample. Absorbance reading was done immediately and again after 25 minutes at 565nm on a BioTek Synergy H1 multimode microplate reader (Agilent, Santa Clara, CA, USA). Samples were run in duplicates. LDH activity in plasma was calculated using the equation: LDH activity = 43.68 x (OD_{S25} – OD_{S0}/ OD_{cal} – OD_{blank}) x n (IU/L). ODS25 and ODS0 are OD565nm sample values at 25 min and 0 min. OD_{cal} and OD_{blank} are the absorbance values of the calibrator and blank.

3.2.1.6 Quantification of serum inflammatory mediators.

Serum levels of pro- and anti-inflammatory cytokines/chemokines were measured using a Milliplex Mouse cytokine/chemokine magnetic bead kit (Millipore Sigma, St. Louis, MO, USA. CAT # MECY2MAG73KPXBK). The analytes measured included IL-6, IL-10, IP-10 (CXCL10), VEGF-A, IFN-γ, MCP-5/CCL12, and MIP-3β/CCL19. The assay was performed on the Luminex xMAP Intelliflex (Millipore Sigma, St. Louis, MO, USA. Model: Intelliflex). The assay procedure is as follows: The xMAP Intelliflex was switched on, and the laser was allowed to warm up, calibrate, and be verified successfully before beginning the assay. Samples were thawed, vortexed, and spun for 10 mins at 10,000xg. Samples were diluted 1:2 with an assay buffer. After sample preparation, 200µL 1x wash buffer was added to each plate (96 well-plate) and shaken at 650rpm

at room temperature. The plate was blotted to dry after priming with wash buffer. The quality controls, serum matrix, and stock standard were reconstituted with 250µL deionized water, vortexed, and allowed to sit for 10 minutes. Seven working standards were prepared by serial dilution, starting with 50µL of the reconstituted standard and 150µL of assay buffer. A final volume of 200µL was prepared for each standard. A volume of 25µL assay buffer (for background and sample wells), each standard, controls were added to the appropriate wells. A volume of 25µL serum matrix was added to the standard and control wells. A volume of 25µL of each sample (in duplicates) was also added to the appropriate wells. Finally, 25µL of pre-mixed beads were added to all wells, bringing each well to a total volume of 75µL. The plate was sealed with film, wrapped in foil, and shaken overnight (18 hours) at 650rpm at 4°C. The next day, the plate was washed three times on a BioTek magnetic plate washer (Agilent, Santa Clara, CA, USA. Model: 405TSRSMML). Detection antibody (25µL) was added to each well. Afterwards, the plate was sealed and shaken for 1 hour at 650rpm at room temperature. After an hour, 25µL Streptavidin-Phycoerythrin was added to each well and shaken for 30mins at 650rpm at room temperature. Two washes are done afterward. A volume of 150µL wash buffer was added to each well, and the plate was shaken for 5 minutes at 650rpm. Reading was done on the xMAP Intelliflex. Serum concentrations were generated using the Belysa™ Immunoassay Curve Fitting Software (Millipore Sigma, St. Louis, MO, USA.)

3.2.1.7 Quantification of urine and serum kidney injury markers.

Kidney injury biomarkers Cystatin C, neutrophil gelatinase-associated lipocalin (NGAL), osteopontin (OPN) and cytoprotective markers, clusterin and epidermal growth factor (EGF) were assessed in urine and serum before and after treatment. NGAL is a sensitive biomarker of renal tubular injury (Soni et al., 2010). NGAL levels in urine and serum increase within few hours after kidney injury, making it an early indicator of acute tubular injury. Cystatin C, a protein produced by host cells, is freely filtered at the glomerulus but completely reabsorbed by renal tubular cells (Madero et al., 2006). Increased urinary cystatin C reflects impaired renal tubule reabsorption function and injury. Increased cystatin C levels in serum is indicative of glomerulopathy (Qiu et al., 2017). Serum cystatin C level is a prognostic marker for glomerular dysfunction (Qiu et al., 2017). Increased cystatin C levels in serum is indicative of glomerulopathy (Qiu et al., 2017). Cytokine osteopontin (OPN) is expressed in the thick ascending limb and distal tubules. OPN promotes renal inflammation and fibrosis and is associated with acute tubular injury (Sinha et al., 2023). Clusterin and epidermal growth factor (EGF) are cytoprotective proteins expressed by the kidney. Clusterin and EGF play roles in renal tissue repair, anti-inflammation, antioxidation, and cytoprotection (Ríos-Silva et al., 2023; Rodríguez-Rivera et al., 2021). Reduced renal expression of these markers is noticed by their low urine levels in kidney disease subjects. Clusterin cannot be filtered by glomeruli because of its size, hence, urinary clusterin is specific to the kidney (van Meer et al., 2014). Elevated urinary clusterin is seen in normal subjects, which reflects continuous renal repair and regeneration processes (Ozer et al., 2010). Epidermal growth factor (EGF) is expressed in the kidney, especially in the loop of Henle and the distal tubules. EGF is nonexistent in blood but present in normal subjects' urine. EGF urinary level decreases in kidney diseases and is associated with deterioration of renal function (Cortvrindt et al., 2022). Both urinary Clusterin

and EGF are kidney-specific with low levels associated with worsened renal injury and disease progression.

The Milliplex mouse kidney injury magnetic bead kit (Millipore Sigma, St. Louis, MO, USA. CAT# MKI2MAG-94K) was used for the assay as follows: The assay was performed on the Luminex xMAP Intelliflex (Millipore Sigma, St. Louis, MO, USA. Model: Intelliflex). The xMapIntelliflex instrument was turned on to allow the lasers to warm up. It was calibrated and verified before the beginning of the assay. Samples were thawed, vortexed, and spun for 10 mins at 10,000xg. Samples were diluted 1:1000 with assay buffer. After sample preparation, 200µL 1x assay buffer was added to each plate (96 well-plate) and shaken at 650rpm at room temperature. The plate was blotted to dry after priming with assay buffer. The quality controls and stock standard were reconstituted with 250µL deionized water, vortexed, and allowed to sit for 10 minutes. Six working standards were prepared by serial dilution, starting with 50µL of the reconstituted standard and 150µL of assay buffer. A final volume of 200µL was ready for each standard. A volume of 25µL assay buffer (for background and sample wells), standard, and controls were added to the appropriate wells. A volume of 25µL assay buffer was added to the wells having the standards and controls. A volume of 25µL samples (in duplicates) was added to the appropriate sample wells. Finally, 25µL of mixed beads solution was added to all wells, bringing each well to a total volume of 75µL. The plate was sealed with film, wrapped in foil, and shaken overnight (18 hours) at 650rpm at 4°C. The next day, the plate was washed twice on a BioTek magnetic plate washer (Agilent, Santa Clara, CA, USA. Model: 405TSRSMML). Detection antibody (25µL) was added to each well. Afterwards, the plate was sealed and shaken for 1 hour at 650rpm at room temperature. After an hour, 25µL Streptavidin-Phycoerythrin was added to each well and shaken for 30mins at 650rpm at room temperature. The plate was washed twice after the 30mins

incubation. A volume of 150µL wash buffer was added to each well, and then the plate was shaken for 5 minutes at 650rpm. A plate reading was done on the Intelliflex. Urine and serum concentrations were generated using the Belysa immunoassay curve fitting software (Millipore Sigma, St. Louis, MO, USA).

3.2.2 Specific Aim 2: To determine the effect of neuregulin-1 treatment on renal histopathologic changes associated with kidney injury in humanized sickle cell mice.

3.2.2.1 Histopathological tissue staining

Tissue sections (4µm) were cut from each tissue block and mounted on slides. Using standard protocols, the slides were stained with hematoxylin and eosin (H&E), Masson trichrome, or periodic acid Schiff (PAS).

Hematoxylin stains the nuclei of cells blue or dark purple, while eosin stains the cytoplasm and proteins pink. Erythrocytes are colored bright red, and all other eosinophilic structures are stained red, pink, or orange. Sections were deparaffinized with xylene for two changes of 5 minutes and rehydrated through 100% ethanol for two changes of 2 minutes, 95% ethanol for 2 minutes. and water washed for 2 minutes. Slides were stained in Mayers hematoxylin for 1 minute. Slides were washed with five changes of distilled water and placed in 1X phosphate buffered solution (PBS) for 1 minute. Afterward, slides were washed with three changes of distilled water and counterstained in alcoholic eosin for 1 minute. Sections were finally dehydrated through three changes of 95% ethanol and two changes of 100% ethanol, 1 minute each, and cleared in three changes of xylene (1 minute each change) and then mounted.

PAS stain was used to report on the thickening of the basement membrane of the Bowman's capsule, as well as to show the brush borders within tubules. Sections were deparaffinized with

xylene for two changes of 5 minutes and rehydrated with 100% ethanol for two changes of 2 minutes, 95% ethanol for 2 minutes. and water washed for 2 minutes. Slides were then oxidized in a 0.5% periodic acid solution for 5 minutes. It was rinsed in three changes of distilled water and placed in Schiff's reagent for 15 minutes. The stained sections were washed in tap water for 5 minutes until they turned dark pink before they were counterstained in Mayer's hematoxylin for 1 minute. Afterward, it was washed with tap water for 5 minutes and rinsed in distilled water. Sections were finally dehydrated through three changes of 95% ethanol and two changes of 100% ethanol, 1 minute each, and cleared in three changes of xylene (1 minute each change) and then mounted using xylene-based mounting media. The basement membrane stained magenta (reddish purple) due to the presence of collagen type IV. Collagen type IV in the basement membrane and brush borders is stained magenta with PAS.

The Masson trichrome stain was used to confirm fibrosis in the glomeruli (glomerulosclerosis). Sections were deparaffinized with xylene for two changes of 5 minutes and rehydrated with 100% ethanol for two changes of 2 minutes, 95% ethanol for 2 minutes. and water washed for 2 minutes. The sections were treated with Bouins's solution (mordant), which was preheated to 58˚C for 15 minutes and washed with distilled water. The sections were stained with modified Weigert's iron hematoxylin (kept in the dark) for 5 minutes**.** They were then washed with four changes of distilled water and differentiated in an acid-alcohol solution for 3-5 seconds. After washing with 3-4 changes of distilled water, the slides were stained with Biebrich Scarlet-Acid Fuchsin solution for 5 minutes and rinsed in three changes of distilled water. Slides were then treated with 1% Phosphomolybdic acid solution for 2 minutes and Aniline Blue solution for 5 minutes; they were rinsed with one change of distilled water, acetic acid solution for 30 seconds, and two changes of distilled water. The slides were finally dehydrated through changes of 70% and 95% ethanol and

two changes of 100% ethanol, 1 minute each, and cleared in three changes of xylene (1 minute each change) and then mounted using xylene-based mounting media. Collagen (fibrosis) stained blue, nuclei black, while red blood cells and smooth muscle stained red.

3.2.2.2 Histopathological analysis

Stained tissue sections were scanned using the Aperio digital pathology slide scanner (Leica Biosystems, Deer Park, IL, USA, Model AT2) at 40X magnification. A pathologist examined and scored the histopathological changes based on criteria previously used to analyze renal histopathology scoring systems in Townes mice (Kasztan et al., 2017, 2019).

Glomerular congestion: At least fifty glomeruli were examined from ten different bright-field areas of the renal cortex on both H&E-stained and Masson trichrome-stained sections using 100X and 400X magnification. Congestion was calculated as the percentage of total glomeruli with congestion in at least 25% of the glomerulus.

Thickened basement membrane: At least fifty glomeruli were examined from ten bright-field areas of the renal cortex on PAS-stained sections using 100X and 400X magnification and calculated as the percentage of total glomeruli with increased basement membrane thickness. The glomerular basement membrane thickening was scored on a 0–4 scale: 0 for no basement membrane thickening, 1 for mild thickening, 2 for moderate thickening, 3 for severe thickening, and 4 for massive thickening.

Glomerulosclerosis: A minimum of fifty glomeruli were examined from ten different bright-field areas of the renal cortex on Masson trichrome-stained sections using 100X and 400X magnification and calculated as the percentage of total glomeruli with disappearance of cellular elements from the tuft, collapse of capillary lumens, and folding of the glomerular basement membrane with entrapment of amorphous eosinophilic material. The grading score was from 0 to

4: 0 for no sclerosis; 1 for sclerosis in up to 25% of the glomerulus; 2 for sclerosis in up to 50% of the glomerulus; 3 for sclerosis in up to 75% of the glomerulus; and 4 for sclerosis in up to 100% of the glomerulus.

Number of glomeruli: Twenty bright fields were examined from the renal cortex on H&E-stained kidney sections using 400X magnification to determine the number of glomeruli per field. The average number of glomeruli per field was calculated. The total number of glomeruli per mm^2 area was then calculated from the average based on the area per field of the 400X magnification.

Vasa recta or medullary congestion: Twenty different bright fields were examined from the renal medulla on H&E-stained and Masson trichrome-stained kidney sections using 100X and 400X magnification for vasa recta congestion. The number of fields with congested vasa recta was expressed as a percentage of the total number of fields.

Loss of brush border: Twenty different fields were examined from the renal cortex on PAS-stained kidney sections using 100X and 400X magnification for the thickness of the brush borders. The percentage of tubules that lost some or all the brush borders was examined. This was scored using a 0 - 4 grading scale: 4 for no changes; 3 for lesions involving <25% of the tubules; 2 for lesions involving 25%–50% of the tubules; 1 for lesions involving >50% of the tubules; and 0 for lesions involving nearly 100% of the tubules. Results were averaged for each animal.

Table 3 Semiquantitative scoring criteria for histopathologic changes in Townes mice renal structures.

Lesion	Criteria/Definition	Scoring Scale / Output
Glomerular congestion	Defined as red blood cell accumulation in ≥25% of the glomerular tuft.	Expressed as % of total glomeruli examined.
Basement membrane thickening	Increased PAS staining of Bowman's capsule or glomerular basement membrane.	0 = none 1 = mild 2 = moderate 3 = severe 4 = massive
Glomerulosclerosis	Collapse of capillary loops, tuft retraction, expansion of extracellular matrix, and deposition of amorphous eosinophilic material.	0 = none 1 = ≤25% of glomerular tuft 2 = ≤50% of glomerular tuft 3 = ≤75% of glomerular tuft 4 = global (100%)
Glomerular number	Average number of glomeruli per field (400×), normalized to area per mm².	Continuous variable (glomeruli/mm²).
Vasa recta congestion	Defined as red blood cell accumulation of vasa recta lumens.	Expressed as % of total fields examined.
Tubular brush border loss	Loss of PAS-positive apical brush border in cortical tubules.	4 = none 3 = <25% tubules 2 = 25–50% tubules 1 = 50–75% tubules 0 = >75% tubules
Tubular iron deposition	Perls' Prussian blue–positive staining in tubular epithelium.	Expressed as % of tubules affected and/or quantified by digital densitometry (pixel intensity).

Histopathological scoring criteria were adapted from established renal pathology scoring systems. For each animal, at least 20 fields were examined at 100X–400X magnification.

3.2.2.3 Perls Prussian blue staining

Perls Prussian solution stains iron blue and was used to show iron deposition within tubules.. Sections were deparaffinized with xylene for two changes of 5 minutes and rehydrated with 100% ethanol for two changes of 2 minutes, 95% ethanol for 2 minutes. and water washed for 2 minutes. Slides were placed in freshly prepared hydrochloric acid-potassium ferrocyanide solution for 30 minutes at room temperature and rinsed in five changes of distilled water. The tissue sections (nuclei) were counterstained with 0.5% aqueous neutral red for 1 minute and washed rapidly in distilled water. After dehydration in graded alcohols, the sections were cleared in xylene, three or four changes, and mounted. Hemosiderin, a breakdown product of haemoglobin that contains ferric acid, can build up in kidney tubules and podocytes in SCD. When ferric ion (Fe^{+3}) in the kidney tissue reacts with the ferrocyanide in Perls Prussian solution, it forms a bright blue pigment called Prussian blue ferric ferrocyanide.

3.2.2.4 Analysis of iron deposition in renal cortex

A histopathologist examined the slides for proportion of cortical tubules with iron deposition within their walls. Twenty different bright fields from the renal cortex were examined using 100X and 400X magnification. The number of cortical tubules with iron deposited within their walls was expressed as a percentage of the total number of tubules observed within the field. The average for the twenty fields was recorded per slide. Slides were then scanned at a magnification of 40X using an Aperio digital pathology slide scanner (Leica Biosystems, Deer Park, IL, USA. Model AT2). The extent of iron deposition within the tubules was also determined by measuring the color intensity of positive stain blue within the cortex. Using the Aperio ImageScope software (Leica Biosystems, Deer Park, IL, USA version 12.4.6.5003), the renal cortex was manually selected with the annotation tool of the software. Positive Pixel Count v9 algorithm software was used to quantify the intensity of blue stains within the tissue. This algorithm is designed to quantify the

amount of a specific stain in a scanned slide image. Analysis thresholds were set at hue value of 0.66 (blue color), hue width of 0.44, and saturation of 0.4 and with default settings for all other parameters. The total pixel intensity was normalized to the total area of the annotated regions (pixel intensity/mm^2).

3.2.3 Specific aim 3: To evaluate the impact of neuregulin-1 treatment on the expression and distribution of heme oxygenase-1 in the kidneys of Townes sickle cell mice.

3.2.3.1 Pre-staining steps: Immunohistochemistry

Slides were deparaffinized and rehydrated by submersing the slides in the following reagents: Xylene (10mins), Xylene (5mins), Xylene (5mins), 100% Ethanol (10mins), 100% Ethanol (5mins), 100% Ethanol (5mins), 95% Ethanol (5mins), 70% Ethanol (5mins) and deionized water (5mins). After rehydration, the slides were transferred into 1X Tris Buffered Saline with Tween 20 buffer (TBST) and stored until the pretreatment process. The antibodies and detection kits, Rabbit Recombinant Monoclonal Heme Oxygenase-1 antibody (Abcam Inc, Waltham, MA, USA. CAT # ab52947), Rabbit ErbB4 polyclonal antibody (Proteintech Group, Inc, Rosemont, IL, USA. CAT # 19943-1-AP) and Rabbit-specific HRP-AEC IHC Detection Kit (Micro-polymer) (Abcam Inc, Waltham, MA, USA. CAT # ab236468), were removed from the freezer and thawed to room temperature. A range of dilutions (1: 100; 1:200; 1:400; 1:500 and 1:1000) was prepared for each antibody using 1X TBST buffer to determine the best dilution rate for each primary antibody. Based on the findings, the optimal concentration for the Rabbit ErbB4 polyclonal antibody (Proteintech Group, Inc, Rosemont, IL, USA. CAT # 19943-1-AP) detection was 1:500 and 1:200 for Rabbit Recombinant Monoclonal Heme Oxygenase-1 antibody (Abcam Inc, Waltham, MA, USA. CAT # ab52947).

3.2.3.2 Pre-Treatment Process: Immunohistochemistry

The Epredia PT Module (Epredia, Kalamazoo, MI, USA) was used to perform the antigen retrieval pre-treatment step required for immuo-detection. A 1500 mL of 1X Dewax and HIER Buffer H (pH 8.6 to 9.0) (Epredia, Kalamazoo, MI, USA. CAT # TA-999-DHBH) was prepared by combining 1400 mL of de-ionized water and 100 mL of Epredia 15X Dewax and HIER Buffer H (pH 8.6 to 9.0). The PT Module tank was filled with 1X HEIR buffer solution, and the warm-up process was initiated. Once the desired temperature was achieved, the slides were placed on the Epredia PT Module staining racks and submerged in the Epredia PT Module tank. The tank was then covered, locked, and programmed to start the antigen retrieval process. After the completion of the pretreatment (antigen retrieval) step, the buffer was allowed to cool to room temperature. Then, the slides were removed and placed in 1X TBST. For the subsequent steps, the slides were not allowed to dry until the final step of the staining and detection process to prevent the development of unwanted artifacts.

3.2.3.3 Staining and Detection: Immunohistochemistry

Slides were carefully removed from the 1X TBST buffer and promptly placed in an incubation chamber to prevent sample drying. The Hydrogen Peroxide Block, from the Rabbit-specific HRP-AEC IHC Detection Kit, was applied to the cover sections and incubated for twenty minutes to block endogenous peroxidase activity. Following this, slides were washed three times in 1X TBST buffer. The Protein Block in the Rabbit-specific HRP-AEC IHC Detection Kit, was then applied to the sections and incubated for twenty minutes at room temperature to block nonspecific binding. Slides were washed once in 1X TBST buffer. Then, each slide was stained with the respective primary antibody at the recommended concentration and incubated for one hour at room temperature. Afterward, slides were washed three times in 1X TBST buffer. The Goat Anti-rabbit HRP Conjugate, provided by the Rabbit-specific HRP-AEC IHC Detection Kit, was applied to the

slides and incubated for thirty minutes. Subsequently, slides were rinsed four times in 1X TBST buffer. AEC Single Solution from the Rabbit-specific HRP-AEC IHC Detection Kit was applied to the sections and incubated for ten minutes. The slides were rinsed four times in 1X TBST buffer. Following the staining, the slides were immersed in Gill's III Hematoxylin for three minutes and rinsed in tap water for nuclear detection. The bluing step was carried out with Scott's Tap Water for two minutes. The slides were then rinsed seven to eight times in tap water. Post-staining, the slides were cover slipped using Aqueous Mounting Media for IHC (Abcam Inc, Waltham, MA, USA. CAT # ab64230) and allowed to dry overnight before imaging. The Autostainer 360 (Epredia, Kalamazoo, MI, USA) protocol was configured to replicate the manually optimized protocol. A minimum of 300µL of each detection reagent was required for the automated detection process per slide. All staining procedures were carried out using the automated system.

3.2.3.4 Imaging and analysis

Stained slides were scanned at a magnification of 40X using an Aperio digital histopathology slide scanner (Leica Biosystems, Deer Park, IL, USA. Model AT2) (Appendix I). Scanned images were examined for positive stain count and stain intensity within the whole tissue was performed using the Aperio ImageScope software (Leica Biosystems, Deer Park, IL, USA. Version 12.4.6.5003). The positive Pixel Count v9 algorithm was used for the analysis. The algorithm was set at hue value = 0 (red), hue width = 0.2 and hue saturation = 0.25 to quantify positive stain pixels and its intensity. Negative controls were used to set thresholds to distinguish signal from background noise. Data was normalized to the total area of the annotated tissue area (Appendix I).

3.3 Statistics.

All statistical analyses were done in GraphPad PRISM version 10.2.2 (GraphPad Software, Boston, MA, USA). The minimum sample size for the study was calculated based on previously reported mean and standard deviations of plasma NGAL levels in untreated and hydroxyurea-treated HbSS mice (Park et al., 2019). For the neuregulin treatment groups, the means and standard deviations were assumed to be equivalent to those observed in the hydroxyurea group. A minimum sample size of 7 mice per group was determined with a Type I error rate of 0.05 and actual statistical power of 83.8%. Statistical analyses were conducted using appropriate methods for each comparison. For two-group comparisons, a two-tailed, unpaired t-test was performed. A two-tailed, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to evaluate differences among the various HbSS treatment groups. To assess the relative changes in urine biomarker levels before and after treatment, a ratio paired t-test (two-tailed) was performed. Pearson's correlation (r) analysis was performed to assess relationships among the measured variables, and results were presented as a correlation matrix. All data are presented as means ± SEM, and statistical significance was defined at $p < 0.05$.

4.0 CHAPTER FOUR: RESULTS.

4.1 Evaluate the effect of Neuregulin-1 treatment on haemolysis, inflammation, and kidney injury biomarkers in humanized sickle cell mice.

4.1.1 Anaemia, leucocytosis, and high haemolytic status in Townes humanized sickle cell mice at baseline.

Anaemia, leucocytosis, and haemolysis have clinical relevance to sickle cell disease pathogenesis. Complete blood count parameters and markers of haemolysis were measured in Townes humanized sickle cell mice (HbSS) and controls (HbAA) aged 12 weeks, so as to establish baseline status before treatment (Figure 6 and 7). Evidence of haemolysis and anaemia in HbSS mice was supported by elevated levels of total plasma heme, lactate dehydrogenase, lower red blood cell count, reduced haemoglobin concentration, lower hematocrit, high reticulocyte counts and increased mean red blood cell volume. Evidence of leucocytosis in HbSS was observed by elevated white blood cell and neutrophil counts. At baseline, both male and female HbSS mice had elevated white blood cell counts (males: 32.00 ± 4.98 x 10^3/µL vs 7.32 ± 0.92 x 10^3/µL, p = 0.0286; females: 30.01 ± 4.35 x 10^3/µL vs 7.28 ± 1.01 x 10^3/µL, p = 0.0266), high neutrophils counts (males: 9.95 ± 2.72 x 10^3/µL vs 1.54 ± 0.16 x 10^3/µL, p = 0.0216; females: 5.46 ± 1.67 x 10^3/µL vs 1.24 ± 0.15 x 10^3/µL, p = 0.0450), increased reticulocyte proportion (males: 7.54 ± 1.41% vs 1.23 ± 0.55%, p = 0.0259; females: 5.72 ± 0.64% vs 0.93 ± 0.07%, p = 0.0046) and increased mean red blood cell volume (males: 38.70 ± 0.51 fL vs 31.40 ± 0.83 fL, p = 0.0078; females: 45.95 ± 2.42 fL vs 31.13 ± 0.40 fL, p = 0.0009) compared to control HbAA mice (Figure 6A-D). Markers of anaemia, thus red blood cell count (males: 8.42 ± 0.66 x 10^6/µL vs 11.80 ± 0.54 x 10^6/µL, p = 0.0081; females: 7.08 ± 0.86 x 10^6/µL vs 11.53 ± 0.20 x 10^6/µL, p = 0.0118), haemoglobin levels (males: 9.87 ± 0.54 g/dL vs 13.25 ± 0.57 g/dL, p = 0.0052; females: 9.20 ± 0.49 g/dL vs 12.8 ± 0.41 g/dL, p = 0.0012), and hematocrit percentages (males: 29.45 ± 1.21 % vs 38.85 ± 1.84 %, p = 0.0073;

females: 30.73 ± 1.50% vs 36.83 ± 0.48%, $p = 0.0221$) were significantly lower in both male and female HbSS compared to control HbAA mice (Figure 6E-G). Platelet counts were significantly lower in HbSS female mice compared to HbAA female mice (422.60 ± 76.97 x 10^3/µL, vs 840.00 ± 82.82 x10^3/µL, $p = 0.0103$) but HbSS and HbAA males had comparable platelet counts (568.3 ± 37.97 x 10^3/µL, vs 690.30 ± 151.90 x10^3/µL, $p = 0.4868$) (Figure 6H). Total plasma heme was significantly higher in HbSS mice compared to HbAA mice (55.43 ± 8.14 µM vs 31.98 ± 1.77 µM; $p = 0.0237$) (Figure 7A). Also, HbSS mice had higher plasma lactate dehydrogenase activity than HbAA mice (120.7 ± 18.17 IU/L vs 58.64 ± 5.99 IU/L; $p = 0.0109$). In summary, at baseline, SCD-related hematological anomalies and haemolysis were evident in HbSS mice, making this murine model clinically relevant for the study.

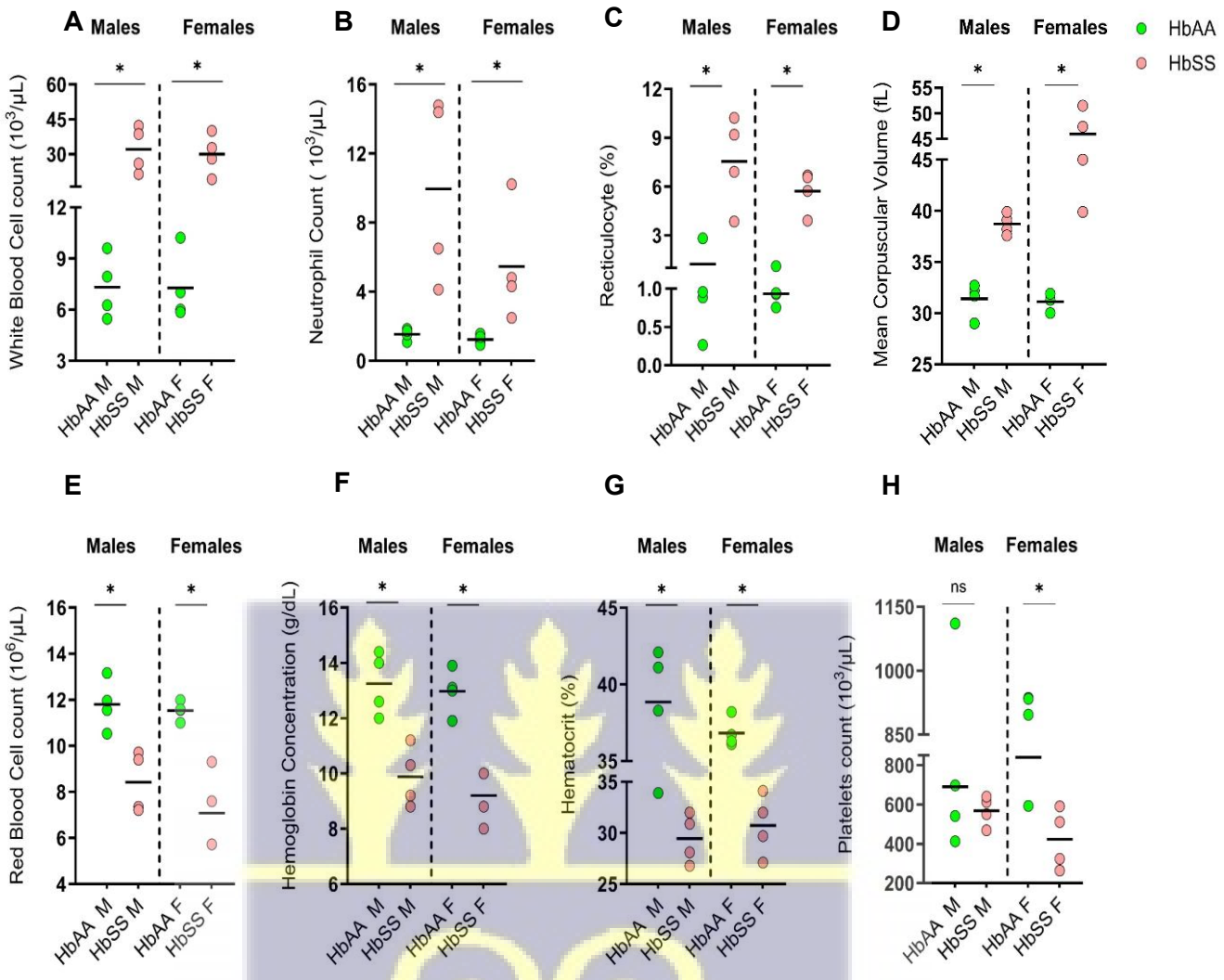


Figure 6 Abnormal hematological profile in Townes HbSS mice compared to HbAA controls.

*Measures of hematological profile;(A). White blood cell count (B). Neutrophil count (C). Reticulocyte percentage (D). Mean corpuscular volume (E). Red blood cell count (F). Haemoglobin concentration (G). Hematocrit and (H). Platelet counts were assessed in Townes HbSS and HbAA mice aged 12 weeks (n = 8 per genotype). Each dot represents a data point. The 2-tailed, unpaired Student t-test or Mann-Whitney test was performed for two groups comparison where applicable. GraphPad Prism 10 software was used. Data is reported as mean ± SEM. P value <0.05 is represented by *.*

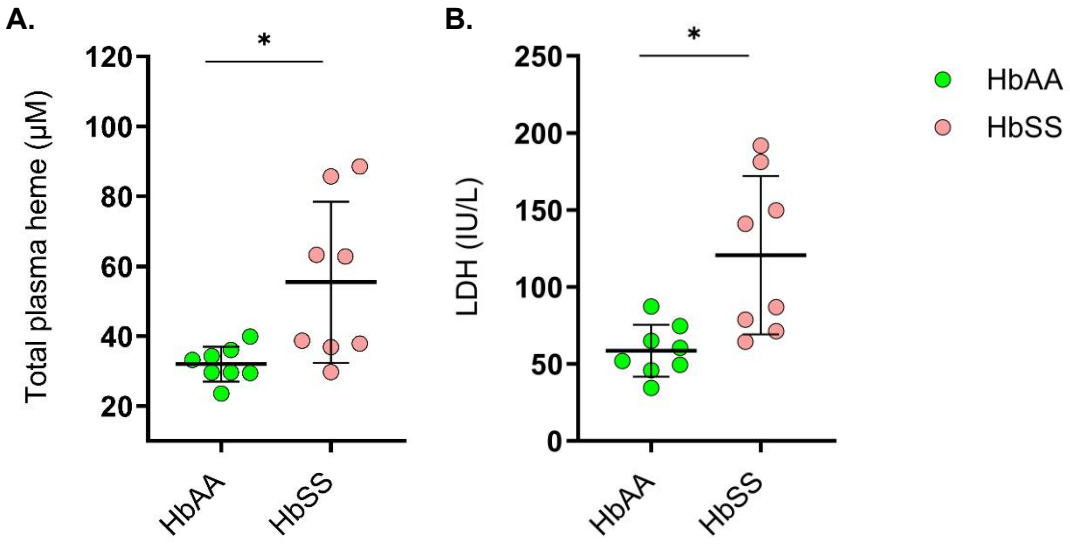


Figure 7 High haemolytic state in Townes HbSS mice compared to HbAA controls.

*Levels of intravascular haemolysis markers (A). Total plasma heme (B). Lactate dehydrogenase (LDH) activity was measured using colorimetric detection in Townes HbSS and HbAA mice aged 12 weeks (n = 8 per group). Each dot represents a data point. The 2-tailed, unpaired Student t-test was performed. GraphPad Prism 10 software was used. Data is reported as mean ± SEM. P-value <0.05 is represented by **.*

4.1.2 Townes humanized sickle cell mice exhibit reduced levels of renal cytoprotective markers and increased tubular injury markers at baseline compared to healthy controls.

Prior to investigating the renal protective effect of neuregulin-1 in sickle cell mice, the state of kidney injury in HbSS compared to controls was determined (Figure 8). Biomarkers of glomerulopathy, tubular injury, renal inflammation, and cytoprotection were measured in serum and urine samples. The findings show that tubular injury is an early-stage renal manifestation in HbSS mice. The study observed significantly elevated levels of urinary cystatin C in HbSS mice compared to HbAA mice (57.66 ± 4.84 ng/mL vs 41.51 ± 3.37 ng/mL; $p = 0.0104$). HbSS mice exhibited increased serum cystatin C levels (808.70 ± 32.04 ng/mL vs 756.10 ± 31.14 ng/mL; $p = 0.2586$) but it was not significant compared to HbAA mice (Figure 8A-B). Tubular injury in HbSS was further shown by the high levels of acute kidney tubule injury marker, neutrophil gelatinase-associated lipocalin (NGAL) in both serum (509.90 ± 21.34 ng/mL vs 188.40 ± 12.75 ng/mL; $p < 0.0001$) and urine (127.70 ± 11.82 ng/mL vs 50.43 ± 10.62 ng/mL; $p = 0.0003$) compared to HbAA mice (Figure 8C-D). Osteopontin levels in HbSS mice serum (203.70 ± 29.56 ng/mL vs 166.80 ± 12.70 ng/mL; $p = 0.2803$) and urine (1310.00 ± 209.70 ng/mL vs 986.40 ± 143.00 ng/mL; $p = 0.2262$) were high compared to HbAA mice. However, the difference was not statistically significant (Figure 8E-F). There was no difference in serum clusterin between the two groups (46.18 ± 5.85 µg/mL vs 49.71 ± 4.62 µg/mL; $p = 0.6437$) while serum EGR was undetectable (Figure 8G – I). Urinary clusterin (910.00 ± 119.20 ng/mL vs 3073.00 ± 322.70 ng/mL; $p = 0.0007$) and urinary EGF (717.60 ± 92.38 ng/mL vs 1302.00 ± 174.70 ng/mL; $p = 0.0135$) levels were significantly reduced in HbSS compared to HbAA mice (Figure 8G – I). Sickle cell mice exhibited evidence of spontaneous nephropathy characterized by marked tubular injury. Reduced urinary levels of the renal cytoprotective biomarkers clusterin and EGF may represent an early risk factor for the progression of kidney in sickle cell disease (SCD).

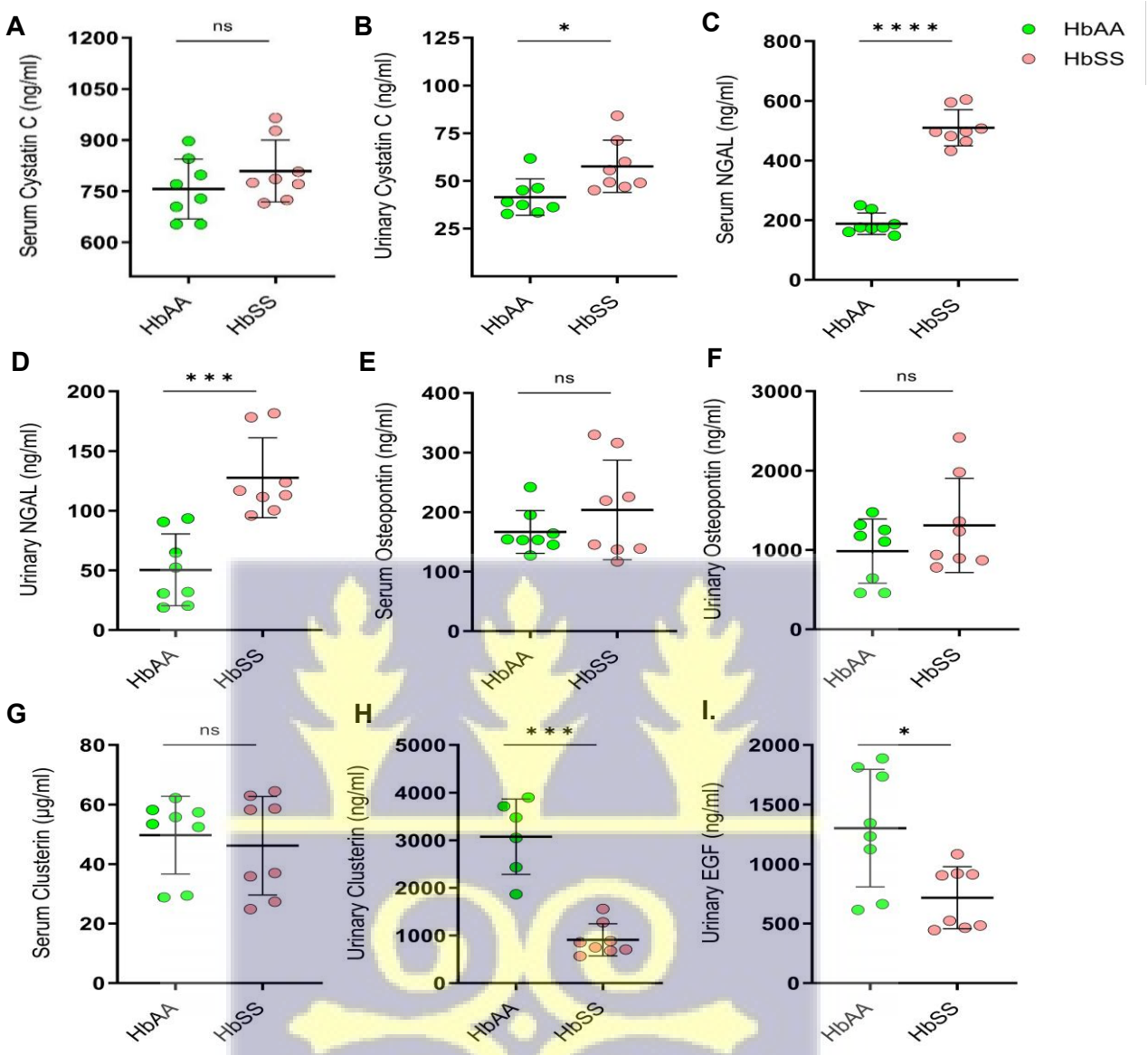


Figure 8 Renal injury and reduced kidney cytoprotection in Townes sickle cell mice compared to controls.

Quantification of serum and urinary kidney injury markers; (A-B). Clusterin (C-D). Neutrophil gelatinase-associated lipocalin (NGAL) (E-F). Osteopontin (OPN) (G-H). Clusterin (I). Epidermal growth factor (EGF) in Townes sickle mice and healthy controls, aged 12 weeks (n = 8 per group). Each dot represents a data point. The 2-tailed, unpaired Student's t-test was performed. GraphPad Prism 10 software was used. Data is reported as mean ± SEM. P-values are denoted as follows: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

4.1.3 Positive correlation between renal tubular injury and haemolysis in Townes humanized sickle cell mice.

In sickle cell disease (SCD), the kidney is continuously exposed to haemolytic toxic molecules, cell-free haemoglobin (Hb) and heme (Ofori-Acquah et al., 2020). Cell-free Hb and heme directly induce cytotoxic and oxidative pathways in renal tubular cells, promoting tubular injury (Avondt et al., 2019). The relationship between byproducts of haemolysis and markers of kidney injury was assessed in HbSS mice. The findings demonstrated strong positive linear relationships between haemolysis and renal injury (Table 4). Total plasma heme showed a strong positive correlation with urinary NGAL ($r = 0.98$, $p = 0.0001$), linking tubular injury with hemolytic stress. Additionally, plasma LDH was strongly and positively correlated with urinary cystatin C ($r = 0.97$, $p < 0.0001$) and urinary OPN ($r = 0.82$, $p = 0.0140$). A significant inverse relationship was observed between plasma LDH and urinary EGF ($r = -0.88$, $p = 0.0040$). and between urinary cystatin C and urinary EGF ($r = 0.80$, $p = 0.0170$)., suggesting that increased hemolysis and tubular injury is associated with reduced regenerative capacity (Table 4).

Table 4 Positive correlation between renal tubular injury and haemolysis in Townes sickle cell mice.

Pearson r	Total plasma heme	Plasma LDH	Urinary Cystatin C	Urinary NGAL	Urinary Clusterin	Urinary EGF	Urinary OPN
Total plasma heme	1.00	0.36	0.35	0.98*	0.31	-0.29	0.47
Plasma LDH	0.36	1.00	0.97*	0.49	0.11	-0.88*	0.82*
Urinary Cystatin C	0.35	0.97	1.00	0.48	0.22	-0.80*	0.88*
Urinary NGAL	0.98	0.49	0.48	1.00	0.27	-0.40	0.53
Urinary Clusterin	0.31	0.11	0.22	0.27	1.00	-0.22	0.62
Urinary EGF	-0.29	-0.88	-0.80	-0.40	-0.22	1.00	-0.76
Urinary OPN	0.47	0.82	0.88	0.53	0.62	-0.76	1.00

A positive relationship exists between haemolysis and kidney injury in Townes sickle cell mice. At age 12 weeks, total plasma heme, plasma lactate dehydrogenase (LDH) in plasma and cystatin C, neutrophil gelatinase-associated lipocalin (NGAL), clusterin, epidermal growth factor (EGF), and osteopontin (OPN) in urine were measured using colorimetric assays and Luminex, respectively. Pearson correlation matrix was computed to measure the strength and direction of the relationship between parameters. Data is reported as Spearman's r correlation coefficients. P-values are denoted as follows: $p < 0.05$ (*).

4.1.4 Neuregulin-1 therapy has similar benefits to hydroxyurea in improving haematological profiles and haemolysis in Townes humanized sickle cell mice, without worsening anaemia like hydroxyurea.

Patients with sickle cell disease and murine models exhibit abnormal hematological profiles and a high level of haemolysis. Reduced red blood cell count, low haemoglobin concentration, higher reticulocyte count, elevated neutrophil and platelet counts, increased plasma lactate dehydrogenase, and excess plasma heme are clinical indicators associated with severe SCD (Nader et al., 2020; Ofori-Acquah, 2020; Torres & Hidalgo, 2023). The study demonstrates that neuregulin-1 (NRG-1) treatment in Townes sickle mice mimics the clinical benefits of hydroxyurea by improving hematological anomalies and reducing haemolysis. Also, NRG-1 had no significant adverse impact on anaemia compared to hydroxyurea.

After two weeks of treating HbAA and HbSS mice with vehicle, neuregulin-1, and hydroxyurea, hematological and haemolysis markers were measured (Figures 9 &10). Vehicle-treated HbSS mice had elevated white blood cell count ($p = 0.0002$) and neutrophil counts ($p = 0.0002$), and a high reticulocyte percentage ($p = < 0.0001$), compared to vehicle-treated HbAA mice. Also, decreased red blood cell count ($p = 0.0009$), reduced haemoglobin concentration ($p = 0.0002$), less hematocrit ($p = 0.0009$), and low platelet count ($p = 0.0002$) were seen in vehicle-treated HbSS mice compared to HbAA controls (Figure 9A-H). Furthermore, HbSS vehicle-treated mice had a hyperhaemolytic status with increased levels of total plasma heme ($p = 0.0061$) and a high lactate dehydrogenase activity ($p = 0.0022$) compared to HbAA controls (Figure 10A-B). The proportion of fetal red blood cells in HbSS mice ($p = 0.0731$) was not statistically different compared to vehicle-treated HbAA controls.

Mice treated with 50mg/kg HU had a significant increase in the percentage circulating fetal haemoglobin containing red blood cells (F-cells) (4.029 ± 0.73% vs 2.58 ± 0.55%, $p = 0.0234$)

compared to vehicle-treated HbSS controls. Interestingly, NRG-1 (25µg/kg dose) treatment showed similar beneficial effects, significantly increasing fetal red blood cells in circulation (4.396 ± 0.88% vs 2.58 ± 0.55%, $p = 0.045$) compared to vehicle-treated HbSS controls. Although NRG-1 (5µg/kg) treated mice also had an increase in fetal cells, the change was not significant (3.023 ± 0.43% vs 2.58 ± 0.55%, $p = 0.3608$) (Figure 9A). These findings suggest that neuregulin-1 can be a potential therapy to augment the induction of HbF, combined with hydroxyurea or alone.

Reduction in the elevated leucocyte and platelet count in SCD is considered a significant clinical improvement. Mice treated with 50mg/kg HU exhibited a significant reduction in white blood cell count (23.57 ± 2.56 x 10^3/µL, vs 31.36 ± 1.84 x10^3/µL, $p = 0.0421$), neutrophil count (3.64 ± 0.82 x 10^3/µL, vs 7.50 ± 1.53 x10^3/µL, $p = 0.0492$), and platelet count (355.3 ± 37.52 x 10^3/µL, vs 542.0 ± 48.02 x10^3/µL, $p = 0.0118$) compared to vehicle-treated HbSS controls (Figure 9B–D). These results are consistent with the clinical benefits attributed to hydroxyurea therapy. HbSS mice treated with NRG-1(5µg/kg) had a blood profile that mimics the clinical benefits of hydroxyurea in SCD. White blood cell count (22.86 ± 1.780 x 10^3/µL, vs 31.36 ± 1.84 x10^3/µL, $p = 0.0118$), neutrophil count (4.020 ± 0.25 x 10^3/µL, vs 7.50 ± 1.53 x10^3/µL, $p = 0.0130$), and platelet count (366.30 ± 52.02 x 10^3/µL, vs 542.0 ± 48.02 x10^3/µL, $p = 0.0421$) were all significantly reduced compared to vehicle-treated HbSS controls. Although neuregulin-1(25µg/kg dose) treated mice also showed improvement in these parameters, it was not statistically significant (Figure 9B–D).

Mice treated with HU had worsened anaemia compared to HbSS controls (Figure 9E-H). Markers of anaemia, red blood cell count (5.99 ± 0.45 x 10^6/µL vs 7.49 ± 1.38 x 10^6/µL; $p = 0.0400$) and haemoglobin levels (8.45 ± 0.44 g/dL vs 9.80 ± 0.35 g/dL, $p = 0.0307$) were further reduced in HU treated mice compared to vehicle-treated HbSS controls. Also, HU significantly suppressed the production of new red blood cells (reticulocyte count) (4.75 ± 0.67% vs 7.98 ± 0.89%, $p = 0.0126$)

compared to vehicle-treated HbSS controls. There was no significant difference in red blood cell count in NRG-1 treated mice (5µg/kg dose; 7.00 ± 0.26 x 10^6/µL vs 7.49 ± 1.38 x 10^6/µL; p = 0.7576); (25µg/kg dose; 6.53 ± 0.31 x 10^6/µL vs 7.49 ± 1.38 x 10^6/µL; p = 0.2525) compared to vehicle-treated HbSS controls. Similarly, there was no difference in haemoglobin concentration between NRG-treated (5µg/kg dose; 9.20 ± 0.19 g/dL vs 9.80 ± 0.35 g/dL, p = 0.4317); (25µg/kg dose; 8.65 ± 0.44 g/dL vs 9.80 ± 0.35 g/dL, p = 0.0601) and vehicle-treated HbSS controls. The percentage of reticulocytes in blood was not different in NRG-1 treated mice and vehicle-treated HbSS controls (Figure 8E-H). Future studies should investigate this clinically relevant finding further.

Total plasma heme was significantly reduced in NRG-1-treated mice (5µg/kg dose; 58.00 ± 5.76 µM vs 90.18 ± 8.66 µM, p = 0.0140); (25µg/kg dose; 40.36 ± 2.52 µM vs 90.18 ± 8.66 µM, p = 0.0025) treated mice compared to vehicle-treated HbSS controls (Figure 10A-B). Similarly, lactate dehydrogenase levels were significantly reduced by NRG-1 (5µg/kg dose) (104.0 ± 17.13 IU/L vs 209.30 ± 33.31 IU/L, p = 0.0260) compared to vehicle-treated HbSS controls. Reduced total plasma heme (39.29 ± 7.10 µM vs 90.18 ± 8.66 µM, p = 0.0012) and lactate dehydrogenase (90.57 ± 29.42 IU/L vs 209.30 ± 33.31 IU/L, p = 0.0234) was also observed in HU-treated mice compared to vehicle-treated HbSS controls (Figure 10A-B). NRG-1's ability to improve abnormal hematological profile and haemolysis in sickle cell mice provides evidence that NRG-1 can have beneficial effects in SCD.

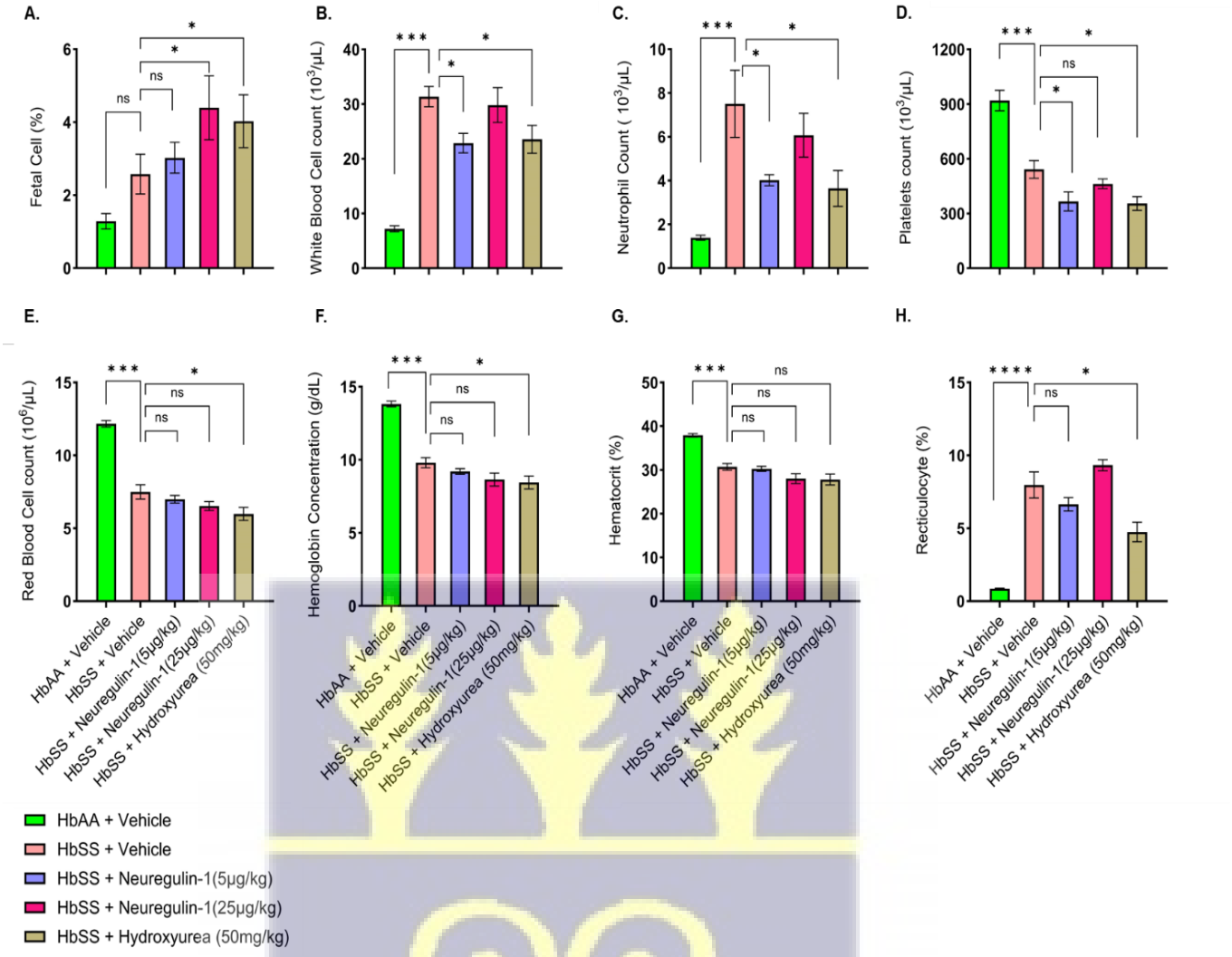


Figure 9 Neuregulin-1 administration improves hematological parameters in Townes humanized sickle cell mice.

Hematological parameters A. Fetal red blood cells B. White blood cell count C. Neutrophil count D. Platelets counts E. Red blood cell count F. Haemoglobin concentration G. Hematocrit H. Reticulocyte count was measured in Townes mice aged 14 weeks after 2 weeks treatment with vehicle, neuregulin, and hydroxyurea (n = 7 - 8 per group). Data is reported as mean ± SEM. The 2-tailed, unpaired t-test was performed for vehicle treated groups comparison. The 2-tailed, one-way analysis of variance with the Tukey post hoc test was performed across the different HbSS treatment groups. GraphPad Prism 10 software was used. P-values are denoted as follows: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

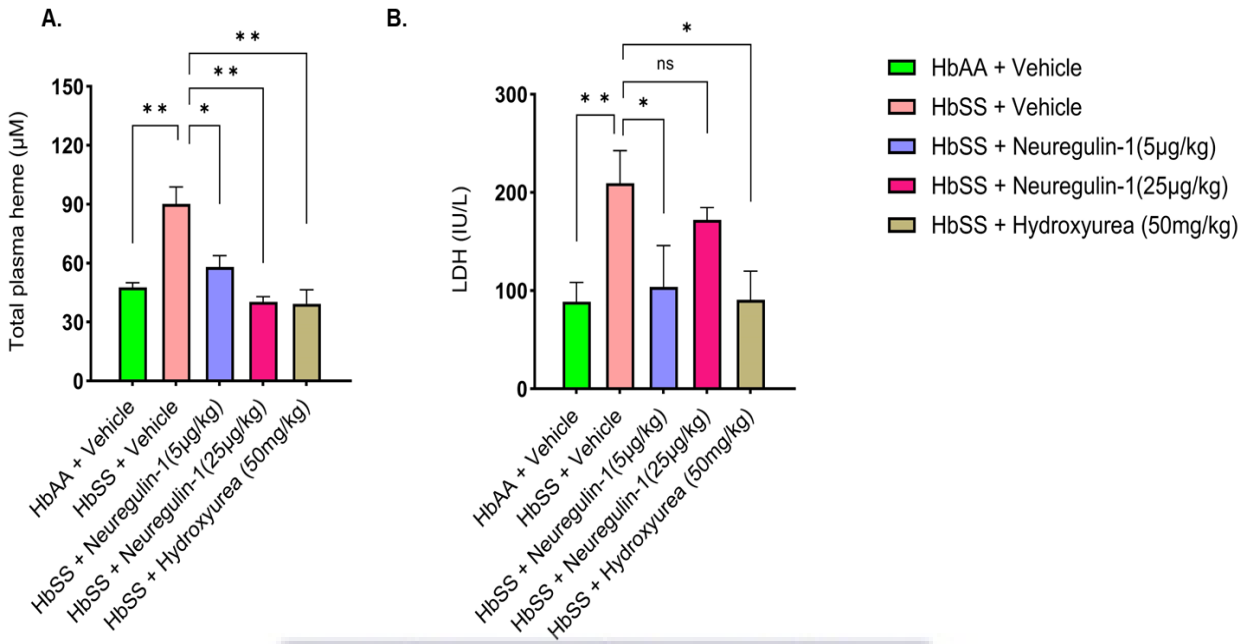


Figure 10 Neuregulin-1 administration improves haemolysis in Townes humanized sickle cell mice.

Total plasma heme (A) and Plasma lactate dehydrogenase (B) were measured in Townes mice aged 14 weeks after 2 weeks of treatment with vehicle, neuregulin, and hydroxyurea (n = 7 - 8 per group). Data is reported as mean ± SEM. The 2-tailed, unpaired t-test was performed for vehicle treated groups comparison. The 2-tailed, one-way analysis of variance with the Tukey post hoc test was performed across the different HbSS treatment groups. GraphPad Prism 10 software was used. GraphPad Prism 10 software was used. P-values are denoted as follows: $p < 0.05$ (*) and $p < 0.01$ (**).

4.1.5 Neuregulin-1 treatment reduces systemic inflammation in Townes humanized sickle cell mice.

Intravascular haemolysis promotes inflammation in SCD. Inflammation is a key contributor to the pathophysiology of SCD kidney injury (Conran & Belcher, 2018). The inflammatory phenotype of SCD is characterized by increased levels of pro-inflammatory cytokines with an unbalanced anti-inflammatory response. Modulating pro-inflammatory cytokine levels with a concomitant increase in anti-inflammatory response is critical in improving clinical outcomes of SCD (Ofori-Acquah, 2020). Neuregulin-1's (NRG-1) ability to modulate systemic SCD inflammatory milieu was evaluated. The findings show that both neuregulin-1 and hydroxyurea significantly improved the high levels of pro-inflammatory cytokines in sickle cell mice (Figure 11).

A heightened pro-inflammatory phenotype was exhibited in HbSS control mice compared to the HbAA counterparts. Serum levels of pro-inflammatory cytokines IFN-γ (7.43 ± 0.36 ng/mL vs 5.36 ± 0.29 ng/mL, $p = 0.013$), CXCL10 (90.67 ± 14.86 ng/mL vs 31.94 ± 1.72 ng/mL, $p = 0.0013$), IL-6 (46.49 ± 3.66 ng/mL vs 6.42 ± 0.73 ng/mL, $p < 0.0001$), VEGF-A (23.31 ± 5.58 ng/mL vs 4.81 ± 0.60 ng/mL, $p = 0.0053$), CCL19 (60.37 ± 3.47 ng/mL vs 38.26 ± 3.91 ng/mL, $p = 0.0011$) and MCP-5 (101.30 ± 6.98 ng/mL vs 63.55 ± 9.71 ng/mL, $p = 0.0189$) were significantly high in HbSS vehicle-treated mice compared to HbAA controls. The anti-inflammatory marker, IL-10 level in HbSS mice was statistically comparable in HbAA mice despite the heightened inflammation (3.09 ± 0.71 ng/mL vs 1.28 ± 0.51 ng/mL, $p = 0.0719$) (Figure 11A-F).

Hydroxyurea's anti-inflammatory effect, whether it is dependent or independent on its HbF inducing mechanism, is not fully explored. Treating HbSS mice with HU for two weeks reduced levels of some circulating pro-inflammatory cytokines compared to HbSS vehicle-treated mice. IFN-γ (4.49 ± 0.59 ng/mL vs 7.43 ± 0.36 ng/mL, $p = 0.0130$), and CXCL10 (45.21 ± 6.20 ng/mL vs 90.67 ± 14.86 ng/mL, $p = 0.0032$) and VEGF-A (7.33 ± 0.74 ng/mL vs 23.31 ± 5.58 ng/mL, p

= 0.0027) were significantly reduced by HU treatment. Levels of CCL19 (51.64 ± 3.86 ng/mL vs 60.37 ± 3.47 ng/mL, p = 0.1148) and MCP-5 (79.22 ± 10.66 ng/mL vs 101.30 ± 6.98 ng/mL vs, p = 0.1089) were reduced by HU treatment but this was not statistically significant. HU did not reduce IL-6, as levels remained high after treatment (Figure 11A-F).

NRG-1's ability to mitigate inflammation in sickle cell disease has not yet been reported. Here, our findings shows that mice treated with NRG-1 (5µg/kg) had a significant reduction in IFN-γ (3.79 ± 0.40 ng/mL vs 7.43 ± 0.36 ng/mL, p = 0.0019), IL-6 (28.78 ± 2.49 ng/mL vs 46.49 ± 3.66 ng/mL, p = 0.0017), CXCL10 (53.04 ± 3.58 ng/mL vs 90.67 ± 14.86 ng/mL, p = 0.0161), VEGF-A (8.030 ± 1.07 ng/mL vs 23.31 ± 5.58 ng/mL, p = 0.0043), CCL19 (36.68 ± 2.97 ng/mL vs 60.37 ± 3.47 ng/mL, p = 0.0001) and MCP-5 (69.28 ± 10.34 ng/mL vs 101.30 ± 6.98 ng/mL vs, p = 0.0244) compared to vehicle-treated HbSS controls. Similarly, mice treated with NRG-1 (25µg/kg) had a significant decrease in VEGF-A (5.45 ± 0.60 ng/mL vs 23.31 ± 5.58 ng/mL, p = 0.0008) and MCP-5 (79.70 ± 2.40 ng/mL vs 101.30 ± 6.98 ng/mL vs, p = 0.0177) compared to vehicle-treated HbSS controls. Interestingly, NRG-1 (5µg/kg dose) treated mice had a significant increase in levels of anti-inflammatory cytokine IL-10 compared to vehicle-treated HbSS mice controls (5.90 ± 0.20 ng/mL vs 3.09 ± 0.71 ng/mL, p = 0.0014). Altogether, these results provide additional evidence of the anti-inflammatory effects of HU. This report provides the first evidence of neuregulin-1's ability to regulate a heightened inflammatory environment in the SCD murine model.

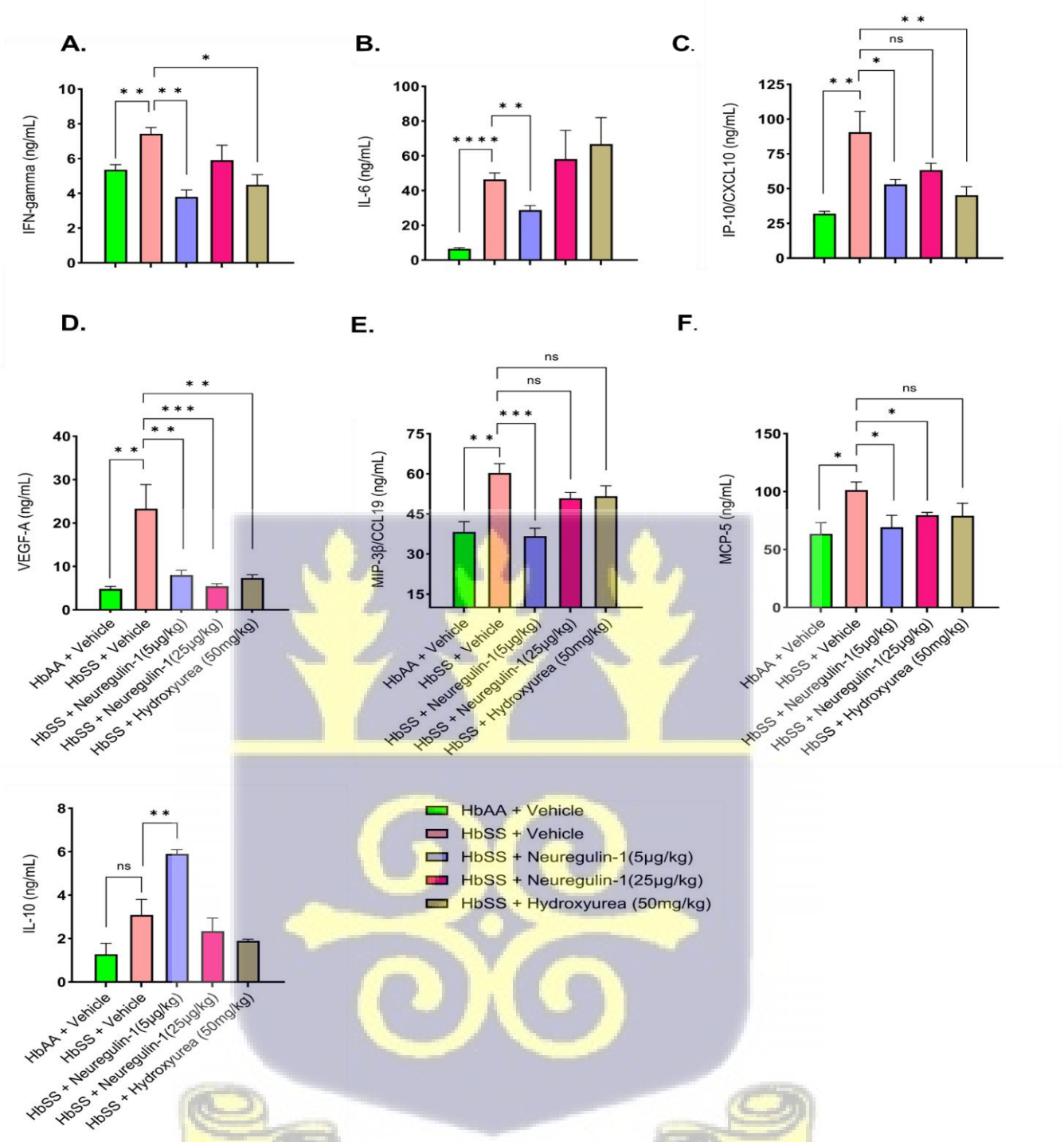


Figure 11 Anti-inflammatory effects of Neuregulin-1 and Hydroxyurea in Townes humanized sickle cell mice.

Pro-inflammatory markers A. Interferon-gamma (IFN-γ) B. Interleukin 6 (IL-6) C. C-X-C motif chemokine ligand 10 (CXCL10) D. Vascular Endothelial Growth Factor-A (VEGF-A) E. Macrophage inflammatory protein 3β (MIP-3β) F. Murine Monocyte Chemoattractant Protein 5

(MCP-5) and anti-inflammatory marker G. Interleukin 10 (IL-10) were measured in Townes mice aged 14 weeks after 2 weeks treatment with vehicle, neuregulin and hydroxyurea (n = 7- 8 per group). Data is reported as mean ± SEM. The 2-tailed, unpaired t-test was performed for vehicle treated groups comparison. The 2-tailed, one-way analysis of variance with the Tukey post hoc test was performed across the different HbSS treatment groups. GraphPad Prism 10 software was used. P-values are denoted as follows: $p < 0.05$ (*) $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

4.1.6 Neuregulin-1 treatment enhances renal cytoprotection and moderately attenuates severe tubular injury in Townes humanized sickle cell mice.

The cytoprotective effect of neuregulin-1 against kidney injury in Townes humanized sickle cell mice was determined. Kidney injury biomarkers Cystatin C, neutrophil gelatinase-associated lipocalin (NGAL), osteopontin (OPN) and cytoprotective markers, clusterin and epidermal growth factor (EGF) were assessed in urine before and after two weeks of treatment. The effect of hydroxyurea on renal injury was compared to neuregulin-1. The study findings showed that neuregulin-1 significantly increased cytoprotective and repair markers and moderately reduced tubular injury markers. Hydroxyurea notably reduced urine and serum biomarkers of tubular and glomerular injury (Figures 12 & 13).

Prior treatment, urinary NGAL levels were significantly elevated in all HbSS mice compared to HbAA controls. After treatment, vehicle-treated sickle cell mice had a fold increment of 5.97 ± 0.19 (p= 0.0060) in urinary levels of cystatin C and 2.92 ± 0.14 (p= 0.0147) in urinary levels of NGAL. HbAA healthy mice did not show any significant change in urine cystatin C (1.77 ± 0.11, p= 0.0786) and NGAL (1.32 ± 0.13, p= 0.4133) (Figure 12A-B).

After treatment, urine cystatin C was further increased in all HbSS mice groups, suggesting that tubular dysfunction is progressive in sickle cell mice (Figure 12A). However, the therapeutic

ability of NRG-1 and HU to reduce the extent of tubular damage was demonstrated by the relatively minimal fold increase in urinary cystatin C levels in treated HbSS mice compared to untreated HbSS controls. The hydroxyurea-treated group had a fold increase of 2.89 ± 0.23 (p = 0.0391) which was relatively lower compared to the vehicle-treated HbSS group 5.97 ± 0.19 (p= 0.0060). Both NRG-1 5µg/kg and 25µg/kg doses mirrored the effect of HU on tubular injury by exhibiting a lower fold increase of 4.82 ± 0.18 (p =0.0066) and 4.99 ± 0.19 (p = 0.0087) respectively, compared to vehicle-treated HbSS controls (Figure 12A).

HU treatment showed an insignificant fold increase in urinary NGAL levels (1.41 ± 0.20, p =0.4923). Like HU effects, mice group treated with low dose NRG-1 (5µg/kg dose) also had an insignificant fold increment of 1.42 ± 0.13 (p = 0.2881) in urinary NGAL levels compared to vehicle-treated HbSS controls. Although 25µg/kg dose neuregulin-1 treated mice exhibited a significant fold increment (2.74 ± 0.09 (p = 0.0031) in urinary NGAL, the increment was however less than that of HbSS controls (Figure 12B).

The study findings showed significantly low levels of clusterin in HbSS mice before treatment compared to HbAA controls. After treatment, clusterin levels remained low in HbSS vehicle-treated mice (1.39 ± 0.04 µg/mL, p = 0.0647) whiles vehicle-treated HbAA controls showed a significant fold increase (2.07 ± 0.03 µg/mL, p = 0.0002). Remarkably, mice treated with 5µg/kg dose NRG-1 had a significant fold increase in urinary clusterin levels (2.17 ± 0.10 µg/mL, p = 0.0179). High dose (25µg/kg dose) neuregulin treatment exhibited an insignificant increase in clusterin levels (1.76 ± 0.13 µg/mL, p = 0.1779) like HbSS control mice. Likewise, hydroxyurea did not have any significant effect on clusterin levels (1.14 ± 0.07 µg/mL, p = 0.4360) (Figure 13A).

After two weeks treatment, EGF levels were significantly elevated in vehicle-treated HbAA mice while levels in vehicle-treated sickle mice remained low. A significant fold increase of 1.55 ± 0.06 (p = 0.0314) in urinary EGF was observed in HbAA mice, while vehicle-treated HbSS mice had no change (1.01 ± 0.05, p = 0.9170) (Figure 13B). This suggests a lack of EGF-mediated regeneration or repair in sickle cell mice amidst ongoing kidney injury. NRG-1 (5µg/kg) treatment had a significant fold increment (1.35 ± 0.02, p = 0.0011) in EGF levels in urine after two weeks therapy. Hydroxyurea treated mice had no significant change in urinary EGF levels (1.23 ± 0.05, p = 0.1108) (Figure 13B). In summary, administering neuregulin-1 to sickle cell mice enhances levels of renal repair and cytoprotection biomarkers and reduces severity of renal injury. The findings also demonstrate that hydroxyurea has a notable effect on minimizing renal injury in Townes sickle cell mice.

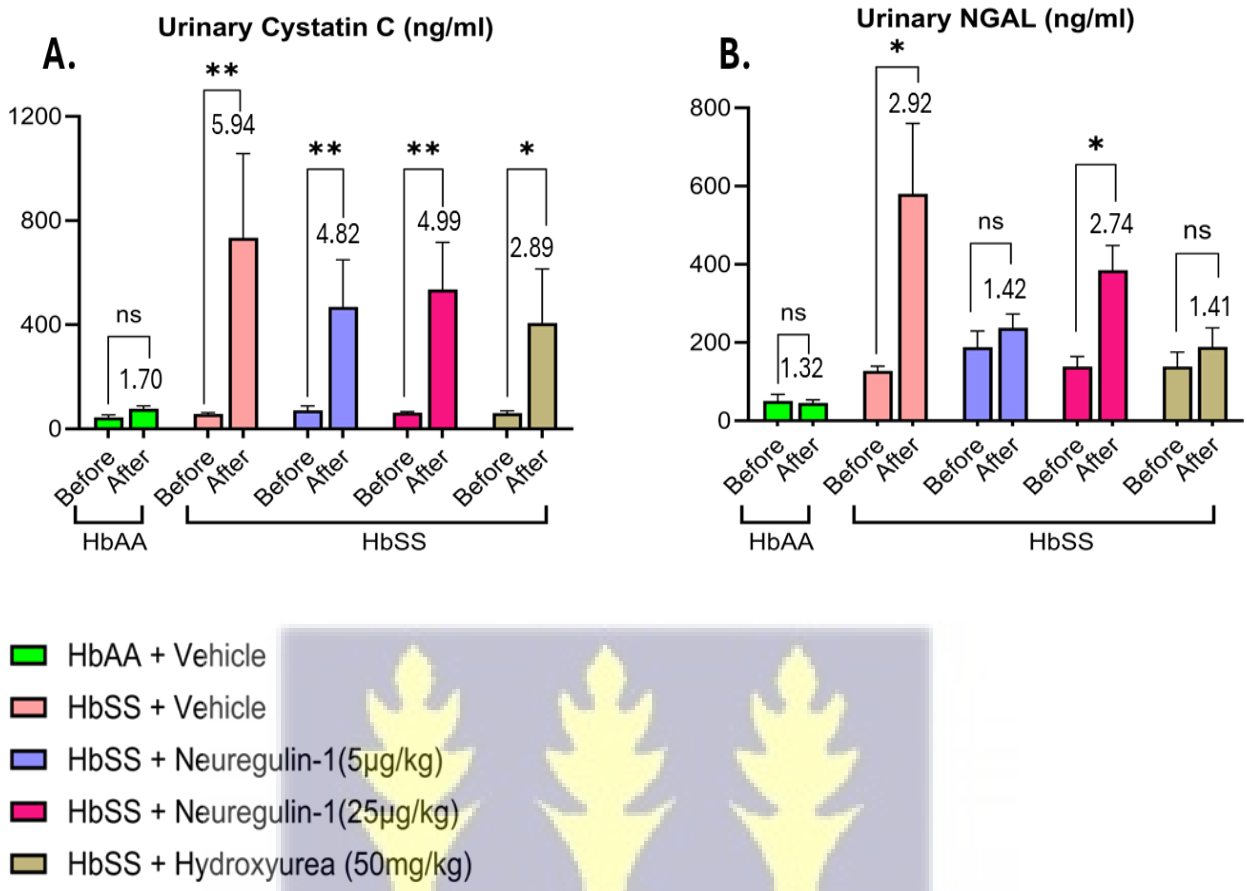


Figure 12 Neuregulin-1 treatment moderately modulates renal tubular injury in Townes humanized sickle cell mice.

Kidney injury biomarkers, urinary cystatin C (A), urinary neutrophil gelatinase-associated lipocalin (NGAL)(B) were assessed in 14-week-old humanized sickle cell mice groups and controls (n = 7 - 8 per group) after 2 weeks treatment with neuregulin-1, hydroxyurea, and vehicle. Data is reported as mean ± SEM. The relative change in levels of biomarkers before and after treatment was performed by ratio paired t-test, two-tailed. P-values are denoted as follows: $p < 0.05$ (*) and $p < 0.01$ (**).

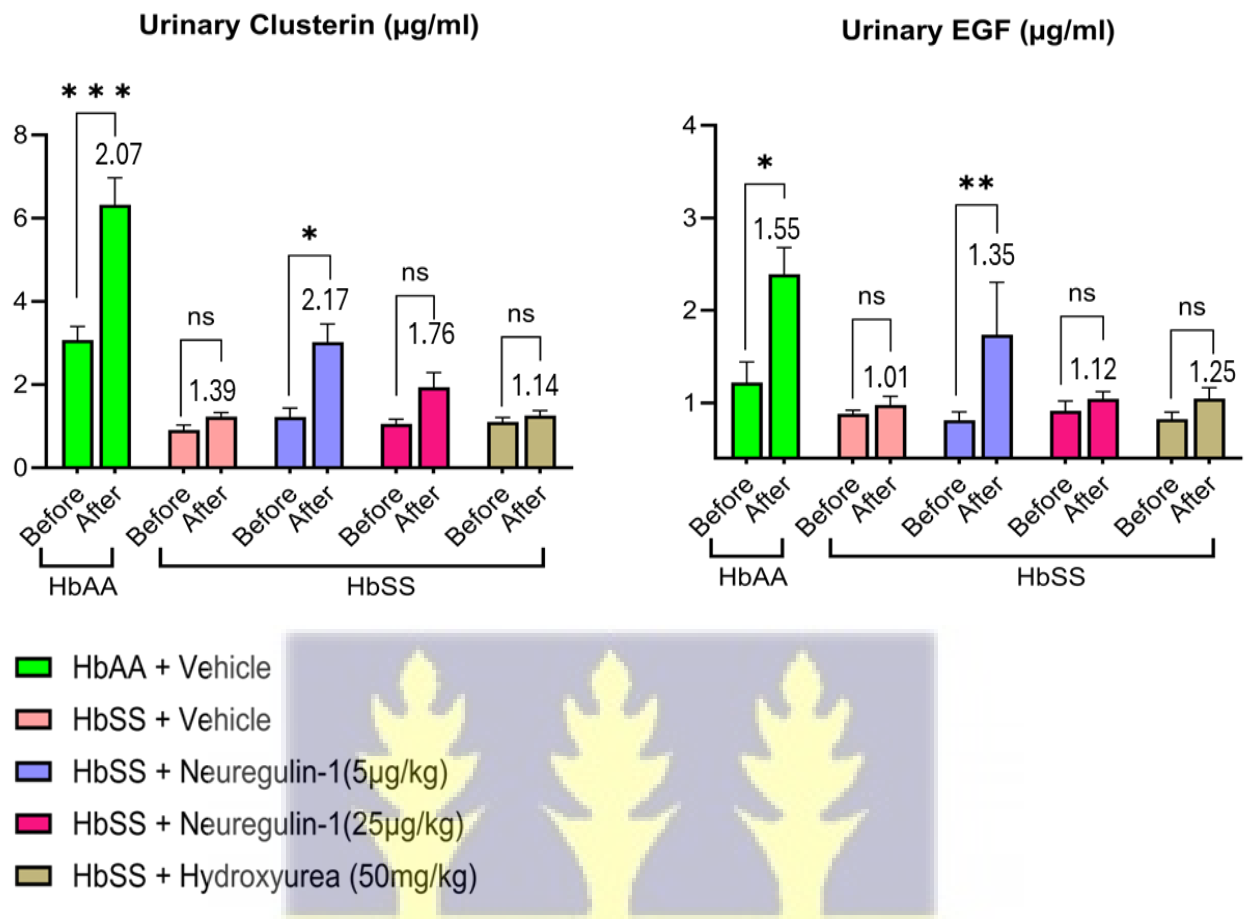


Figure 13 Neuregulin-1 treatment increases renal protective biomarkers in Townes humanized sickle cell mice.

Urinary levels of renal protective biomarkers clusterin (A) and epidermal growth factor (EGF) (B) were assessed in 14-week-old humanized sickle cell mice groups and controls (n = 7 - 8 per group) after 2 weeks of treatment with neuregulin-1, hydroxyurea, and vehicle. Data is reported as mean ± SEM. Relative change in levels of biomarkers before and after treatment was performed by ratio paired t-test, two-tailed. P-values are denoted as follows: $p < 0.05$ (*) and $p < 0.01$ (**).

4.2 Results on specific Aim 2: Determine the effect of Neuregulin-1 treatment on renal histopathological changes associated with kidney injury in humanized sickle cell mice.

4.2.1 Townes humanized sickle cell mice develop severe renal vascular congestion, glomerular/tubular damage, and multi-organ structural changes compared to healthy mice.

Gross architectural changes were observed in the organs of sickle cell mice. While HbAA mice had grossly unremarkable organs, HbSS mice exhibited mild to severe hypertrophic heart, moderately congested lung, severely enlarged liver and spleen, and mild edematous brain The HbSS mice's kidneys had mild to severe dark appearance with mild-to-moderate hypertrophy (Figure 14).

Histological examination of the kidneys confirmed histopathologic changes in HbSS mice at baseline, compared to HbAA healthy controls. Glomerular defects were characterized by glomerular capillary congestion, thickening of the bowman's capsule, glomerulosclerosis, and the number of glomeruli. Tubular defects and injury were assessed by tubular epithelium brush border thickness and accumulation of iron deposits in tubular epithelial cells. Medullary changes were examined by vasa recta congestion as an indicator of vasculopathy.

Sickle cell mice had significant congestion in the vasa recta and glomerular capillaries. About one-third of the glomerular capillaries were occluded with sickled red blood cells in HbSS mice (34.170 ± 10.12% vs 6.250 ± 10.12%, $p = 0.0429$) compared to HbAA controls (Figure 15, 19A). Vascular congestion was more pronounced in the vasa recta. More than two-thirds of the renal medulla vessels were occluded in HbSS mice (70.830 ± 4.17% vs. 5.000 ± 2.87%, $p < 0.0001$), but this was nearly absent in HbAA mice (Figure 18; 19E).

Glomerulosclerosis was observed in HbSS mice with 25% to 50% of glomeruli being sclerotic while this was absent in HbAA mice (1.800 ± 0.20 score vs 0.000 ± 0.000 score, $p = 0.008$) (Figure

17; 19C). On average, HbSS mice exhibited mild thickening of the bowman's capsule, but this was insignificant compared to HbAA controls (0.917 ± 0.08 score vs 0.625 ± 0.24 score, $p = 0.4000$) (Figure 16; 19B). Also, individual sickle cell mice showed mild-to-moderate loss of epithelium brush border thickness; but on average, this was also statistically insignificant compared to HbAA mice (2.917 ± 0.49 score vs. 3.500 ± 0.29 score, $p = 0.5810$) (Figure 16; 19F). There was no difference in the estimated number of glomeruli per mm^2 between HbSS and HbAA. Iron deposition within the cortical tubules was nearly absent in HbAA mice. However, tubular iron deposition was a remarkable feature in the kidneys of HbSS mice where 25.83 ± 0.49% of HbSS cortical tubules had iron accumulated in them versus 1.25 ± 0.75% in HbAA mice ($p = 0.0095$) (Figure 18; 19G). The positive iron stain intensity was also significantly higher in HbSS mice compared to HbAA mice (42.35 ± 12.77 vs 4.167 ± 0.83 x 10^3 pixels/mm^2, $p = 0.0304$) (Figure 19H). The accumulation of iron deposits within cortical tubules reflects excess uptake of heme proteins. This is a risk factor for developing tubular injury (Nath et al., 2022; Ofori-Acquah et al., 2020). These findings underscore the relevance of sickle cell mice to develop natural course kidney damage without external manipulations as a good model for studying critical aspects of renal disease. The marked vascular congestion observed in HbSS provides compelling evidence that it is a key contributor to the development of spontaneous nephropathy in sickle cell disease.

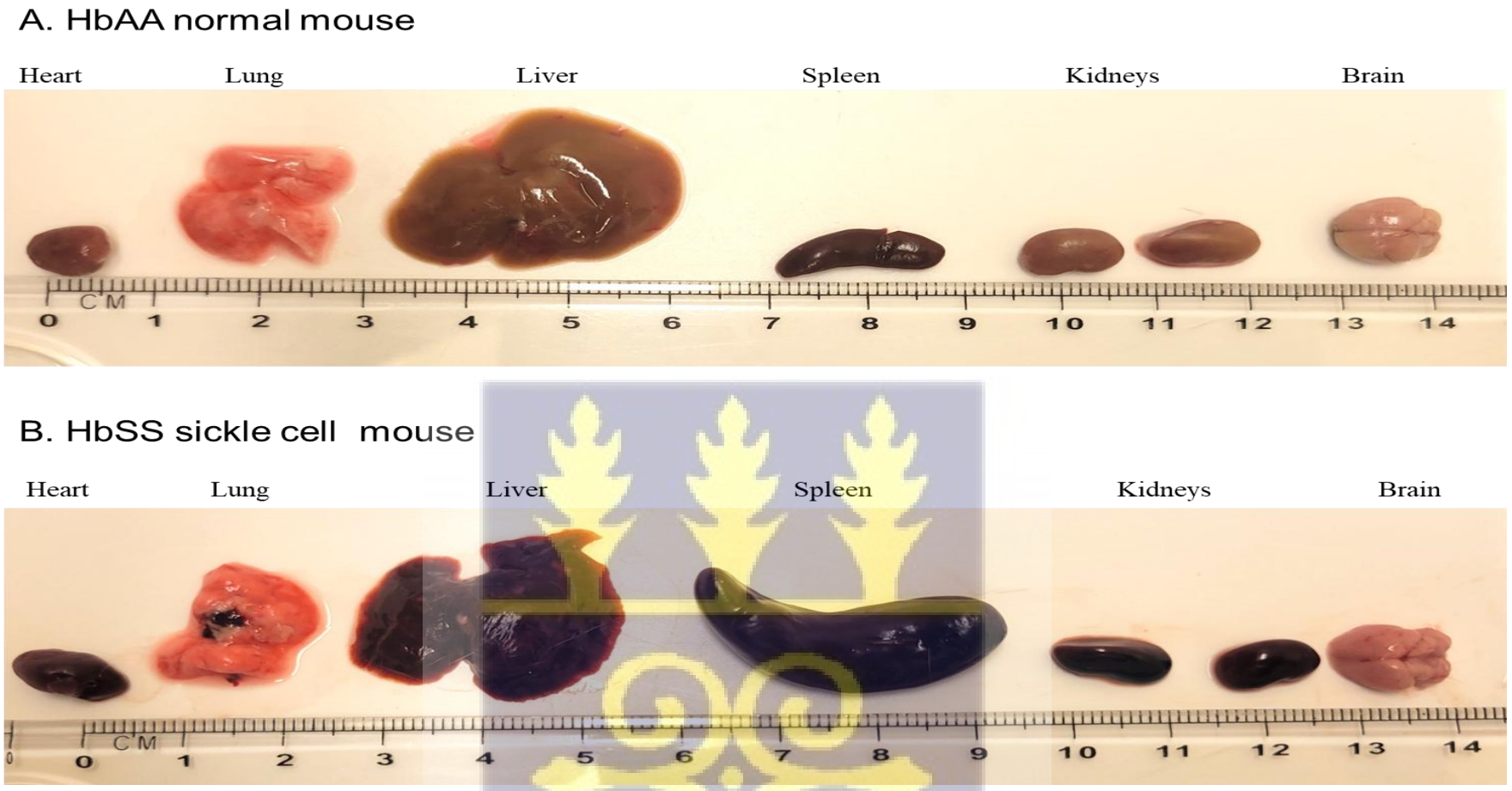


Figure 14 Multi-organ structural changes in Townes humanized mice.

Representative images of organs harvested from Townes sickle cell (HbSS) mice and control (HbAA) mice at 12 weeks old. A) Depicts unremarkable organs from a representative HbAA mouse. B) Depicts hypertrophic heart, congested lung, enlarged liver and spleen, congested and hypertrophic kidneys, and edematous brain from a representative HbSS mouse.

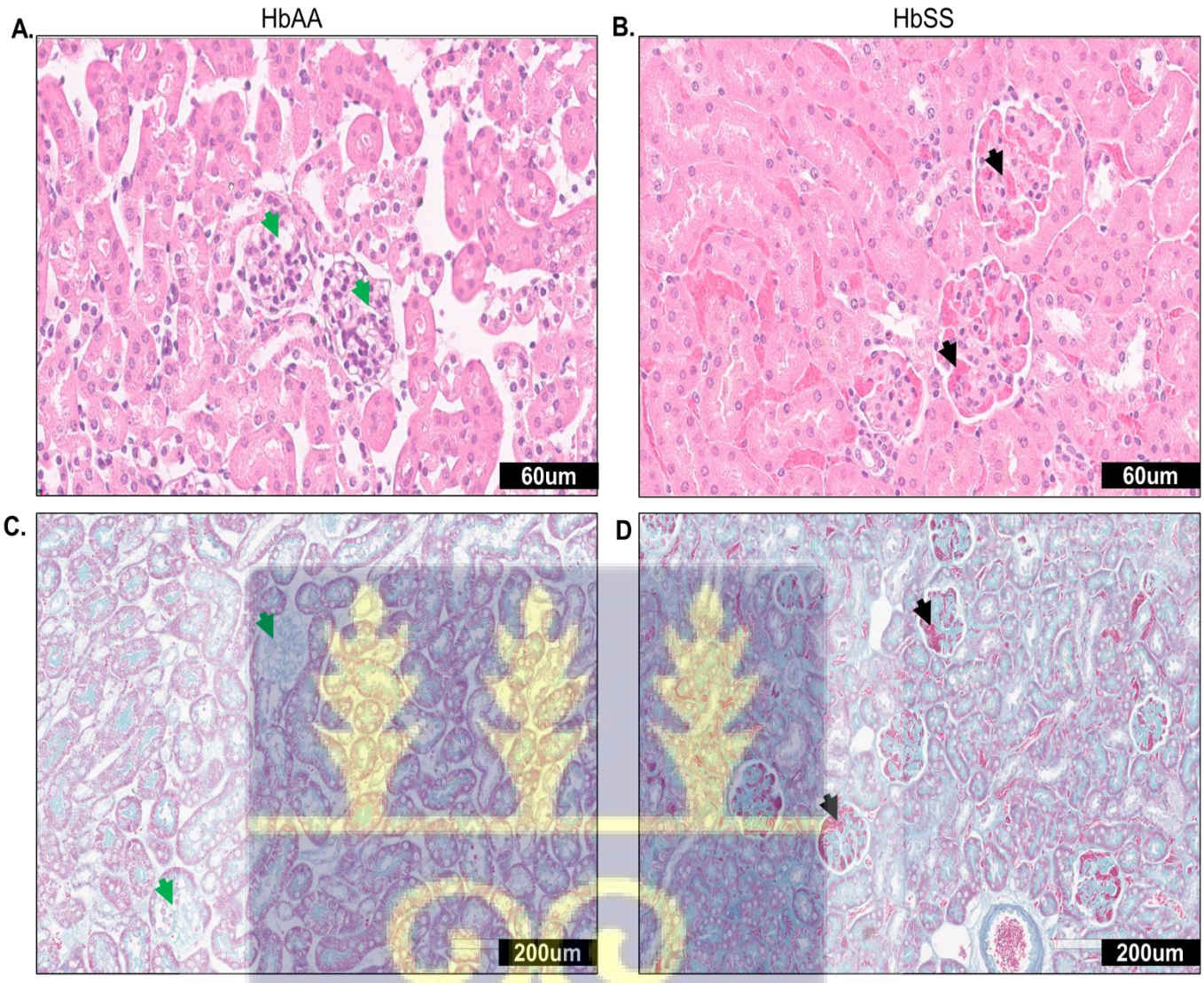


Figure 15 Glomerular congestion in the kidney of Townes humanized sickle mice (HbSS) compared to heathy HbAA mice.

Panels A and C display representative renal cortex sections from HbAA mouse, stained with Hematoxylin–and–eosin (Panel A) and Masson's trichrome (Panel C), with green arrowheads indicating normal glomerulus with no congestion. Panels B and D display representative renal cortex sections from HbSS mouse, stained with Hematoxylin–and–eosin (Panel B) and Masson's trichrome (Panel D), highlighting areas of glomerular congestion (black arrowheads). Each panel represents Townes humanized sickle cell mice and healthy HbAA controls (n = 7 – 8 per group) at 12 weeks old. Original magnification x 40.

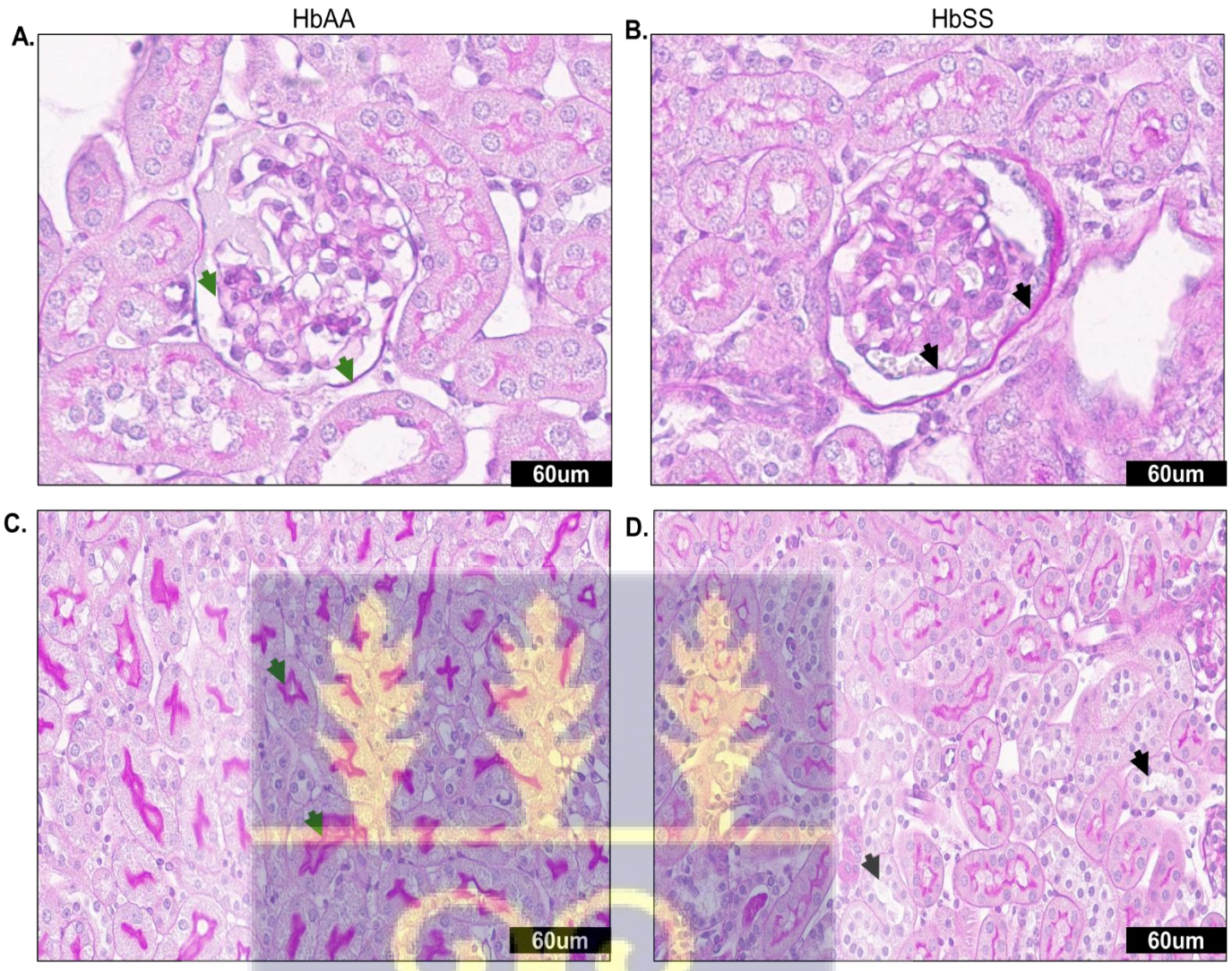


Figure 16. Bowman's capsule membrane thickening and tubular brush border loss in the kidney of Townes humanized sickle mice (HbSS) compared to healthy HbAA mice.

Each panel represents kidney sections from Townes humanized sickle cell mice and healthy HbAA controls (n = 7 – 8 per group) at 12 weeks old stained with Periodic acid-Schiff (PAS). Panel A display representative renal cortex from HbAA mouse with green arrowheads indicating normal Bowman's capsule membrane. Panel B displays representative renal cortex from HbSS mouse, highlighting a thickened Bowman's capsule membrane (black arrowheads). Panel C displays representative renal cortex from HbAA mouse with green arrowheads indicating presence of tubular brush borders. Panel D displays representative renal cortex from HbSS mouse, highlighting loss of tubular brush borders (black arrowheads). Original magnification x 40.

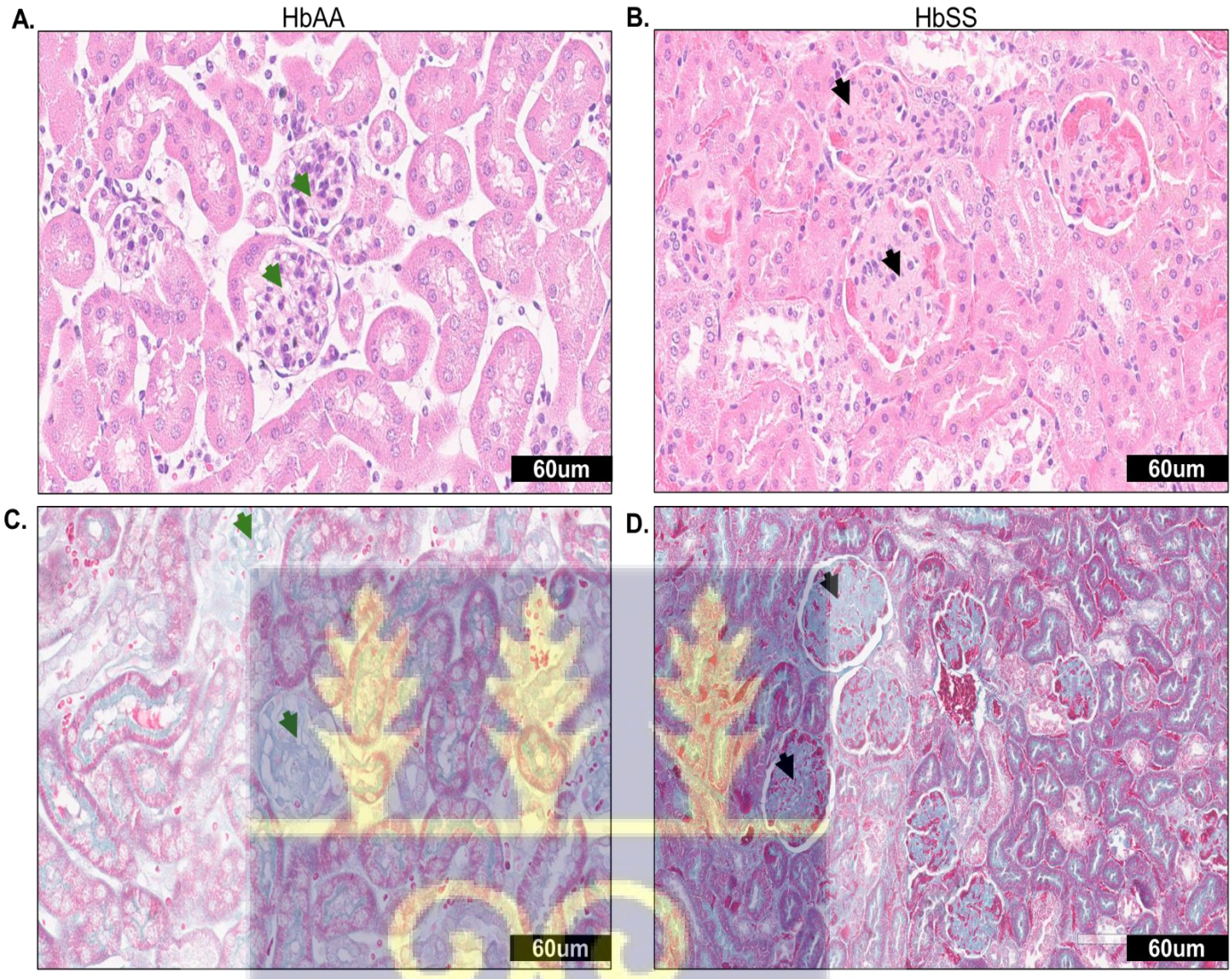


Figure 17 Glomerulosclerosis in the kidney of Townes humanized sickle mice (HbSS) compared to heathy HbAA mice.

Panels A and C display representative renal cortex sections from HbAA mouse, stained with Hematoxylin–and–eosin (Panel A) and Masson's trichrome (Panel C), with green arrowheads indicating normal glomerulus. Panels B and D display representative renal cortex sections from HbSS mouse, stained with Hematoxylin–and–eosin (Panel B) and Masson's trichrome (Panel D), highlighting areas of sclerosis in glomerulus (black arrowheads). Each panel represents Townes humanized sickle cell mice and healthy HbAA controls (n = 7 – 8 per group) at 12 weeks old. Original magnification x 40

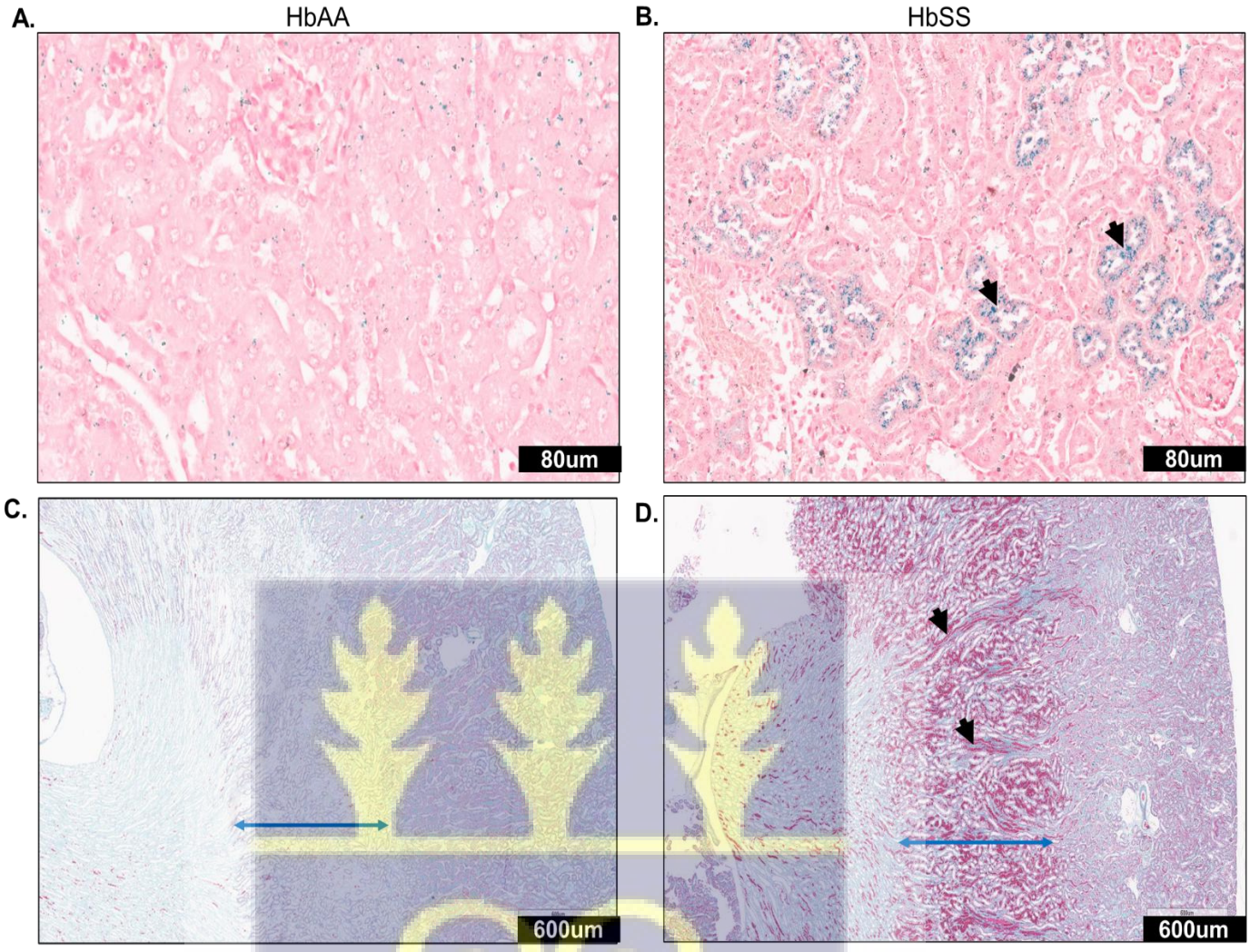


Figure 18 Renal tubular iron accumulation and medulla congestion in the kidney of Townes humanized sickle mice (HbSS) compared to healthy HbAA mice.

Each panel represents kidney sections from Townes humanized sickle cell mice and healthy HbAA controls (n = 7 – 8 per group) at 12 weeks old. Panel A display representative renal cortex section from HbAA mouse stained with Perl's Prussian blue with no tubular iron deposits. Panel B displays representative renal cortex section from HbSS mouse stained with Perl's Prussian blue, highlighting iron deposits within cortical tubules (black arrowheads). Panel C displays representative kidney section from HbAA mouse stained with Masson's trichrome, indicating absence of congestion in medulla (blue double-headed arrow region). Panel D displays representative kidney section from HbSS mouse, highlighting severe congestion (black arrowheads) within the medulla (blue double-headed arrow region). Original magnification x 40.

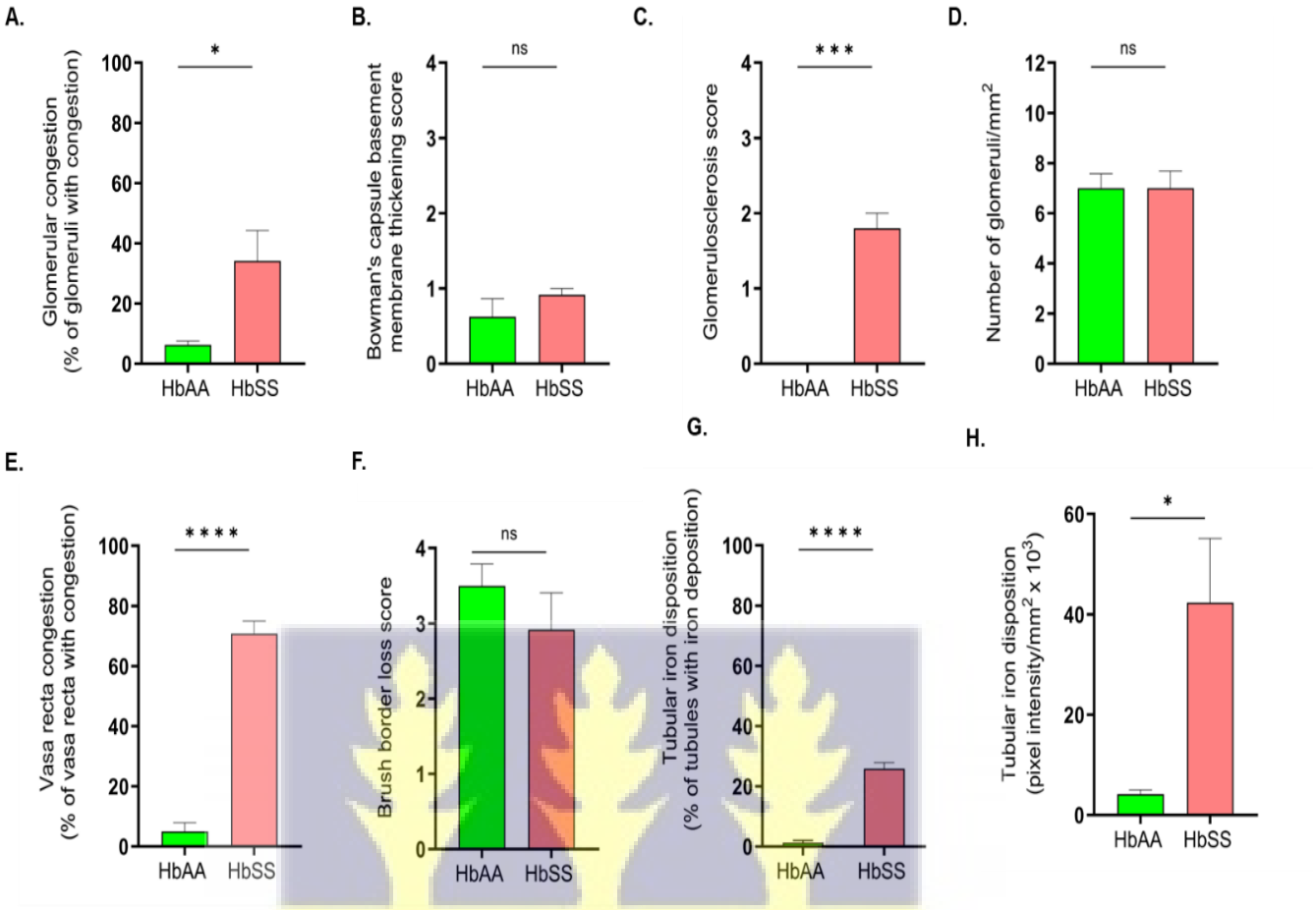


Figure 19 Spontaneous histopathologic manifestations in the kidney of Townes humanized (HbSS) sickle cell mice compared to healthy HbAA controls.

Histopathologic analysis of kidney sections from humanized sickle cell mice and healthy controls (n = 7 - 8 per group) at 12 weeks old. HbSS naturally developed mild to moderate pathologic changes, which are represented by semi-quantitative scoring of (A) glomerular congestion, (B) thickening of the Bowman's capsule basement membrane, (C) glomerular sclerosis, (D) number of glomeruli (E) congestion of the vasa recta (F) thickness of the tubular epithelium brush border and (G -H). Iron deposition in the tubular epithelium. Data is represented by Mean ± SEM. The 2-tailed, unpaired Student t-test was performed. P-values are denoted as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.001$ (****).

4.2.2 Neuregulin-1 treatment reduces glomerular damage in Townes humanized sickle cell mice.

To evaluate the beneficial effect of neuregulin-1 (NRG-1) on SCD glomerulopathy, histopathologic changes associated with glomerular injury were examined after two weeks of administering NRG-1 to humanized sickle cell mice (HbSS) and non-sickle HbAA genetic controls. Hydroxyurea was concomitantly administered to sickle cell mice. These results provide preliminary evidence demonstrating that neuregulin-1 treatment improves glomerular damage in SCD.

Vehicle-treated HbSS mice exhibited significant glomerular damage compared to vehicle-treated HbAA control mice. Glomeruli from the HbSS mice had pronounced glomerular congestion (51.250 ± 6.02% vs 3.750 ± 1.57%, $p = 0.0002$), thickened Bowman's capsule membrane (2.250 ± 0.21 score vs 0.500 ± 0.18 score, $p = 0.0007$), and were sclerotic (1.500 ± 0.19 score vs 0.167 ± 0.01 score, $p = 0.0003$) (Figures 20-22).

Neuregulin-1 treatment significantly reduced congestion within glomerular capillaries in HbSS mice (5µg/kg/dose; 15.00 ± 9.82% vs 51.250 ± 6.02%, $p = 0.0025$) (25µg/kg/dose; 7.50 ± 1.64% $p = 0.0003$) compared to vehicle-treated HbSS mice. Hydroxyurea treatment also reduced congestion, which was nearly significant compared to HbSS controls (35.00 ± 5.70% vs 51.250 ± 6.02%, $p = 0.0875$) (Figure 20). The glomerulus undergoes ischemic stress because of vessel congestion, resulting in injury. These findings indicate that neuregulin treatment protects the glomerulus by reducing congestion.

The thickening of the Bowman's capsule membrane is a notable sign of nephropathy. HbSS mice treated with neuregulin-1 (5µg/kg/dose; 1.500 ± 0.13 score vs 2.250 ± 0.21 score, $p = 0.0228$) and hydroxyurea (1.313 ± 0.27 score vs 2.250 ± 0.21 score, $p = 0.0365$) exhibited mild thickening of

their Bowman's capsule membrane compared to the moderate thickening seen in HbSS control mice (Figure 21).

Injured glomeruli without effective cellular repair become sclerotic. The study observed that over 25% of the glomeruli in the vehicle-treated HbSS mice kidneys had developed severe sclerosis. However, treatment with neuregulin-1 or hydroxyurea significantly reduced glomerulosclerosis. Less than 25% of the glomeruli in mice treated with NRG-1 (5µg/kg/dose; 0.563 ± 0.06 score, p = 0.0002, 25µg/kg/dose; 0.688 ± 0.06 score, p = 0.0011), and hydroxyurea (0.87 ± 0.16 score, p = 0.0139) exhibited mild sclerosis compared to HbSS controls (Figure 22). There was no significant loss in glomeruli number between HbAA mice and all HbSS mice groups.The findings suggest that combined neuregulin and hydroxyurea treatment may have enhanced effects in attenuating glomerular damage in SCD patients.

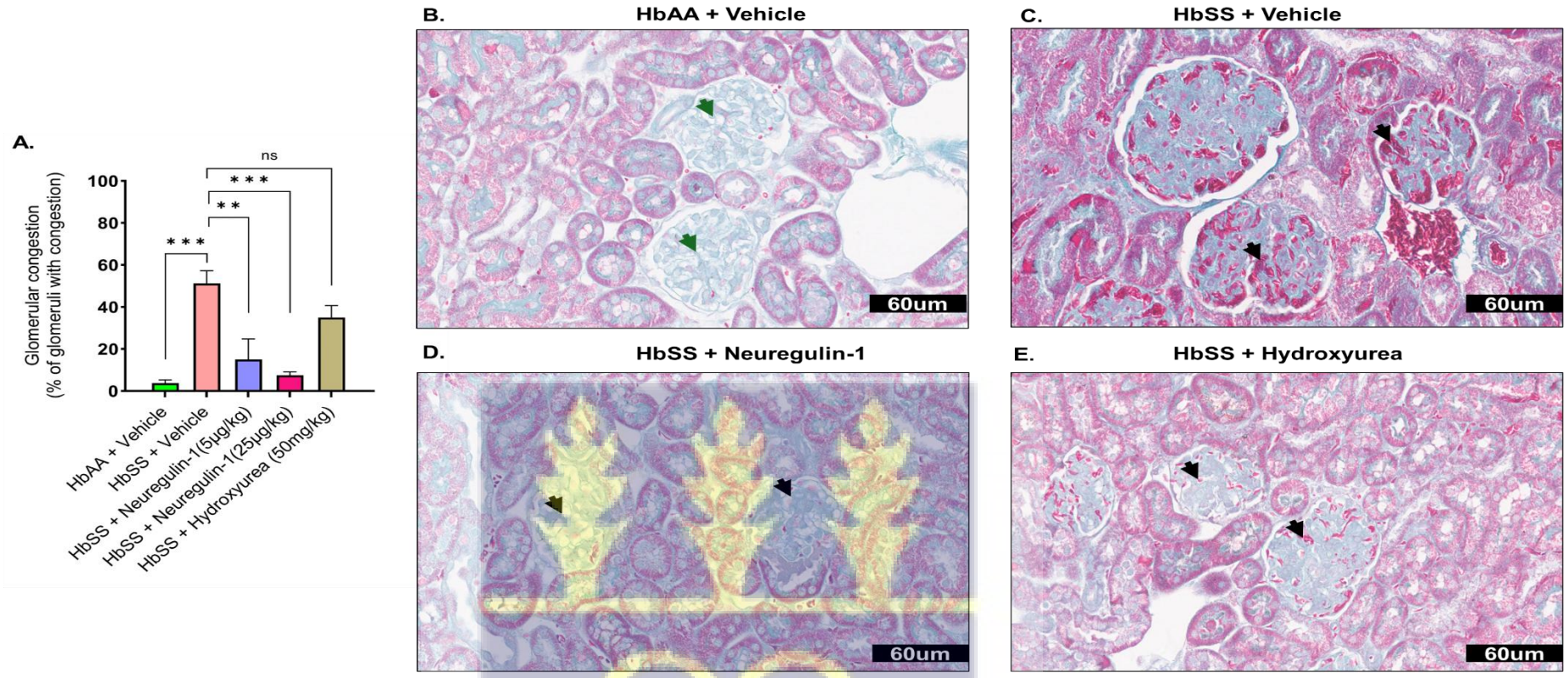


Figure 20 Neuregulin-1 treatment reduces glomerular congestion in Townes humanized sickle cell mice.

Kidney sections stained with Masson's trichome from sickle cell (HbSS) and control (HbAA) mice treated with vehicle, neuregulin-1, and hydroxyurea over two weeks from 12 weeks of age (n = 7-8 per group) are shown. A. Semi-quantitative scoring of glomerular congestion. B. Representative image of normal, uncongested glomeruli in vehicle-treated HbAA mouse (green arrowheads). C-E. Representative images of severe, mild, and moderate congestion in vehicle, neuregulin-1 and hydroxyurea-treated HbSS mouse (black arrowheads), respectively. Statistics involved two-tailed unpaired t-tests and one-way ANOVA with Tukey's post hoc test. The data are reported as Mean ± SEM. P-values are denoted as follows: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Magnification x40

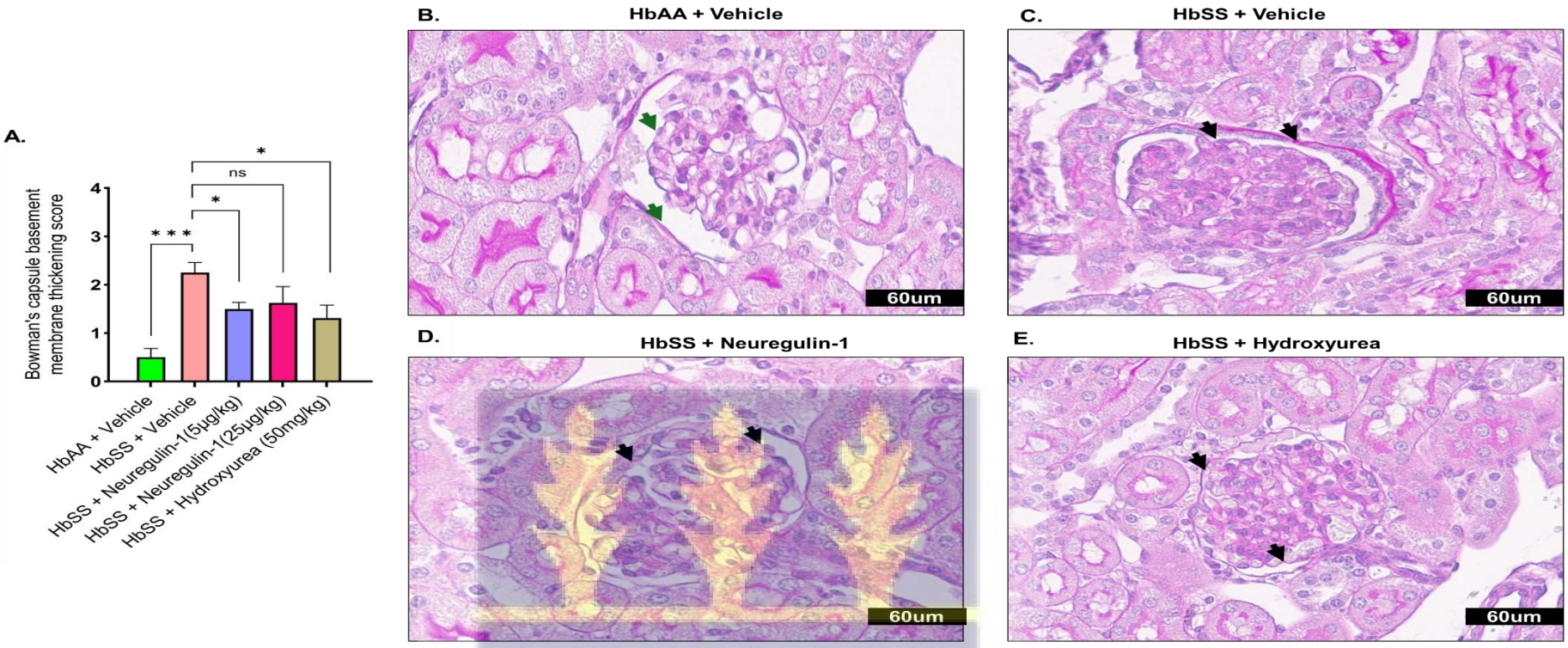


Figure 21 Neuregulin-1 and hydroxyurea treatment reduces thickening of Bowman's capsule membrane in Townes humanized sickle cell mice.

Kidney sections stained with Periodic acid-Schiff (PAS) from sickle cell (HbSS) and control (HbAA) mice treated with vehicle, neuregulin-1, and hydroxyurea over two weeks from 12 weeks of age (n = 7-8 per group) are shown. A. Semi-quantitative scoring of thickened Bowman's capsule membrane. Representative images: B. Normal Bowman's capsule membrane in vehicle-treated HbAA mouse (green arrowheads), C. Moderate thickening in vehicle-treated HbSS mouse, D-E. Mild thickening in neuregulin-1 and hydroxyurea-treated HbSS mouse (black arrowheads). Statistics involved two-tailed unpaired t-tests and one-way ANOVA with Tukey's post hoc test. Data reported as Mean ± SEM. P-values are denoted as follows: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Magnification x40.

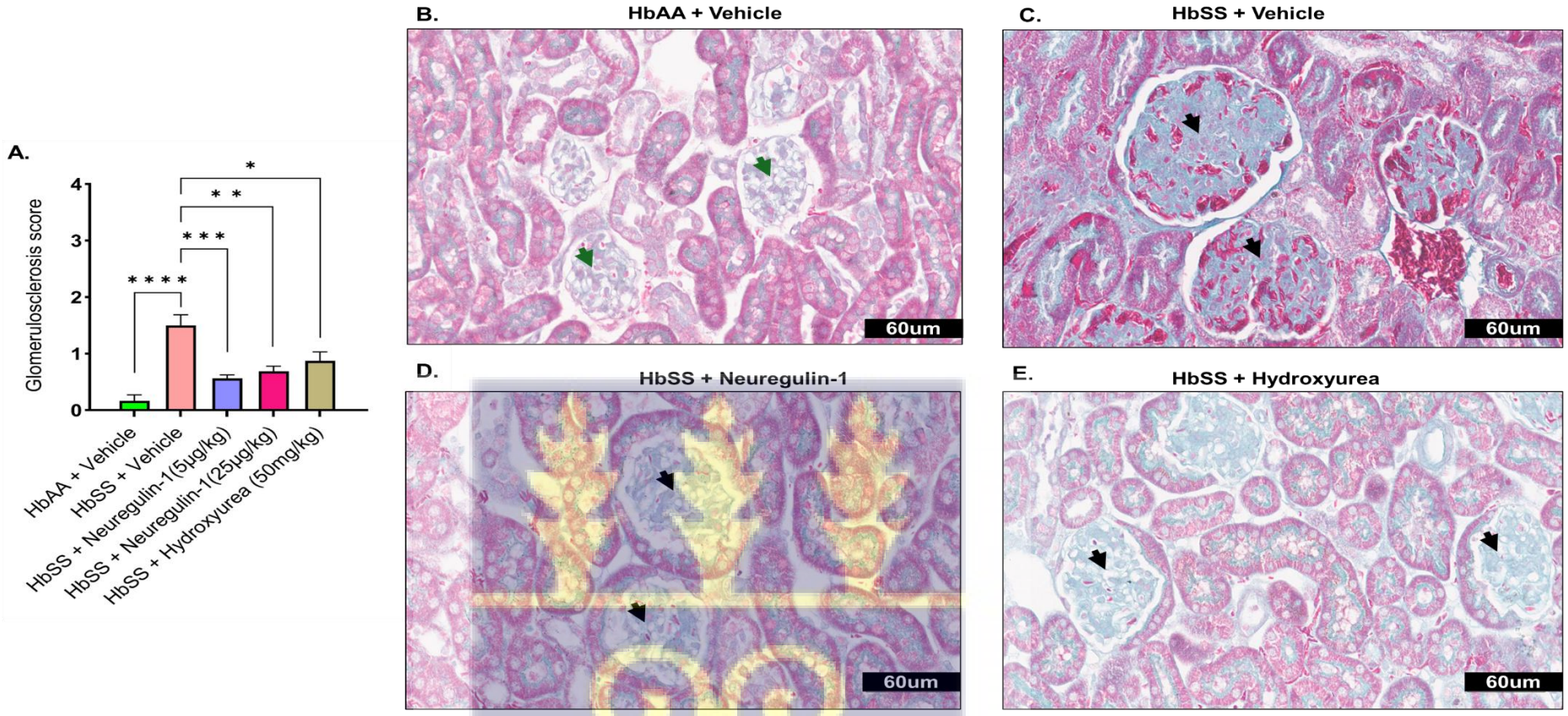


Figure 22 Neuregulin-1 and hydroxyurea treatment reduces glomerulosclerosis in Townes humanized sickle cell mice.

Kidney sections stained with Masson's trichome from sickle cell (HbSS) and control (HbAA) mice treated with vehicle, neuregulin-1, and hydroxyurea over two weeks from 12 weeks of age (n = 7-8 per group) are shown. A. Semi-quantitative scoring of glomerulosclerosis. Representative images: B Normal glomeruli with no sclerosis in vehicle-treated HbAA mouse (green arrowheads), C. Severe glomerulosclerosis in vehicle-treated HbSS mouse, D-E. Mild glomerulosclerosis in neuregulin-1 and hydroxyurea-treated HbSS mouse (black arrowheads). Statistics involved two-tailed unpaired t-tests and one-way ANOVA with Tukey's post hoc test. Data reported as Mean ± SEM. P-values are denoted as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.001$ (****). Magnification X40

4.2.3 Hydroxyurea treatment significantly reduces medullary vascular congestion compared to neuregulin-1 treatment in Townes humanized sickle cell mice.

Renal medulla blood vessels are susceptible to sickle cell erythrocyte occlusion because of the hypoxic, acidotic, and hyperosmolar environment within the medulla. Congestion within the medulla blood vessel, vasa recta, when persistent and pronounced, causes ischemic-induced renal injury. (Bissonnette et al., 2016). Medulla congestion was assessed in humanized sickle mice (HbSS) and healthy control (HbAA) treated with vehicle, neuregulin-1, and hydroxyurea for two weeks beginning at 12 weeks of age was examined (Figure 23). Congestion in the vasa recta was significantly severe in vehicle-treated HbSS mice compared to the HbAA controls (82.500 ± 2.83% score vs 13.330 ± 6.03 score, $p < 0.0001$). Neuregulin-1 treatments did not significantly improve vasa recta congestion (75.000 ± 1.34, $p = 0.0875$ vs HbSS). Interestingly, hydroxyurea-treated mice exhibited a notable significant reduction in the proportion of congested vasa recta compared to vehicle-treated HbSS controls. (46.250 ± 8.80 score vs 82.50 ± 2.83 score, $p = 0.0023$) (Figure 23).

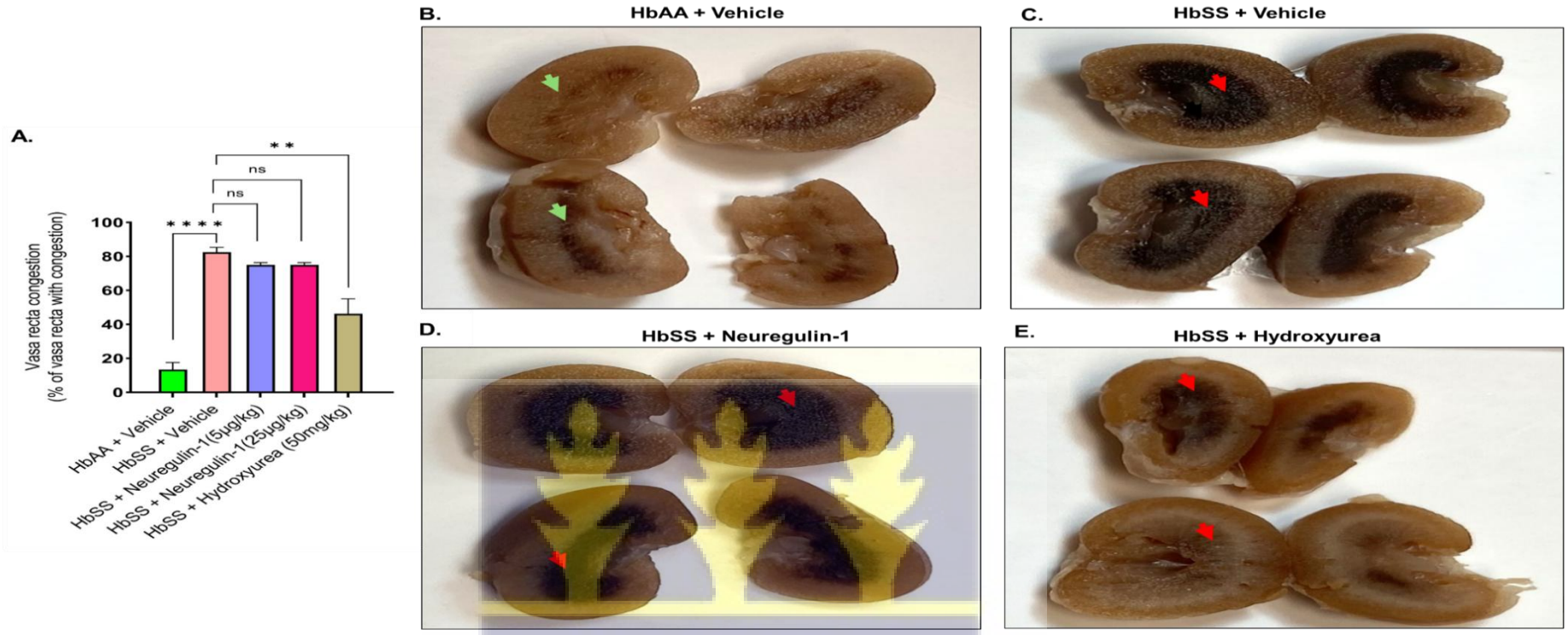


Figure 23 Hydroxyurea treatment significantly reduces vasa recta congestion compared to neuregulin-1 treatment in humanized sickle cell mice.

Gross appearance of kidney halves from sickle cell (HbSS) and healthy control (HbAA) mice treated with vehicle, neuregulin-1, and hydroxyurea over two weeks from 12 weeks of age (n = 7-8 per group) are shown. A. Semi-quantitative scoring of glomerulosclerosis. Representative images: B Medulla with mild congestion in vehicle-treated HbAA mouse (green arrowheads), C-D. Severe medulla congestion in vehicle and neuregulin-treated HbSS mouse, respectively (black arrowheads)., E. Medulla with mild congestion in hydroxyurea-treated HbSS mouse (black arrowheads). Statistics involved two-tailed unpaired t-tests and one-way ANOVA with Tukey's post hoc test. Data reported as Mean ± SEM. P-values are denoted as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.001$ (****).

4.2.4 Neuregulin-1 mitigates severe tubular brush border loss and iron accumulation in humanized sickle cell mice.

The cytoprotective effects of Neuregulin-1 (NRG-1) against heme-mediated histopathological changes in the renal tubules of humanized sickle mice HbSS and genetic HbAA controls was assessed. Hydroxyurea (HU) treatment in HbSS mice was done concomitantly. Heme-mediated renal tubular epithelial injury is characterized by histopathological changes, including excessive iron accumulation and reduced epithelial brush border thickness.

Both treated and untreated sickle cell mice exhibited marked iron accumulation within the tubular walls, and the number of cortical tubules showing iron deposition did not differ significantly among the HbSS groups (Figure 24). However, neuregulin-1 treatment significantly reduced the intensity of iron staining in the tubules (5µg/kg/; 32.820 ± 11.22 vs 165.000 ± 26.79, x 10^3 pixels intensity/mm^2, $p = 0.0012$). While treatment with 25µg/kg NRG-1(77.720 ± 32.34 vs 165.000 ± 26.79, x 10^3 pixels intensity/mm^2, $p = 0.0566$) and hydroxyurea (117.400 ± 36.17 vs 165.000 ± 26.79, x 10^3 pixels intensity/mm^2, $p = 0.3098$) also resulted in reduced iron deposition, these changes were not statistically significant compared to HbSS controls (Figure 24).

Loss of the renal epithelial brush border is a histopathological feature indicative of tubular necrosis. More than half of the cortical tubules in the kidneys of vehicle-treated HbSS mice exhibited marked loss of brush borders compared to vehicle-treated HbAA controls (1.625 ± 0.16 score vs 3.33 ± 0.11 score, $p < 0.0001$). Treatment with neuregulin-1 and hydroxyurea improved the extent of tubular brush border loss compared to vehicle-treated sickle cell mice. Mice treated with NRG-1 (5µg/kg/dose) had less than 25% of their tubular cells losing their brush borders (3.125 ± 0.08, $p < 0.0001$). Additionally, mice treated with HU (2.813 ± 0.13, $p < 0.0001$) and NRG-1 (25µg/kg/dose; 2.625 ± 0.20, $p = 0.0019$ exhibited loss of brush borders in over 25% of

their cortical tubules. However, this effect was significantly less severe than in untreated HbSS controls (Figure 25).

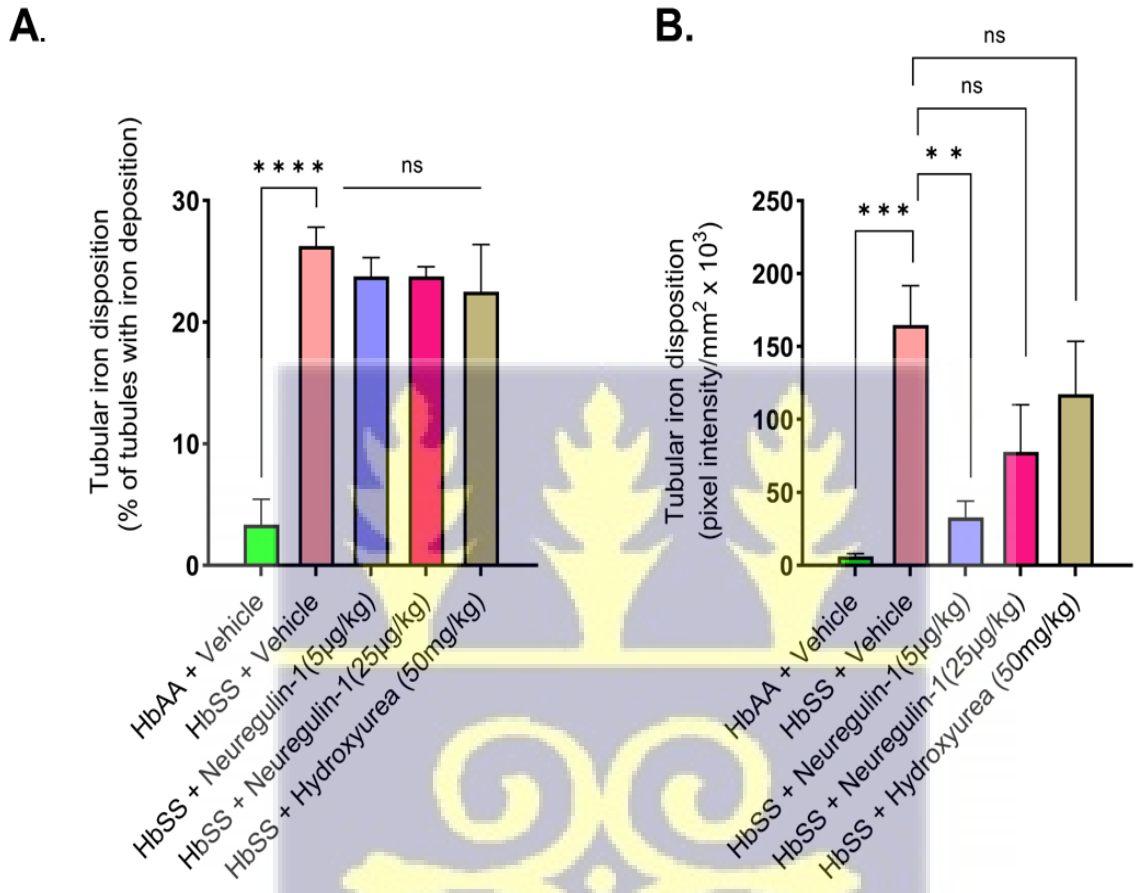


Figure 24 Neuregulin-1 treatment reduces intensity of iron deposits in kidneys of Townes humanized sickle cell mice.

Kidney sections stained with Perl's Prussian blue from sickle cell (HbSS) and control (HbAA) mice treated with vehicle, neuregulin-1, and hydroxyurea over two weeks from 12 weeks of age (n = 7-8 per group) were examined for tubular iron deposits. Semi-quantitative scoring of: A. Percentage of tubules with iron deposition, and B. Quantification of the intensity of iron deposits. Statistical analysis involved two-tailed unpaired t-tests and one-way ANOVA with Tukey's post hoc test. Data reported as Mean ± SEM. P-values are denoted as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.001$ (****).

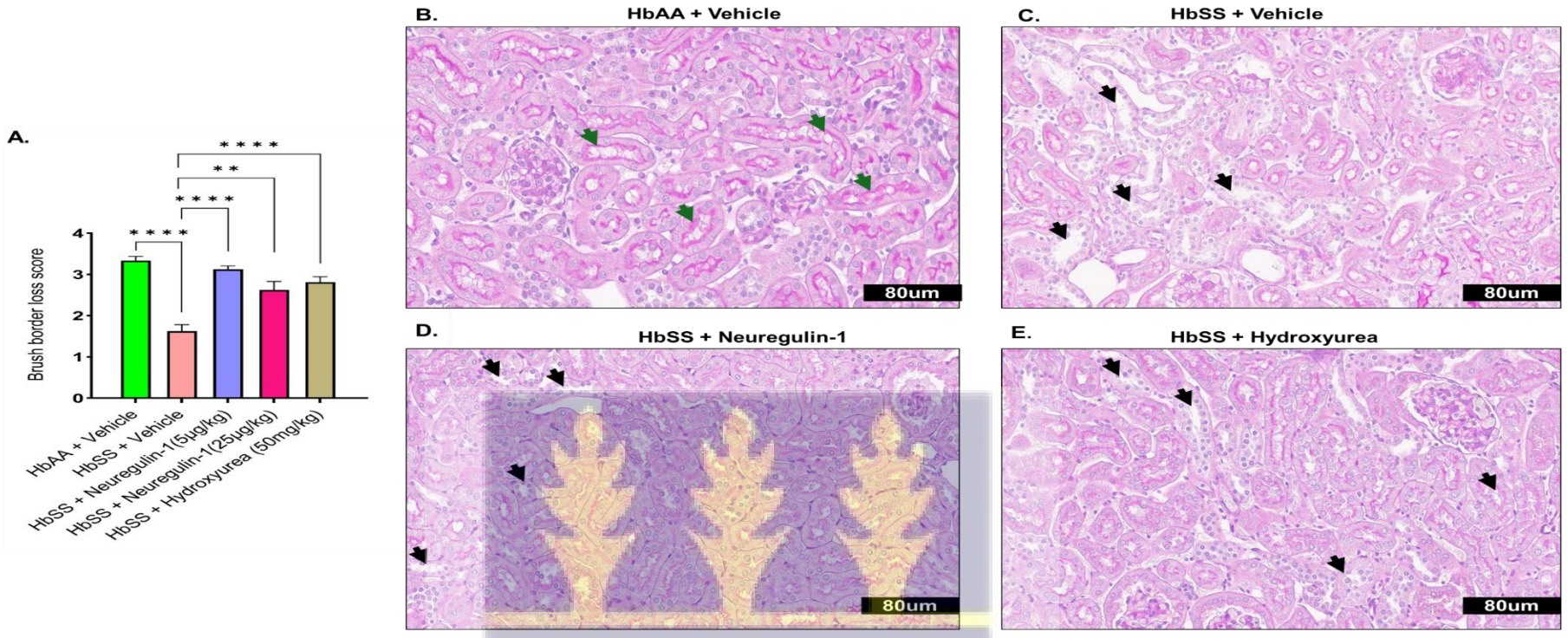


Figure 25 Neuregulin-1 and hydroxyurea treatment reduces loss of renal tubular brush border in Townes humanized sickle cell mice.

Kidney sections stained with Periodic acid-Schiff (PAS) from sickle cell (HbSS) and control (HbAA) mice treated with vehicle, neuregulin-1, and hydroxyurea over two weeks from 12 weeks of age (n = 7-8 per group) are shown. A. Semi-quantitative scoring of tubular brush border loss. Representative images: B. Cortical tubules with stained brush border in vehicle-treated HbAA mouse (green arrowheads), C. Severe tubular brush border loss in vehicle-treated HbSS mouse, D-E. Mild tubular brush border loss in neuregulin-1 and hydroxyurea-treated HbSS mouse (black arrowheads). Statistics involved two-tailed unpaired t-tests and one-way ANOVA with Tukey's post hoc test. Data reported as Mean ± SEM. P-values are denoted as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.001$ (****).

4.3 Specific aim 3 - To evaluate the impact of neuregulin-1 treatment on the expression and distribution of heme oxygenase-1 in the kidneys of humanized sickle cell mice.

4.3.1 Neuregulin-1 treatment increases the expression of heme oxygenase-1 in the renal tubules of Townes humanized sickle cell mice.

Heme oxygenase-1 (HO-1), the rate-limiting enzyme that catalyzes heme degradation is upregulated during acute haemolytic stress, serves as a critical nephroprotective factor that mitigates heme-mediated oxidative and inflammatory pathways (Grunenwald et al., 2021). To evaluate the capacity of neuregulin-1 (NRG-1) to induce renal HO-1 expression, humanized sickle cell (HbSS) mice and healthy HbAA controls were treated with NRG-1 for two weeks; hydroxyurea was administered concomitantly to HbSS mice. Notably, NRG-1 treatment markedly increased renal HO-1 expression in HbSS mice relative to untreated HbSS cohorts.

In both HbSS and HbAA mice, HO-1 positive stains were mostly located in the renal cortical tubules (Figure 26). Also, weak and isolated positive stains were observed in the glomeruli of some HbSS mice but were absent in the glomeruli of HbAA mice. HO-1 positive stains were not observed in the inner medulla of both HbSS and HbAA mice. Prior treatment, sickle cell mice had increased HO-1 expression with a higher positive stain count (1.893 ± 0.45 vs 0.206 ± 0.08 x 10^3 pixels count/mm^2, $p = 0.0302$) and stain intensity (0.157 ± 0.04 vs 0.016 ± 0.01, megapixels/mm^2, $p = 0.0406$) compared to HbAA mice (Figure 26).

Remarkably, mice treated with neuregulin-1 exhibited a significant increased HO-1 positive stain (12.060 ± 7.35 vs 4.97 ± 1.945 x 10^3 pixels count/mm^2, $p = 0.0070$) and stain intensity (3.257 ± 0.57 vs 0.191 ± 0.02, megapixels/mm^2, $p = 0.0002$) compared to HbSS controls (Figure 27). Hydroxyurea treatment did not have any significant effect on positive pixel count (1.586 ± 0.42 vs 2.261 pixels count/mm^2, $p = 0.9905$) and stain intensity (0.450 ± 0.32 megapixels/mm^2, $p = 0.7552$) compared to vehicle-treated HbSS mice (Figure 27).

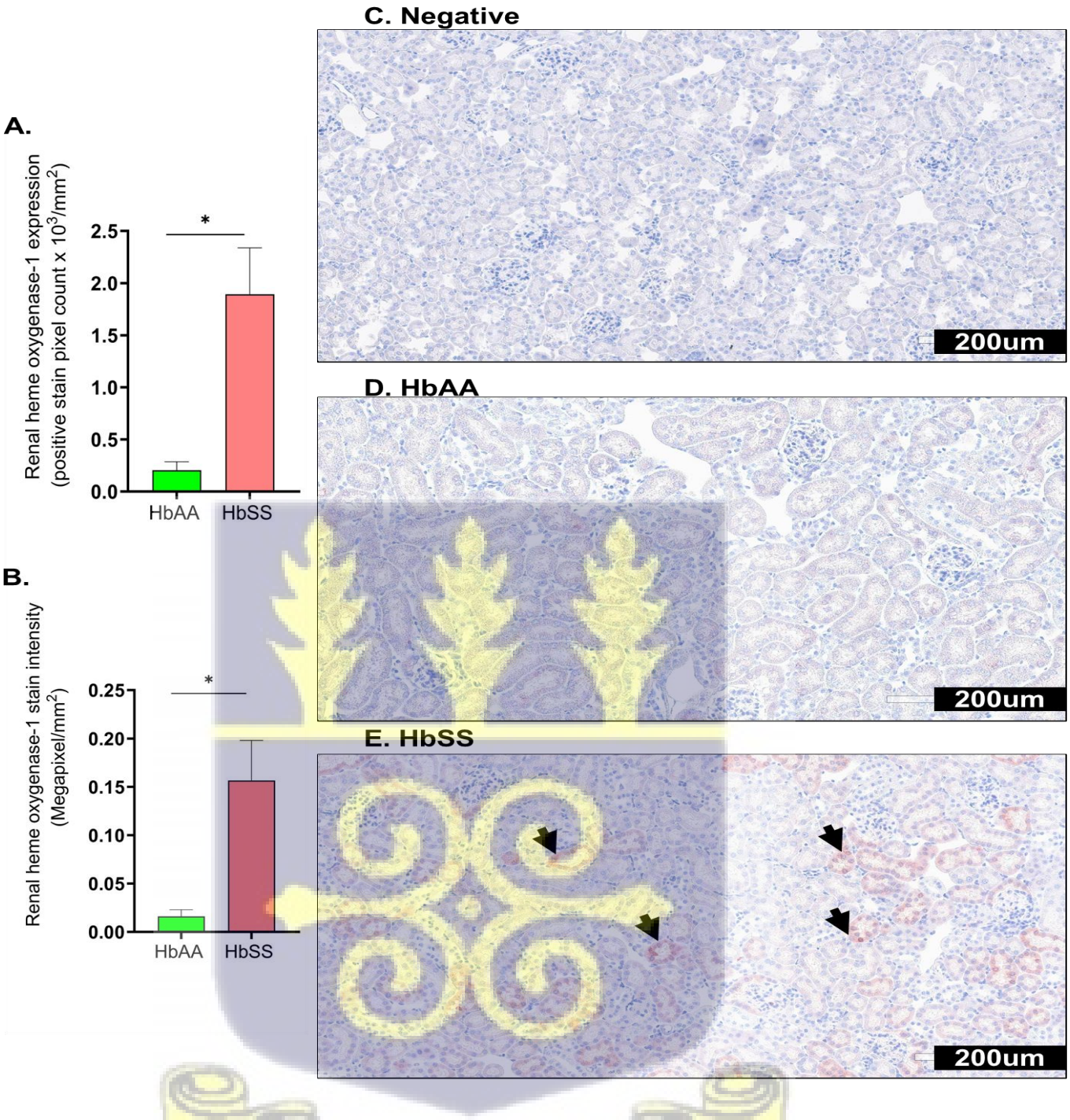
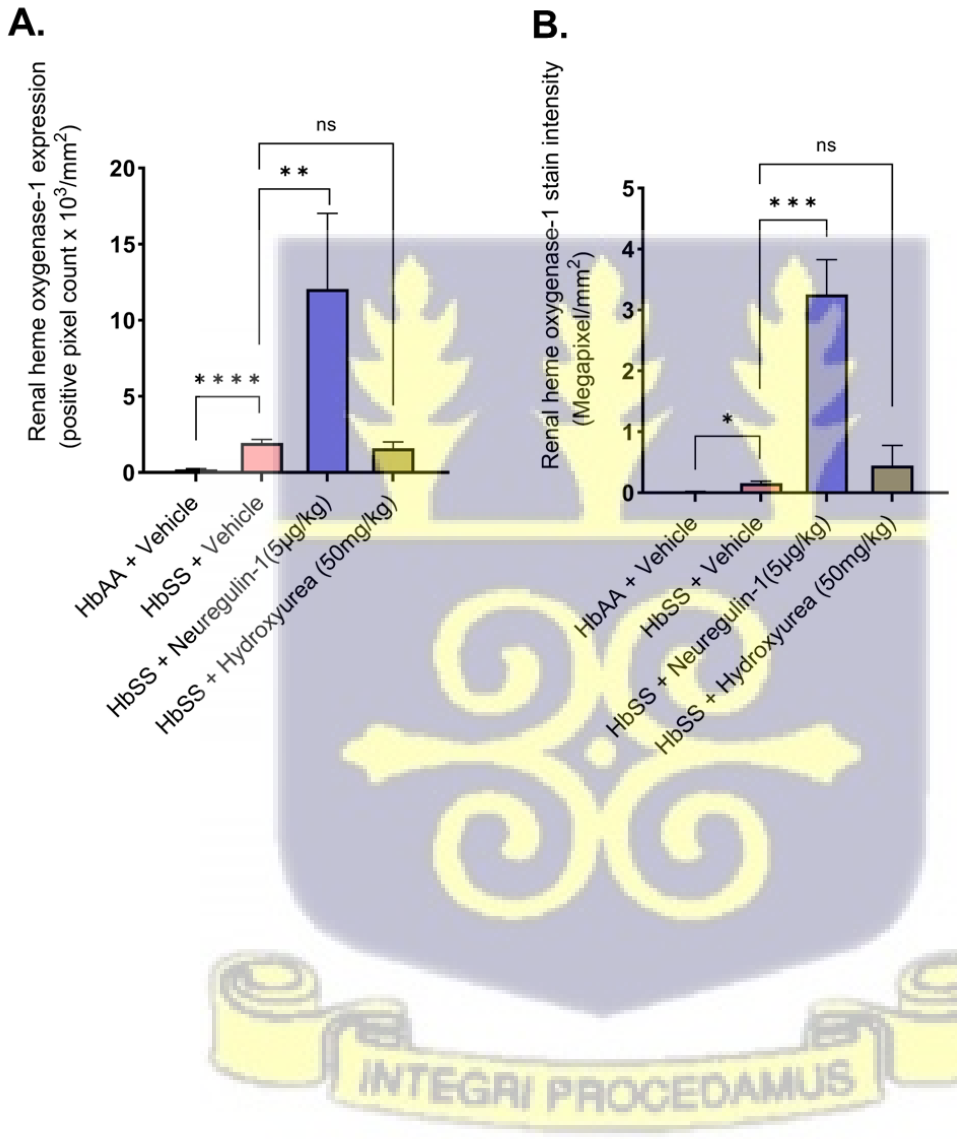


Figure 26 Increased heme oxygenase-1 expression in the kidney of Townes humanized sickle cell mice compared to healthy controls.

Anti-heme oxygenase-1 immunohistochemical analysis of kidney sections from sickle cell (HbSS) and healthy control (HbAA) mice at 12 weeks of age (n = 7-8 per group) are shown. A-B. Quantification of renal heme oxygenase-1 expression in HbAA versus HbSS mice, C – E.

Representative cortex images of anti-heme oxygenase-1-stained renal sections of HbAA and HbSS mouse, with dark arrowheads showing increased expression of heme oxygenase-1 in HbSS (Panel E) cortical tubules compared to HbAA (Panel D). Statistics involved two-tailed unpaired t-tests. Data reported as Mean ± SEM. P-values $p < 0.05$ (*). Original magnification X40.



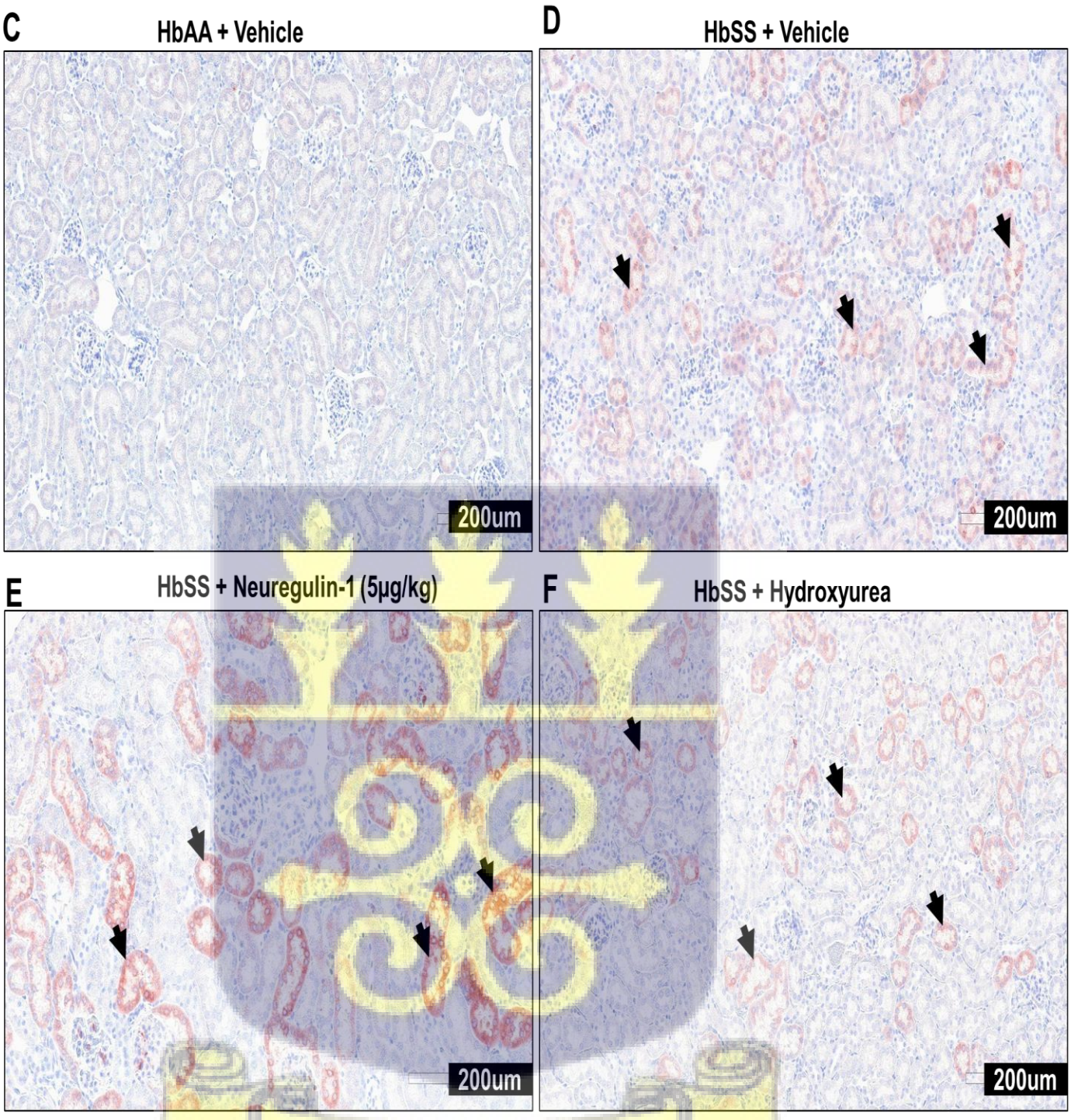


Figure 27 Neuregulin-1 treatment increases heme oxygenase-1 expression in the kidney of Townes humanized sickle cell mice.

Anti-heme oxygenase-1 immunohistochemical analysis of kidney sections from sickle cell (HbSS) and control (HbAA) mice treated with vehicle, neuregulin-1, and hydroxyurea over two weeks from

12 weeks of age (n = 7-8 per group) are shown. A-B. Quantification of renal heme oxygenase-1 (HO-1) expression and intensity among treatment groups, Representative images: Panel C shows nearly absent HO-1 expression in vehicle-treated HbAA mouse, Panels D-F displays HO-1 expression among HbSS groups with neuregulin-1-treated HbSS mouse showing a higher expression (black arrowheads). Statistics involved two-tailed unpaired t-tests and one-way ANOVA with Tukey's post hoc test. Data reported as Mean ± SEM. P-values are denoted as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.001$ (****). Original magnification X40.

4.3.2 Increased expression of neuregulin-1 receptor ErbB4 localized to the apical surfaces of renal tubules in Townes humanized sickle cell mice.

Given the observed upregulation of renal heme oxygenase-1 (HO-1) expression by neuregulin-1 (NRG-1), it was important to identify the kidney cell types that NRG-1 may influence through ligand–receptor interactions. NRG-1 is known to directly bind and activate the receptor tyrosine kinase ErbB4, a member of the epidermal growth factor receptor family. To characterize its renal distribution, ErbB4 expression and localization were examined in the kidneys of humanized sickle cell (HbSS) and healthy (HbAA) mice using immunohistochemistry. The results revealed that ErbB4 is abundantly expressed in the kidneys of HbSS mice, predominantly localized to the apical surface of renal tubular epithelial cells.

ErbB4 expression and localization exhibited a similar pattern in both sickle (HbSS) and normal (HbAA) mice (Figure 20A). ErbB4 was predominantly detected in the tubular segments of the outer medulla, encompassing the proximal straight tubules, thick ascending limbs, thin limbs of the loop of Henle, and collecting ducts (Figure 28). The receptor was mainly localized to the apical surface of tubular epithelial cells, with weak intracellular staining observed in cortical tubules and glomeruli (Figure 28).

Interestingly, increased Erb4 positive stains (253.50 ± 41.51 vs 99.87 ± 12.81 x 10^3 pixels count/mm^2, $p = 0.0164$) with higher intensity (18.33 ± 4.43 vs 8.14 ± 1.25 megapixels/mm^2, $p = 0.0442$) was observed in the sickle cell mice compared with HbAA controls (Figure 29). ErbB4 expression did not significantly differ in HbSS mice groups after treatment (Appendix 2). Taken together, the increased expression of ErbB4 on sickle cell mice's kidney tubules suggests a critical role of ErbB4 signaling in protecting against ongoing tubular injury.

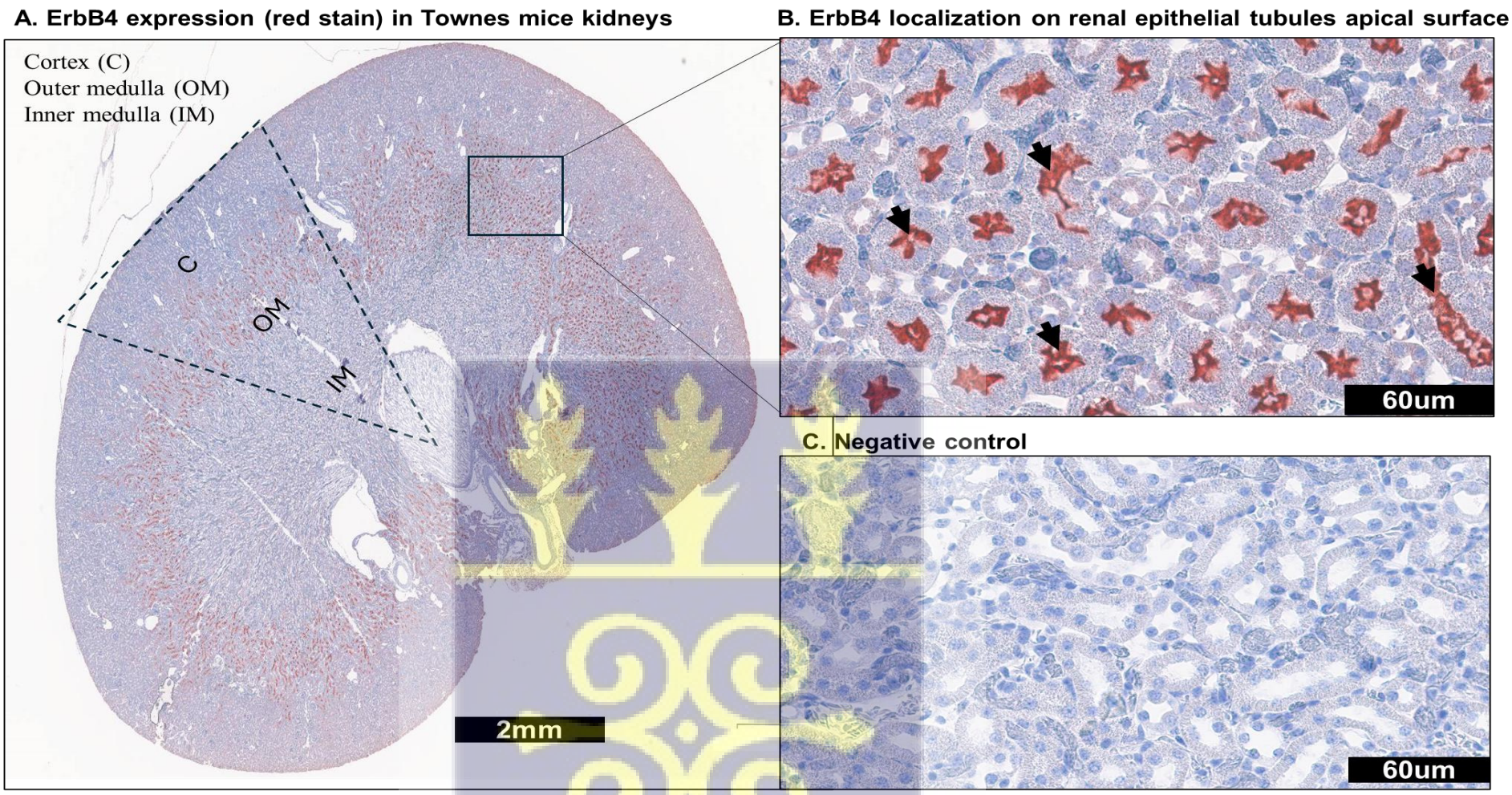


Figure 28 Expression of neuregulin-1 receptor, ErbB4 in the kidneys of Townes HbSS and HbAA mice.

ErbB4, belonging to the epidermal growth factor family expression and localization (black arrowheads) on the apical surface of renal tubular epithelial cells *were detected in the kidneys of humanized sickle cell mice and normal mice by immunohistochemistry.*

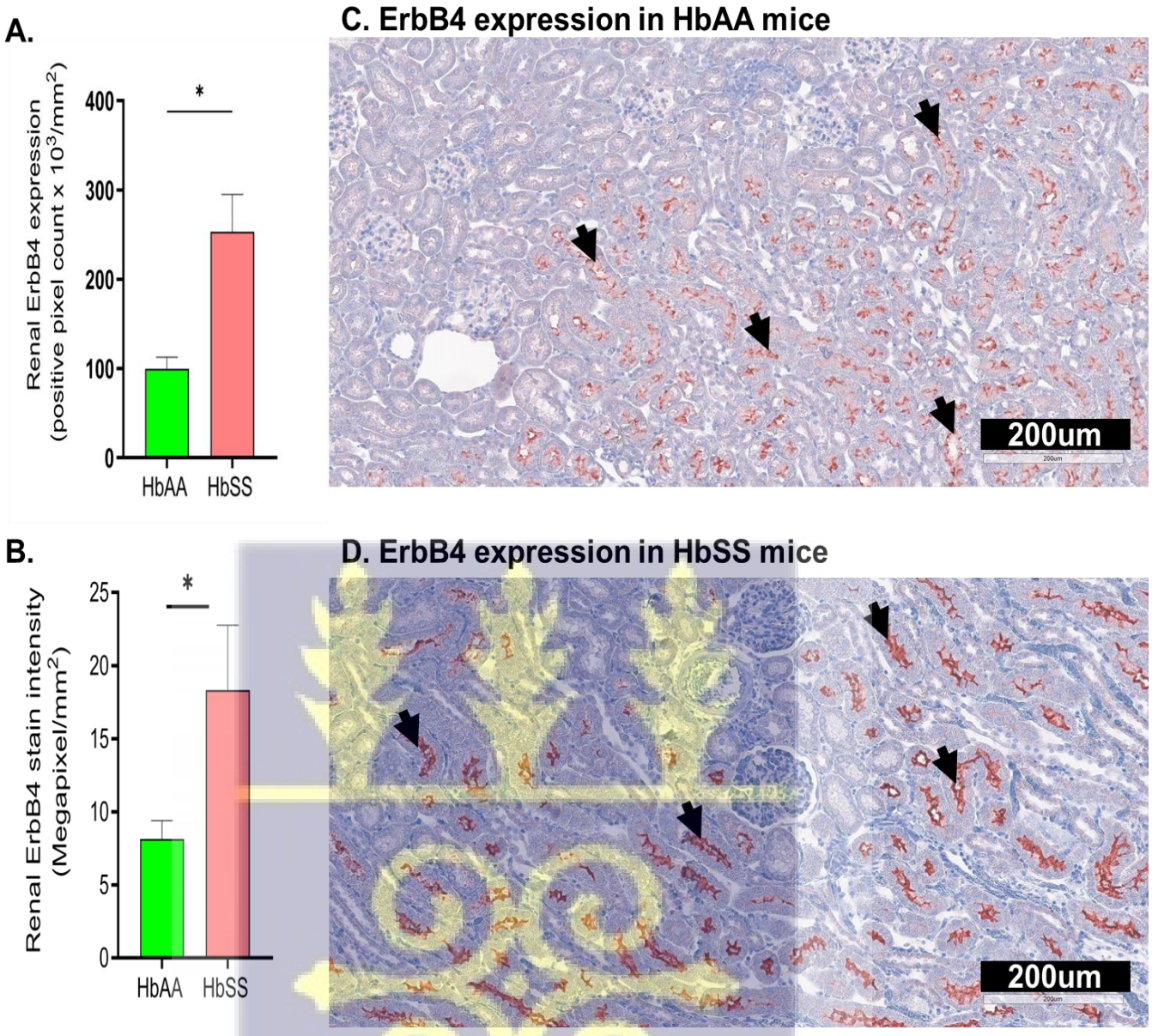


Figure 29 Increased expression of neuregulin-1 receptor, ErbB4 in the kidneys of Townes sickle cell mice compared to healthy controls.

Anti-ErbB4 immunohistochemical analysis was conducted on kidney sections from Townes sickle cell (HbSS) and control (HbAA) mice at 12 weeks of age (n = 7-8). Panel A-B depicts quantification of renal ErbB4 expression, and Panel C-D are representative images illustrating differences in expression intensity between HbSS and HbAA mice, indicated by black arrowheads. Statistical analyses employed two-tailed unpaired t-tests, with data expressed as Mean ± SEM. Significant differences were noted at P-values < 0.05 (*).

5.0 CHAPTER FIVE: DISCUSSION.

Neuregulin-1 (NRG-1), a versatile signaling molecule, plays a critical role in cytoprotection through the induction of anti-inflammatory, anti-cytotoxic, and pro-survival mechanisms (Cespedes et al., 2018). This project investigated NRG-1's therapeutic potential to attenuate haemolysis-mediated kidney injury in the Townes humanized sickle cell murine model. The study's rationale was based on evidence from human and experimental studies suggesting that NRG-1 could provide renal protective benefits in sickle cell disease (SCD) (Chambliss et al., 2023; Daniels et al., 2021; Gao et al., 2010; Harbuzariu et al., 2019, 2022; Jabbour et al., 2011; Liu et al., 2018; Solomon et al., 2014). Concurrently, hydroxyurea (HU), the standard treatment for SCD, was administered to HbSS mice, comparing an established therapy with NRG-1's novel intervention.

The dosages used for NRG-1 and HU treatment were based on previous studies in murine models. The study findings showed that a high dose of NRG-1 (25 µg/kg/day) did not produce enhanced effects compared to the low dose (5 µg/kg/day). Most of the renal protective effects of NRG-1 were observed in the low-dose group, though the reason for this is unclear. Similar observations have been reported in studies where higher doses of NRG-1 did not yield greater effects compared to lower doses in experimental cerebral malaria models (Liu et al., 2018; Solomon et al., 2014). In a clinical trial, a low dose of NRG-1 (0.6 µg/kg) improved heart function in patients, while no improvement was seen with the high dose (1.2 µg/kg). Various NRG-1 doses have been used to modulate inflammation and cell death in murine models, including 1 µg/kg/day (Shahsavani et al., 2021), 3µg/kg/day (Li et al., 2015; Wang et al., 2021), 5µg/kg/day (Liu et al., 2018; Vega-Torres et al., 2022), 10µg/kg/day (Guo et al., 2012; Liu et al., 2020; Zhou et al., 2016), and 35µg/kg/day (Xu et al., 2017). The effective concentration of NRG-1 appears to vary depending on the cell type and disease model (Gui et al., 2007; Rohrbach et al., 2005; Xu et al., 2012). For example, high

doses of NRG-1 have been found to be detrimental to neural function in experimental studies(Guertin et al., 2005; Syed et al., 2010; Xu et al., 2012; Zanazzi et al., 2001). The observation that most effects of NRG-1 occurred at the low dose likely reflect dose-dependent regulatory feedback rather than toxicity. Excessive ErbB4 receptor activation can trigger feedback mechanisms to prevent pathway overstimulation. Thus, the higher dose may have activated negative feedback mechanisms that limited the therapeutic response (Fry et al., 2009). Further research is needed to establish the optimal NRG-1 dose in humanized sickle cell mice. A maximum tolerable dose of 50 mg/kg/day of HU was used, based on previous dose-finding experiments (Lebensburger et al., 2010). This dose has been shown to mitigate kidney damage in humanized sickle cell mice and confer disease-modifying benefits, similar to results seen in humans (Park et al., 2019; Taylor et al., 2019). In humans, hydroxyurea treatment is typically initiated at a low dose and gradually increased to the maximum tolerated dose to confer clinical benefits while minimizing treatment-related toxicity (Akingbola et al., 2019).

The sickle cell mice used in this study spontaneously developed kidney damage features before treatment initiation. The natural progression of renal disease in humanized sickle cell mice closely resembles that of human renal disease. In humans, renal disease typically begins in early childhood with a phase of hyperfiltration, followed by a progressive decline in kidney function (Aygun et al., 2013; Nath & Hebbel, 2015; Vazquez et al., 2014; Wang et al., 2011; Ware et al., 2010). Similarly, in sickle cell mice, renal manifestations typically begin around 12 weeks of age, starting with glomerular hyperfiltration and progressing to a loss of renal function (Kasztan et al., 2017, 2019). Glomerular hyperfiltration is common in children and young SCD patients and is associated with elevated renal injury biomarkers (Alvarez et al., 2008; Aygun et al., 2011; Becton et al., 2010; Heimlich et al., 2018). The HbSS mice in this study displayed early renal damage features that

paralleled those seen in humans, as indicated by increased levels of injury biomarkers at 12 weeks old. This confirmed that, prior to treatment, HbSS mice had renal tubular injury. These findings are consistent with previous studies (Kasztan et al., 2017, 2019; Taylor et al., 2019). The understanding is that hyperfiltration increases the filtration of heme proteins, which are then taken up by tubular epithelial cells (Ghosh et al., 2017; Saraf et al., 2015). Endocytosed heme proteins mediate cytotoxicity within these cells, causing injury. Although tubular injury is often associated with glomerular injury (Sundaram et al., 2011), serum cystatin C, a glomerular injury marker, was not significantly different between HbSS and HbAA mice. However, mild histopathologic changes were noted in the glomeruli of HbSS mice, suggesting the onset of glomerular damage. Serum cystatin C may therefore not be a sensitive biomarker for detecting early glomerular injury. Sickle cell nephropathy progresses through a tubuloglomerular mechanism, where tubular injury precedes significant glomerular injury (Kasztan et al., 2019). The 12-week-old Townes sickle cell mice used in this study served as a good model for investigating the therapeutic potential of NRG-1 in modulating early kidney injury, as seen in humans.

A positive correlation was established between urinary tubular injury markers (NGAL and cystatin C) and serum haemolysis markers (heme and lactate dehydrogenase) in HbSS mice. This finding agrees with previous studies in humans and mice, emphasizing the central role of intravascular haemolysis in SCD-related kidney injury (Avondt et al., 2019; Ghosh et al., 2017; Hamideh et al., 2014; Ofori-Acquah et al., 2020; Tolosano et al., 1999). Acute haemolysis results in excess amounts of heme proteins released into circulation, which are then filtered by the kidneys, exposing renal tubular cells to the toxic effects of heme (Avondt et al., 2019).

The study demonstrated that renal tubular injury, characterized by elevated levels of NGAL in serum or plasma and urine cystatin C, can be reduced by hydroxyurea treatment in SCD mice. This

observation is consistent with prior studies in which hydroxyurea improved renal tubular injury (Park et al., 2019; Taylor et al., 2019). Although the exact renal tubular protective mechanism of hydroxyurea is yet to be fully understood, the study findings suggest that its ability to reduce tubular injury is likely linked to its primary benefit of reducing haemolysis. This is supported by studies in humans and mice, where elevated levels of lactate dehydrogenase or heme are significantly associated with kidney injury or impaired function (Aygun et al., 2013; Gurkan et al., 2010; Hamideh et al., 2014; Haymann et al., 2010; Laurin et al., 2014; Maier-Redelsperger et al., 2010).

Though the exact mechanism by which inflammatory cytokines trigger kidney injury in SCD is unclear, anti-inflammatory strategies have been proposed as protective for SCD kidneys (Obadina et al., 2023). The literature on hydroxyurea's anti-inflammatory effects is limited and conflicting (Conran et al., 2004; Keikhaei et al., 2013; Lanaro et al., 2009; Penkert et al., 2018; Zahran et al., 2020). For example, IL-6, a kidney injury–associated cytokine, was elevated in hydroxyurea-treated HbSS mice, consistent with Keikhaei et al. (2013), but contradicts with the findings by Zahran et al., (2020) where hydroxyurea therapy decreased IL-6 serum levels.

This is the first study demonstrating the renal beneficial effects of NRG-1 in SCD, using the Townes sickle cell model. After two weeks of treatment, neuregulin-1 moderately attenuated the severity of tubular injury by reducing urinary NGAL and cystatin C levels compared to vehicle-treated HbSS mice. In a similar novel finding, NRG-1 treatment reduced NGAL urine levels in a diabetic nephropathy mouse model (Vandekerckhove et al., 2016). These findings on the renoprotective ability of NRG-1 in mice models are supported by a study identifying NRG-1 as a predictive renal biomarker. Higher circulating levels of neuregulin-1 are strongly associated with kidney recovery and survival in acute kidney injury patients (Daniels et al., 2021). Drawing from

observations that early initiation of hydroxyurea treatment in SCD children results in better renal protection (Zahr et al., 2019), it would be valuable to investigate early NRG-1 treatment in a sickle cell mice model before the onset of renal disease.

Although the precise mechanism of NRG-1's renal tubular protection in SCD remains to be determined, the observed reduction in plasma heme and lactate dehydrogenase levels together with decreased inflammatory cytokine expression in NRG-1-treated HbSS mice suggests that its protective effect is mediated, at least in part, through enhanced heme clearance and suppression of inflammation.. NRG-1 significantly reduced haemolytic markers such as plasma heme and lactate dehydrogenase in HbSS mice. Similarly, neuregulin markedly reduced plasma lactate dehydrogenase in a rat model of myocardial ischemia (Fang et al., 2010). In a mouse model of cerebral malaria, which shares a similar haemolytic milieu with SCD, exogenous NRG-1 administration attenuated organ damage and mortality related to heme-mediated pathways (Liu et al., 2018; Solomon et al., 2014). The effect of NRG-1 on plasma heme clearance is likely linked to its downstream signaling via nuclear factor erythroid 2-related factor 2 (Nrf2) (Park et al., 2021). Nrf2 activation in sickle cell mice increases the expression of heme-neutralizing proteins such as hemopexin and heme oxygenase in the liver and blood (Belcher et al., 2017; Krishnamoorthy et al., 2017).

Haemolysis is a key mediator of vascular inflammation in SCD (Ofori-Acquah, 2020). Serum levels of pro-inflammatory cytokines are associated with acute kidney injury and repair (Mulay et al., 2016). Thus, reducing serum pro-inflammatory cytokines was anticipated as a strategy for mitigating renal injury in the sickle cell kidney. As expected, neuregulin-1 significantly reduced serum pro-inflammatory markers. Importantly, NRG-1 reduced IL-6 cytokine, an acute kidney injury biomarker. This observation aligns with studies where NRG-1 treatment reduced serum IL-

6 levels in other disease models (Dreymueller et al., 2014; Kang et al., 2019; Liu et al., 2020; Zhou et al., 2016). Additionally, NRG-1 significantly reduced serum levels of IFN-γ, IP-10, VEGFA, CCL19, and MCP-5 in HbSS mice. It has been previously demonstrated that NRG-1 suppresses the release of pro-inflammatory cytokines including MCP-1 (the human/rat ortholog of mouse MCP-5) expression in a rat model of ischemic stroke (Simmons et al., 2016). Alizadeh et al. (2018) also demonstrated that chronic NRG-1 administration reduced IP-10 levels in a rat model of spinal cord injury (Alizadeh et al., 2018). These anti-inflammatory effects of NRG-1 are particularly relevant to SCD-related kidney injury, as high levels of IL-6, MCP-1, IP-10, and other cytokines contribute to the emergence or persistence of albuminuria in sickle cell children (Belisário et al., 2020). Also, IL-6, IFN-γ, and MCP-1 (human ortholog of MCP-5) have been identified in other studies as renal biomarkers associated with poor outcomes in acute kidney injury (Burne et al., 2001; Chen et al., 2020; Moledina et al., 2019; Nechemia-Arbely et al., 2008; Wu et al., 2016). While pro-inflammatory cytokine levels were elevated in untreated HbSS mice, the anti-inflammatory cytokine IL-10 was at normal levels compared to HbAA mice, indicating a lack of effective regulation of inflammation in sickle cell mice. Remarkably, NRG-1 treatment significantly increased serum IL-10 levels. This increase is relevant to renal protection, as IL-10 deficiency has been shown to lead to elevated inflammatory cytokines and is associated with kidney inflammation and fibrosis in mice (Jin et al., 2013). Taking it together, the results support the hypothesis that dysregulated inflammatory processes in SCD worsen kidney injury, and NRG-1 treatment, through its anti-inflammatory effects, has the potential to attenuate this damage.

The study observed that renal injury repair and regeneration biomarkers were reduced in HbSS mice compared to HbAA controls. Specifically, low urinary levels of clusterin (CLU) and epidermal growth factor (EGF) were detected, reflecting decreased renal expression. Contrary to

the study findings, Belisário et al. (2021) reported that urinary CLU and EGF were elevated in sickle cell anaemia children with albuminuria compared to healthy controls (Belisário et al., 2020). However, the study findings are consistent with those from studies on critically ill children with acute kidney injury (Askenazi et al., 2016) and adult patients with acute kidney injury, where urinary EGF levels were significantly reduced (Ono et al., 2024). Since urinary CLU and EGF levels reflect the repair and recovery response following acute kidney injury, it would be relevant to assess these levels in a cohort of SCD patients with acute kidney injury and relate the findings to this study. More importantly, this finding raises interest in understanding the role of renal cytoprotective, repair, and recovery factors following AKI in SCD. It is increasingly recognized that complete recovery from AKI is not uniform among patient populations (Coca et al., 2009; Goldberg & Dennen, 2008). Sickle cell patients, who are vulnerable to frequent episodes of acute kidney injury, may have an impaired renal recovery response, which could explain the increased prevalence of chronic kidney disease in SCD. Investigating the renal recovery and repair response to AKI in hospitalized SCD patients would be a worthwhile pursuit. This idea is supported by a previous observation where sickle cell mice failed to upregulate pro-survival signaling proteins and cytoprotective genes in response to renal ischemia (Juncos et al., 2010).

Remarkably, NRG-1 treatment significantly increased urinary CLU and EGF levels in HbSS mice, suggesting that upregulating renal injury repair and restorative mechanisms could be explored to improve AKI recovery in SCD. While induction of individual renoprotective factors, such as heme oxygenase-1, has shown some improvement in kidney injury (Toda et al., 2002), a concerted approach involving multiple factors remains unexplored. Future studies should determine the impact of NRG-1 on other renoprotective factors not assessed in this study. Urinary CLU and EGF are relevant biomarkers for studying acute kidney injury because they are kidney-specific.

Clusterin is not filtered by the glomerulus, so its presence in urine is of kidney origin (Rodríguez-Rivera et al., 2021). Similarly, EGF is expressed in the loop of Henle and distal tubules and is present in the urine of healthy individuals but is barely detectable in plasma (Cortvrindt et al., 2022). Clusterin overexpression in glomeruli and tubules after kidney injury plays roles in anti-apoptosis, anti-inflammation, cell proliferation, and cell debris clearance (Jung et al., 2012; Weng et al., 2021). Insufficient renal clusterin expression has been linked to worsened renal inflammation (Weng et al., 2021), kidney tissue fibrosis (Guo et al., 2016; Jung et al., 2012), oxidative stress-induced podocyte apoptosis (He et al., 2020), progressive glomerulopathy (Rosenberg et al., 2002), apoptosis following ischemia-reperfusion renal injury (Zhou et al., 2010) as well as impaired renal tissue repair (Nguan et al., 2014). EGF, on the other hand, activates repair and regeneration pathways after renal injury and plays a critical role in recovery following acute tubular injury. Genetic deletion of EGF in renal tubules has been shown to delay recovery from AKI (Chen et al., 2012). Decreased urinary EGF levels, which reflect intrarenal EGF expression, are correlated with an increased risk of kidney damage (Mejia-Vilet et al., 2021; Norvik et al., 2021).

The study's findings, consistent with prior clinical and murine studies have shown that hydroxyurea therapy increases circulating fetal haemoglobin containing red blood cells (F-cells) and reduces neutrophil and platelet counts. Also, it has an adverse effect that can worsen anaemia if not monitored (Lebensburger et al., 2010; Steinberg et al., 1997; Strouse & Heeney, 2012). To manage this adverse effect, frequent blood monitoring is required, but this is challenging in resource-limited settings like Africa, where frequent blood tests are not feasible (Akingbola et al., 2019). This limitation in using hydroxyurea, especially in Africa, underscores the need to evaluate new drugs that can provide similar benefits without the associated risks.

A novel observation in this study is that NRG-1 treatment mimicked the hematological benefits of hydroxyurea but did not worsen anaemia. The induction of fetal red blood cell production by NRG-1 may be linked to its downstream signaling effector, nuclear factor erythroid 2-related factor 2 (Nrf2) (Park et al., 2021). In erythroid progenitors, Nrf2 induces fetal haemoglobin expression by directly binding to the γ-globin promoter and modifying the chromatin structure in the β-locus (Li et al., 2019; Macari & Lowrey, 2011; Zhu et al., 2017, 2018, 2019). Similar to this study's findings, chemical molecules that activate Nrf2 have been shown to increase fetal haemoglobin in sickle cell mice (Krishnamoorthy et al., 2017; Xi et al., 2024). NRG-1 treatment did not exacerbate anaemia in the mice, as observed in hydroxyurea-treated mice. Krishnamoorthy *et al*. (2017) demonstrated that activating the Nrf2 pathway increases red blood cell count and total haemoglobin, improving anaemia in sickle cell mice (Krishnamoorthy et al., 2017). This suggests that the Neuregulin-1-Nrf2 signaling pathway could be a potential therapeutic target for SCD, promoting HbF production, improving hematological profiles, and activating cytoprotective factors. Additionally, NRG-1 reduced leukocyte and platelet counts, which has not been previously reported. As shown by Krishnamoorthy *et al*., Nrf2 also regulates leukocyte function, where the genetic deletion of *Nrf2* in HbSS mice increases total white blood cells in blood (Zhu et al., 2018). Future studies linking neuregulin-induced Nrf2 activation with improvements in anaemia, heme detoxification, and decreased production of leukocytes and platelets, as well as reduced vascular inflammation, will be essential to fully understand its multifaceted therapeutic potential.

Severe renal medulla congestion was a prominent histopathological change observed in untreated HbSS mice. Medulla congestion is a well-established feature in sickle cell disease and is central to the onset of renal damage (Bissonnette et al., 2016; Khalighi et al., 2014; Nath et al., 2005). The unique environment of the renal medulla, characterized by hypoxia, acidosis, hyperosmolality, and

reduced blood flow, promotes erythrocyte sickling in the vasa recta, leading to congestion (Nath & Hebbel, 2015). This congestion and resultant ischemia damage the vasa recta and impair the kidney's ability to concentrate urine, a common issue in SCD patients (Miller et al., 2010). Additionally, renal medulla ischemia can cause significant damage to the glomeruli and tubular cells (Nath & Hebbel, 2015). Hydroxyurea treatment significantly reduced renal medulla congestion, consistent with previous observations in sickle cell mice following acute hydroxyurea treatment (Park et al., 2019). Hydroxyurea's ability to reduce vascular congestion has been linked to its effects on increasing nitric oxide bioavailability, reducing endothelial adhesion molecule expression, decreasing leukocyte adhesion, and inducing fetal haemoglobin (Agrawal et al., 2014; Taylor et al., 2021). The reduction in medullary congestion may be a mechanism by which hydroxyurea protects against severe tubular injury and glomerular damage (Han et al., 2020; Laurin et al., 2014; Park et al., 2019; Taylor et al., 2019). NRG-1 treatment did not significantly reduce renal medulla congestion. However, in a cerebral malaria mouse model, NRG-1 was shown to reduce vascular congestion in brain tissues (Solomon et al., 2014). Future studies will need to explore whether long-term NRG-1 treatment could reduce renal medulla congestion, given the unique conditions in the medulla that promote severe occlusion.

Significant iron deposition was observed in the renal cortical tubules of all HbSS mice, consistent with several other studies reporting iron accumulation in the kidneys of sickle cell patients and mice (Falk et al., 1992; Lusco et al., 2016; Nguyen et al., 2014; Ofori-Acquah et al., 2020; Taylor et al., 2019; Zahr et al., 2019). Acute haemolysis leads to increased uptake of filtered heme proteins by renal tubular cells, resulting in heme-dependent iron overload, particularly in the proximal tubules (Denton et al., 2021; Schein et al., 2008; Vasavda et al., 2012). Labile cellular iron causes direct tubular injury through oxidative stress-dependent ferroptosis and mitochondrial dysfunction

(Fortuna et al., 2024). Hydroxyurea did not significantly reduce iron accumulation compared with untreated HbSS mice, which is consistent with findings by Taylor *et al.* (2020), who demonstrated that short-term hydroxyurea treatment did not reduce renal iron accumulation in sickle cell mice (Taylor et al., 2019). However, in one sickle cell patient, long-term hydroxyurea therapy resulted in the disappearance of iron deposits in the kidney (Stehlé et al., 2016). This suggests that further research is needed to determine whether long-term hydroxyurea treatment can significantly reduce iron deposition in the kidneys of humanized sickle cell mice.

Interestingly, two weeks of neuregulin-1 treatment significantly reduced iron accumulation, as indicated by lower pixel intensity in iron stains compared with untreated HbSS mice. This is the first report of neuregulin-1 therapy attenuating tissue iron deposition. In the literature, protective mechanisms against heme-dependent iron accumulation involve heme degradation by the heme oxygenase-1 (HO-1) enzyme and the increased synthesis of iron-binding (ferritin) or iron-exporting proteins, which safely sequester labile cellular iron within tissues (Nath, 2010; Shi et al., 2016). The expression of HO-1 is regulated by Nrf2. Belcher *et al.* demonstrated a 4.6-fold increase in HO-1 expression in the kidneys of HbSS-Townes mice through Nrf2 activation (Belcher et al., 2017). Similarly, the genetic deletion of *Nrf2* in sickle cell mice significantly decreased HO-1 levels in the spleen (Zhu et al., 2018). Thus, it is believed that the reduction in iron accumulation observed with neuregulin-1 treatment may be linked to Nrf2 activation and subsequent upregulation of HO-1, contributing to improved iron detoxification and protection against renal injury.

Histopathologic changes in the glomerulus are common manifestations of renal damage in sickle cell disease (Ataga et al., 2014). The severity of glomerular congestion, Bowman's capsule basement membrane thickening, and glomerulosclerosis in HbSS mice was significantly improved

by both neuregulin-1 and hydroxyurea treatments. Consistent with these findings, Taylor *et al.* (2019) showed that hydroxyurea treatment reduced glomerulosclerosis in sickle cell mice (Taylor et al., 2019). The attenuation of glomerular injury by hydroxyurea is further supported by clinical studies, where indicators of glomerulopathy—such as reduced glomerular filtration rates, albuminuria, or proteinuria—improved with hydroxyurea therapy (Aygun et al., 2013; Laurin et al., 2014; McKie et al., 2007; Zahr et al., 2019). Similarly, the observation that neuregulin-1 administration significantly protects against severe glomerulopathy in sickle cell mice is also reported in other disease murine models. In a diabetic model of kidney damage, neuregulin-1 treatment attenuated glomerulosclerosis (Vandekerckhove et al., 2016). NRG-1 treatment also reduced glomerular enlargement and renal fibrosis in both a chronic kidney disease model and a nitric oxide-deficient mouse model (Sárközy et al., 2023; Shakeri et al., 2018). While the glomerular-protective mechanism of hydroxyurea remains unclear, and the mechanism of NRG-1 has yet to be fully elucidated, several studies have identified factors such as renal endothelial dysfunction (Ataga et al., 2010, 2016; Caughey et al., 2015; Chen et al., 2024), oxidative stress (Roy et al., 2013), nitric oxide deficiency (Baylis, 2008) and haemolysis (Day et al., 2012; Gurkan et al., 2010; Kato et al., 2007; Maier-Redelsperger et al., 2010) as key contributors to glomerulopathy in sickle cell disease. Both hydroxyurea and NRG-1 have been independently shown to reduce haemolysis, decrease oxidative stress, increase nitric oxide bioavailability, and improve endothelial function in murine models and human patients (Chenou et al., 2021; Johnson & Telen, 2008; Shakeri et al., 2021; Siegert et al., 2024; Taylor et al., 2019; Torres et al., 2012; Vinhaes et al., 2020; Wang et al., 2021). Carefully designed clinical studies are required to establish the glomerular-protective mechanisms of both hydroxyurea and NRG-1 in sickle cell disease,

linking the roles of endothelial dysfunction, oxidative stress, nitric oxide bioavailability, and haemolysis.

HO-1 is an indispensable cytoprotectant, neutralizing the harmful effects of heme and other circulating stressors on organ function (Belcher et al., 2006; Gbotosho et al., 2020). HO-1 expression was increased in the kidneys of HbSS mice, consistent with earlier reports where the HbSS mouse kidney expressed HO-1 at 10-fold higher levels than normal kidneys (Belcher et al., 2017; Ghosh et al., 2011; Nath, Grande, et al., 2001). Likewise, in kidney biopsies from sickle cell patients, HO-1 expression was significantly higher compared to healthy kidneys (Nath, Grande, et al., 2001; Nath, Vercellotti, et al., 2001). This phenomenon reflects the HO-1 response in defending the kidney against heme stress (Nath et al., 2001; Ofori-Acquah et al., 2020). HO-1 was primarily expressed in the cortical tubular epithelium, which may relate to the differential exposure, sensitivity, and response of renal tubules to heme and other stressors. Proximal tubules, in particular, are more susceptible to damage and rely on increased HO-1 expression for protection (Yang et al., 2003). Kidney biopsies from patients with proteinuria have also shown high HO-1 expression in renal epithelial cells (Shimizu et al., 2005). In a rare case of human HO-1 deficiency, tubular injury and massive iron deposition were prominent pathological features, highlighting the vulnerability of renal tubules in the absence of HO-1 (Ohta et al., 2000).

Endothelial cells are the first line of defense against heme exposure, and HO-1 expression is essential for their survival (Kim et al., 2011). Surprisingly, HO-1 expression was nearly absent in the glomeruli of HbSS mice and undetectable in normal mice. A similar lack of glomerular HO-1 expression has been observed in kidney biopsies from patients with various renal diseases (Morimoto et al., 2001). In sickle cell patients, glomeruli sometimes show weak and focal HO-1 expression, primarily located within cells identified as infiltrating macrophages or mesangial cells

(Nath et al., 2001). Despite the increased exposure of glomerular endothelial cells to heme, it remains unclear why HO-1 expression is nearly absent in the glomerulus. Studies suggest that glomerular endothelial cells may lack the capacity to induce HO-1 expression. For example, under heme exposure or haemolytic conditions, microvascular endothelial cells, particularly of the glomeruli, fail to express HO-1, which is upregulated in macrovascular endothelial cells (May et al., 2018). The vulnerability of glomerular endothelial cells to heme exposure and their capacity for HO-1 induction requires further investigation. This study also found that HO-1 expression was negative in the inner medulla of HbSS mice, which contrasts with reports of higher HO-1 expression in the medulla of hypertensive rats (Zou et al., 2000). Overall, data on HO-1 expression in different renal cell populations in both sickle cell patients and mice models remain limited. Further research is needed to identify which renal cell populations can upregulate HO-1 and under what conditions, as this information is crucial for therapeutic design.

The protective effect of up-regulated HO-1 expression in the kidney has been demonstrated in various renal diseases (Grunenwald et al., 2021; Zhai et al., 2023). Of particular interest are reports on the beneficial effects of HO-1 induction against kidney injury mediated by heme proteins (Grunenwald et al., 2021; Lever et al., 2016). Sickle cell patients with specific GT tandem repeat polymorphisms in the HO-1 gene promoter, which are associated with increased HO-1 expression, have a reduced risk of developing kidney injury (Saraf et al., 2015, 2018). Unfortunately, renal tubular epithelial cells (RTECs) show a decline in HO-1 expression when persistently exposed to heme, rather than an increase (Yang et al., 2003). This suggests that in chronic haemolytic conditions like sickle cell disease, the HO-1 response to heme may be insufficient to counteract ongoing haemolytic events.

For the first time, this study demonstrates that exogenous neuregulin-1 can significantly increase both the number of cells expressing HO-1 and the intensity of that expression in Townes sickle cell mice. This is a novel finding, as neuregulin-1 has not previously been shown to increase HO-1 expression in any mouse model. Although this observation is intriguing, it aligns with the known role of neuregulin-1, which acts through the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (Park et al., 2021; Zhang et al., 2018). Nrf2 is the primary regulator of HO-1 expression (Grunenwald et al., 2021), and its activation has been shown to increase HO-1 expression in sickle cell mice, leading to attenuation of organ damage (Belcher et al., 2017; Ghosh et al., 2018). This novel finding is highly relevant, as neuregulin-1 could serve as a potential therapeutic strategy against kidney injury in sickle cell disease, particularly for RTECs during acute haemolysis. Hydroxyurea (HU) treatment, however, did not have a significant effect on HO-1 expression compared to untreated HbSS mice. *In vitro* studies have shown that HU does not affect HO-1 expression, with or without heme challenge, in human peripheral blood mononuclear cells and human umbilical vein endothelial cells (Santana et al., 2020). However, the effect of HU on HO-1 expression is worth paying attention to in future studies. HU in the presence of heme groups is metabolized to nitric oxide, and nitric oxide is potent activator of antioxidant enzymes gene transcription including HO-1 (Cho et al., 2010; Santana et al., 2020). While this study did not assess heme content in the kidneys, it is possible that hydroxyurea therapy reduces the levels of heme exposure to renal tubules, which could explain the lower number of tubular cells expressing HO-1. Hydroxyurea's primary benefit in sickle cell disease lies in its ability to increase the percentage of fetal haemoglobin (HbF) cells (Chenou et al., 2021). Despite hydroxyurea's reported ability to improve renal dysfunction in sickle cell disease, its direct effects on renal cells, independent of HbF induction, deserve further exploration.

This study also demonstrates that the ErbB4 receptor is expressed in the kidneys of the Townes mice model. Expression of ErbB4 in the kidney, along with other tissues such as the brain, heart, and muscles, has been previously documented (Segers et al., 2020). Among the epidermal growth factor receptor family (EGFR, ErbB2, ErbB3, ErbB4), ErbB4 is the main receptor expressed in the kidney, suggesting it plays a key role in signaling necessary for maintaining kidney function (Veikkolainen et al., 2012a). The observed expression of ErbB4 in the outer medulla is consistent with previous findings in both humans and mic hybridization and immunohistochemistry analyses show that ErbB4 is expressed in renal epithelial structures, including the proximal tubules, thick ascending limbs, descending and ascending thin limbs of the loop of Henle, and the collecting ducts (Elenius et al., 1997; Veikkolainen et al., 2012; Zeng et al., 2018). Similar to the observed localization of ErbB4 to the apical surfaces of tubular cells, ErbB2, which forms heterodimers with ErbB4, is primarily localized to the apical surfaces of renal epithelial cells in human polycystic kidneys (Nakanishi et al., 2001). Based on the expression pattern of ErbB4 in Townes mice kidney, it is predicted that neuregulin-1 activation of its receptor will induce cytoprotective signals, particularly in kidney epithelial cells. This finding may explain the observed upregulation of cytoprotective biomarkers, clusterin and EGF in NRG-1 treated mice. Future studies should investigate the impact of the NRG-1/ErbB4 interaction on renoprotective factors expressed and secreted by the tubules. Similar to the observations in this study, perinuclear staining of ErbB4 in proximal tubule and mesangial (glomerular) cells has been reported previously (Vandekerckhove et al., 2016; Veikkolainen et al., 2012). This is attributed to the report that only the ErbB4 JM-a isoform exists in the kidney (Veikkolainen et al., 2012). The ErbB4 JM-a isoform is cleavable, producing a soluble intracellular domain that translocates to the nucleus to regulate transcription (Williams et al., 2004). Future research should aim to link the attenuation of glomerular damage

and the upregulation of cortical tubule heme oxygenase-1 levels by NRG-1 to the expression and activation of ErbB4 in these cells.

Of note, ErbB4 expression is increased in sickle cell mice compared to normal mice. This finding is consistent with other studies. Activated ErbB4 expression increases with tubular injury in a mouse model of ischemia-reperfusion injury (Zeng et al., 2018). Similarly, ErbB4 expression increases with the progression of chronic kidney disease in mice (Zeng et al., 2014). In humans, ErbB4 expression inversely correlates with the degree of renal fibrosis, regardless of the underlying cause (Zeng et al., 2018). These findings suggest that renal ErbB4 expression may reflect a compensatory response to tubular injury, underscoring the importance of ErbB4 signaling in maintaining kidney function. *In vitro* studies have demonstrated that ErbB4 expression promotes tubule formation and proliferation in kidney-derived epithelial cells (Veikkolainen et al., 2012). Likewise, ErbB4 expression regulates and maintains renal epithelial formation, size, and cellular functions in mice (Veikkolainen et al., 2012). The importance of renal ErbB4 expression is further confirmed in mouse models of tubular injury, where the global deletion of ErbB4 worsened tubular injury, increased levels of renal cell death, and accelerated the progression of renal fibrosis compared to wild-type mice (Zeng et al., 2014, 2018).

Reproducing the outcomes of this project in human sickle cell disease could be feasible, as the recombinant human neuregulin-1 used in this study (which has undergone safety and efficacy testing in clinical trials) is known to activate mouse ErbB4. The mouse ErbB4 receptor is highly homologous to the human version, differing by only one out of sixty-nine nucleotides (Elenius et al., 1997; Veikkolainen et al., 2012).

6.0 CHAPTER SIX: CONCLUSION, RECOMMENDATION AND LIMITATIONS.

6.1 SUMMARY

Using the clinically relevant Townes sickle cell mouse model, this study evaluated the renal effects of neuregulin-1 (NRG-1) and hydroxyurea (HU) in the context of haemolysis and inflammation mediated kidney injury. Both agents reduced kidney injury and ameliorated renal histopathologic changes. NRG-1 lowered levels of inflammatory cytokines and improved haematologic indices, including circulating fetal haemoglobin containing red blood cells (F-cells). Urinary clusterin (CLU) and epidermal growth factor (EGF) were lower in HbSS mice than in HbAA controls, and NRG-1 treatment increased CLU and EGF, indicating enhancement of renal repair/regeneration markers. Renal heme oxygenase-1 (HO-1) expression was higher in HbSS kidneys, predominantly within cortical tubules. NRG-1 further increased the number of HO-1 positive tubular cells and staining intensity and coincided with reduced renal iron staining. ErbB4, the receptor for NRG-1, was detected on the apical surface of renal epithelial structures in Townes mice.

6.2 CONCLUSION

Taken together, these findings support further exploration of neuregulin-1 (NRG-1) as a kidney-targeted therapeutic candidate in sickle cell disease. The recombinant human NRG-1 used here has undergone clinical safety evaluation, supporting its translational relevance in SCD. The complementary renoprotective effects observed with NRG-1 and hydroxyurea support further evaluation of their combined use to improve kidney outcomes in SCD.

6.3 RECOMMENDATIONS

1. The preliminary data reported in this study demonstrate that NRG-1's pleiotropic effects can be beneficial in mitigating organ damage in SCD. It is recommended that further studies be conducted on other organs to establish the protective role of neuregulin-1 in addressing SCD-related organ damage.
2. Neuregulin-1's activation of nuclear factor erythroid 2-related factor 2 (Nrf2) represents a promising therapeutic pathway. Future studies should explore its potential in inducing fetal haemoglobin expression, either alone or in combination with hydroxyurea.
3. The findings in the Townes HbSS mice suggest a possible impaired renal recovery response in sickle cell patients with AKI, a phenomenon that has yet to be fully investigated. It is recommended that carefully designed prospective, longitudinal cohort study of SCD patients with AKI to assess renal repair and regeneration biomarker levels at presentation and through recovery, testing whether their trajectories predict renal recovery and CKD progression.
4. Based on this study's preliminary data, it is recommended that future research investigate neuregulin-1 as a potential intervention to activate multiple renal recovery biomarkers, improving kidney recovery following AKI in SCD patients.
5. There is limited data on HO-1 expression in different renal cell populations from kidney biopsies. Future research should focus on identifying which renal cell populations can upregulate HO-1 and under what conditions, as this will be important for developing targeted therapies.
6. Future studies should also establish the mechanistic relationship between the attenuation of renal damage, the upregulation of cortical tubule HO-1 levels by NRG-1, and the expression and activation of ErbB4 in these cells.

6.4 LIMITATION

1. The Townes sickle cell mouse model has been instrumental in preclinical testing of various therapeutics for sickle cell disease. While it shares many relevant pathophysiological similarities with human SCD, it does not fully capture the complexity of the disease in humans. As a result, the data presented in this study have limitations when it comes to translating these findings to clinical practice in humans.
2. Sex differences influence the manifestation of sickle cell disease in Townes mice. This study did not report on sex-dependent differences in renal disease severity and therapeutic response.
3. Further research is needed to establish the optimal NRG-1 dose in humanized sickle cell mice, particularly to determine the threshold at which pathway activation transitions from therapeutic to self-limiting.

7.0 REFERENCES.

Aban, I., Baddam, S., Hilliard, L. M., Howard, T. H., Feig, D. I., & Lebensburger, J. D. (2017). Severe anemia early in life as a risk factor for sickle-cell kidney disease. *Blood*, *129*(3), 385–387. https://doi.org/10.1182/blood-2016-09-738104

Abbasi, M., Srivastava, A., & Saraf, S. L. (2024). Management of Kidney Disease with Sickle Cell Disease. *Journal of the American Society of Nephrology*, 10.1681/ASN.0000000804. https://doi.org/10.1681/ASN.0000000804

Adisa, O. A., Hu, Y., Ghosh, S., Aryee, D., Osunkwo, I., & Ofori-Acquah, S. F. (2013). Association between plasma free haem and incidence of vaso-occlusive episodes and acute chest syndrome in children with sickle cell disease. *British Journal of Haematology*, *162*(5), 702–705. https://doi.org/10.1111/bjh.12445

Afangbedji, N., & Jerebtsova, M. (2022). Glomerular filtration rate abnormalities in sickle cell disease. *Frontiers in Medicine*, *9*, 1029224. https://doi.org/10.3389/fmed.2022.1029224

Agrawal, R. K., Patel, R. K., shah, V., Nainiwal, L., & Trivedi, B. (2014). Hydroxyurea in Sickle Cell Disease: Drug Review. *Indian Journal of Hematology & Blood Transfusion*, *30*(2), 91–96. https://doi.org/10.1007/s12288-013-0261-4

Akingbola, T. S., Tayo, B. O., Ezekekwu, C. A., Sonubi, O., Zhang, X., Saraf, S. L., Molokie, R., Hsu, L. L., Han, J., Cooper, R. S., & Gordeuk, V. R. (2019). "Maximum tolerated dose" vs "fixed low-dose" hydroxyurea for treatment of adults with sickle cell anemia. *American Journal of Hematology*, *94*(4), E112–E115. https://doi.org/10.1002/ajh.25412

Aleem, A., Al-Sultan, A., Alsuwaida, A., Alsaleh, K., Algahtani, F., Almomen, A., Sharif, M., & Alotaibi, G. S. (2023). Response to Lisinopril in Patients with Sickle Cell Anemia and Proteinuria. *Saudi Journal of Kidney Diseases and Transplantation: An Official Publication of the Saudi Center for Organ Transplantation, Saudi Arabia*, *34*(6), 531–536. https://doi.org/10.4103/sjkdt.sjkdt_312_22

Alizadeh, A., Santhosh, K. T., Kataria, H., Gounni, A. S., & Karimi-Abdolrezaee, S. (2018). Neuregulin-1 elicits a regulatory immune response following traumatic spinal cord injury. *Journal of Neuroinflammation*, *15*(1), 53. https://doi.org/10.1186/s12974-018-1093-9

Alvarez, O., Lopez-Mitnik, G., & Zilleruelo, G. (2008). Short-term follow-up of patients with sickle cell disease and albuminuria. *Pediatric Blood & Cancer*, *50*(6), 1236–1239. https://doi.org/10.1002/pbc.21520

Alvarez, O., Miller, S. T., Wang, W. C., Luo, Z., McCarville, M. B., Schwartz, G. J., Thompson, B., Howard, T., Iyer, R. V., Rana, S. R., Rogers, Z. R., Sarnaik, S. A., Thornburg, C. D., & Ware, R. E. (2012). Effect of Hydroxyurea Treatment on Renal Function Parameters: Results from the Multi-Center Placebo-Controlled BABY HUG Clinical Trial for Infants with Sickle Cell Anemia. *Pediatric Blood & Cancer*, *59*(4), 668–674. https://doi.org/10.1002/pbc.24100

Alvarez, O., Montane, B., Lopez, G., Wilkinson, J., & Miller, T. (2006). Early blood transfusions protect against microalbuminuria in children with sickle cell disease. *Pediatric Blood & Cancer*, *47*(1), 71–76. https://doi.org/10.1002/pbc.20645

An, T., Huang, Y., Zhou, Q., Wei, B. Q., Zhang, R. C., Yin, S. J., Zou, C. H., Zhang, Y. H., & Zhang, J. (2013). Neuregulin-1 attenuates doxorubicin-induced autophagy in neonatal rat cardiomyocytes. *Journal of Cardiovascular Pharmacology*, *62*(2), 130–137. https://doi.org/10.1097/FJC.0b013e318291c094

Anto, E. O., Obirikorang, C., Acheampong, E., Adua, E., Donkor, S., Afranie, B. O., Ofori, M., Asiamah, E. A., & Adu, E. A. (2019). Renal abnormalities among children with sickle cell conditions in highly resource-limited setting in Ghana. *PLoS ONE*, *14*(11), e0225310. https://doi.org/10.1371/journal.pone.0225310

Aranda-Rivera, A. K., Cruz-Gregorio, A., Pedraza-Chaverri, J., & Scholze, A. (2022). Nrf2 Activation in Chronic Kidney Disease: Promises and Pitfalls. *Antioxidants (Basel, Switzerland)*, *11*(6), 1112. https://doi.org/10.3390/antiox11061112

Askenazi, D. J., Koralkar, R., Patil, N., Halloran, B., Ambalavanan, N., & Griffin, R. (2016). Acute Kidney Injury Urine Biomarkers in Very Low-Birth-Weight Infants. *Clinical Journal of the American Society of Nephrology: CJASN*, *11*(9), 1527–1535. https://doi.org/10.2215/CJN.13381215

Ataga, K. I., Brittain, J. E., Moore, D., Jones, S. K., Hulkower, B., Strayhorn, D., Adam, S., Redding-Lallinger, R., Nachman, P., & Orringer, E. P. (2010a). Urinary Albumin Excretion is Associated with Pulmonary Hypertension in Sickle Cell Disease: Potential Role of Soluble Fms-Like Tyrosine Kinase-1. *European Journal of Haematology*, *85*(3), 257–263. https://doi.org/10.1111/j.1600-0609.2010.01471.x

Ataga, K. I., Brittain, J. E., Moore, D., Jones, S. K., Hulkower, B., Strayhorn, D., Adam, S., Redding-Lallinger, R., Nachman, P., & Orringer, E. P. (2010b). Urinary albumin

excretion is associated with pulmonary hypertension in sickle cell disease: Potential role of soluble fms-like tyrosine kinase-1. *European Journal of Haematology*, *85*(3), 257–263. https://doi.org/10.1111/j.1600-0609.2010.01471.x

Ataga, K. I., Derebail, V. K., & Archer, D. R. (2014). The glomerulopathy of sickle cell disease. *American Journal of Hematology*, *89*(9), 907–914. https://doi.org/10.1002/ajh.23762

Ataga, K. I., Derebail, V. K., Caughey, M., Elsherif, L., Shen, J. H., Jones, S. K., Maitra, P., Pollock, D. M., Cai, J., Archer, D. R., & Hinderliter, A. L. (2016a). Albuminuria Is Associated with Endothelial Dysfunction and Elevated Plasma Endothelin-1 in Sickle Cell Anemia. *PloS One*, *11*(9), e0162652. https://doi.org/10.1371/journal.pone.0162652

Ataga, K. I., Derebail, V. K., Caughey, M., Elsherif, L., Shen, J. H., Jones, S. K., Maitra, P., Pollock, D. M., Cai, J., Archer, D. R., & Hinderliter, A. L. (2016b). Albuminuria Is Associated with Endothelial Dysfunction and Elevated Plasma Endothelin-1 in Sickle Cell Anemia. *PLOS ONE*, *11*(9), e0162652. https://doi.org/10.1371/journal.pone.0162652

Ataga, K. I., Saraf, S. L., & Derebail, V. K. (2022). The nephropathy of sickle cell trait and sickle cell disease. *Nature Reviews. Nephrology*, *18*(6), 361–377. https://doi.org/10.1038/s41581-022-00540-9

Audard, V., Bartolucci, P., & Stehlé, T. (2017). Sickle cell disease and albuminuria: Recent advances in our understanding of sickle cell nephropathy. *Clinical Kidney Journal*, *10*(4), 475–478. https://doi.org/10.1093/ckj/sfx027

Avondt, K. V., Nur, E., & Zeerleder, S. (2019). Mechanisms of haemolysis-induced kidney injury. *Nature Reviews. Nephrology*, *15*(11), 671–692. https://doi.org/10.1038/s41581-019-0181-0

Aygun, B., Mortier, N. A., Smeltzer, M. P., Hankins, J. S., & Ware, R. E. (2011). Glomerular hyperfiltration and albuminuria in children with sickle cell anemia. *Pediatric Nephrology (Berlin, Germany)*, *26*(8), 1285–1290. https://doi.org/10.1007/s00467-011-1857-2

Aygun, B., Mortier, N. A., Smeltzer, M. P., Shulkin, B. L., Hankins, J. S., & Ware, R. E. (2013). Hydroxyurea treatment decreases glomerular hyperfiltration in children with sickle cell anemia. *American Journal of Hematology*, *88*(2), 116–119. https://doi.org/10.1002/ajh.23365

Azar, S., & Wong, T. E. (2017). Sickle Cell Disease: A Brief Update. *Medical Clinics of North America*, *101*(2), 375–393. https://doi.org/10.1016/j.mcna.2016.09.009

Bábíčková, J., Klinkhammer, B. M., Buhl, E. M., Djudjaj, S., Hoss, M., Heymann, F., Tacke, F., Floege, J., Becker, J. U., & Boor, P. (2017). Regardless of etiology, progressive renal disease causes ultrastructural and functional alterations of peritubular capillaries. *Kidney International*, *91*(1), 70–85. https://doi.org/10.1016/j.kint.2016.07.038

Baddam, S., Aban, I., Hilliard, L., Howard, T., Askenazi, D., & Lebensburger, J. D. (2017). Acute kidney injury during a pediatric sickle cell vaso-occlusive pain crisis. *Pediatric Nephrology (Berlin, Germany)*, *32*(8), 1451–1456. https://doi.org/10.1007/s00467-017-3623-6

Bartolucci, P., Habibi, A., Stehlé, T., Di Liberto, G., Rakotoson, M. G., Gellen-Dautremer, J., Loric, S., Moutereau, S., Sahali, D., Wagner-Ballon, O., Remy, P., Lang, P., Grimbert, P., Audureau, E., Godeau, B., Galacteros, F., & Audard, V. (2016). Six Months of Hydroxyurea Reduces Albuminuria in Patients with Sickle Cell Disease. *Journal of the American Society of Nephrology : JASN*, *27*(6), 1847–1853. https://doi.org/10.1681/ASN.2014111126

Batte, A., Menon, S., Ssenkusu, J., Kiguli, S., Kalyesubula, R., Lubega, J., Mutebi, E. I., Opoka, R. O., John, C. C., Starr, M. C., & Conroy, A. L. (2022). Acute kidney injury in hospitalized children with sickle cell anemia. *BMC Nephrology*, *23*(1), 110. https://doi.org/10.1186/s12882-022-02731-9

Baylis, C. (2008). Nitric oxide deficiency in chronic kidney disease. *American Journal of Physiology. Renal Physiology*, *294*(1), F1-9. https://doi.org/10.1152/ajprenal.00424.2007

Becton, L. J., Kalpatthi, R. V., Rackoff, E., Disco, D., Orak, J. K., Jackson, S. M., & Shatat, I. F. (2010). Prevalence and clinical correlates of microalbuminuria in children with sickle cell disease. *Pediatric Nephrology (Berlin, Germany)*, *25*(8), 1505–1511. https://doi.org/10.1007/s00467-010-1536-8

Belcher, J. D., Chen, C., Nguyen, J., Milbauer, L., Abdulla, F., Alayash, A. I., Smith, A., Nath, K. A., Hebbel, R. P., & Vercellotti, G. M. (2014a). Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood*, *123*(3), 377–390. https://doi.org/10.1182/blood-2013-04-495887

Belcher, J. D., Chen, C., Nguyen, J., Milbauer, L., Abdulla, F., Alayash, A. I., Smith, A., Nath, K. A., Hebbel, R. P., & Vercellotti, G. M. (2014b). Heme triggers TLR4 signaling leading to

endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood*, *123*(3), 377–390. https://doi.org/10.1182/blood-2013-04-495887

Belcher, J. D., Chen, C., Nguyen, J., Zhang, P., Abdulla, F., Nguyen, P., Killeen, T., Xu, P., O'Sullivan, G., Nath, K. A., & Vercellotti, G. M. (2017). Control of Oxidative Stress and Inflammation in Sickle Cell Disease with the Nrf2 Activator Dimethyl Fumarate. *Antioxidants & Redox Signaling*, *26*(14), 748–762. https://doi.org/10.1089/ars.2015.6571

Belcher, J. D., Mahaseth, H., Welch, T. E., Otterbein, L. E., Robert P. Hebbel, & Vercellotti, G. M. (2006). Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice. *The Journal of Clinical Investigation*, *116*(3), 808–816. https://doi.org/10.1172/JCI26857

Belisário, A. R., Vieira, É. L. M., de Almeida, J. A., Mendes, F. G., Miranda, A. S., Rezende, P. V., Viana, M. B., & Simões E Silva, A. C. (2020). Evidence for interactions between inflammatory markers and renin-angiotensin system molecules in the occurrence of albuminuria in children with sickle cell anemia. *Cytokine*, *125*, 154800. https://doi.org/10.1016/j.cyto.2019.154800

Bissonnette, M. L. Z., Henriksen, K. J., Delaney, K., Stankus, N., & Chang, A. (2016). Medullary Microvascular Thrombosis and Injury in Sickle Hemoglobin C Disease. *Journal of the American Society of Nephrology: JASN*, *27*(5), 1300–1304. https://doi.org/10.1681/ASN.2015040399

Borg, J. P., Marchetto, S., Le Bivic, A., Ollendorff, V., Jaulin-Bastard, F., Saito, H., Fournier, E., Adélaïde, J., Margolis, B., & Birnbaum, D. (2000). ERBIN: A basolateral PDZ protein that interacts with the mammalian ERBB2/HER2 receptor. *Nature Cell Biology*, *2*(7), 407–414. https://doi.org/10.1038/35017038

Boutaud, O., & Roberts, L. J. (2011). Mechanism-based therapeutic approaches to rhabdomyolysis-induced renal failure. *Free Radical Biology & Medicine*, *51*(5), 1062–1067. https://doi.org/10.1016/j.freeradbiomed.2010.10.704

Brero, A., Ramella, R., Fitou, A., Dati, C., Alloatti, G., Gallo, M. P., & Levi, R. (2010). Neuregulin-1beta1 rapidly modulates nitric oxide synthesis and calcium handling in rat cardiomyocytes. *Cardiovascular Research*, *88*(3), 443–452. https://doi.org/10.1093/cvr/cvq238

Burkholder, T., Foltz, C., Karlsson, E., Linton, C. G., & Smith, J. M. (2012). Health Evaluation of Experimental Laboratory Mice. *Current Protocols in Mouse Biology*, *2*, 145–165. https://doi.org/10.1002/9780470942390.mo110217

Burne, M. J., Daniels, F., El Ghandour, A., Mauiyyedi, S., Colvin, R. B., O'Donnell, M. P., & Rabb, H. (2001). Identification of the CD4(+) T cell as a major pathogenic factor in ischemic acute renal failure. *The Journal of Clinical Investigation*, *108*(9), 1283–1290. https://doi.org/10.1172/JCI12080

Cançado, R. D., Costa, F. F., Lobo, C., Migliavaca, C. B., Falavigna, M., Souza Filho, H. C. R., Bueno, C. T., & Silva-Pinto, A. C. (2023). Estimated mortality rates of individuals with sickle cell disease in Brazil: Real-world evidence. *Blood Advances*, *7*(15), 3783–3792. https://doi.org/10.1182/bloodadvances.2022008938

Cardoso, E. C., Silva-Neto, P. V., Hounkpe, B. W., Chenou, F., Albuquerque, C. C. M. X., Garcia, N. P., Silva-Junior, A. L., Malheiro, A., Cesar, P., de Lima, F., De Paula, E. V., & Fraiji, N. A. (2023). Changes in Heme Levels During Acute Vaso-occlusive Crisis in Sickle Cell Anemia. *Hematology/Oncology and Stem Cell Therapy*, *16*(2), 124–132. https://doi.org/10.1016/j.hemonc.2021.08.002

Caughey, M., Derebail, V. K., Jones, S. K., Shen, J., Maitra, P., Cai, J., Hinderliter, A. L., Archer, D. R., & Ataga, K. I. (2015). Albuminuria Is Associated with Endothelial Dysfunction in Sickle Cell Disease. *Blood*, *126*(23), 2186. https://doi.org/10.1182/blood.V126.23.2186.2186

Cecchini, J., Lionnet, F., Djibré, M., Parrot, A., Stojanovic, K. S., Girot, R., & Fartoukh, M. (2014). Outcomes of Adult Patients With Sickle Cell Disease Admitted to the ICU: A Case Series*. *Critical Care Medicine*, *42*(7), 1629. https://doi.org/10.1097/CCM.0000000000000316

Cerqueira, B. A. V., Boas, W. V., Zanette, A. D., Reis, M. G., & Goncalves, M. S. (2011). Increased concentrations of IL-18 and uric acid in sickle cell anemia: Contribution of hemolysis, endothelial activation and the inflammasome. *Cytokine*, *56*(2), 471–476. https://doi.org/10.1016/j.cyto.2011.08.013

Cespedes, J. C., Liu, M., Harbuzariu, A., Nti, A., Onyekaba, J., Cespedes, H. W., Bharti, P. K., Solomon, W., Anyaoha, P., Krishna, S., Adjei, A., Botchway, F., Ford, B., & Stiles, J. K.

(2018). Neuregulin in Health and Disease. *International Journal of Brain Disorders and Treatment*, *4*(1), 024. https://doi.org/10.23937/2469-5866/1410024

Chambliss, C., Stiles, J. K., & Gee, B. E. (2023). Neuregulin-1 attenuates hemolysis- and ischemia induced-cerebrovascular inflammation associated with sickle cell disease. *Journal of Stroke and Cerebrovascular Diseases: The Official Journal of National Stroke Association*, *32*(2), 106912. https://doi.org/10.1016/j.jstrokecerebrovasdis.2022.106912

Chaturvedi, S., Ghafuri, D. L., Glassberg, J., Kassim, A. A., Rodeghier, M., & DeBaun, M. R. (2016). Rapidly progressive acute chest syndrome in individuals with sickle cell anemia: A distinct acute chest syndrome phenotype. *American Journal of Hematology*, *91*(12), 1185–1190. https://doi.org/10.1002/ajh.24539

Chen, J., Chen, J.-K., & Harris, R. C. (2012). Deletion of the epidermal growth factor receptor in renal proximal tubule epithelial cells delays recovery from acute kidney injury. *Kidney International*, *82*(1), 45–52. https://doi.org/10.1038/ki.2012.43

Chen, Q., Hazra, R., Crosby, D., Lenhart, D., Lenhart, S. C., Mondal, P., Zhang, Y., Nouraie, S. M., Tan, R. J., Esmon, C. T., Rao, L. V. M., Kim, K., & Ghosh, S. (2024). Heme induced loss of renovascular endothelial protein C receptor promotes chronic kidney disease in sickle mice. *Blood*. https://doi.org/10.1182/blood.2023023528

Chen, Q. M. (2022). Nrf2 for protection against oxidant generation and mitochondrial damage in cardiac injury. *Free Radical Biology & Medicine*, *179*, 133–143. https://doi.org/10.1016/j.freeradbiomed.2021.12.001

Chen, Z., Chen, L., Yao, G., Yang, W., Yang, K., & Xiong, C. (2020). Novel Blood Cytokine-Based Model for Predicting Severe Acute Kidney Injury and Poor Outcomes After Cardiac Surgery. *Journal of the American Heart Association*, *9*(22), e018004. https://doi.org/10.1161/JAHA.120.018004

Chenou, F., Hounkpe, B. W., Domingos, I. de F., Tonassé, W. V., Batista, T. H. C., Santana, R. M., Arcanjo, G. da S., Alagbe, A. E., Araújo, A. da S., Lucena-Araújo, A. R., Bezerra, M. A. C., Costa, F. F., Sonati, M. de F., De Paula, E. V., & Dos Santos, M. N. N. (2021a). Effect of hydroxyurea therapy on intravascular hemolysis and endothelial dysfunction markers in sickle cell anemia patients. *Annals of Hematology*, *100*(11), 2669–2676. https://doi.org/10.1007/s00277-021-04636-3

Chenou, F., Hounkpe, B. W., Domingos, I. de F., Tonassé, W. V., Batista, T. H. C., Santana, R. M., Arcanjo, G. da S., Alagbe, A. E., Araújo, A. da S., Lucena-Araújo, A. R., Bezerra, M. A. C., Costa, F. F., Sonati, M. de F., De Paula, E. V., & Dos Santos, M. N. N. (2021b). Effect of hydroxyurea therapy on intravascular hemolysis and endothelial dysfunction markers in sickle cell anemia patients. *Annals of Hematology*, *100*(11), 2669–2676. https://doi.org/10.1007/s00277-021-04636-3

Cho, C.-S., Kato, G. J., Yang, S. H., Bae, S. W., Lee, J. S., Gladwin, M. T., & Rhee, S. G. (2010). Hydroxyurea-induced expression of glutathione peroxidase 1 in red blood cells of individuals with sickle cell anemia. *Antioxidants & Redox Signaling*, *13*(1), 1–11. https://doi.org/10.1089/ars.2009.2978

Chonat, S., Jones, J. A., Patel, S. R., Zerra, P. E., Jeffers, L. A., Jajosky, R. P., Arthur, C. M., Koval, M., Joiner, C. H., Archer, D. R., & Stowell, S. R. (2022). Complement Drives Acute Chest Syndrome in a Model of Sickle Cell Disease. *Blood*, *140*(Supplement 1), 8221–8222. https://doi.org/10.1182/blood-2022-170830

Chowdhury, I., Branch, A., Mehrabi, S., Ford, B. D., & Thompson, W. E. (2017). Gonadotropin-Dependent Neuregulin-1 Signaling Regulates Female Rat Ovarian Granulosa Cell Survival. *Endocrinology*, *158*(10), 3647–3660. https://doi.org/10.1210/en.2017-00065

Citri, A., Gan, J., Mosesson, Y., Vereb, G., Szollosi, J., & Yarden, Y. (2004). Hsp90 restrains ErbB-2/HER2 signalling by limiting heterodimer formation. *EMBO Reports*, *5*(12), 1165–1170. https://doi.org/10.1038/sj.embor.7400300

Coca, S. G., Yusuf, B., Shlipak, M. G., Garg, A. X., & Parikh, C. R. (2009). Long-term risk of mortality and other adverse outcomes after acute kidney injury: A systematic review and meta-analysis. *American Journal of Kidney Diseases: The Official Journal of the National Kidney Foundation*, *53*(6), 961–973. https://doi.org/10.1053/j.ajkd.2008.11.034

Conran, N., & Belcher, J. D. (2018). Inflammation in Sickle Cell Disease. *Clinical Hemorheology and Microcirculation*, *68*(2–3), 263–299. https://doi.org/10.3233/CH-189012

Conran, N., Fattori, A., Saad, S. T. O., & Costa, F. F. (2004). Increased levels of soluble ICAM-1 in the plasma of sickle cell patients are reversed by hydroxyurea. *Am J Hematol*, *76*(4), 343–347. https://doi.org/10.1002/ajh.20129

Cortvrindt, C., Speeckaert, R., Delanghe, J. R., & Speeckaert, M. M. (2022). Urinary Epidermal Growth Factor: A Promising "Next Generation" Biomarker in Kidney Disease. *American Journal of Nephrology*, *53*(5), 372–387. https://doi.org/10.1159/000524586

Cui, W., Tao, J., Wang, Z., Ren, M., Zhang, Y., Sun, Y., Peng, Y., & Li, R. (2013). Neuregulin1beta1 antagonizes apoptosis via ErbB4-dependent activation of PI3-kinase/Akt in APP/PS1 transgenic mice. *Neurochemical Research*, *38*(11), 2237–2246. https://doi.org/10.1007/s11064-013-1131-z

Daniels, J. R., Ma, J. Z., Cao, Z., Beger, R. D., Sun, J., Schnackenberg, L., Pence, L., Choudhury, D., Palevsky, P. M., Portilla, D., & Yu, L.-R. (2021). Discovery of Novel Proteomic Biomarkers for the Prediction of Kidney Recovery from Dialysis-Dependent AKI Patients. *Kidney360*, *2*(11), 1716–1727. https://doi.org/10.34067/KID.0002642021

Day, T. G., Drasar, E. R., Fulford, T., Sharpe, C. C., & Thein, S. L. (2012). Association between hemolysis and albuminuria in adults with sickle cell anemia. *Haematologica*, *97*(2), 201–205. https://doi.org/10.3324/haematol.2011.050336

Denton, C. C., Detterich, J. A., Coates, T. D., & Wood, J. C. (2021). Kidney iron deposition by R2* is associated with hemolysis and urinary iron. *British Journal of Haematology*, *193*(3), 633–636. https://doi.org/10.1111/bjh.17085

Deuel, J. W., Schaer, C. A., Boretti, F. S., Opitz, L., Garcia-Rubio, I., Baek, J. H., Spahn, D. R., Buehler, P. W., & Schaer, D. J. (2016). Hemoglobinuria-related acute kidney injury is driven by intrarenal oxidative reactions triggering a heme toxicity response. *Cell Death & Disease*, *7*(1), e2064. https://doi.org/10.1038/cddis.2015.392

di Masi, A., De Simone, G., Ciaccio, C., D'Orso, S., Coletta, M., & Ascenzi, P. (2020). Haptoglobin: From hemoglobin scavenging to human health. *Molecular Aspects of Medicine*, *73*, 100851. https://doi.org/10.1016/j.mam.2020.100851

Di Segni, A., Farin, K., & Pinkas-Kramarski, R. (2006). ErbB4 activation inhibits MPP+-induced cell death in PC12-ErbB4 cells: Involvement of PI3K and Erk signaling. *Journal of Molecular Neuroscience: MN*, *29*(3), 257–267. https://doi.org/10.1385/JMN:29:3:257

Di Segni, A., Shaharabani, E., Stein, R., & Pinkas-Kramarski, R. (2005). Neuregulins rescue PC12-ErbB-4 cells from cell death induced by beta-amyloid peptide: Involvement of PI3K and PKC. *Journal of Molecular Neuroscience: MN*, *26*(1), 57–69. https://doi.org/10.1385/JMN:26:1:057

Dimayuga, F. O., Ding, Q., Keller, J. N., Marchionni, M. A., Seroogy, K. B., & Bruce-Keller, A. J. (2003). The neuregulin GGF2 attenuates free radical release from activated microglial cells. *Journal of Neuroimmunology*, *136*(1–2), 67–74. https://doi.org/10.1016/s0165-5728(03)00003-1

Dodson, M., Shakya, A., Anandhan, A., Chen, J., Garcia, J. G. N., & Zhang, D. D. (2022). NRF2 and Diabetes: The Good, the Bad, and the Complex. *Diabetes*, *71*(12), 2463–2476. https://doi.org/10.2337/db22-0623

Doss, J. F., Jonassaint, J. C., Garrett, M. E., Ashley-Koch, A. E., Telen, M. J., & Chi, J.-T. (2016). Phase 1 Study of a Sulforaphane-Containing Broccoli Sprout Homogenate for Sickle Cell Disease. *PloS One*, *11*(4), e0152895. https://doi.org/10.1371/journal.pone.0152895

Dreymueller, D., Martin, C., Schumacher, J., Groth, E., Boehm, J. K., Reiss, L. K., Uhlig, S., & Ludwig, A. (2014). Smooth muscle cells relay acute pulmonary inflammation via distinct ADAM17/ErbB axes. *Journal of Immunology (Baltimore, Md.: 1950)*, *192*(2), 722–731. https://doi.org/10.4049/jimmunol.1302496

Elenius, K., Corfas, G., Paul, S., Choi, C. J., Rio, C., Plowman, G. D., & Klagsbrun, M. (1997). A novel juxtamembrane domain isoform of HER4/ErbB4. Isoform-specific tissue distribution and differential processing in response to phorbol ester. *The Journal of Biological Chemistry*, *272*(42), 26761–26768. https://doi.org/10.1074/jbc.272.42.26761

Ephraim, R. K. D., Osakunor, D. N. M., Cudjoe, O., Oduro, E. A., Asante-Asamani, L., Mitchell, J., Agbodzakey, H., & Adoba, P. (2015). Chronic kidney disease is common in sickle cell disease: A cross-sectional study in the Tema Metropolis, Ghana. *BMC Nephrology*, *16*(1), 75. https://doi.org/10.1186/s12882-015-0072-y

Erdei, J., Tóth, A., Balogh, E., Nyakundi, B. B., Bányai, E., Ryffel, B., Paragh, G., Cordero, M. D., & Jeney, V. (2018). Induction of NLRP3 Inflammasome Activation by Heme in Human Endothelial Cells. *Oxidative Medicine and Cellular Longevity*, *2018*, 4310816. https://doi.org/10.1155/2018/4310816

Falk, R. J., Scheinman, J., Phillips, G., Orringer, E., Johnson, A., & Jennette, J. C. (1992). Prevalence and pathologic features of sickle cell nephropathy and response to inhibition of angiotensin-converting enzyme. *The New England Journal of Medicine*, *326*(14), 910–915. https://doi.org/10.1056/NEJM199204023261402

Fang, S.-J., Wu, X.-S., Han, Z.-H., Zhang, X.-X., Wang, C.-M., Li, X.-Y., Lu, L.-Q., & Zhang, J.-L. (2010). Neuregulin-1 preconditioning protects the heart against ischemia/reperfusion injury through a PI3K/Akt-dependent mechanism. *Chinese Medical Journal*, *123*(24), 3597–3604.

Farina, M., Vieira, L. E., Buttari, B., Profumo, E., & Saso, L. (2021). The Nrf2 Pathway in Ischemic Stroke: A Review. *Molecules (Basel, Switzerland)*, *26*(16), 5001. https://doi.org/10.3390/molecules26165001

Fernandez, P. A., Tang, D. G., Cheng, L., Prochiantz, A., Mudge, A. W., & Raff, M. C. (2000). Evidence that axon-derived neuregulin promotes oligodendrocyte survival in the developing rat optic nerve. *Neuron*, *28*(1), 81–90. https://doi.org/10.1016/s0896-6273(00)00087-8

Fortuna, V., Lima, J., Oliveira, G. F., Oliveira, Y. S., Getachew, B., Nekhai, S., Aschner, M., & Tizabi, Y. (2024). Ferroptosis as an emerging target in sickle cell disease. *Current Research in Toxicology*, *7*, 100181. https://doi.org/10.1016/j.crtox.2024.100181

Fry, W. H. D., Kotelawala, L., Sweeney, C., & Carraway, K. L. (2009). Mechanisms of ErbB receptor negative regulation and relevance in cancer. *Experimental Cell Research*, *315*(4), 697–706. https://doi.org/10.1016/j.yexcr.2008.07.022

Fujihara, C. K., Antunes, G. R., Mattar, A. L., Malheiros, D. M. A. C., Vieira, J. M., & Zatz, R. (2007). Chronic inhibition of nuclear factor-kappaB attenuates renal injury in the 5/6 renal ablation model. *American Journal of Physiology. Renal Physiology*, *292*(1), F92-99. https://doi.org/10.1152/ajprenal.00184.2006

G Bardallo, R., Panisello-Roselló, A., Sanchez-Nuno, S., Alva, N., Roselló-Catafau, J., & Carbonell, T. (2022). Nrf2 and oxidative stress in liver ischemia/reperfusion injury. *The FEBS Journal*, *289*(18), 5463–5479. https://doi.org/10.1111/febs.16336

Galadanci, N., Phillips, S., Schlenz, A., Ivankova, N., & Kanter, J. (2024). Current Methods of Newborn Screening Follow-Up for Sickle Cell Disease Are Highly Variable and without Quality Assurance: Results from the ENHANCE Study. *International Journal of Neonatal Screening*, *10*(1), 22. https://doi.org/10.3390/ijns10010022

Gao, R., Zhang, J., Cheng, L., Wu, X., Dong, W., Yang, X., Li, T., Liu, X., Xu, Y., Li, X., & Zhou, M. (2010). A Phase II, Randomized, Double-Blind, Multicenter, Based on Standard Therapy, Placebo-Controlled Study of the Efficacy and Safety of Recombinant Human

Neuregulin-1 in Patients With Chronic Heart Failure. *Journal of the American College of Cardiology*, *55*(18), 1907–1914. https://doi.org/10.1016/j.jacc.2009.12.044

Gardner, K., Douiri, A., Drasar, E., Allman, M., Mwirigi, A., Awogbade, M., & Thein, S. L. (2016). Survival in adults with sickle cell disease in a high-income setting. *Blood*, *128*(10), 1436–1438. https://doi.org/10.1182/blood-2016-05-716910

Gauthier, M.-K., Kosciuczyk, K., Tapley, L., & Karimi-Abdolrezaee, S. (2013). Dysregulation of the neuregulin-1-ErbB network modulates endogenous oligodendrocyte differentiation and preservation after spinal cord injury. *The European Journal of Neuroscience*, *38*(5), 2693–2715. https://doi.org/10.1111/ejn.12268

Gbotosho, O. T., Kapetanaki, M. G., & Kato, G. J. (2020). The Worst Things in Life are Free: The Role of Free Heme in Sickle Cell Disease. *Frontiers in Immunology*, *11*, 561917. https://doi.org/10.3389/fimmu.2020.561917

Ghosh, S., Adisa, O. A., Chappa, P., Tan, F., Jackson, K. A., Archer, D. R., & Ofori-Acquah, S. F. (2013). Extracellular hemin crisis triggers acute chest syndrome in sickle mice. *The Journal of Clinical Investigation*, *123*(11), 4809–4820. https://doi.org/10.1172/JCI64578

Ghosh, S., Hazra, R., Ihunnah, C. A., Weidert, F., Flage, B., & Ofori-Acquah, S. F. (2018). Augmented NRF2 activation protects adult sickle mice from lethal acute chest syndrome. *British Journal of Haematology*, *182*(2), Article 2. https://doi.org/10.1111/bjh.15401

Ghosh, S., Ihunnah, C. A., Hazra, R., Walker, A. L., Hansen, J. M., Archer, D. R., Owusu-Ansah, A. T., & Ofori-Acquah, S. F. (2016). Nonhematopoietic Nrf2 dominantly impedes adult progression of sickle cell anemia in mice. *JCI Insight*, *1*(4), e81090, 81090. https://doi.org/10.1172/jci.insight.81090

Ghosh, S., & Ofori-Acquah, S. F. (2010). Acute Chest Syndrome In Transgenic Mouse Models of Sickle Cell Disease Triggered by Free Heme. *Blood*, *116*(21), 944. https://doi.org/10.1182/blood.V116.21.944.944

Ghosh, S., Orikogbo, O., Hazra, R., Flage, B., Crosby, D., & Ofori-Acquah, S. F. (2019). Hemopexin Replacement Therapy Protects Sickle Cell Disease Mice from Acute Kidney Injury. *Blood*, *134*(Supplement_1), 78. https://doi.org/10.1182/blood-2019-127161

Ghosh, S., Paintsil, V., Hazra, R., Orikogbo, O. O., Flage, B., Ackah, E. B., Owusu-Dabo, E., & Ofori-Acquah, S. F. (2017). Enhanced Renal Clearance of Heme Triggers Acute Kidney

Injury in Sickle Cell Disease. *Blood*, *130*, 441. https://doi.org/10.1182/blood.V130.Suppl_1.441.441

Ghosh, S., Tan, F., & Ofori-Acquah, S. F. (2012). Spatiotemporal dysfunction of the vascular permeability barrier in transgenic mice with sickle cell disease. *Anemia*, *2012*, 582018. https://doi.org/10.1155/2012/582018

Ghosh, S., Tan, F., Yu, T., Li, Y., Adisa, O., Mosunjac, M., & Ofori-Acquah, S. F. (2011). Global Gene Expression Profiling of Endothelium Exposed to Heme Reveals an Organ-Specific Induction of Cytoprotective Enzymes in Sickle Cell Disease. *PLoS ONE*, *6*(3), e18399. https://doi.org/10.1371/journal.pone.0018399

Giraud, M.-N., Flück, M., Zuppinger, C., & Suter, T. M. (2005). Expressional reprogramming of survival pathways in rat cardiocytes by neuregulin-1beta. *Journal of Applied Physiology (Bethesda, Md.: 1985)*, *99*(1), 313–322. https://doi.org/10.1152/japplphysiol.00609.2004

Gladwin, M. T., & Ofori-Acquah, S. F. (2014). Erythroid DAMPs drive inflammation in SCD. *Blood*, *123*(24), 3689–3690. https://doi.org/10.1182/blood-2014-03-563874

Goldberg, R., & Dennen, P. (2008). Long-term outcomes of acute kidney injury. *Advances in Chronic Kidney Disease*, *15*(3), 297–307. https://doi.org/10.1053/j.ackd.2008.04.009

Goldshmit, Y., Erlich, S., & Pinkas-Kramarski, R. (2001). Neuregulin rescues PC12-ErbB4 cells from cell death induced by H(2)O(2). Regulation of reactive oxygen species levels by phosphatidylinositol 3-kinase. *The Journal of Biological Chemistry*, *276*(49), 46379–46385. https://doi.org/10.1074/jbc.M105637200

Gonzalez-Michaca, L., Farrugia, G., Croatt, A. J., Alam, J., & Nath, K. A. (2004). Heme: A determinant of life and death in renal tubular epithelial cells. *American Journal of Physiology-Renal Physiology*, *286*(2), F370–F377. https://doi.org/10.1152/ajprenal.00300.2003

Grunenwald, A., Roumenina, L. T., & Frimat, M. (2021). Heme Oxygenase 1: A Defensive Mediator in Kidney Diseases. *International Journal of Molecular Sciences*, *22*(4), 2009. https://doi.org/10.3390/ijms22042009

Guarda, C. C. da, Santiago, R. P., Fiuza, L. M., Aleluia, M. M., Ferreira, J. R. D., Figueiredo, C. V. B., Yahouedehou, S. C. M. A., Oliveira, R. M. de, Lyra, I. M., & Gonçalves, M. de S. (2017). Heme-mediated cell activation: The inflammatory puzzle of sickle cell anemia.

Expert Review of Hematology, *10*(6), 533–541. https://doi.org/10.1080/17474086.2017.1327809

Guertin, A. D., Zhang, D. P., Mak, K. S., Alberta, J. A., & Kim, H. A. (2005). Microanatomy of Axon/Glial Signaling during Wallerian Degeneration. *Journal of Neuroscience*, *25*(13), 3478–3487. https://doi.org/10.1523/JNEUROSCI.3766-04.2005

Gui, C., Wang, J. A., He, A. N., Chen, T. L., Luo, R. H., Jiang, J., Hu, X. Y., & Xie, X. J. (2007). Heregulin protects mesenchymal stem cells from serum deprivation and hypoxia-induced apoptosis. *Molecular and Cellular Biochemistry*, *305*(1–2), 171–178. Scopus. https://doi.org/10.1007/s11010-007-9541-3

Guo, J., Guan, Q., Liu, X., Wang, H., Gleave, M. E., Nguan, C. Y. C., & Du, C. (2016). Relationship of clusterin with renal inflammation and fibrosis after the recovery phase of ischemia-reperfusion injury. *BMC Nephrology*, *17*(1), 133. https://doi.org/10.1186/s12882-016-0348-x

Guo, Y., Zhang, X., Liu, Y., Duan, H., Jie, B., & Wu, X. (2012). Neuregulin-1 attenuates mitochondrial dysfunction in a rat model of heart failure. *Chinese Medical Journal*, *125*(5), 807–814.

Gurkan, S., Scarponi, K. J., Hotchkiss, H., Savage, B., & Drachtman, R. (2010). Lactate dehydrogenase as a predictor of kidney involvement in patients with sickle cell anemia. *Pediatric Nephrology (Berlin, Germany)*, *25*(10), 2123–2127. https://doi.org/10.1007/s00467-010-1560-8

Habib, R. (2021). Multifaceted roles of Toll-like receptors in acute kidney injury. *Heliyon*, *7*(3), e06441. https://doi.org/10.1016/j.heliyon.2021.e06441

Hamideh, D., Raj, V., Harrington, T., Li, H., Margolles, E., Amole, F., Garcia-Buitrago, M., Ruiz, P., Zilleruelo, G., & Alvarez, O. (2014). Albuminuria correlates with hemolysis and NAG and KIM-1 in patients with sickle cell anemia. *Pediatric Nephrology (Berlin, Germany)*, *29*(10), 1997–2003. https://doi.org/10.1007/s00467-014-2821-8

Han, J., Srisuwananukorn, A., Gowhari, M., Hussain, F. A., Njoku, F., Molokie, R. E., Gordeuk, V. R., & Saraf, S. L. (2020). Effects of Hydroxyurea and Renin-Angiotensin Blockade on Kidney Function in Sickle Cell Disease. *Blood*, *136*, 21–22. https://doi.org/10.1182/blood-2020-138753

Harbuzariu, A., Nti, A., Harp, K. O., Cespedes, J. C., Driss, A., & Stiles, J. K. (2022). Neuregulin-1/ErbB4 signaling modulates Plasmodium falciparum HRP2-induced damage to brain cortical organoids. *iScience*, *25*(6), 104407. https://doi.org/10.1016/j.isci.2022.104407

Harbuzariu, A., Pitts, S., Cespedes, J. C., Harp, K. O., Nti, A., Shaw, A. P., Liu, M., & Stiles, J. K. (2019). Modelling heme-mediated brain injury associated with cerebral malaria in human brain cortical organoids. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-55631-8

Haymann, J.-P., Stankovic, K., Levy, P., Avellino, V., Tharaux, P.-L., Letavernier, E., Grateau, G., Baud, L., Girot, R., & Lionnet, F. (2010). Glomerular Hyperfiltration in Adult Sickle Cell Anemia: A Frequent Hemolysis Associated Feature. *Clinical Journal of the American Society of Nephrology*, *5*(5), 756. https://doi.org/10.2215/CJN.08511109

He, J., Dijkstra, K. L., Bakker, K., Bus, P., Bruijn, J. A., Scharpfenecker, M., & Baelde, H. J. (2020). Glomerular clusterin expression is increased in diabetic nephropathy and protects against oxidative stress-induced apoptosis in podocytes. *Scientific Reports*, *10*(1), 14888. https://doi.org/10.1038/s41598-020-71629-z

Hedhli, N., Dobrucki, L. W., Kalinowski, A., Zhuang, Z. W., Wu, X., Russell, R. R., III, Sinusas, A. J., & Russell, K. S. (2012). Endothelial-derived neuregulin is an important mediator of ischaemia-induced angiogenesis and arteriogenesis. *Cardiovascular Research*, *93*(3), Article 3. https://doi.org/10.1093/cvr/cvr352

Hegemann, L., Narasimhan, V., Marfo, K., Kuma-Aboagye, P., Ofori-Acquah, S., & Odame, I. (2023). Bridging the Access Gap for Comprehensive Sickle Cell Disease Management Across Sub-Saharan Africa: Learnings for Other Global Health Interventions? *Annals of Global Health*, *89*(1), 76. https://doi.org/10.5334/aogh.4132

Heimlich, J. B., Chipoka, G., Elsherif, L., David, E., Ellis, G., Kamthunzi, P., Krysiak, R., Mafunga, P., Zhou, Q., Cai, J., Gopal, S., Key, N. S., & Ataga, K. I. (2018). Nephrin as a biomarker of sickle cell glomerulopathy in Malawi. *Pediatric Blood & Cancer*, *65*(6), e26993. https://doi.org/10.1002/pbc.26993

Heimlich, J. B., Speed, J. S., O'Connor, P. M., Pollock, J. S., Townes, T. M., Meiler, S. E., Kutlar, A., & Pollock, D. M. (2016). Endothelin-1 contributes to the progression of renal

injury in sickle cell disease via reactive oxygen species. *British Journal of Pharmacology*, *173*(2), 386–395. https://doi.org/10.1111/bph.13380

Henderson, S. J., Timbs, A. T., McCarthy, J., Gallienne, A. E., Proven, M., Rugless, M. J., Lopez, H., Eglinton, J., Dziedzic, D., Beardsall, M., Khalil, M. S. M., & Old, J. M. (2016). Ten Years of Routine α- and β-Globin Gene Sequencing in UK Hemoglobinopathy Referrals Reveals 60 Novel Mutations. *Hemoglobin*, *40*(2), 75–84. https://doi.org/10.3109/03630269.2015.1113990

Herrick, J. B. (2001). Peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia. 1910. *The Yale Journal of Biology and Medicine*, *74*(3), 179–184.

Herrick, J. B. (2014). Peculiar Elongated and Sickle-Shaped Red Blood Corpuscles in a Case of Severe Anemia. *JAMA*, *312*(10), 1063. https://doi.org/10.1001/jama.2014.11011

Horie, T., Ono, K., Nishi, H., Nagao, K., Kinoshita, M., Watanabe, S., Kuwabara, Y., Nakashima, Y., Takanabe-Mori, R., Nishi, E., Hasegawa, K., Kita, T., & Kimura, T. (2010). Acute doxorubicin cardiotoxicity is associated with miR-146a-induced inhibition of the neuregulin-ErbB pathway. *Cardiovascular Research*, *87*(4), 656–664. https://doi.org/10.1093/cvr/cvq148

Howard, J. (2016). Sickle cell disease: When and how to transfuse. *Hematology: The American Society of Hematology Education Program*, *2016*(1), 625–631.

Hyacinth, H. I., Adams, R. J., Voeks, J. H., Hibbert, J. M., & Gee, B. E. (2014). Frequent red cell transfusions reduced vascular endothelial activation and thrombogenicity in children with sickle cell anemia and high stroke risk. *American Journal of Hematology*, *89*(1), 47–51. https://doi.org/10.1002/ajh.23586

Imig, J. D., & Ryan, M. J. (2013). Immune and Inflammatory Role in Renal Disease. *Comprehensive Physiology*, *3*(2), 957–976. https://doi.org/10.1002/cphy.c120028

Indrák, K., Divoká, M., Pospíšilová, D., Čermák, J., Beličková, M., Horváthová, M., & Divoký, V. (2018). Hemoglobinopathies. *Vnitrni Lekarstvi*, *64*(5), 476–487.

Jabbour, A., Hayward, C. S., Keogh, A. M., Kotlyar, E., McCrohon, J. A., England, J. F., Amor, R., Liu, X., Li, X. Y., Zhou, M. D., Graham, R. M., & Macdonald, P. S. (2011). Parenteral administration of recombinant human neuregulin-1 to patients with stable chronic heart failure produces favourable acute and chronic haemodynamic responses. *European Journal of Heart Failure*, *13*(1), 83–92. https://doi.org/10.1093/eurjhf/hfq152

James, J., Srivastava, A., Varghese, M. V., Eccles, C. A., Zemskova, M., Rafikova, O., & Rafikov, R. (2020). Heme induces rapid endothelial barrier dysfunction via the MKK3/p38MAPK axis. *Blood*, *136*(6), 749–754. https://doi.org/10.1182/blood.2019003986

Jana, S., Mitra, P., & Roy, S. (2022). Proficient Novel Biomarkers Guide Early Detection of Acute Kidney Injury: A Review. *Diseases*, *11*(1), 8. https://doi.org/10.3390/diseases11010008

Ji, Y., Teng, L., Zhang, R., Sun, J., & Guo, Y. (2017). NRG-1β exerts neuroprotective effects against ischemia reperfusion-induced injury in rats through the JNK signaling pathway. *Neuroscience*, *362*, 13–24. https://doi.org/10.1016/j.neuroscience.2017.08.032

Jiang, H., Shen, Z., Zhuang, J., Lu, C., Qu, Y., Xu, C., Yang, S., & Tian, X. (2024). Understanding the podocyte immune responses in proteinuric kidney diseases: From pathogenesis to therapy. *Frontiers in Immunology*, *14*, 1335936. https://doi.org/10.3389/fimmu.2023.1335936

Jiang, Q., Chen, S., Hu, C., Huang, P., Shen, H., & Zhao, W. (2016). Neuregulin-1 (Nrg1) signaling has a preventive role and is altered in the frontal cortex under the pathological conditions of Alzheimer's disease. *Molecular Medicine Reports*, *14*(3), 2614–2624. https://doi.org/10.3892/mmr.2016.5542

Jie, B., Zhang, X., Wu, X., Xin, Y., Liu, Y., & Guo, Y. (2012). Neuregulin-1 suppresses cardiomyocyte apoptosis by activating PI3K/Akt and inhibiting mitochondrial permeability transition pore. *Molecular and Cellular Biochemistry*, *370*(1), 35–43. https://doi.org/10.1007/s11010-012-1395-7

Jin, Y., Liu, R., Xie, J., Xiong, H., He, J. C., & Chen, N. (2013). Interleukin-10 deficiency aggravates kidney inflammation and fibrosis in the unilateral ureteral obstruction mouse model. *Laboratory Investigation*, *93*(7), 801–811. https://doi.org/10.1038/labinvest.2013.64

Johnson, C., & Telen, M. J. (2008). Adhesion molecules and hydroxyurea in the pathophysiology of sickle cell disease. *Haematologica*, *93*(4), Article 4. https://doi.org/10.3324/haematol.12734

Juncos, J. P., Grande, J. P., Croatt, A. J., Hebbel, R. P., Vercellotti, G. M., Katusic, Z. S., & Nath, K. A. (2010). Early and prominent alterations in hemodynamics, signaling, and gene

expression following renal ischemia in sickle cell disease. *American Journal of Physiology - Renal Physiology*, *298*(4), F892–F899. https://doi.org/10.1152/ajprenal.00631.2009

Jung, G.-S., Kim, M.-K., Jung, Y.-A., Kim, H.-S., Park, I.-S., Min, B.-H., Lee, K.-U., Kim, J.-G., Park, K.-G., & Lee, I.-K. (2012). Clusterin Attenuates the Development of Renal Fibrosis. *Journal of the American Society of Nephrology : JASN*, *23*(1), 73–85. https://doi.org/10.1681/ASN.2011010048

Kang, W., Cheng, Y., Zhou, F., Wang, L., Zhong, L., Li, H. T., Wang, X., Dang, S., & Wang, X. (2019). Neuregulin-1 protects cardiac function in septic rats through multiple targets based on endothelial cells. *International Journal of Molecular Medicine*, *44*(4), 1255–1266. https://doi.org/10.3892/ijmm.2019.4309

Kasztan, M., Fox, B. M., Lebensburger, J. D., Hyndman, K. A., Speed, J. S., Pollock, J. S., & Pollock, D. M. (2019). Hyperfiltration predicts long-term renal outcomes in humanized sickle cell mice. *Blood Advances*, *3*(9), 1460–1475. https://doi.org/10.1182/bloodadvances.2018028878

Kasztan, M., Fox, B. M., Speed, J. S., De Miguel, C., Gohar, E. Y., Townes, T. M., Kutlar, A., Pollock, J. S., & Pollock, D. M. (2017a). Long-Term Endothelin-A Receptor Antagonism Provides Robust Renal Protection in Humanized Sickle Cell Disease Mice. *Journal of the American Society of Nephrology: JASN*, *28*(8), 2443–2458. https://doi.org/10.1681/ASN.2016070711

Kasztan, M., Fox, B. M., Speed, J. S., De Miguel, C., Gohar, E. Y., Townes, T. M., Kutlar, A., Pollock, J. S., & Pollock, D. M. (2017b). Long-Term Endothelin-A Receptor Antagonism Provides Robust Renal Protection in Humanized Sickle Cell Disease Mice. *Journal of the American Society of Nephrology : JASN*, *28*(8), 2443–2458. https://doi.org/10.1681/ASN.2016070711

Kato, G. J., Gladwin, M. T., & Steinberg, M. H. (2007). Deconstructing sickle cell disease: Reappraisal of the role of hemolysis in the development of clinical subphenotypes. *Blood Reviews*, *21*(1), 37–47. https://doi.org/10.1016/j.blre.2006.07.001

Kato, G. J., McGowan, V., Machado, R. F., Little, J. A., Taylor, J., Morris, C. R., Nichols, J. S., Wang, X., Poljakovic, M., Morris, S. M., & Gladwin, M. T. (2006). Lactate dehydrogenase as a biomarker of hemolysis-associated nitric oxide resistance, priapism,

leg ulceration, pulmonary hypertension, and death in patients with sickle cell disease. *Blood*, *107*(6), 2279–2285. https://doi.org/10.1182/blood-2005-06-2373

Kato, G. J., Piel, F. B., Reid, C. D., Gaston, M. H., Ohene-Frempong, K., Krishnamurti, L., Smith, W. R., Panepinto, J. A., Weatherall, D. J., Costa, F. F., & Vichinsky, E. P. (2018). Sickle cell disease. *Nature Reviews Disease Primers*, *4*, 18010. https://doi.org/10.1038/nrdp.2018.10

Kato, G. J., Steinberg, M. H., & Gladwin, M. T. (2017). Intravascular hemolysis and the pathophysiology of sickle cell disease. *The Journal of Clinical Investigation*, *127*(3), 750–760. https://doi.org/10.1172/JCI89741

Kauf, T. L., Coates, T. D., Huazhi, L., Mody-Patel, N., & Hartzema, A. G. (2009). The cost of health care for children and adults with sickle cell disease. *American Journal of Hematology*, *84*(6), 323–327. https://doi.org/10.1002/ajh.21408

Keikhaei, B., Mohseni, A. R., Norouzirad, R., Alinejadi, M., Ghanbari, S., Shiravi, F., & Solgi, G. (2013). Altered levels of pro-inflammatory cytokines in sickle cell disease patients during vaso-occlusive crises and the steady state condition. *Eur Cytokine Netw*, *24*(1), 45–52. https://doi.org/10.1684/ecn.2013.0328

Keleku-Lukwete, N., Suzuki, M., Otsuki, A., Tsuchida, K., Katayama, S., Hayashi, M., Naganuma, E., Moriguchi, T., Tanabe, O., Engel, J. D., Imaizumi, M., & Yamamoto, M. (2015). Amelioration of inflammation and tissue damage in sickle cell model mice by Nrf2 activation. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(39), Article 39. https://doi.org/10.1073/pnas.1509158112

Keleku-Lukwete, N., Suzuki, M., Panda, H., Otsuki, A., Katsuoka, F., Saito, R., Saigusa, D., Uruno, A., & Yamamoto, M. (2019). Nrf2 activation in myeloid cells and endothelial cells differentially mitigates sickle cell disease pathology in mice. *Blood Advances*, *3*(8), 1285–1297. https://doi.org/10.1182/bloodadvances.2018017574

Kellum, J. A., Romagnani, P., Ashuntantang, G., Ronco, C., Zarbock, A., & Anders, H.-J. (2021). Acute kidney injury. *Nature Reviews Disease Primers*, *7*(1), 1–17. https://doi.org/10.1038/s41572-021-00284-z

Khalighi, M. A., Meehan, S. M., & Chang, A. (2014). Medullary peritubular capillary thrombi: A harbinger of sickle cell nephropathy. *Kidney International*, *86*(4), 861. https://doi.org/10.1038/ki.2014.94

Khargekar, N., Banerjee, A., Athalye, S., Mahajan, N., Kargutkar, N., Tapase, P., & Madkaikar, M. (2024). Role of hydroxyurea therapy in the prevention of organ damage in sickle cell disease: A systematic review and meta-analysis. *Systematic Reviews*, *13*(1), 60. https://doi.org/10.1186/s13643-024-02461-z

Kida, Y., Tchao, B. N., & Yamaguchi, I. (2014). Peritubular capillary rarefaction: A new therapeutic target in chronic kidney disease. *Pediatric Nephrology*, *29*(3), 333–342. https://doi.org/10.1007/s00467-013-2430-y

Kim, H.-B., Yoo, J.-Y., Yoo, S.-Y., Lee, J.-H., Chang, W., Kim, H.-S., Baik, T.-K., & Woo, R.-S. (2020a). Neuregulin-1 inhibits CoCl2-induced upregulation of excitatory amino acid carrier 1 expression and oxidative stress in SH-SY5Y cells and the hippocampus of mice. *Molecular Brain*, *13*(1), 153. https://doi.org/10.1186/s13041-020-00686-2

Kim, H.-B., Yoo, J.-Y., Yoo, S.-Y., Lee, J.-H., Chang, W., Kim, H.-S., Baik, T.-K., & Woo, R.-S. (2020b). Neuregulin-1 inhibits CoCl2-induced upregulation of excitatory amino acid carrier 1 expression and oxidative stress in SH-SY5Y cells and the hippocampus of mice. *Molecular Brain*, *13*(1), 153. https://doi.org/10.1186/s13041-020-00686-2

Kim, Y.-M., Pae, H.-O., Park, J. E., Lee, Y. C., Woo, J. M., Kim, N.-H., Choi, Y. K., Lee, B.-S., Kim, S. R., & Chung, H.-T. (2011). Heme oxygenase in the regulation of vascular biology: From molecular mechanisms to therapeutic opportunities. *Antioxidants & Redox Signaling*, *14*(1), 137–167. https://doi.org/10.1089/ars.2010.3153

Krishnamoorthy, S., Pace, B., Gupta, D., Sturtevant, S., Li, B., Makala, L., Brittain, J., Moore, N., Vieira, B. F., Thullen, T., Stone, I., Li, H., Hobbs, W. E., & Light, D. R. (2017). Dimethyl fumarate increases fetal hemoglobin, provides heme detoxification, and corrects anemia in sickle cell disease. *JCI Insight*, *2*(20), e96409, 96409. https://doi.org/10.1172/jci.insight.96409

Lanaro, C., Franco-Penteado, C. F., Albuqueque, D. M., Saad, S. T. O., Conran, N., & Costa, F. F. (2009). Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy. *Journal of Leukocyte Biology*, *85*(2), 235–242. https://doi.org/10.1189/jlb.0708445

Lanzkron, S., Carroll, C. P., & Haywood, C. (2013). Mortality Rates and Age at Death from Sickle Cell Disease: U.S., 1979–2005. *Public Health Reports*, *128*(2), 110–116.

Larsson, J., Allhorn, M., & Kerström, B. (2004). The lipocalin alpha(1)-microglobulin binds heme in different species. *Archives of Biochemistry and Biophysics*, *432*(2), 196–204. https://doi.org/10.1016/j.abb.2004.09.021

Laurin, L.-P., Nachman, P. H., Desai, P. C., Ataga, K. I., & Derebail, V. K. (2014). Hydroxyurea is associated with lower prevalence of albuminuria in adults with sickle cell disease. *Nephrology Dialysis Transplantation*, *29*(6), 1211–1218. https://doi.org/10.1093/ndt/gft295

Lebensburger, J. D., Palabindela, P., Howard, T. H., Feig, D. I., Aban, I., & Askenazi, D. J. (2016). Prevalence of Acute Kidney Injury During Pediatric Admissions for Acute Chest Syndrome. *Pediatric Nephrology (Berlin, Germany)*, *31*(8), 1363–1368. https://doi.org/10.1007/s00467-016-3370-0

Lebensburger, J. D., Pestina, T. I., Ware, R. E., Boyd, K. L., & Persons, D. A. (2010a). Hydroxyurea therapy requires HbF induction for clinical benefit in a sickle cell mouse model. *Haematologica*, *95*(9), 1599–1603. https://doi.org/10.3324/haematol.2010.023325

Lebensburger, J. D., Pestina, T. I., Ware, R. E., Boyd, K. L., & Persons, D. A. (2010b). Hydroxyurea therapy requires HbF induction for clinical benefit in a sickle cell mouse model. *Haematologica*, *95*(9), 1599–1603. https://doi.org/10.3324/haematol.2010.023325

Lebensburger, J., Johnson, S. M., Askenazi, D. J., Rozario, N. L., Howard, T. H., & Hilliard, L. M. (2011). Protective role of hemoglobin and fetal hemoglobin in early kidney disease for children with sickle cell anemia. *American Journal of Hematology*, *86*(5), 430–432. https://doi.org/10.1002/ajh.21994

Lee, J.-H., Yoo, J.-Y., Kim, H., Yoo, H.-I., Song, D.-Y., Min, S. S., Baik, T.-K., & Woo, R.-S. (2019). Neuregulin1 Attenuates H2O2-Induced Reductions in EAAC1 Protein Levels and Reduces H2O2-Induced Oxidative Stress. *Neurotoxicity Research*, *35*(2), 401–409. https://doi.org/10.1007/s12640-018-9965-4

Legendre, C. M., Licht, C., Muus, P., Greenbaum, L. A., Babu, S., Bedrosian, C., Bingham, C., Cohen, D. J., Delmas, Y., Douglas, K., Eitner, F., Feldkamp, T., Fouque, D., Furman, R. R., Gaber, O., Herthelius, M., Hourmant, M., Karpman, D., Lebranchu, Y., … Loirat, C. (2013). Terminal complement inhibitor eculizumab in atypical hemolytic-uremic syndrome. *The New England Journal of Medicine*, *368*(23), 2169–2181. https://doi.org/10.1056/NEJMoa1208981

Leung, K. C. W., Tonelli, M., & James, M. T. (2013). Chronic kidney disease following acute kidney injury-risk and outcomes. *Nature Reviews. Nephrology*, *9*(2), 77–85. https://doi.org/10.1038/nrneph.2012.280

Lever, J. M., Boddu, R., George, J. F., & Agarwal, A. (2016). Heme Oxygenase-1 in Kidney Health and Disease. *Antioxidants & Redox Signaling*, *25*(3), 165–183. https://doi.org/10.1089/ars.2016.6659

Li, B., Zheng, Z., Wei, Y., Wang, M., Peng, J., Kang, T., Huang, X., Xiao, J., Li, Y., & Li, Z. (2011). Therapeutic effects of neuregulin-1 in diabetic cardiomyopathy rats. *Cardiovascular Diabetology*, *10*, 69. https://doi.org/10.1186/1475-2840-10-69

Li, B., Zhu, X., Ward, C. M., Starlard-Davenport, A., Takezaki, M., Berry, A., Ward, A., Wilder, C., Neunert, C., Kutlar, A., & Pace, B. S. (2019). MIR-144-mediated NRF2 gene silencing inhibits fetal hemoglobin expression in sickle cell disease. *Experimental Hematology*, *70*, 85-96.e5. https://doi.org/10.1016/j.exphem.2018.11.002

Li, Y., Lein, P. J., Ford, G. D., Liu, C., Stovall, K. C., White, T. E., Bruun, D. A., Tewolde, T., Gates, A. S., Distel, T. J., Surles-Zeigler, M. C., & Ford, B. D. (2015). Neuregulin-1 inhibits neuroinflammatory responses in a rat model of organophosphate-nerve agent-induced delayed neuronal injury. *Journal of Neuroinflammation*, *12*, 64. https://doi.org/10.1186/s12974-015-0283-y

Li, Y., Ma, K., Han, Z., Chi, M., Sai, X., Zhu, P., Ding, Z., Song, L., & Liu, C. (2021). Immunomodulatory Effects of Heme Oxygenase-1 in Kidney Disease. *Frontiers in Medicine*, *8*, 708453. https://doi.org/10.3389/fmed.2021.708453

Li, Y., Tennekoon, G. I., Birnbaum, M., Marchionni, M. A., & Rutkowski, J. L. (2001). Neuregulin Signaling through a PI3K/Akt/Bad Pathway in Schwann Cell Survival. *Molecular and Cellular Neuroscience*, *17*(4), 761–767. https://doi.org/10.1006/mcne.2000.0967

Li, Y., Xu, Z., Ford, G. D., Croslan, D. R., Cairobe, T., Li, Z., & Ford, B. D. (2007). Neuroprotection by neuregulin-1 in a rat model of permanent focal cerebral ischemia. *Brain Research*, *1184*, 277–283. https://doi.org/10.1016/j.brainres.2007.09.037

Lin, W., Wu, X., Wen, J., Fei, Y., Wu, J., Li, X., Zhang, Q., Dong, Y., Xu, T., Fan, Y., & Wang, N. (2021). Nicotinamide retains Klotho expression and ameliorates rhabdomyolysis-induced

acute kidney injury. *Nutrition (Burbank, Los Angeles County, Calif.)*, *91–92*, 111376. https://doi.org/10.1016/j.nut.2021.111376

Liu, H., Weng, X., Yao, J., Zheng, J., Lv, X., Zhou, X., Jiang, H., & Li, S. (2020). Neuregulin-1*β* Protects the Rat Diaphragm during Sepsis against Oxidative Stress and Inflammation by Activating the PI3K/Akt Pathway. *Oxidative Medicine and Cellular Longevity*, *2020*, e1720961. https://doi.org/10.1155/2020/1720961

Liu, M., Solomon, W., Cespedes, J. C., Wilson, N. O., Ford, B., & Stiles, J. K. (2018). Neuregulin-1 attenuates experimental cerebral malaria (ECM) pathogenesis by regulating ErbB4/AKT/STAT3 signaling. *Journal of Neuroinflammation*, *15*(1), Article 1. https://doi.org/10.1186/s12974-018-1147-z

Lok, J., Zhao, S., Leung, W., Seo, J. H., Navaratna, D., Wang, X., Whalen, M. J., & Lo, E. H. (2012). Neuregulin-1 effects on endothelial and blood-brain-barrier permeability after experimental injury. *Translational Stroke Research*, *3 Suppl 1*(S1), S119-124. https://doi.org/10.1007/s12975-012-0157-x

López Rubio, M., & Argüello Marina, M. (2024). The Current Role of Hydroxyurea in the Treatment of Sickle Cell Anemia. *Journal of Clinical Medicine*, *13*(21), 6404. https://doi.org/10.3390/jcm13216404

Lu, Y., Gao, Y., Tao, R., Liao, M., Huang, J., Wu, G., Han, F., & Li, X. (2016). Calpain-Dependent ErbB4 Cleavage Is Involved in Brain Ischemia-Induced Neuronal Death. *Molecular Neurobiology*, *53*(4), 2600–2609. https://doi.org/10.1007/s12035-015-9275-2

Lusco, M. A., Fogo, A. B., Najafian, B., & Alpers, C. E. (2016). AJKD Atlas of Renal Pathology: Sickle Cell Nephropathy. *American Journal of Kidney Diseases: The Official Journal of the National Kidney Foundation*, *68*(1), e1-3. https://doi.org/10.1053/j.ajkd.2016.05.003

Ma, Y., Fan, P., Zhao, R., Zhang, Y., Wang, X., & Cui, W. (2022). Neuregulin-1 regulates the conversion of M1/M2 microglia phenotype via ErbB4-dependent inhibition of the NF-κB pathway. *Molecular Biology Reports*, *49*(5), 3975–3986. https://doi.org/10.1007/s11033-022-07249-9

Ma, Y., Yang, X., Chatterjee, V., Meegan, J. E., Beard, R. S., & Yuan, S. Y. (2019). Role of Neutrophil Extracellular Traps and Vesicles in Regulating Vascular Endothelial Permeability. *Frontiers in Immunology*, *10*, 1037. https://doi.org/10.3389/fimmu.2019.01037

Macari, E. R., & Lowrey, C. H. (2011). Induction of human fetal hemoglobin via the NRF2 antioxidant response signaling pathway. *Blood*, *117*(22), 5987–5997. https://doi.org/10.1182/blood-2010-10-314096

Macharia, A. W., Mochamah, G., Uyoga, S., Ndila, C. M., Nyutu, G., Makale, J., Tendwa, M., Nyatichi, E., Ojal, J., Shebe, M., Awuondo, K. O., Mturi, N., Peshu, N., Tsofa, B., Scott, J. A. G., Maitland, K., & Williams, T. N. (2018). The clinical epidemiology of sickle cell anemia In Africa. *American Journal of Hematology*, *93*(3), 363–370. https://doi.org/10.1002/ajh.24986

Madero, M., Sarnak, M. J., & Stevens, L. A. (2006). Serum cystatin C as a marker of glomerular filtration rate. *Current Opinion in Nephrology and Hypertension*, *15*(6), 610. https://doi.org/10.1097/01.mnh.0000247505.71915.05

Maier-Redelsperger, M., Lévy, P., Lionnet, F., Stankovic, K., Haymann, J.-P., Lefèvre, G., Avellino, V., Perol, J.-P., Girot, R., & Elion, J. (2010). Strong association between a new marker of hemolysis and glomerulopathy in sickle cell anemia. *Blood Cells, Molecules, and Diseases*, *45*(4), 289–292. Scopus. https://doi.org/10.1016/j.bcmd.2010.08.001

Mandal, A. K., Mitra, A., & Das, R. (2020). Sickle Cell Hemoglobin. *Sub-Cellular Biochemistry*, *94*, 297–322. https://doi.org/10.1007/978-3-030-41769-7_12

May, O., Merle, N. S., Grunenwald, A., Gnemmi, V., Leon, J., Payet, C., Robe-Rybkine, T., Paule, R., Delguste, F., Satchell, S. C., Mathieson, P. W., Hazzan, M., Boulanger, E., Dimitrov, J. D., Fremeaux-Bacchi, V., Frimat, M., & Roumenina, L. T. (2018). Heme Drives Susceptibility of Glomerular Endothelium to Complement Overactivation Due to Inefficient Upregulation of Heme Oxygenase-1. *Frontiers in Immunology*, *9*, 3008. https://doi.org/10.3389/fimmu.2018.03008

McClellan, A. C., Luthi, J.-C., Lynch, J. R., Soucie, J. M., Kulkarni, R., Guasch, A., Huff, E. D., Gilbertson, D., McClellan, W. M., & DeBaun, M. R. (2012). High one year mortality in adults with sickle cell disease and end-stage renal disease. *British Journal of Haematology*, *159*(3), 360–367. https://doi.org/10.1111/bjh.12024

McCormick, M., Richardson, T., Warady, B. A., Novelli, E. M., & Kalpatthi, R. (2020). Acute kidney injury in paediatric patients with sickle cell disease is associated with increased morbidity and resource utilization. *British Journal of Haematology*, *189*(3), 559–565. https://doi.org/10.1111/bjh.16384

McGann, P. T., & Ware, R. E. (2015). Hydroxyurea therapy for sickle cell anemia. *Expert Opinion on Drug Safety*, *14*(11), 1749–1758. https://doi.org/10.1517/14740338.2015.1088827

McKie, K. T., Hanevold, C. D., Hernandez, C., Waller, J. L., Ortiz, L., & McKie, K. M. (2007). Prevalence, prevention, and treatment of microalbuminuria and proteinuria in children with sickle cell disease. *Journal of Pediatric Hematology/Oncology*, *29*(3), 140–144. https://doi.org/10.1097/MPH.0b013e3180335081

Meegan, J. E., Bastarache, J. A., & Ware, L. B. (2021). Toxic effects of cell-free hemoglobin on the microvascular endothelium: Implications for pulmonary and nonpulmonary organ dysfunction. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, *321*(2), L429–L439. https://doi.org/10.1152/ajplung.00018.2021

Mejia-Vilet, J. M., Shapiro, J. P., Zhang, X. L., Cruz, C., Zimmerman, G., Méndez-Pérez, R. A., Cano-Verduzco, M. L., Parikh, S. V., Nagaraja, H. N., Morales-Buenrostro, L. E., & Rovin, B. H. (2021). Association Between Urinary Epidermal Growth Factor and Renal Prognosis in Lupus Nephritis. *Arthritis & Rheumatology (Hoboken, N.J.)*, *73*(2), 244–254. https://doi.org/10.1002/art.41507

Mencel, M., Nash, M., & Jacobson, C. (2013). Neuregulin upregulates microglial α7 nicotinic acetylcholine receptor expression in immortalized cell lines: Implications for regulating neuroinflammation. *PloS One*, *8*(7), e70338. https://doi.org/10.1371/journal.pone.0070338

Mendonça, R., Silveira, A. A. A., & Conran, N. (2016). Red cell DAMPs and inflammation. *Inflammation Research*, *65*(9), 665–678. https://doi.org/10.1007/s00011-016-0955-9

Merle, N. S., Grunenwald, A., Figueres, M.-L., Chauvet, S., Daugan, M., Knockaert, S., Robe-Rybkine, T., Noe, R., May, O., Frimat, M., Brinkman, N., Gentinetta, T., Miescher, S., Houillier, P., Legros, V., Gonnet, F., Blanc-Brude, O. P., Rabant, M., Daniel, R., … Roumenina, L. T. (2018). Characterization of Renal Injury and Inflammation in an Experimental Model of Intravascular Hemolysis. *Frontiers in Immunology*, *9*, 179. https://doi.org/10.3389/fimmu.2018.00179

Miller, S. T., Wang, W. C., Iyer, R., Rana, S., Lane, P., Ware, R. E., Li, D., & Rees, R. C. (2010). Urine concentrating ability in infants with sickle cell disease: Baseline data from the

phase III trial of hydroxyurea (BABY HUG). *Pediatric Blood & Cancer*, *54*(2), 265–268. https://doi.org/10.1002/pbc.22189

Mòdol-Caballero, G., Santos, D., Navarro, X., & Herrando-Grabulosa, M. (2018). Neuregulin 1 Reduces Motoneuron Cell Death and Promotes Neurite Growth in an in Vitro Model of Motoneuron Degeneration. *Frontiers in Cellular Neuroscience*, *11*. https://www.frontiersin.org/articles/10.3389/fncel.2017.00431

Moerdler, S., & Manwani, D. (2018). New insights into the pathophysiology and development of novel therapies for sickle cell disease. *Hematology. American Society of Hematology. Education Program*, *2018*(1), 493–506. https://doi.org/10.1182/asheducation-2018.1.493

Moledina, D. G., Mansour, S. G., Jia, Y., Obeid, W., Thiessen-Philbrook, H., Koyner, J. L., McArthur, E., Garg, A. X., Wilson, F. P., Shlipak, M. G., Coca, S. G., & Parikh, C. R. (2019). Association of T Cell–Derived Inflammatory Cytokines With Acute Kidney Injury and Mortality After Cardiac Surgery. *Kidney International Reports*, *4*(12), 1689–1697. https://doi.org/10.1016/j.ekir.2019.09.003

Montecinos, L., Eskew, J. D., & Smith, A. (2019). What Is Next in This "Age" of Heme-Driven Pathology and Protection by Hemopexin? An Update and Links with Iron. *Pharmaceuticals*, *12*(4), Article 4. https://doi.org/10.3390/ph12040144

Morimoto, K., Ohta, K., Yachie, A., Yang, Y., Shimizu, M., Goto, C., Toma, T., Kasahara, Y., Yokoyama, H., Miyata, T., Seki, H., & Koizumi, S. (2001). Cytoprotective role of heme oxygenase (HO)-1 in human kidney with various renal diseases. *Kidney International*, *60*(5), 1858–1866. https://doi.org/10.1046/j.1523-1755.2001.01000.x

Mulay, S. R., Holderied, A., Kumar, S. V., & Anders, H.-J. (2016). Targeting Inflammation in So-Called Acute Kidney Injury. *Seminars in Nephrology*, *36*(1), 17–30. https://doi.org/10.1016/j.semnephrol.2016.01.006

Murakami, S., Kusano, Y., Okazaki, K., Akaike, T., & Motohashi, H. (2023). NRF2 signalling in cytoprotection and metabolism. *British Journal of Pharmacology*. https://doi.org/10.1111/bph.16246

Nader, E., Conran, N., Romana, M., & Connes, P. (2021). Vasculopathy in Sickle Cell Disease: From Red Blood Cell Sickling to Vascular Dysfunction. In *Comprehensive Physiology* (pp. 1785–1803). John Wiley & Sons, Ltd. https://doi.org/10.1002/cphy.c200024

Nader, E., Romana, M., & Connes, P. (2020). The Red Blood Cell—Inflammation Vicious Circle in Sickle Cell Disease. *Frontiers in Immunology*, *11*. https://doi.org/10.3389/fimmu.2020.00454

Nakanishi, K., Sweeney, W. E., & Avner, E. D. (2001). Segment-specific c-ErbB2 expression in human autosomal recessive polycystic kidney disease. *Journal of the American Society of Nephrology: JASN*, *12*(2), 379–384. https://doi.org/10.1681/ASN.V122379

Nath, K. A. (2010). Heme, Iron, and the Kidney. *Blood*, *116*(21), SCI-26. https://doi.org/10.1182/blood.V116.21.SCI-26.SCI-26

Nath, K. A. (2014). Heme oxygenase-1 and acute kidney injury. *Current Opinion in Nephrology and Hypertension*, *23*(1), 17–24. https://doi.org/10.1097/01.mnh.0000437613.88158.d3

Nath, K. A., Balla, G., Vercellotti, G. M., Balla, J., Jacob, H. S., Levitt, M. D., & Rosenberg, M. E. (1992). Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. *Journal of Clinical Investigation*, *90*(1), 267–270.

Nath, K. A., Belcher, J. D., Nath, M. C., Grande, J. P., Croatt, A. J., Ackerman, A. W., Katusic, Z. S., & Vercellotti, G. M. (2018). Role of TLR4 signaling in the nephrotoxicity of heme and heme proteins. *American Journal of Physiology - Renal Physiology*, *314*(5), F906–F914. https://doi.org/10.1152/ajprenal.00432.2017

Nath, K. A., Grande, J. P., Croatt, A. J., Frank, E., Caplice, N. M., Hebbel, R. P., & Katusic, Z. S. (2005). Transgenic Sickle Mice Are Markedly Sensitive to Renal Ischemia-Reperfusion Injury. *The American Journal of Pathology*, *166*(4), 963–972. https://doi.org/10.1016/S0002-9440(10)62318-8

Nath, K. A., Grande, J. P., Haggard, J. J., Croatt, A. J., Katusic, Z. S., Solovey, A., & Hebbel, R. P. (2001). Oxidative Stress and Induction of Heme Oxygenase-1 in the Kidney in Sickle Cell Disease. *The American Journal of Pathology*, *158*(3), 893–903.

Nath, K. A., Haggard, J. J., Croatt, A. J., Grande, J. P., Poss, K. D., & Alam, J. (2000). The indispensability of heme oxygenase-1 in protecting against acute heme protein-induced toxicity in vivo. *The American Journal of Pathology*, *156*(5), 1527–1535. https://doi.org/10.1016/S0002-9440(10)65024-9

Nath, K. A., & Hebbel, R. P. (2015). Sickle cell disease: Renal manifestations and mechanisms. *Nature Reviews. Nephrology*, *11*(3), 161–171. https://doi.org/10.1038/nrneph.2015.8

Nath, K. A., Singh, R. D., Croatt, A. J., & Adams, C. M. (2022). Heme Proteins and Kidney Injury: Beyond Rhabdomyolysis. *Kidney360*, *3*(11), 1969–1979. https://doi.org/10.34067/KID.0005442022

Nath, K. A., Vercellotti, G. M., Grande, J. P., Miyoshi, H., Paya, C. V., Manivel, J. C., Haggard, J. J., Croatt, A. J., Payne, W. D., & Alam, J. (2001). Heme protein-induced chronic renal inflammation: Suppressive effect of induced heme oxygenase-1. *Kidney International*, *59*(1), 106–117. https://doi.org/10.1046/j.1523-1755.2001.00471.x

Nath, M., & Agarwal, A. (2020). New insights into the role of heme oxygenase-1 in acute kidney injury. *Kidney Research and Clinical Practice*, *39*(4), 387–401. https://doi.org/10.23876/j.krcp.20.091

National Research Council (US) Committee for the Update of the Guide for theCare and Use of Laboratory Animals. (2011). *Guide for the Care and Use of Laboratory Animals* (8th ed.). National Academies Press (US). http://www.ncbi.nlm.nih.gov/books/NBK54050/

Nechemia-Arbely, Y., Barkan, D., Pizov, G., Shriki, A., Rose-John, S., Galun, E., & Axelrod, J. H. (2008). IL-6/IL-6R Axis Plays a Critical Role in Acute Kidney Injury. *Journal of the American Society of Nephrology : JASN*, *19*(6), 1106–1115. https://doi.org/10.1681/ASN.2007070744

Nezu, M., & Suzuki, N. (2020). Roles of Nrf2 in Protecting the Kidney from Oxidative Damage. *International Journal of Molecular Sciences*, *21*(8), 2951. https://doi.org/10.3390/ijms21082951

Nguan, C. Y. C., Guan, Q., Gleave, M. E., & Du, C. (2014). Promotion of cell proliferation by clusterin in the renal tissue repair phase after ischemia-reperfusion injury. *American Journal of Physiology. Renal Physiology*, *306*(7), F724-733. https://doi.org/10.1152/ajprenal.00410.2013

Nguyen, J., Abdulla, F., Chen, C., Nguyen, P., Nguyen, M., Tittle, B., O'Sullivan, G., Belcher, J. D., & Vercellotti, G. M. (2014). Phenotypic Characterization the Townes Sickle Mice. *Blood*, *124*(21), 4916. https://doi.org/10.1182/blood.V124.21.4916.4916

Njoku, F., Pugh, N., Brambilla, D., Kroner, B., Shah, N., Treadwell, M., Gibson, R., Hsu, L. L., Gordeuk, V. R., Glassberg, J., Hankins, J. S., Kutlar, A., King, A. A., & Kanter, J. (2024). Mortality in adults with sickle cell disease: Results from the sickle cell disease

implementation consortium (SCDIC) registry. *American Journal of Hematology*, *99*(5), 900–909. https://doi.org/10.1002/ajh.27279

Noll, J. M., Sherafat, A. A., Ford, G. D., & Ford, B. D. (2024). The case for neuregulin-1 as a clinical treatment for stroke. *Frontiers in Cellular Neuroscience*, *18*. https://doi.org/10.3389/fncel.2024.1325630

Norvik, J. V., Harskamp, L. R., Nair, V., Shedden, K., Solbu, M. D., Eriksen, B. O., Kretzler, M., Gansevoort, R. T., Ju, W., & Melsom, T. (2021). Urinary excretion of epidermal growth factor and rapid loss of kidney function. *Nephrology, Dialysis, Transplantation: Official Publication of the European Dialysis and Transplant Association - European Renal Association*, *36*(10), 1882–1892. https://doi.org/10.1093/ndt/gfaa208

Obadina, M., Wilson, S., Derebail, V. K., & Little, J. (2023). Emerging Therapies and Advances in Sickle Cell Disease with a Focus on Renal Manifestations. *Kidney360*, *4*(7), 997–1005. https://doi.org/10.34067/KID.0000000000000162

Ofori-Acquah, S. F. (2020). Sickle cell disease as a vascular disorder. *Expert Review of Hematology*, *13*(6), 645–653. https://doi.org/10.1080/17474086.2020.1758555

Ofori-Acquah, S. F., Hazra, R., Orikogbo, O. O., Crosby, D., Flage, B., Ackah, E. B., Lenhart, D., Tan, R. J., Vitturi, D. A., Paintsil, V., Owusu-Dabo, E., Ghosh, S., & SickleGenAfrica Network. (2020a). Hemopexin deficiency promotes acute kidney injury in sickle cell disease. *Blood*, *135*(13), 1044–1048. https://doi.org/10.1182/blood.2019002653

Ofori-Acquah, S. F., Hazra, R., Orikogbo, O. O., Crosby, D., Flage, B., Ackah, E. B., Lenhart, D., Tan, R. J., Vitturi, D. A., Paintsil, V., Owusu-Dabo, E., Ghosh, S., & SickleGenAfrica Network. (2020b). Hemopexin deficiency promotes acute kidney injury in sickle cell disease. *Blood*, *135*(13), 1044–1048. https://doi.org/10.1182/blood.2019002653

Ohta, K., Yachie, A., Fujimoto, K., Kaneda, H., Wada, T., Toma, T., Seno, A., Kasahara, Y., Yokoyama, H., Seki, H., & Koizumi, S. (2000). Tubular injury as a cardinal pathologic feature in human heme oxygenase-1 deficiency. *American Journal of Kidney Diseases: The Official Journal of the National Kidney Foundation*, *35*(5), 863–870. https://doi.org/10.1016/s0272-6386(00)70256-3

Olaniran, K. O., Allegretti, A. S., Zhao, S. H., Nigwekar, S. U., & Kalim, S. (2021). Acute Kidney Injury among Black Patients with Sickle Cell Trait and Sickle Cell Disease.

Clinical Journal of the American Society of Nephrology : CJASN, *16*(3), 348–355. https://doi.org/10.2215/CJN.06960520

Olaniran, K. O., Eneanya, N. D., Zhao, S. H., Ofsthun, N. J., Maddux, F. W., Thadhani, R. I., Dalrymple, L. S., & Nigwekar, S. U. (2020). Mortality and Hospitalizations among Sickle Cell Disease Patients with End-Stage Kidney Disease Initiating Dialysis. *American Journal of Nephrology*, *51*(12), 995–1003. https://doi.org/10.1159/000513012

Ono, K., Maeshima, A., Nagayama, I., Kubo, T., Yagisawa, T., & Nagata, D. (2024). Urinary Epidermal Growth Factor Level as a Noninvasive Indicator of Tubular Repair in Patients with Acute Kidney Injury. *Diagnostics*, *14*(9), 947. https://doi.org/10.3390/diagnostics14090947

Oron, A. P., Chao, D. L., Ezeanolue, E. E., Ezenwa, L. N., Piel, F. B., Ojogun, O. T., Uyoga, S., Williams, T. N., & Nnodu, O. E. (2020). Caring for Africa's sickle cell children: Will we rise to the challenge? *BMC Medicine*, *18*, 92. https://doi.org/10.1186/s12916-020-01557-2

Osei-Yeboah, C. T., & Rodrigues, O. (2011). Renal Status of Children With Sickle Cell Disease in Accra, Ghana. *Ghana Medical Journal*, *45*(4), 155–160.

Ou, G., Lin, W., & Zhao, W. (2021). Neuregulins in Neurodegenerative Diseases. *Frontiers in Aging Neuroscience*, *13*, 662474. https://doi.org/10.3389/fnagi.2021.662474

Owusu-Ansah, A., Ihunnah, C. A., Walker, A. L., & Ofori-Acquah, S. F. (2016). Inflammatory targets of therapy in sickle cell disease. *Translational Research*, *167*(1), 281–297. https://doi.org/10.1016/j.trsl.2015.07.001

Ozer, J. S., Dieterle, F., Troth, S., Perentes, E., Cordier, A., Verdes, P., Staedtler, F., Mahl, A., Grenet, O., Roth, D. R., Wahl, D., Legay, F., Holder, D., Erdos, Z., Vlasakova, K., Jin, H., Yu, Y., Muniappa, N., Forest, T., … Gerhold, D. L. (2010). A panel of urinary biomarkers to monitor reversibility of renal injury and a serum marker with improved potential to assess renal function. *Nature Biotechnology*, *28*(5), 486–494. https://doi.org/10.1038/nbt.1627

Panda, H., Keleku-Lukwete, N., Kuga, A., Fuke, N., Suganuma, H., Suzuki, M., & Yamamoto, M. (2019). Dietary supplementation with sulforaphane attenuates liver damage and heme overload in a sickle cell disease murine model. *Experimental Hematology*, *77*, 51-60.e1. https://doi.org/10.1016/j.exphem.2019.08.001

Parasuraman, S., & Raveendran, R. (2022). Biological Sample Collection from Experimental Animals. In M. Lakshmanan, D. G. Shewade, & G. M. Raj (Eds.), *Introduction to Basics of Pharmacology and Toxicology: Volume 3: Experimental Pharmacology: Research Methodology and Biostatistics* (pp. 45–63). Springer Nature. https://doi.org/10.1007/978-981-19-5343-9_4

Park, F., Soni, H., Pressly, J. D., & Adebiyi, A. (2019). Acute hydroxyurea treatment reduces tubular damage following bilateral ischemia-reperfusion injury in a mouse model of sickle cell disease. *Biochemical and Biophysical Research Communications*, *515*(1), 72–76. https://doi.org/10.1016/j.bbrc.2019.05.116

Park, J.-Y., Saeidi, S., Kim, E.-H., Kim, D.-H., Na, H.-K., Keum, J.-S., & Surh, Y.-J. (2021a). Heregulin-β1 Activates NF-E2-related Factor 2 and Induces Manganese Superoxide Dismutase Expression in Human Breast Cancer Cells via Protein Kinase B and Extracellular Signal-regulated Protein Kinase Signaling Pathways. *Journal of Cancer Prevention*, *26*(1), Article 1. https://doi.org/10.15430/JCP.2021.26.1.54

Park, J.-Y., Saeidi, S., Kim, E.-H., Kim, D.-H., Na, H.-K., Keum, J.-S., & Surh, Y.-J. (2021b). Heregulin-β1 Activates NF-E2-related Factor 2 and Induces Manganese Superoxide Dismutase Expression in Human Breast Cancer Cells via Protein Kinase B and Extracellular Signal-regulated Protein Kinase Signaling Pathways. *Journal of Cancer Prevention*, *26*(1), Article 1. https://doi.org/10.15430/JCP.2021.26.1.54

Parry, T. J., Ganguly, A., Troy, E. L., Luis Guerrero, J., Iaci, J. F., Srinivas, M., Vecchione, A. M., Button, D. C., Hackett, C. S., Zolty, R., Sawyer, D. B., & Caggiano, A. O. (2017). Effects of neuregulin GGF2 (cimaglermin alfa) dose and treatment frequency on left ventricular function in rats following myocardial infarction. *European Journal of Pharmacology*, *796*, 76–89. https://doi.org/10.1016/j.ejphar.2016.12.024

Pascual-Gil, S., Abizanda, G., Iglesias, E., Garbayo, E., Prósper, F., & Blanco-Prieto, M. J. (2019). NRG1 PLGA MP locally induce macrophage polarisation toward a regenerative phenotype in the heart after acute myocardial infarction. *Journal of Drug Targeting*, *27*(5–6), 573–581. https://doi.org/10.1080/1061186X.2018.1531417

Pavan, A. R., Terroni, B., & Dos Santos, J. L. (2024). Endothelial dysfunction in Sickle Cell Disease: Strategies for the treatment. *Nitric Oxide*, *149*, 7–17. https://doi.org/10.1016/j.niox.2024.05.003

Penkert, R. R., Hurwitz, J. L., Thomas, P., Rosch, J., Dowdy, J., Sun, Y., Tang, L., & Hankins, J. S. (2018). Inflammatory molecule reduction with hydroxyurea therapy in children with sickle cell anemia. *Haematologica*, *103*(2), e50–e54. https://doi.org/10.3324/haematol.2017.177360

Piel, F. B., Hay, S. I., Gupta, S., Weatherall, D. J., & Williams, T. N. (2013). Global Burden of Sickle Cell Anaemia in Children under Five, 2010–2050: Modelling Based on Demographics, Excess Mortality, and Interventions. *PLoS Medicine*, *10*(7), e1001484. https://doi.org/10.1371/journal.pmed.1001484

Piel, F. B., Steinberg, M. H., & Rees, D. C. (2017). Sickle cell disease. *New England Journal of Medicine*, *376*(16), 1561–1573.

Piotrowska, M., Swierczynski, M., Fichna, J., & Piechota-Polanczyk, A. (2021). The Nrf2 in the pathophysiology of the intestine: Molecular mechanisms and therapeutic implications for inflammatory bowel diseases. *Pharmacological Research*, *163*, 105243. https://doi.org/10.1016/j.phrs.2020.105243

Puri, L., Nottage, K. A., Hankins, J. S., & Anghelescu, D. L. (2018). State of the Art Management of Acute Vaso-occlusive Pain in Sickle Cell Disease. *Pediatric Drugs*, *20*(1), 29–42. https://doi.org/10.1007/s40272-017-0263-z

Qian, Q., Nath, K. A., Wu, Y., Daoud, T. M., & Sethi, S. (2010). Hemolysis and Acute Kidney Failure. *American Journal of Kidney Diseases*, *56*(4), 780–784. https://doi.org/10.1053/j.ajkd.2010.03.025

Qin, X.-F., Shan, Y.-G., Dou, M., Li, F.-X., & Guo, Y.-X. (2023). Notch1 signaling activation alleviates coronary microvascular dysfunction through histone modification of Nrg-1 via the interaction between NICD and GCN5. *Apoptosis: An International Journal on Programmed Cell Death*, *28*(1–2), 124–135. https://doi.org/10.1007/s10495-022-01777-2

Qiu, X., Liu, C., Ye, Y., Li, H., Chen, Y., Fu, Y., Liu, Z., Huang, X., Zhang, Y., Liao, X., Liu, H., Zhao, W., & Liu, X. (2017). The diagnostic value of serum creatinine and cystatin c in evaluating glomerular filtration rate in patients with chronic kidney disease: A systematic literature review and meta-analysis. *Oncotarget*, *8*(42), 72985–72999. https://doi.org/10.18632/oncotarget.20271

Quinn, C. T., Saraf, S. L., Gordeuk, V. R., Fitzhugh, C. D., Creary, S. E., Bodas, P., George, A., Raj, A. B., Nero, A. C., Terrell, C. E., McCord, L., Lane, A., Ackerman, H. C., Yang, Y.,

Niss, O., Taylor, M. D., Devarajan, P., & Malik, P. (2017). Losartan for the Nephropathy of Sickle Cell Anemia: A Phase-2, Multi-Center Trial. *American Journal of Hematology*, *92*(9), E520–E528. https://doi.org/10.1002/ajh.24810

Raij, L., Tian, R., Wong, J. S., He, J. C., & Campbell, K. N. (2016). Podocyte injury: The role of proteinuria, urinary plasminogen, and oxidative stress. *American Journal of Physiology - Renal Physiology*, *311*(6), F1308–F1317. https://doi.org/10.1152/ajprenal.00162.2016

Ramos, S., Jeney, V., Figueiredo, A., Paixão, T., Sambo, M. R., Quinhentos, V., Martins, R., Gouveia, Z., Carlos, A. R., Ferreira, A., Pais, T. F., Lainé, H., Faísca, P., Rebelo, S., Cardoso, S., Tolosano, E., Penha-Gonçalves, C., & Soares, M. P. (2024). Targeting circulating labile heme as a defense strategy against malaria. *Life Science Alliance*, *7*(4). https://doi.org/10.26508/lsa.202302276

Ríos-Silva, M., Huerta, M., Mendoza-Cano, O., Murillo-Zamora, E., Cárdenas, Y., Bricio-Barrios, J. A., Diaz, Y., Ibarra, I., & Trujillo, X. (2023). Urinary epidermal growth factor in kidney disease: A systematic review. *Nefrología*, *43*(4), 413–426. https://doi.org/10.1016/j.nefro.2022.10.003

Rodríguez-Rivera, C., Garcia, M. M., Molina-Álvarez, M., González-Martín, C., & Goicoechea, C. (2021). Clusterin: Always protecting. Synthesis, function and potential issues. *Biomedicine & Pharmacotherapy*, *134*, 111174. https://doi.org/10.1016/j.biopha.2020.111174

Rohrbach, S., Muller-Werdan, U., Werdan, K., Koch, S., Gellerich, N. F., & Holtz, J. (2005). Apoptosis-modulating interaction of the neuregulin/erbB pathway with antracyclines in regulating Bcl-xS and Bcl-xL in cardiomyocytes. *Journal of Molecular and Cellular Cardiology*, *38*(3), 485–493. https://doi.org/10.1016/j.yjmcc.2004.12.013

Rosenberg, M. E., Girton, R., Finkel, D., Chmielewski, D., Barrie, A., Witte, D. P., Zhu, G., Bissler, J. J., Harmony, J. A. K., & Aronow, B. J. (2002). Apolipoprotein J/clusterin prevents a progressive glomerulopathy of aging. *Molecular and Cellular Biology*, *22*(6), 1893–1902. https://doi.org/10.1128/MCB.22.6.1893-1902.2002

Roumenina, L. T., Rayes, J., Lacroix-Desmazes, S., & Dimitrov, J. D. (2016). Heme: Modulator of Plasma Systems in Hemolytic Diseases. *Trends in Molecular Medicine*, *22*(3), Article 3. https://doi.org/10.1016/j.molmed.2016.01.004

Roy, N. B., Carpenter, A., Dale-Harris, I., Dorée, C., & Estcourt, L. J. (2023). Interventions for chronic kidney disease in people with sickle cell disease. *The Cochrane Database of Systematic Reviews*, *8*(8), CD012380. https://doi.org/10.1002/14651858.CD012380.pub3

Roy, S., Konstantinidis, D. G., Rizvi, T., Chang, K.-H., Sundaram, N., Wourms, M., Anastacia, L., Song, L., Burke, K., Inagami, T., Ratner, N., Witte, D., Kalfa, T. A., Cancelas, J. A., Shanmukhappa, S. K., & Malik, P. (2013). Increased Oxidative Stress In Sickle Cell Disease Activates The Renin-Angiotensin-TGF-β Pathway To Mediate Sickle Nephropathy. *Blood*, *122*(21), 2211. https://doi.org/10.1182/blood.V122.21.2211.2211

Rubio-Navarro, A., Sanchez-Niño, M. D., Guerrero-Hue, M., García-Caballero, C., Gutiérrez, E., Yuste, C., Sevillano, Á., Praga, M., Egea, J., Román, E., Cannata, P., Ortega, R., Cortegano, I., de Andrés, B., Gaspar, M. L., Cadenas, S., Ortiz, A., Egido, J., & Moreno, J. A. (2018). Podocytes are new cellular targets of haemoglobin-mediated renal damage. *The Journal of Pathology*, *244*(3), 296–310. https://doi.org/10.1002/path.5011

Runge, A., Brazel, D., & Pakbaz, Z. (2022). Stroke in sickle cell disease and the promise of recent disease modifying agents. *Journal of the Neurological Sciences*, *442*, 120412. https://doi.org/10.1016/j.jns.2022.120412

Ryzhov, S., Matafonov, A., Galindo, C. L., Zhang, Q., Tran, T.-L., Lenihan, D. J., Lenneman, C. G., Feoktistov, I., & Sawyer, D. B. (2017). ERBB signaling attenuates proinflammatory activation of nonclassical monocytes. *American Journal of Physiology-Heart and Circulatory Physiology*, *312*(5), Article 5. https://doi.org/10.1152/ajpheart.00486.2016

Salgar, S., Bolívar, B. E., Flanagan, J. M., Anum, S. J., & Bouchier-Hayes, L. (2023). The NLRP3 inflammasome fires up heme-induced inflammation in hemolytic conditions. *Translational Research*, *252*, 34–44. https://doi.org/10.1016/j.trsl.2022.08.011

Santana, S. S., Pitanga, T. N., de Santana, J. M., Zanette, D. L., Vieira, J. de J., Yahouédéhou, S. C. M. A., Adanho, C. S. A., Viana, S. de M., Luz, N. F., Borges, V. M., & Goncalves, M. S. (2020). Hydroxyurea Scavenges Free Radicals and Induces the Expression of Antioxidant Genes in Human Cell Cultures Treated With Hemin. *Frontiers in Immunology*, *11*, 1488. https://doi.org/10.3389/fimmu.2020.01488

Santaterra, V. A. G., Fiusa, M. M. L., Hounkpe, B. W., Chenou, F., Tonasse, W. V., da Costa, L. N. G., Garcia-Weber, D., Domingos, I. de F., de Lima, F., Borba-Junior, I. T., Araújo, A. da S., Lucena-Araújo, A. R., Bezerra, M. A. C., Dos Santos, M. N. N., Costa, F. F.,

Millán, J., & De Paula, E. V. (2020). Endothelial Barrier Integrity Is Disrupted In Vitro by Heme and by Serum From Sickle Cell Disease Patients. *Frontiers in Immunology*, *11*, 535147. https://doi.org/10.3389/fimmu.2020.535147

Santiago, R. P., Guarda, C. C., Figueiredo, C. V. B., Fiuza, L. M., Aleluia, M. M., Adanho, C. S. A., Carvalho, M. O. S., Pitanga, T. N., Zanette, D. L., Lyra, I. M., Nascimento, V. M. L., Vercellotti, G. M., Belcher, J. D., & Goncalves, M. S. (2018). Serum Haptoglobin and Hemopexin Levels Are Depleted in Pediatric Sickle Cell Disease Patients. *Blood Cells, Molecules & Diseases*, *72*, 34–36. https://doi.org/10.1016/j.bcmd.2018.07.002

Saraf, S. L. (2020). Heme A1M'ed at the kidney in sickle cell disease. *Blood*, *135*(13), 979–981. https://doi.org/10.1182/blood.2020005134

Saraf, S. L., Viner, M., Rischall, A., Raslan, R., Shah, B. N., Zhang, X., Han, J., Gowhari, M., Jain, S., Molokie, R. E., Machado, R. F., Lash, J. P., & Gordeuk, V. R. (2018). HMOX1 and acute kidney injury in sickle cell anemia. *Blood*, *132*(15), 1621. https://doi.org/10.1182/blood-2018-05-853929

Saraf, S. L., Zhang, X., Shah, B., Kanias, T., Gudehithlu, K. P., Kittles, R., Machado, R. F., Arruda, J. A. L., Gladwin, M. T., Singh, A. K., & Gordeuk, V. R. (2015a). Genetic variants and cell-free hemoglobin processing in sickle cell nephropathy. *Haematologica*, *100*(10), Article 10. https://doi.org/10.3324/haematol.2015.124875

Saraf, S. L., Zhang, X., Shah, B., Kanias, T., Gudehithlu, K. P., Kittles, R., Machado, R. F., Arruda, J. A. L., Gladwin, M. T., Singh, A. K., & Gordeuk, V. R. (2015b). Genetic variants and cell-free hemoglobin processing in sickle cell nephropathy. *Haematologica*, *100*(10), 1275–1284. https://doi.org/10.3324/haematol.2015.124875

Sarakpi, T., Mesic, A., & Speer, T. (2023). Leukocyte–endothelial interaction in CKD. *Clinical Kidney Journal*, *16*(11), 1845–1860. https://doi.org/10.1093/ckj/sfad135

Sárközy, M., Watzinger, S., Kovács, Z. Z. A., Acar, E., Márványkövi, F., Szűcs, G., Lauber, G. Y., Galla, Z., Siska, A., Földesi, I., Fintha, A., Kriston, A., Kovács, F., Horváth, P., Kővári, B., Cserni, G., Krenács, T., Szabó, P. L., Szabó, G. T., … Kiss, A. (2023a). Neuregulin-1β Improves Uremic Cardiomyopathy and Renal Dysfunction in Rats. *JACC: Basic to Translational Science*. https://doi.org/10.1016/j.jacbts.2023.03.003

Sárközy, M., Watzinger, S., Kovács, Z. Z. A., Acar, E., Márványkövi, F., Szűcs, G., Lauber, G. Y., Galla, Z., Siska, A., Földesi, I., Fintha, A., Kriston, A., Kovács, F., Horváth, P.,

Kővári, B., Cserni, G., Krenács, T., Szabó, P. L., Szabó, G. T., … Kiss, A. (2023b). Neuregulin-1β Improves Uremic Cardiomyopathy and Renal Dysfunction in Rats. *JACC: Basic to Translational Science*, *8*(9), 1160–1176. https://doi.org/10.1016/j.jacbts.2023.03.003

Schein, A., Enriquez, C., Coates, T. D., & Wood, J. C. (2008). Magnetic resonance detection of kidney iron deposition in sickle cell disease: A marker of chronic hemolysis. *Journal of Magnetic Resonance Imaging: JMRI*, *28*(3), 698–704. https://doi.org/10.1002/jmri.21490

Schmidt, H., Kelley, E., & Straub, A. (2018). 56—Excess circulating free heme triggers immense elevation in plasma xanthine oxidase activity. *Free Radical Biology and Medicine*, *128*, S40. https://doi.org/10.1016/j.freeradbiomed.2018.10.058

Scindia, Y., Dey, P., Thirunagari, A., Liping, H., Rosin, D. L., Floris, M., Okusa, M. D., & Swaminathan, S. (2015). Hepcidin Mitigates Renal Ischemia-Reperfusion Injury by Modulating Systemic Iron Homeostasis. *Journal of the American Society of Nephrology: JASN*, *26*(11), 2800–2814. https://doi.org/10.1681/ASN.2014101037

Segers, V. F. M., Dugaucquier, L., Feyen, E., Shakeri, H., & De Keulenaer, G. W. (2020). The role of ErbB4 in cancer. *Cellular Oncology*, *43*(3), 335–352. https://doi.org/10.1007/s13402-020-00499-4

Shahriary, G. M., Kataria, H., & Karimi-Abdolrezaee, S. (2019). Neuregulin-1 Fosters Supportive Interactions between Microglia and Neural Stem/Progenitor Cells. *Stem Cells International*, *2019*, e8397158. https://doi.org/10.1155/2019/8397158

Shahsavani, N., Alizadeh, A., Kataria, H., & Karimi-Abdolrezaee, S. (2021). Availability of neuregulin-1beta1 protects neurons in spinal cord injury and against glutamate toxicity through caspase dependent and independent mechanisms. *Experimental Neurology*, *345*, 113817. https://doi.org/10.1016/j.expneurol.2021.113817

Shakeri, H., Boen, J. R. A., De Moudt, S., Hendrickx, J. O., Leloup, A. J. A., Jacobs, G., De Meyer, G. R. Y., De Keulenaer, G. W., Guns, P.-J. D. F., & Segers, V. F. M. (2021). Neuregulin-1 compensates for endothelial nitric oxide synthase deficiency. *American Journal of Physiology-Heart and Circulatory Physiology*, *320*(6), H2416–H2428. https://doi.org/10.1152/ajpheart.00914.2020

Shakeri, H., Gevaert, A. B., Schrijvers, D. M., De Meyer, G. R. Y., De Keulenaer, G. W., Guns, P.-J. D. F., Lemmens, K., & Segers, V. F. (2018). Neuregulin-1 attenuates stress-induced

vascular senescence. *Cardiovascular Research*, *114*(7), 1041–1051. https://doi.org/10.1093/cvr/cvy059

Shankar, K., Shah, D., Huffman, D. L., Peterson, C., Bhagavatula, R., Shankar, K., Shah, D., Huffman, D. L., Peterson, C., & Bhagavatula, R. (2021). Hyperhemolysis Syndrome in a Patient With Sickle Cell Disease and Acute Chest Syndrome. *Cureus*, *13*(1). https://doi.org/10.7759/cureus.13017

Shi, L., & Bergson, C. M. (2020). Neuregulin 1: An intriguing therapeutic target for neurodevelopmental disorders. *Translational Psychiatry*, *10*(1), 1–11. https://doi.org/10.1038/s41398-020-00868-5

Shi, P. A., Choi, E., Chintagari, N. R., Nguyen, J., Guo, X., Yazdanbakhsh, K., Mohandas, N., Alayash, A. I., Manci, E. A., Belcher, J. D., & Vercellotti, G. M. (2016a). Sustained treatment of sickle cell mice with haptoglobin increases HO-1 and H-ferritin expression and decreases iron deposition in the kidney without improvement in kidney function. *British Journal of Haematology*, *175*(4), 714–723. https://doi.org/10.1111/bjh.14280

Shi, P. A., Choi, E., Chintagari, N. R., Nguyen, J., Guo, X., Yazdanbakhsh, K., Mohandas, N., Alayash, A. I., Manci, E. A., Belcher, J. D., & Vercellotti, G. M. (2016b). Sustained treatment of sickle cell mice with haptoglobin increases HO-1 and H-ferritin expression and decreases iron deposition in the kidney without improvement in kidney function. *British Journal of Haematology*, *175*(4), 714–723. https://doi.org/10.1111/bjh.14280

Shi, Y., Xu, L., Tang, J., Fang, L., Ma, S., Ma, X., Nie, J., Pi, X., Qiu, A., Zhuang, S., & Liu, N. (2017). Inhibition of HDAC6 protects against rhabdomyolysis-induced acute kidney injury. *American Journal of Physiology - Renal Physiology*, *312*(3), F502–F515. https://doi.org/10.1152/ajprenal.00546.2016

Shimizu, M., Ohta, K., Yang, Y., Nakai, A., Toma, T., Saikawa, Y., Kasahara, Y., Yachie, A., Yokoyama, H., Seki, H., & Koizumi, S. (2005). Glomerular Proteinuria Induces Heme Oxygenase-1 Gene Expression within Renal Epithelial Cells. *Pediatric Research*, *58*(4), 666–671. https://doi.org/10.1203/01.PDR.0000180557.68222.5A

Siegert, T. F., Opoka, R. O., Nakafeero, M., Carman, A., Mellencamp, K. A., Latham, T., Hume, H., Lane, A., Ware, R. E., Ssenkusu, J. M., John, C. C., & Conroy, A. L. (2024). Angiopoietin-2 is associated with sickle cell complications, including stroke risk, and

decreases with hydroxyurea therapy. *Blood Vessels, Thrombosis & Hemostasis*, *1*(1), 100001. https://doi.org/10.1016/j.bvth.2024.100001

Simmons, L. J., Surles-Zeigler, M. C., Li, Y., Ford, G. D., Newman, G. D., & Ford, B. D. (2016a). Regulation of inflammatory responses by neuregulin-1 in brain ischemia and microglial cells in vitro involves the NF-kappa B pathway. *Journal of Neuroinflammation*, *13*(1), 237. https://doi.org/10.1186/s12974-016-0703-7

Simmons, L. J., Surles-Zeigler, M. C., Li, Y., Ford, G. D., Newman, G. D., & Ford, B. D. (2016b). Regulation of inflammatory responses by neuregulin-1 in brain ischemia and microglial cells in vitro involves the NF-kappa B pathway. *Journal of Neuroinflammation*, *13*(1), 237. https://doi.org/10.1186/s12974-016-0703-7

Sinha, S. K., Mellody, M., Carpio, M. B., Damoiseaux, R., & Nicholas, S. B. (2023). Osteopontin as a Biomarker in Chronic Kidney Disease. *Biomedicines*, *11*(5), Article 5. https://doi.org/10.3390/biomedicines11051356

Solomon, W., Wilson, N. O., Anderson, L., Pitts, S., Patrickson, J., Liu, M., Ford, B. D., & Stiles, J. K. (2014). Neuregulin-1 attenuates mortality associated with experimental cerebral malaria. *Journal of Neuroinflammation*, *11*, 9. https://doi.org/10.1186/1742-2094-11-9

Soni, S. S., Cruz, D., Bobek, I., Chionh, C. Y., Nalesso, F., Lentini, P., de Cal, M., Corradi, V., Virzi, G., & Ronco, C. (2010). NGAL: A biomarker of acute kidney injury and other systemic conditions. *Int Urol Nephrol*, *42*(1), 141–150. https://doi.org/10.1007/s11255-009-9608-z

Stehlé, T., Bartolucci, P., Bouanane, M., Galacteros, F., Dudreuilh, C., Grimbert, P., Deux, J.-F., & Audard, V. (2016). Reversible kidney iron accumulation in a patient with sickle cell disease treated with hydroxyurea. *American Journal of Hematology*, *91*(12), 1283–1284. https://doi.org/10.1002/ajh.24544

Steinberg, M. H. (2020). Fetal hemoglobin in sickle cell anemia. *Blood*, *136*(21), 2392–2400. https://doi.org/10.1182/blood.2020007645

Steinberg, M. H., Lu, Z. H., Barton, F. B., Terrin, M. L., Charache, S., & Dover, G. J. (1997). Fetal hemoglobin in sickle cell anemia: Determinants of response to hydroxyurea. Multicenter Study of Hydroxyurea. *Blood*, *89*(3), 1078–1088.

Strouse, J. J., & Heeney, M. M. (2012). Hydroxyurea for the Treatment of Sickle Cell Disease: Efficacy, Barriers, Toxicity, and Management in Children. *Pediatric Blood & Cancer*, *59*(2), 365–371. https://doi.org/10.1002/pbc.24178

Sundaram, N., Bennett, M., Wilhelm, J., Kim, M.-O., Atweh, G., Devarajan, P., & Malik, P. (2011). Biomarkers for early detection of sickle nephropathy. *American Journal of Hematology*, *86*(7), 559–566. https://doi.org/10.1002/ajh.22045

Sundd, P., Gladwin, M. T., & Novelli, E. M. (2019). Pathophysiology of Sickle Cell Disease. *Annual Review of Pathology*, *14*, 263–292. https://doi.org/10.1146/annurev-pathmechdis-012418-012838

Syed, N., Reddy, K., Yang, D. P., Taveggia, C., Salzer, J. L., Maurel, P., & Kim, H. A. (2010). Soluble Neuregulin-1 Has Bifunctional, Concentration-Dependent Effects on Schwann Cell Myelination. *Journal of Neuroscience*, *30*(17), 6122–6131. https://doi.org/10.1523/JNEUROSCI.1681-09.2010

Taylor, C., Kasztan, M., Tao, B., Pollock, J. S., & Pollock, D. M. (2019a). Combined Hydroxyurea and Eta Receptor Blockade Reduces Renal Injury in the Humanized Sickle Cell Mouse. *Acta Physiologica*, *225*(2), e13178. https://doi.org/10.1111/apha.13178

Taylor, C., Kasztan, M., Tao, B., Pollock, J. S., & Pollock, D. M. (2019b). Combined hydroxyurea and ETA receptor blockade reduces renal injury in the humanized sickle cell mouse. *Acta Physiologica*, *225*(2), e13178. https://doi.org/10.1111/apha.13178

Taylor, C. M., Kasztan, M., Sedaka, R., Molina, P. A., Dunaway, L. S., Pollock, J. S., & Pollock, D. M. (2021). Hydroxyurea improves nitric oxide bioavailability in humanized sickle cell mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *320*(5), R630–R640. https://doi.org/10.1152/ajpregu.00205.2020

Thein, S. L. (2017). Genetic Basis and Genetic Modifiers of β-Thalassemia and Sickle Cell Disease. *Advances in Experimental Medicine and Biology*, *1013*, 27–57. https://doi.org/10.1007/978-1-4939-7299-9_2

Thein, S. L., & Howard, J. (2018a). How I treat the older adult with sickle cell disease. *Blood*, *132*(17), 1750–1760. https://doi.org/10.1182/blood-2018-03-818161

Thein, S. L., & Howard, J. (2018b). How I treat the older adult with sickle cell disease. *Blood*, *132*(17), 1750–1760. https://doi.org/10.1182/blood-2018-03-818161

Thomson, A. M., McHugh, T. A., Oron, A. P., Teply, C., Lonberg, N., Vilchis Tella, V., Wilner, L. B., Fuller, K., Hagins, H., Aboagye, R. G., Aboye, M. B., Abu-Gharbieh, E., Abu-Zaid, A., Addo, I. Y., Ahinkorah, B. O., Ahmad, A., AlRyalat, S. A. S., Amu, H., Aravkin, A. Y., … Kassebaum, N. J. (2023). Global, regional, and national prevalence and mortality burden of sickle cell disease, 2000–2021: A systematic analysis from the Global Burden of Disease Study 2021. *The Lancet Haematology*, *10*(8), e585–e599. https://doi.org/10.1016/S2352-3026(23)00118-7

Timolati, F., Ott, D., Pentassuglia, L., Giraud, M.-N., Perriard, J.-C., Suter, T. M., & Zuppinger, C. (2006). Neuregulin-1 beta attenuates doxorubicin-induced alterations of excitation–contraction coupling and reduces oxidative stress in adult rat cardiomyocytes. *Journal of Molecular and Cellular Cardiology*, *41*(5), 845–854. https://doi.org/10.1016/j.yjmcc.2006.08.002

Toda, N., Takahashi, T., Mizobuchi, S., Fujii, H., Nakahira, K., Takahashi, S., Yamashita, M., Morita, K., Hirakawa, M., & Akagi, R. (2002). Tin chloride pretreatment prevents renal injury in rats with ischemic acute renal failure. *Critical Care Medicine*, *30*(7), 1512–1522. https://doi.org/10.1097/00003246-200207000-00020

Tolosano, E., Hirsch, E., Patrucco, E., Camaschella, C., Navone, R., Silengo, L., & Altruda, F. (1999). Defective recovery and severe renal damage after acute hemolysis in hemopexin-deficient mice. *Blood*, *94*(11), 3906–3914.

Torres, L. de S., da Silva, D. G. H., Belini Junior, E., de Almeida, E. A., Lobo, C. L. de C., Cançado, R. D., Ruiz, M. A., & Bonini-Domingos, C. R. (2012). The influence of hydroxyurea on oxidative stress in sickle cell anemia. *Revista Brasileira de Hematologia e Hemoterapia*, *34*(6), 421–425. https://doi.org/10.5581/1516-8484.20120106

Torres, L. S., & Hidalgo, A. (2023). Neutrophils as drivers of vascular injury in sickle cell disease. *Immunological Reviews*, *314*(1), 302–312. https://doi.org/10.1111/imr.13146

Tran, H., Gupta, M., & Gupta, K. (2017). Targeting novel mechanisms of pain in sickle cell disease. *Hematology: The American Society of Hematology Education Program*, *2017*(1), 546–555.

Twumasi, S., Anto, E. O., Obirikorang, C., Ephraim, R. K. D., Sackey, B., Paintsil, V., Ansah, R. O., Effah, A., Ayirebi, A. A., Opoku, A., Scott, G. Y., Osei, L., Duku, J., Adjei, E. A., & Boateng, L. A. (2025). Neutrophil gelatinase-associated lipocalin and fibrinogen-to-

albumin ratio are indicators of kidney disease in sickle cell disease patients with microalbuminuria: A multicentre case-control study in Ghana. *BMC Nephrology*, *26*(1), 499. https://doi.org/10.1186/s12882-025-04427-2

Usmani, A., & Machado, R. F. (2018). Vascular complications of sickle cell disease. *Clinical Hemorheology and Microcirculation*, *68*(2–3), 205. https://doi.org/10.3233/CH-189008

Valle, J., Lebensburger, J. D., Garimella, P. S., & Gopal, S. (2024). Prevalence, Mortality, and Access to Care for Chronic Kidney Disease in Medicaid-Enrolled Adults With Sickle Cell Disease in California: Retrospective Cohort Study. *JMIR Public Health Surveill*, *10*, e57290. https://doi.org/10.2196/57290

Vallelian, F., Buehler, P. W., & Schaer, D. J. (2022). Hemolysis, free hemoglobin toxicity and scavenger protein therapeutics. *Blood*. https://doi.org/10.1182/blood.2022015596

van Meer, L., Moerland, M., Cohen, A. F., & Burggraaf, J. (2014). Urinary kidney biomarkers for early detection of nephrotoxicity in clinical drug development. *British Journal of Clinical Pharmacology*, *77*(6), 947–957. https://doi.org/10.1111/bcp.12282

Vandekerckhove, L., Vermeulen, Z., Liu, Z. Z., Boimvaser, S., Patzak, A., Segers, V. F. M., & De Keulenaer, G. W. (2016a). Neuregulin-1 attenuates development of nephropathy in a type 1 diabetes mouse model with high cardiovascular risk. *American Journal of Physiology - Endocrinology and Metabolism*, *310*(7), E495–E504. https://doi.org/10.1152/ajpendo.00432.2015

Vandekerckhove, L., Vermeulen, Z., Liu, Z. Z., Boimvaser, S., Patzak, A., Segers, V. F. M., & De Keulenaer, G. W. (2016b). Neuregulin-1 attenuates development of nephropathy in a type 1 diabetes mouse model with high cardiovascular risk. *American Journal of Physiology - Endocrinology and Metabolism*, *310*(7), E495–E504. https://doi.org/10.1152/ajpendo.00432.2015

Vasavda, N., Gutiérrez, L., House, M. J., Drašar, E., St Pierre, T. G., & Thein, S. L. (2012). Renal iron load in sickle cell disease is influenced by severity of haemolysis. *British Journal of Haematology*, *157*(5), 599–605. https://doi.org/10.1111/j.1365-2141.2012.09093.x

Vazquez, B., Shah, B., Zhang, X., Lash, J. P., Gordeuk, V. R., & Saraf, S. L. (2014). Hyperfiltration is associated with the development of microalbuminuria in patients with sickle cell anemia. *American Journal of Hematology*, *89*(12), 1156–1157. https://doi.org/10.1002/ajh.23817

Vega-Torres, J. D., Ontiveros-Angel, P., Terrones, E., Stuffle, E. C., Solak, S., Tyner, E., Oropeza, M., dela Peña, I., Obenaus, A., Ford, B. D., & Figueroa, J. D. (2022). Short-term exposure to an obesogenic diet during adolescence elicits anxiety-related behavior and neuroinflammation: Modulatory effects of exogenous neuregulin-1. *Translational Psychiatry*, *12*(1), Article 1. https://doi.org/10.1038/s41398-022-01788-2

Veikkolainen, V., Naillat, F., Railo, A., Chi, L., Manninen, A., Hohenstein, P., Hastie, N., Vainio, S., & Elenius, K. (2012a). ErbB4 Modulates Tubular Cell Polarity and Lumen Diameter during Kidney Development. *Journal of the American Society of Nephrology*, *23*(1), 112. https://doi.org/10.1681/ASN.2011020160

Veikkolainen, V., Naillat, F., Railo, A., Chi, L., Manninen, A., Hohenstein, P., Hastie, N., Vainio, S., & Elenius, K. (2012b). ErbB4 Modulates Tubular Cell Polarity and Lumen Diameter during Kidney Development. *Journal of the American Society of Nephrology*, *23*(1), 112. https://doi.org/10.1681/ASN.2011020160

Vichinsky, E. (2017). Chronic organ failure in adult sickle cell disease. *Hematology: The American Society of Hematology Education Program*, *2017*(1), 435–439.

Vinhaes, C. L., Teixeira, R. S., Monteiro-Júnior, J. A. S., Tibúrcio, R., Cubillos-Angulo, J. M., Arriaga, M. B., Sabarin, A. G., de Souza, A. J., Silva, J. J., Lyra, I. M., Ladeia, A. M., & Andrade, B. B. (2020). Hydroxyurea treatment is associated with reduced degree of oxidative perturbation in children and adolescents with sickle cell anemia. *Scientific Reports*, *10*, 18982. https://doi.org/10.1038/s41598-020-76075-5

Wagener, F. A., Eggert, A., Boerman, O. C., Oyen, W. J., Verhofstad, A., Abraham, N. G., Adema, G., van Kooyk, Y., de Witte, T., & Figdor, C. G. (2001). Heme is a potent inducer of inflammation in mice and is counteracted by heme oxygenase. *Blood*, *98*(6), 1802–1811. https://doi.org/10.1182/blood.v98.6.1802

Wang, F., Wang, H., Liu, X., Yu, H., Huang, X., Huang, W., & Wang, G. (2021). Neuregulin-1 alleviate oxidative stress and mitigate inflammation by suppressing NOX4 and NLRP3/caspase-1 in myocardial ischaemia-reperfusion injury. *Journal of Cellular and Molecular Medicine*, *25*(3), 1783–1795. https://doi.org/10.1111/jcmm.16287

Wang, J., Zhou, J., Ding, X., Zhu, L., Jiang, K., Fu, M., Wang, S., Hu, K., & Ge, J. (2015). Qiliqiangxin improves cardiac function and attenuates cardiac remodeling in rats with

experimental myocardial infarction. *International Journal of Clinical and Experimental Pathology*, *8*(6), 6596–6606.

Wang, L., & He, C. (2022). Nrf2-mediated anti-inflammatory polarization of macrophages as therapeutic targets for osteoarthritis. *Frontiers in Immunology*, *13*, 967193. https://doi.org/10.3389/fimmu.2022.967193

Wang, W. C., Ware, R. E., Miller, S. T., Iyer, R. V., Casella, J. F., Minniti, C. P., Rana, S., Thornburg, C. D., Rogers, Z. R., Kalpatthi, R. V., Barredo, J. C., Brown, R. C., Sarnaik, S. A., Howard, T. H., Wynn, L. W., Kutlar, A., Armstrong, F. D., Files, B. A., Goldsmith, J. C., … BABY HUG investigators. (2011). Hydroxycarbamide in very young children with sickle-cell anaemia: A multicentre, randomised, controlled trial (BABY HUG). *Lancet (London, England)*, *377*(9778), 1663–1672. https://doi.org/10.1016/S0140-6736(11)60355-3

Wang, Y., Wei, J., Zhang, P., Zhang, X., Wang, Y., Chen, W., Zhao, Y., & Cui, X. (2022). Neuregulin-1, a potential therapeutic target for cardiac repair. *Frontiers in Pharmacology*, *13*, 945206. https://doi.org/10.3389/fphar.2022.945206

Wang, Y., Wen, J., Almoiliqy, M., Wang, Y., Liu, Z., Yang, X., Lu, X., Meng, Q., Peng, J., Lin, Y., & Sun, P. (2021). Sesamin Protects against and Ameliorates Rat Intestinal Ischemia/Reperfusion Injury with Involvement of Activating Nrf2/HO-1/NQO1 Signaling Pathway. *Oxidative Medicine and Cellular Longevity*, *2021*, 5147069. https://doi.org/10.1155/2021/5147069

Wang, Z., Li, B., & Sun, Y. (2012). [Effects of neuregulin-1β1 on CD44, TNF-α and IL-1β of astrocytes after oxygen-glucose deprivation]. *Zhonghua Yi Xue Za Zhi*, *92*(45), 3227–3231.

Ware, K., Qamri, Z., Ozcan, A., Satoskar, A. A., Nadasdy, G., Rovin, B. H., Hebert, L. A., Nadasdy, T., & Brodsky, S. V. (2013). N-acetylcysteine ameliorates acute kidney injury but not glomerular hemorrhage in an animal model of warfarin-related nephropathy. *American Journal of Physiology. Renal Physiology*, *304*(12), F1421-1427. https://doi.org/10.1152/ajprenal.00689.2012

Ware, R. E., Rees, R. C., Sarnaik, S. A., Iyer, R. V., Alvarez, O. A., Casella, J. F., Shulkin, B. L., Shalaby-Rana, E., Strife, C. F., Miller, J. H., Lane, P. A., Wang, W. C., Miller, S. T., & BABY HUG Investigators. (2010). Renal function in infants with sickle cell anemia:

Baseline data from the BABY HUG trial. *The Journal of Pediatrics*, *156*(1), 66-70.e1. https://doi.org/10.1016/j.jpeds.2009.06.060

Weng, X., Zhao, H., Guan, Q., Shi, G., Feng, S., Gleave, M. E., Nguan, C. C., & Du, C. (2021). Clusterin regulates macrophage expansion, polarization and phagocytic activity in response to inflammation in the kidneys. *Immunology & Cell Biology*, *99*(3), 274–287. https://doi.org/10.1111/imcb.12405

Williams, C. C., Allison, J. G., Vidal, G. A., Burow, M. E., Beckman, B. S., Marrero, L., & Jones, F. E. (2004). The ERBB4/HER4 receptor tyrosine kinase regulates gene expression by functioning as a STAT5A nuclear chaperone. *The Journal of Cell Biology*, *167*(3), 469–478. https://doi.org/10.1083/jcb.200403155

Woo, R.-S., Lee, J.-H., Kim, H.-S., Baek, C.-H., Song, D.-Y., Suh, Y.-H., & Baik, T.-K. (2012). Neuregulin-1 protects against neurotoxicities induced by Swedish amyloid precursor protein via the ErbB4 receptor. *Neuroscience*, *202*, 413–423. https://doi.org/10.1016/j.neuroscience.2011.11.026

Wu, L., Walas, S., Leung, W., Sykes, D. B., Wu, J., Lo, E. H., & Lok, J. (2015). Neuregulin1-β decreases IL-1β-induced neutrophil adhesion to human brain microvascular endothelial cells. *Translational Stroke Research*, *6*(2), Article 2. https://doi.org/10.1007/s12975-014-0347-9

Wu, L.-C., Sun, C.-W., Ryan, T. M., Pawlik, K. M., Ren, J., & Townes, T. M. (2006). Correction of sickle cell disease by homologous recombination in embryonic stem cells. *Blood*, *108*(4), 1183–1188. https://doi.org/10.1182/blood-2006-02-004812

Wu, Y., Wang, L., Meng, L., Cao, G.-K., & Zhang, Y. (2016). MIP-1α and NF-κB as indicators of acute kidney injury secondary to acute lung injury in mechanically ventilated patients. *European Review for Medical and Pharmacological Sciences*, *20*(18), 3830–3834.

Xi, C., Palani, C., Takezaki, M., Shi, H., Horuzsko, A., Pace, B. S., & Zhu, X. (2024). Simvastatin-Mediated Nrf2 Activation Induces Fetal Hemoglobin and Antioxidant Enzyme Expression to Ameliorate the Phenotype of Sickle Cell Disease. *Antioxidants*, *13*(3), 337. https://doi.org/10.3390/antiox13030337

Xie, X., Liu, Y., Perkovic, V., Li, X., Ninomiya, T., Hou, W., Zhao, N., Liu, L., Lv, J., Zhang, H., & Wang, H. (2016). Renin-Angiotensin System Inhibitors and Kidney and Cardiovascular Outcomes in Patients With CKD: A Bayesian Network Meta-analysis of

Randomized Clinical Trials. *American Journal of Kidney Diseases: The Official Journal of the National Kidney Foundation*, *67*(5), 728–741. https://doi.org/10.1053/j.ajkd.2015.10.011

Xiong, J., & Zhao, J. (2024). Pyroptosis: The Determinator of Cell Death and Fate in Acute Kidney Injury. *Kidney Diseases (Basel, Switzerland)*, *10*(2), 118–131. https://doi.org/10.1159/000535894

Xu, C., Lv, L., Zheng, G., Li, B., Gao, L., & Sun, Y. (2012). Neuregulin1β1 protects oligodendrocyte progenitor cells from oxygen glucose deprivation injury induced apoptosis via ErbB4-dependent activation of PI3-kinase/Akt. *Brain Research*, *1467*, 104–112. https://doi.org/10.1016/j.brainres.2012.05.044

Xu, J., Hu, C., Chen, S., Shen, H., Jiang, Q., Huang, P., & Zhao, W. (2017). Neuregulin-1 protects mouse cerebellum against oxidative stress and neuroinflammation. *Brain Research*, *1670*, 32–43. https://doi.org/10.1016/j.brainres.2017.06.012

Xu, J., Sun, P., Zhao, X., Meng, L., Wang, X., Qin, X., Zhou, Y., Zhou, M., & Cui, Y. (2023). Safety, Tolerability, and Pharmacokinetics of Recombinant Human Neuregulin-1 in Healthy Chinese Subjects. *American Journal of Cardiovascular Drugs*, *23*(4), 419–428. https://doi.org/10.1007/s40256-023-00585-6

Xu, M., Wu, X., Jie, B., Zhang, X., Zhang, J., Xin, Y., & Guo, Y. (2014). Neuregulin-1 protects myocardial cells against H2 O2 -induced apoptosis by regulating endoplasmic reticulum stress. *Cell Biochemistry and Function*, *32*(5), 464–469. https://doi.org/10.1002/cbf.3038

Xu, W., Mimnaugh, E., Rosser, M. F. N., Nicchitta, C., Marcu, M., Yarden, Y., & Neckers, L. (2001). Sensitivity of Mature ErbB2 to Geldanamycin Is Conferred by Its Kinase Domain and Is Mediated by the Chaperone Protein Hsp90*. *Journal of Biological Chemistry*, *276*(5), 3702–3708. https://doi.org/10.1074/jbc.M006864200

Xu, W., Yuan, X., Xiang, Z., Mimnaugh, E., Marcu, M., & Neckers, L. (2005). Surface charge and hydrophobicity determine ErbB2 binding to the Hsp90 chaperone complex. *Nature Structural & Molecular Biology*, *12*(2), 120–126. https://doi.org/10.1038/nsmb885

Xu, Z., Ford, G. D., Croslan, D. R., Jiang, J., Gates, A., Allen, R., & Ford, B. D. (2005). Neuroprotection by neuregulin-1 following focal stroke is associated with the attenuation of ischemia-induced pro-inflammatory and stress gene expression. *Neurobiology of Disease*, *19*(3), 461–470. https://doi.org/10.1016/j.nbd.2005.01.027

Xu, Z., Jiang, J., Ford, G., & Ford, B. D. (2004). Neuregulin-1 is neuroprotective and attenuates inflammatory responses induced by ischemic stroke. *Biochemical and Biophysical Research Communications*, *322*(2), 440–446. https://doi.org/10.1016/j.bbrc.2004.07.149

Yan, F., Tan, X., Wan, W., Dixon, B. J., Fan, R., Enkhjargal, B., Li, Q., Zhang, J., Chen, G., & Zhang, J. H. (2017). ErbB4 protects against neuronal apoptosis via activation of YAP/PIK3CB signaling pathway in a rat model of subarachnoid hemorrhage. *Experimental Neurology*, *297*, 92–100. https://doi.org/10.1016/j.expneurol.2017.07.014

Yang, Y., Ohta, K., Shimizu, M., Morimoto, K., Goto, C., Nakai, A., Toma, T., Kasahara, Y., Yachie, A., Seki, H., & Koizumi, S. (2003). Selective protection of renal tubular epithelial cells by heme oxygenase (HO)-1 during stress-induced injury. *Kidney International*, *64*(4), 1302–1309. https://doi.org/10.1046/j.1523-1755.2003.00231.x

Yang, Z., Jiang, Q., Chen, S.-X., Hu, C.-L., Shen, H.-F., Huang, P.-Z., Xu, J.-P., Mei, J.-P., Zhang, B.-P., & Zhao, W.-J. (2016). Differential changes in Neuregulin-1 signaling in major brain regions in a lipopolysaccharide-induced neuroinflammation mouse model. *Molecular Medicine Reports*, *14*(1), 790–796. https://doi.org/10.3892/mmr.2016.5325

Yee, M. E., Lane, P. A., Archer, D. R., Joiner, C. H., Eckman, J. R., & Guasch, A. (2018). Losartan therapy decreases albuminuria with stable glomerular filtration and permselectivity in sickle cell anemia. *Blood Cells, Molecules & Diseases*, *69*, 65–70. https://doi.org/10.1016/j.bcmd.2017.09.006

Yeruva, S. L. H., Paul, Y., Oneal, P., & Nouraie, M. (2016). Renal Failure in Sickle Cell Disease: Prevalence, Predictors of Disease, Mortality and Effect on Length of Hospital Stay. *Hemoglobin*, *40*(5), 295–299. https://doi.org/10.1080/03630269.2016.1224766

Yoo, J.-Y., Kim, H.-B., Baik, T.-K., Lee, J.-H., & Woo, R.-S. (2021). Neuregulin 1/ErbB4/Akt signaling attenuates cytotoxicity mediated by the APP-CT31 fragment of amyloid precursor protein. *Experimental and Molecular Pathology*, *120*, 104622. https://doi.org/10.1016/j.yexmp.2021.104622

Young, G.-H., Huang, T.-M., Wu, C.-H., Lai, C.-F., Hou, C.-C., Peng, K.-Y., Liang, C.-J., Lin, S.-L., Chang, S.-C., Tsai, P.-R., Wu, K.-D., Wu, V.-C., & Ko, W.-J. (2014). Hemojuvelin Modulates Iron Stress During Acute Kidney Injury: Improved by Furin Inhibitor. *Antioxidants & Redox Signaling*, *20*(8), 1181–1194. https://doi.org/10.1089/ars.2013.5366

Youssry, I., Makar, S., Fawzy, R., Wilson, M., AbdAllah, G., Fathy, E., & Sawires, H. (2015). Novel marker for the detection of sickle cell nephropathy: Soluble FMS-like tyrosine kinase-1 (sFLT-1). *Pediatric Nephrology (Berlin, Germany)*, *30*(12), 2163–2168. https://doi.org/10.1007/s00467-015-3172-9

Zahr, R. S., Hankins, J. S., Kang, G., Li, C., Wang, W. C., Lebensburger, J., & Estepp, J. H. (2019). Hydroxyurea prevents onset and progression of albuminuria in children with sickle cell anemia. *American Journal of Hematology*, *94*(1), E27–E29. https://doi.org/10.1002/ajh.25329

Zahr, R. S., & Saraf, S. L. (2023). Sickle Cell Disease and CKD: An Update. *American Journal of Nephrology*, *55*(1), 56–71. https://doi.org/10.1159/000534865

Zahr, R. S., Yee, M. E., Weaver, J., Twombley, K., Matar, R. B., Aviles, D., Sreedharan, R., Rheault, M. N., Malatesta-Muncher, R., Stone, H., Srivastava, T., Kapur, G., Baddi, P., Volovelsky, O., Pelletier, J., Gbadegesin, R., Seeherunvong, W., Patel, H. P., & Greenbaum, L. A. (2019). Kidney biopsy findings in children with sickle cell disease: A Midwest Pediatric Nephrology Consortium study. *Pediatric Nephrology*, *34*(8), 1435–1445. https://doi.org/10.1007/s00467-019-04237-3

Zahran, A. M., Nafady, A., Saad, K., Hetta, H. F., Abdallah, A.-E. M., Abdel-Aziz, S. M., Embaby, M. M., Abo Elgheet, A. M., Darwish, S. F., Abo-Elela, M. G. M., Elhoufey, A., & Elsayh, K. I. (2020). Effect of Hydroxyurea Treatment on the Inflammatory Markers Among Children With Sickle Cell Disease. *Clinical and Applied Thrombosis/Hemostasis*, *26*, 1076029619895111. https://doi.org/10.1177/1076029619895111

Zanazzi, G., Einheber, S., Westreich, R., Hannocks, M.-J., Bedell-Hogan, D., Marchionni, M. A., & Salzer, J. L. (2001). Glial Growth Factor/Neuregulin Inhibits Schwann Cell Myelination and Induces Demyelination. *Journal of Cell Biology*, *152*(6), 1289–1300. https://doi.org/10.1083/jcb.152.6.1289

Zeng, F., Miyazawa, T., Kloepfer, L. A., & Harris, R. C. (2014a). Deletion of ErbB4 accelerates polycystic kidney disease progression in cpk mice. *Kidney International*, *86*(3), 538–547. https://doi.org/10.1038/ki.2014.84

Zeng, F., Miyazawa, T., Kloepfer, L. A., & Harris, R. C. (2014b). Deletion of ErbB4 accelerates polycystic kidney disease progression in cpk mice. *Kidney International*, *86*(3), 538–547. https://doi.org/10.1038/ki.2014.84

Zeng, F., Miyazawa, T., Kloepfer, L. A., & Harris, R. C. (2018). ErbB4 deletion accelerates renal fibrosis following renal injury. *American Journal of Physiology-Renal Physiology*, *314*(5), F773–F787. https://doi.org/10.1152/ajprenal.00260.2017

Zgorzynska, E., Dziedzic, B., & Walczewska, A. (2021). An Overview of the Nrf2/ARE Pathway and Its Role in Neurodegenerative Diseases. *International Journal of Molecular Sciences*, *22*(17), 9592. https://doi.org/10.3390/ijms22179592

Zhai, H., Ni, L., & Wu, X. (2023). The roles of heme oxygenase-1 in renal disease. *Frontiers in Nephrology*, *3*. https://www.frontiersin.org/articles/10.3389/fneph.2023.1156346

Zhang, D., Xu, C., Manwani, D., & Frenette, P. S. (2016). Neutrophils, platelets, and inflammatory pathways at the nexus of sickle cell disease pathophysiology. *Blood*, *127*(7), 801–809. https://doi.org/10.1182/blood-2015-09-618538

Zhang, T.-T., Qu, N., Sun, G.-H., Zhang, L., Wang, Y.-J., Mu, X.-M., Wei, W.-J., Wang, Y.-L., Wang, Y., Ji, Q.-H., Zhu, Y.-X., & Shi, R.-L. (2018). NRG1 regulates redox homeostasis via NRF2 in papillary thyroid cancer. *International Journal of Oncology*, *53*(2), Article 2. https://doi.org/10.3892/ijo.2018.4426

Zhou, J., Zheng, Q., & Chen, Z. (2022). The Nrf2 Pathway in Liver Diseases. *Frontiers in Cell and Developmental Biology*, *10*, 826204. https://doi.org/10.3389/fcell.2022.826204

Zhou, Q., Pan, X., Wang, L., Wang, X., & Xiong, D. (2016). The protective role of neuregulin-1: A potential therapy for sepsis-induced cardiomyopathy. *European Journal of Pharmacology*, *788*, 234–240. https://doi.org/10.1016/j.ejphar.2016.06.042

Zhou, W., Guan, Q., Kwan, C. C. H., Chen, H., Gleave, M. E., Nguan, C. Y. C., & Du, C. (2010). Loss of clusterin expression worsens renal ischemia-reperfusion injury. *American Journal of Physiology. Renal Physiology*, *298*(3), F568-578. https://doi.org/10.1152/ajprenal.00399.2009

Zhu, X., Li, B., & Pace, B. S. (2017). NRF2 mediates γ-globin gene regulation and fetal hemoglobin induction in human erythroid progenitors. *Haematologica*, *102*(8), e285–e288. https://doi.org/10.3324/haematol.2016.160788

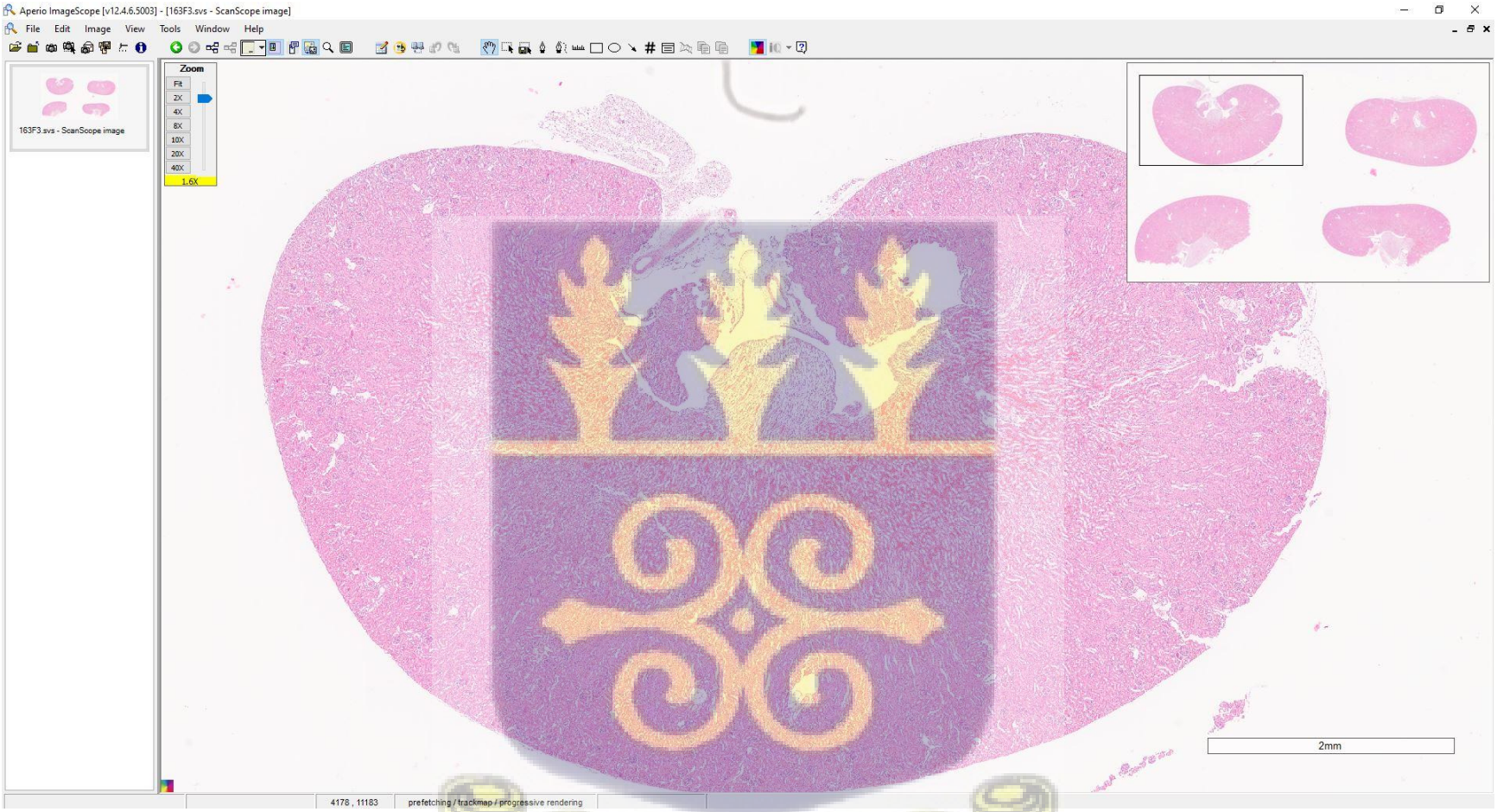
Zhu, X., Oseghale, A. R., Nicole, L. H., Li, B., & Pace, B. S. (2019). Mechanisms of NRF2 activation to mediate fetal hemoglobin induction and protection against oxidative stress in sickle cell disease. *Experimental Biology and Medicine*, *244*(2), Article 2. https://doi.org/10.1177/1535370219825859

Zhu, X., Xi, C., Thomas, B., & Pace, B. S. (2018). Loss of NRF2 function exacerbates the pathophysiology of sickle cell disease in a transgenic mouse model. *Blood*, *131*(5), Article 5. https://doi.org/10.1182/blood-2017-10-810531

Zou, A. P., Billington, H., Su, N., & Cowley, A. W. (2000). Expression and actions of heme oxygenase in the renal medulla of rats. *Hypertension (Dallas, Tex.: 1979)*, *35*(1 Pt 2), 342–347. https://doi.org/10.1161/01.hyp.35.1.342

8.0 APPENDIX.

8.1 Appendix I: Representative Aperio ImageScope image analysis of sample kidneys



8.2 Appendix II: Expression of neuregulin-1 receptor, ErbB4 in the kidneys of Townes sickle cell mice.

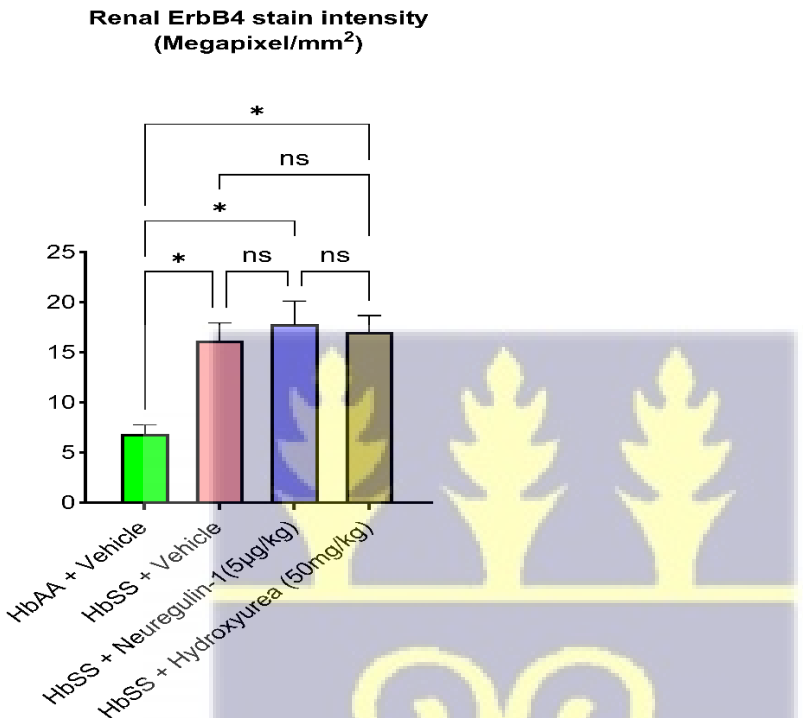


Figure 30 Expression of neuregulin-1 receptor, ErbB4 in the kidneys of Townes sickle cell mice.

Anti-ErbB4 immunohistochemical analysis was conducted on kidney sections from sickle cell (HbSS)and control (HbAA) mice treated with vehicle, neuregulin-1, and hydroxyurea over two weeks from 12 weeks of age (n = 7-8 per group). Quantification of renal ErbB4 expression stain intensity among treatment groups. Statistics involved two-tailed unpaired t-tests and one-way ANOVA with Tukey's post hoc test. Data reported as Mean ± SEM. P-values are denoted as follows: p <0.05 (*).