GENETIC VARIANTS OF ERYTHROPOIETIN AND THEIR BIOLOGICAL SIGNIFICANCE

by

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Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
July 2023

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Abstract

Erythropoietin (EPO) is a hematopoietic cytokine with a multitude of functions, that are erythropoietic, metabolic or cytoprotective. The erythropoietic functions of EPO have led to the development of many erythropoietic stimulating agents (ESAs) that are recombinant human EPOs (rhEPO) biologic therapies. ESAs have primarily been used to treat anemia, but have also been investigated to treat ischemic injuries, such as acute myocardial infarction and stroke. ESAs can vary in their glycosylation pattern that can affect their pharmacodynamics. Genetic variants in EPO have also been associated with microvascular complications and anemia in diabetes. In our study we investigated the regulation of EPO by single nucleotide polymorphisms (SNPs) and post transcriptionally by intron retention. We explore the potential differences in EPO's function in different ESAs formulations *in vitro*. We sequenced the rs1617640, rs507392 and rs551238 SNPs in EPO in a cardiovascular surgery population. We found the C allele in rs1617640 correlated with anemia and the GG genotype in rs507392 correlated with dyslipidemia. In an *in vitro* cell model, intron retention was found to be increased in response to ischemia in brain cells, but not kidney and liver. The variation in intron retention also shows temporal and circadian changes. Epoetin α, Peprotech are rhEPOs with three N-glycosylation sites compared to five in darbepoetin α (which also has five amino acid substitutions). Epoietin α has similar erythropoietic functions compared to a recombinant mouse Epo (rmEpo). In H9c2 these showed a higher degree of proliferation compared to darbepoetin α, with Peprotech being in between. H9c2 cells treated with epoetin α for 24 hours resulted in an increase in mitochondrial oxygen consumption. Pre-treatment with epoetin α or Peprotech, but not darbeopoetin α, provide cytoprotection against hydrogen peroxide, but not against staurosporine or hemin. Taken together, our study shows variations in EPO at the various molecular levels have physiological implications, which require further study.

List of abbreviations and symbols used

2-OG	2-oxglutarate
ActD	ActinomycinD
ADP	adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
AMI	acute myocardial infarction
AngII	Angiotension II
ARNT	hydrocarbon receptor nuclear translocator
AUE	anemia of unknown etiology
BFU-E	blast forming unit-erythroid
BHK	baby hamster kidney
BM	bone marrow
BMI	body mass index
bp	base pairs
BPS	branch point sequence
BRE	B recognition elements
BSA	bovine serum albumin
cDNA	complimentary DNA
CERA	continuous erythropoiesis receptor activator
CFSE	carboxyfluorescien succinimidyl ester
CFU-E	colony forming unit-erythroid
CHF	chronic heart failure
CHO	Chinese hamster ovary

CKD	chronic kidney disease
CO_2	carbon dioxide
CPEO	carbamylated EPO
CVD	cardiovascular disease
DNA	Deoxyribonucleic acid
dN/dS	Ratio of non-synonymous to synonymous substitutions
DTT	Dithiothreitol
DTX	docetaxel
E2	estrogen 17 β
EC_{50}	Half maximal effective concentration
ELISA	enzyme linked immmunosorbent assay
E_{max}	Maximum effect
EMP	EPO mimetic peptides
EPO	Erythropoietin
EPOR	erythropoietin receptor
ER	endoplasmic reticulum
ERBP	EPO mRNA-binding protein
ESA	erythropoiesis stimulating agent
ESE	exon splicing enhancer
ESS	exon splicing silencer
eQTL	expression quantitative trait loci
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FDA	Food and Drug Administration

FIH	factor inhibiting HIF
GalNac	N-acetylgalactosamine
gDNA	genomic DNA
GlcNAc	N-acetyl glucosamine
GWAS	Genome wide association study
H_2O_2	hydrogen peroxide
Hb	hemoglobin
Hct	Hematocrit
HDL	high-density lipoprotein
HFpEF	Heart failure with preserved ejection fraction
HIF	hypoxia inducible factors
HIF-PHI	Hypoxia inducible factor prolyl hyodroxylase inhibitors
HMOX-1	Heme oxygenase 1
HNF-4	hepatocyte nuclear factor 4 alpha
hnRNP	heterogenous nuclear RNA protein
HRE	hypoxia response elements
HRP	horse radish peroxidase
IC_{50}	Half maximal inhibitory concentration
IE	intron excised
IR	intron retention
IRP	International Reference Preparation
ISE	intronic splicing enhancer
ISS	intron splicing silencer

IU	international units
IV	intravenous
JAK2	Janus family tyrosine kinases 2
kb	kilobase
Kd	dissociation constant
kDa	kilodalton
kg	kilogram
KIE	kidney inducible element
KO	knock out
LDL	low-density lipoprotein
LIE	liver inducible element
LPL	lipoprotein lipase
LVEF	left ventricular ejection fraction
m7G	7-methylguanosine
MAPK	mitogen-activated protein kinase
MBTB	Maritime Brain Tissue Bank
MDS	Myelodysplastic syndrome
miRNA	microRNA
MNC	mononuclear cells
mRNA	messenger RNA
mU	milli units
MW	molecular weight
Neu5Ac	N-acetylneuraminic

NIBSC	National Institute for Biological Standards and Controls
NIH	National Institute of Health
NLR	neutrophil-lymphocyte-ratio
NMD	non-sense mediated decay
no-RT	no reverse transcriptase control
nts	nucleotides
NYHA	New York Heart association
O_2	oxygen
OGA	O-GLcNAcase
OGT	O-GlcNAc-transferase
OPOS	Obesity on Postoperative Outcomes following cardiac Surgery
OR	odds ratio
PCR	polymerase chain reaction
PHD	prolyl hydroxylase
Pi3K	phosphatidylinositol 3-kinase
poly(A)	polyadenylated
PPIs	peptidyl prolyl isomerase
pre-mRNA	Precursor mRNA
PTC	premature termination codon
PTM	post translational modification
PTT	polypyrimidine tract
pVHL	vonHippel-Lindeau
RAAS	renin-angiotensin-aldosterone system

RBP	RNA binding protein
rEPO	recombinant EPO
rhEPO	recombinant human EPO
rmEPO	recombinant mouse EPO
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RNP	ribonucleoprotein
ROS	reactive oxygen species
rtPCR	reverse transcriptase PCR
SC	subcutaneous
SCF	stem cell factor
SCL	stem-cell leukemia
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM	standard error of the mean
SF1	splicing factor 1
SHP-1	Src homology region 2 domain-containing phosphatase-1
SNP	single nucleotide polymorphism
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
SNS	sympathetic nervous system
SR	serine/arginine-rich
SS	splice site

STAT	signal transducers and activators of transcription
T2DM	Type 2 diabetes mellitus
TAE	tris acetate EDTA
TBS-T	Tris buffered saline-Tween20
TF	transcription factor
TfR	transferrin
TPR	tissue protective receptor
TSS	Transcriptional start site
U	units
UTR	untranslated region
VEGF	vascular endothelial growth factor
VUS	variant of unknown significance
WHO	world health organization
β-CR	beta common receptor (CD131)

Acknowledgements

I would like to thank my supervisor Dr. Keith Brunt for his encouragement, support and guidance over the past few years. I appreciate your support in both my scientific endeavours throughout me thesis and in pursuit of training to aide in my future career. Even outside the lab many of the discussions both regarding science and other topics has always provided great food for thought. Your guidance and support have improved my abilities as a scientist, communicator and colleague and always pushed me to improve. Thank you for your support and always having faith in me, even when I may not have.

I would also like to thank my various collaborators. Dr. Jeremy Simpson for his guidance and feedback throughout my thesis and in some ways acted as a second supervisor. You have provided me with great insights and always challenged to improve my scientific thinking. Dr. Brittany Edgett for your assistance and guidance throughout my thesis. Dr. Lester Perez for his guidance and training. Dr. Tobias Karakach for his assistance in bioinformatics as well as general support. Dr. Deniz Top for his guidance on circadian rhythm. I would also like to thank my supervisory committee Dr. Morgan Langille and Dr. Susan Howlett for your advice and guidance over the years.

I have been fortunate to have many great lab mates both at Dalhousie Medicine New Brunswick and the University of Guelph. Ashley Eadie for your comradery throughout our PhD's. Also, MinJi Kim, Tori Nelson, Benny Habiyambere, and Kyle Wells for your comradery.

I would also like to thank my many supporters at the Saint John Regional Hospital who have supported me through my PhD and encouraged me towards my future career

prospects. Dr. Nancy Carson for your guidance for my future career in Clinical genetics. Dr. Doha Itani and Dr. Ather Naseemuddin for your support and guidance over the past couple of year. Dr. Jennifer Shea, Dr. Tarek Rahmeh, Dr. Cenk Acar for your consistent support. Dr. Ken Obenson for your support and finally convincing me I should go do my PhD.

I am thankful to my family for their love and support throughout this. My parents and stepparents for your consistent encouragement and faith in me. To my siblings and nephews for your love and support. Finally, I would like to thank my husband, Eric. Your love and support has meant the world to me. You have probably put up with more throughout this process than I will ever be able to thank you enough for. Thank you for being there for the good and bad and I will always love you for your unwavering support and patience.

The ideas, hypotheses, and experiments presented were a collaboration between Victoria Northrup and Dr. Keith Brunt, with input from Dr. Jeremy Simpson, Dr. Lester Perez, Dr, Brittany Edgett. Unless otherwise stated, Victoria Northrup was responsible for the experimental design, experimentation and data analysis presented. Ashley Eadie performed the ELISA presented in Chapter 3, Tori Nelson assisted in the immunoblotting in Figure 5.10 and Angella Mercer assisted with the Oxygraphy in Figure 5.5. StemCell Technologies were directed to perform the CFU-E assays (Figure 5.2).

Chapter 1 - Introduction

1.1 Genetic variants in hormone regulation

Hormones are molecules that act as physiological messengers to elicit homeostasis in the body.[1] The expression and function of hormones are regulated at multiple levels, including the epigenetic level, the genomic DNA (gDNA) level, transcriptional level (i.e. messenger RNA (mRNA)), translational level (i.e. protein) or through post translational modifications (PTMs). Epigenetic modifications can include changes to DNA methylation, acetylation, sumoylation, histone modification, nucleosome availability, heritable variation or acquired that alone or together affect mRNA or microRNA (miRNA) expression and translation.[2,3] Portela and Esteller provide a detailed review of epigenetic modifications.[2] Herein, we will focus on the variants at the levels of gDNA, mRNA, and protein focusing on PTMs.

1.1.1 Hormone regulation through genomic DNA elements

Hormones are encoded by genes that are composed of multiple structures that affect their regulation and/or their protein product. Vertebrate genes are typically composed of short exons (approximately 170 nucleotides (nts)) and longer introns (>300 nts).[4–6] The exons are the coding regions to the final protein product. Typically, exon 1 contains a 5' untranslated region (UTR), while the last exon contains a 3' UTR. The UTRs are not translated into the protein product but contain critical regulatory elements. The 5' UTR of eukaryotic genes is critical to start codon choice and recruitment of the mRNA to the ribosome.[7] The 5' UTR is ended by the transcription start site that begins the coding region.[8] The 3' UTR contains *cis*-elements that recruit RNA-binding proteins

that regulate mRNA fate, translation, protein-protein interactions (facilitated by co-translational protein complex formation) and compartmentalization.[9] The 3'UTR is adjacent to the termination codon that ends the coding region and contains the code for the polyadenylated (poly (A)) tail.[8]

Gene expression is regulated by multiple elements located either upstream or downstream of the gene. The promoter region is typically located upstream of the gene and contains elements that regulate transcription initiation. The promoter contains a TATA box of approximately 25 base pairs (bp) upstream of the transcription initiation site, which is surrounded by GC-rich sequences. Other elements found in the promoter region include: B recognition element (BRE), transcription factor (TF) recognition elements, initiator sequence (at the start of transcription), downstream promoter elements (approximately 30 bp 3' of transcription initiation site), and the CCAAT box (50-200 bp upstream) that binds multiple TFs.[8] Other regulatory elements, such as enhancers, silencers and insulators, can act on the promoter to regulate gene expression.

Enhancers, silencers, and insulator elements can be *cis* or *trans*-acting.[10] *Cis*-acting elements are located in close proximity to the gene, whereas *trans*-acting elements act on distant genes.[11–13] Enhancers are activated by the binding of one or more TFs and TF co-factors that together coordinate to act upon the promoter to initiate gene transcription. Enhancers are important in regulating tissue-specific gene activation, with some genes having different enhancers (in combination with TFs) depending on the tissue. Silencers act to repress gene activity by either looping their target promoter or competing with TF binding for the promoter site. Some genes also have insulators that are boundaries in which gene regulatory elements reside.[10] Together, these regulatory

elements ensure the proper expression of a gene within appropriate cellular context.

1.1.2 Transcriptional regulation of hormones

1.1.2.1 RNA splicing

Precursor mRNA (pre-mRNA) is generated by transcription from gDNA and contains both introns and exons. Splicing of the pre-mRNA typically results in the removal of introns to form the mature mRNA. A mature mRNA will also have a 7-methylguanosine (m7G) cap (commonly referred to as the 5'cap)[14] and a poly(A) tail that aids in translation and mRNA stability.[15] Splicing is performed by the spliceosome and is a highly regulated process requiring both *cis* and *trans*-acting elements. The introns of pre-mRNA contain three key *cis*-acting elements for the recognition of the spliceosome: 1) exon/intron junction, also known as the splice site (SS), 2) the branch point sequence (BPS), and 3) polypyrimidine tract (PTT). The SS is located at the 5' and 3' ends of the intron. The BPS and PTT are located upstream of the 3'SS, with the PTT located between the BPS and 3'SS. The most common consensus sequence for 5' SS is CAG/GURAGU in the -3 to +6 position. The 5'SS consensus sequence is recognized by Watson-Crick base pairing by the small nuclear RNA (snRNA) in the small nuclear ribonucleoprotein (snRNP). For the 3'SS, the most common consensus sequence is AG/G that are recognized along with the PPT by U2AF65/U2AF25 heterodimers.[16] Additionally, enhancers and silencers are *cis*-acting elements in the pre-mRNA that regulate splicing. Enhancers include the exon splicing enhancer (ESE) and intronic splicing enhancer (ISE). Silencers include the exon splicing silencer (ESS) and the intron splicing silencer (ISS). Secondary structures formed by the pre-mRNA are also

implicated in splicing regulation.[17]

The *cis*-acting elements recruit *trans*-acting elements that enhance and/or silence splicing. Enhancers are generally recognized by proteins in the serine/arginine-rich (SR) family and facilitate splicing by interacting with the spliceosome. Silencing elements are generally recognized by the heterogenous nuclear RNA protein (hnRNP) family. The exact mechanism of inhibition has not been elucidated, but is hypothesized to repress splicing by steric hindrance.[17]

The spliceosome is a metalloribozyme, multi-megaDalton ribonucleoprotein (RNP) that catalyzes the splicing of pre-mRNA. The spliceosome is comprised of RNA components and as many as 200 proteins incorporating two catalytic metal ions (M1 and M2). The proteins in the spliceosome can be categorized into five classes: 1) uridine-rich RNPs (U1, U2, U4, U5 and U6 snRNPs), 2) NineTeen complex and NineTeen-related complex, 3) various splicing factors, 4) RNA-dependant ATPase-helicases, and 5) other regulatory and auxiliary proteins including kinases/phosphatases and peptidyl-prolyl isomerases (PPIs).[18]

Assembly of the spliceosome involves multi-stepped recruitment of the various components and conformation changes. Splicing is initiated by the binding of the U1 snRNP to the intron's 5'SS and splicing factor 1 (SF1) binding to the BPS region and heterodimer U2AF65/U2AF35 to the PPT to form the 3' SS Complex E. Spliceosome recruitment is facilitated by Complex E acting as a bridge to bring the 5' and 3' ends of the intron in close proximity.[17,19,20] Splicing occurs through a sequence of recruitments and conformational changes that ultimately result in the splicing as the 5' SS followed by the 3'SS through *trans*-esterification reactions. The end-product is an intron lariat

(spliced out intron) that joins the exons to generate the mature mRNA. Wahl *et al.*[17] provides a detailed review of the major spliceosome assembly.

There also exists a minor spliceosome that recognizes approximately 0.5 % of all introns. The minor spliceosome contains four specific snRNPs (U11, U12, U4atac and U6 atac corresponding to U1, U2, U4 and U6 in the major spliceosome, respectively). Although there is variation in the snRNPs, the major and minor spliceosome share similarities in secondary structures.[21] Whereas the major spliceosome is primarily located in the nucleus, the minor spliceosome has been detected in the cytoplasm,[22] and this suggests at least some splicing occurs in the cytoplasm.

1.1.2.2 Alternative splicing

Alternative splicing is an important regulatory mechanism allowing a single gene to result in multiple gene products. Alternative splicing is more prevalent in higher eukaryotes and aids in explaining the increased protein complexity without a proportional increase in genome size.[23] *In silico* analysis predicted that as much as 95% of human genes have multiple splice forms.[24,25] Yet, high-resolution spectrometry has only detected isoforms in approximately 37% of genes.[26] This discrepancy could be due to over-prediction *in silico,* low abundant isoforms not being detected using the high-resolution spectrometry, or not all splice variants producing a different product. Regardless, both the *in silico* and high-resolution spectrometry demonstrate that alternative splicing is common in the human genome.

Alternative splicing is an essential mechanism of development and adaptation with both time and tissue specificity.[27] RNA-sequencing (RNA-Seq) experiments have

shown over 400 differential splicing events between embryonic and adult mouse cortical cortex, with 31% of these genes exhibiting no change in overall gene expression.[28] Alternative splicing has also been shown to increase with age in humans,[29] and response to nutrient restriction, suggesting that alternative splicing is physiologically responsive to stimuli and developmental programs.[30–32]

There are five main types of alternative splicing: 1) cassette (exon) exclusion/inclusion, 2) alternative 5' or 3' SS, 3) mutually exclusive exons, 4) alternative promoter and polyadenylation sites, and 5) intron retention (Figure 1.1).[27,30] Three main elements influence alternative splicing: 1) strength of the SS, 2) *cis*-regulatory sequences in the pre-mRNA that affect exon recognition, and 3) levels of *trans*-acting factors, such as RNA binding proteins (RBPs) and splicing factors.[27] The strength of the SS is determined by their sequence with consensus sequence being considered strong SS. Strong SSs lead to constitutive splicing resulting in the exon being included in all transcripts. Weak SS requires *cis*-acting sequences, such as splicing enhancers (ISEs and ESEs), and appropriate cellular context to be recognized by the splicesome.[33] There has been significantly more study into the first four forms of alternative splicing, whereas intron retention has only recently begun to be studied in mammals.

1.1.2.2.1 Intron Retention

Intron retention is a form of alternative splicing that results in mature mRNA retaining one or more introns. Intron retention was initially considered to only occur in plants, fungi, or viruses but has now been described as an important mechanism of gene regulation in mammals.[34,35] Transcriptomics studies have identified that 50% to 80% of multi-exonic genes utilize intron retention in vertebrates.[36,37] Intron retention has shown

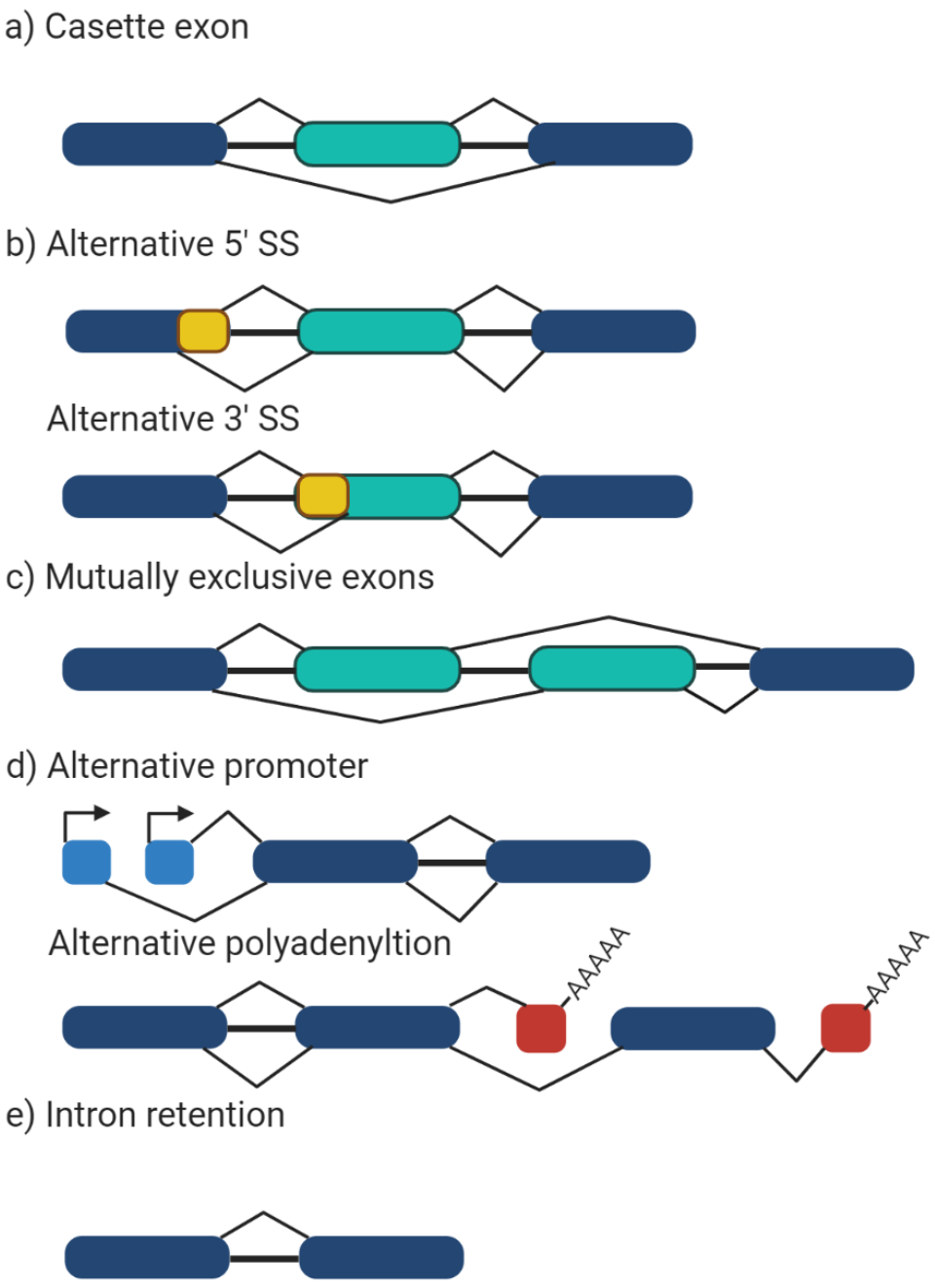


Figure 1.1- Different types of alternative splicing.
Exons are shown by rectangles and introns by solid lines. The constitutive splicing is indicated by the lines joining exons above the transcript. The alternative splice form is shown below the transcript by lines joining the exons that are joined. A) Cassette exon, B) alterative splice sites, C) mutually exclusive exons, D) alternative promoter/polyadenylation, E) intron retention. Created with BioRender.com

tissue specificity and changes during development.[35,36,38,39] Although intron retention in mammals has been neglected until recently, it is being demonstrated as an important form of alternative splicing.

Intron retention is an evolutionary conserved mechanism of post-transcriptional gene regulation.[36,40] Intron retention has been shown to affect similar biological processes between mouse and human.[34,40,41] Cross species analysis has shown an inverse correlation with the number of intron-retaining genes and protein coding genes, suggesting it is a mechanism to increase transcriptomic complexity. Additionally, retained introns are more commonly found proximal to the 3'UTR or have miRNA binding sites, suggesting an indirect gene regulatory mechanism through miRNA. This suggests intron retention may have evolved as a mechanism of gene regulation to improve adaptation of organisms.[41]

There are characteristics that are common among introns that are retained. Retained introns are typically short, with high GC content and weak 5' and 3' SSs.[36,41–44] In addition to the characteristics of the intron, an increased GC content of the flanking exon, the ratio of the length of the intron to flanking exons and the position of the intron in the gene affect intron retention.[36] Both the intron and flanking exons tend to have more RNA-associated protein binding sites for enhancers and repressors. A decrease in splicing factors, such as SR proteins, or spliceosome subunits (such as U1 and U2) are associated with increased intron retention.[45] A slower elongation of RNA Pol II, such as with secondary structures caused by the high GC content, favours intron retention.[36,41,42] High CpG density or reduced CpG methylation also favours intron retention. Histones can affect intron retention, with H33K36me3 histone modification being associated with

increased intron retention.[46–48] The cooperation of the epigenetic and splicing machinery accounts for the mechanisms of approximately 20% of intron retention events.[35] Though we have gained a great deal of insight into intron retention recently, there are still significant gaps in our understanding of this important gene regulatory mechanism.

The fate of intron retaining transcripts is also varied. Intron-retaining transcripts can be: 1) degraded, 2) held in reserve to be translated later, or 3) generate new protein isoforms entirely (Figure 1.2). Intron-retaining transcripts can either remain in the nucleus (sometimes referred to as intron-detained) or be exported to the cytoplasm.[34,35] Intron-retaining transcripts that are exported to the cytoplasm can be degraded by nonsense-mediated decay (NMD) if a premature termination codon (PTC) is present.[49,50] Other intron-retaining transcripts that are exported to the cytoplasm have signals in the intron that can specify subcellular localization,[51] or may be translated to form new protein isoforms.[52,53] Intron-detained transcripts that remain in the nucleus until a stimulus is applied, can be viewed as a reserve of mRNA. Once a stimulus is applied it can either cause splicing of the intron to form the canonical protein or be exported to the cytoplasm. Neurons have been shown to use intron retention to retain a reserve of intron-retaining mRNA for their larger mRNAs, which likely aligns defensively with any fluctuation of critical metabolic substrate bioavailability. The retained introns are promptly spliced out during neuronal activation, which allows for a more rapid response than generating *de novo* mRNA.[54] Therefore, intron retention is an important mechanism in fine-tuning gene regulation and adaptation to maintain homeostasis in sensitive/precious cell types.

Hypoxia has been shown to increase intron retention. Memon *et al*.[55] induced hypoxia (1% oxygen) in HCT116 (human colorectal carcinoma) cells and found an

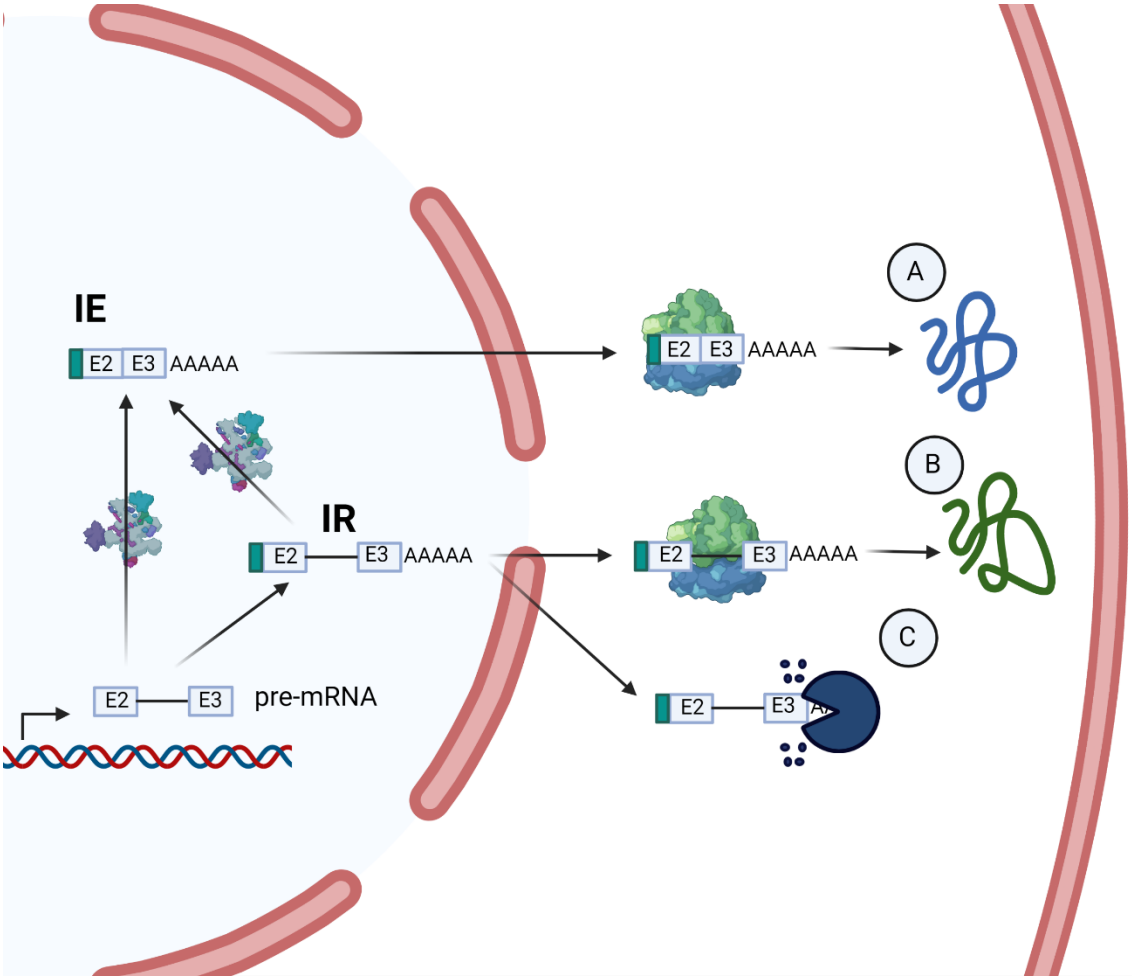


Figure 1.2- Intron retention is a mechanism of post-translational gene regulation. The pre-mRNA with introns and exons is produced by the transcription of the gDNA. Pre-mRNA is spliced to remove introns and add the 5'cap and poly(A) tail then exported to the cytoplasm for translation to protein (IE). Intron-retained transcripts retain one or more introns and have a 5' cap and a poly(A) tail (IR). The intron-retained transcript can be held in reserve in the nucleus (also referred to as intron-detained) until a stimulus is applied. The stimulus can cause the intron-retained transcript to either be spliced and then exported to the cytoplasm for translation (A) or exported to the cytoplasm with the intron. If the intron is retained in the cytoplasm the transcript can either by decayed by non-sense mediated decay (NMD) (C), or is translated into a new protein isoform (B). Created with BioRender.com

overall increase in intron retention. That increase also resulted in a decrease in protein content. This led the authors to conclude that mRNAs are being held-back from translation until cells are in favourable conditions as a means of conserving energy during acute hypoxic exposure. Introns have also been shown to be important in nutrient deprivation. Parenteau *et al*[56] developed a budding yeast that lacked introns. Although the yeast were viable under normal growth conditions, they were not able to tolerate nutrient withdrawal compared to the intron-containing yeast. This reinforces that introns have a highly conserved role in adaptation to stress. Although further study is needed, intron retention may be a mechanism that helps with adaptation to environmental stresses or pathological conditions, such as ischemia that boasts elements of both nutrient deprivation and tissue hypoxia.

1.1.3 Translation and post-translational regulation of hormones

The m7G 5' cap on the mature mRNA aids in the ribosome recognition of the mRNA to allow translation initiation.[14] Translational controls are essential to ensure the mRNA is accurately translated to a protein sequence. Aberrantly translated proteins can result in altered or misfolded proteins, which can impact their function or contribute to the unfolded protein endoplasmic reticulum stress response pathways. Ribosome-associated protein "quality control systems" have evolved to control the biogenesis of functional proteins. These regulatory systems can include folding chaperones, control of translational rate, or "quality control" of the polypeptides produced.[57–59] Many proteins will also undergo PTMs that are important in their localization and function.[59–62]

There are over 700 proteins that are known to undergo over 20 different types of PTMs.[61,62] PTMs are important to allow cells to rapidly respond to extracellular stimuli

or insults to ensure homeostasis is maintained. PTMs can be divided into two main groups: 1) addition of chemical groups (enzymatic) or 2) formation of covalent linkage (non-enzymatic). The enzymatic PTMs included glycosylation, phosphorylation, detyrosination, ubiquitination, hydroxylation, acetylation or methylation occurring at specific amino acids. The machinery that regulates the site specificity and abundance of enzymatic PTMs can be referred to as writers (addition) or erasers (removal). The formation of covalent linkage includes ubiquitination, and SUMOylation, and occurs on monomeric or polymeric protein moieties. Non-enzymatic PTMs are thought to be generated spontaneously between nucleophilic amino acids and electrophilic metabolites. However, there is some evidence of non-enzymatic PTM regulation by secondary enzymatic processes.[59,61] Due to a large amount of PTMs a full review is outside the scope of this thesis, but many are reviewed by Jennings *et al*.[61] and Buuh *et al*.[60] Phosphorylation and glycosylation are among the most common forms of PTMs[60] and will be discussed briefly here.

1.1.3.1 Phosphorylation

Phosphorylation is mediated by phosphate kinases and phosphatases.[60] Phosphorylation is one of the most common forms of PTMs with an estimated one third of protein at any given time being phosphorylated.[63] Phosphorylation plays a role in almost all cellular functions including metabolism, cell cycle and apoptosis. The majority of phosphorylation sites are on Serine (Ser), Threonine (Thr) and Tyrosine (Tyr) residues.[60,61] The biological functions of phosphorylation are vast and can include allosteric regulation of stability and activity or recognition by ubiquitin-ligase. Phosphorylation may also enhance or antagonize tandem PTMs.[60] Therefore

phosphorylation is an important form of PTMs that can regulate hormones.

1.1.3.2 Glycosylation

Glycosylation is another important PTM with an estimated 20% of proteins being glycosylated.[64] Glycosylation is responsible for the stability and function of many cellular processes, adding mono- and oligosaccharides to proteins. There are three types of glycosylation: *N*-linked glycosylation, *O*-linked glycosylation and *C*-linked glycosylation. *N*-linked and *O*-linked glycosylation are the most common glycosylation[65] and will be the focus herein.

N-linked glycosylation acts on Asn residues through the nitrogen atom in the side chain.[60,61,65] Generally, the Asn residue is found in the consensus sequence N-X-S/T (where X is any amino acid except proline).[66] *N*-linked glycosylation commonly occurs in the endoplasmic reticulum (ER) where a core glycan with *N*-acetyl glucosamine (GluNAc) is transferred to the protein.[60,65,67] Although most mature N-glycans will have the core pentasaccharide structure, they can vary in structure depending on cell type, protein localization and protein structure.[68]

O-linked glycosylation acts on the oxygen atom on Ser on Thr residues.[65] *O*-linked glycosylation commonly occurs in the golgi[67] where N-acetylgalactosamine (GalNAc) is transferred to the protein.[60,65] The glycan transfer is facilitated by *O*-GlcNAc-transferase (OGT) and can be reversed by *O*-GlcNAcase (OGA).[60] *O*-linked glycans do not share a universal core but the most common type is mucin-type.[65] The variations in both *N*-linked and *O*-linked glycosylation plays a significant role in the stability and function of many glycohormones, such as erythropoietin (EPO).[60,61,65]

1.2 History of Erythropoietin

In 1863, Jourdanet was the first to observe an increase in hematocrit (Hct) in people travelling to the high altitudes in Mexico City[69], linking erythropoiesis with high altitude. The increase in Hct at high altitudes was also observed in travelers to Peru by Viault in 1890.[70] Carnot and Deflandre hypothesized in 1906 there was a humoral factor responsible for the increased Hct called "hemopoietine".[71] Carnot and Deflandres' hypothesis was confirmed and renamed erythropoietin (EPO) by Bonsdorff and Jalvisto in 1948.[72]

The observation of lower body hypoxia in anemic polycythemia patients led to the search for the organ responsible for EPO production to organs below the diaphragm.[73,74] At the time it was assumed only one organ was responsible for the production of EPO. In 1957, Jacobson *et al.*[75] published their ablation study that suggested the kidney was the source of EPO. However, also in 1957, Mirand and Prentice[76] showed the presence of plasma EPO in nephrectomised and splenectomised hypoxic rats, suggesting the kidney could not be the sole organ responsible for EPO production. Findings by Jacobson *et al* garnered more attention and were reinforced by the clinical association of anemia with chronic kidney disease (CKD), so the kidney was enshrined as being the main, if not the sole, producer of EPO of significance for over 60 years. However, it has since been discovered that EPO is expressed in many tissues including brain, liver, spleen, lungs, testis, and ovaries and has diverse paracrine and hormone function beyond erythropoietic regulation.[77]

EPO was first purified in 1977 from the urine of patients with aplastic anemia.[78] The *EPO* gene was cloned in 1983[79] and led to the ability to produce recombinant human

EPO (rhEPO) in Chinese hamster ovary (CHO) cells. rhEPO was first used to treat anemia in CKD in 1986,[80] receiving Food and Drug Administration (FDA) approval in 1989, and later approved for anemia from myelosuppressive chemotherapy in 1993.[81] Subsequently rhEPO has been investigated for use in other forms of anemia and as a cytoprotectant to ischemic injuries.[82,83]

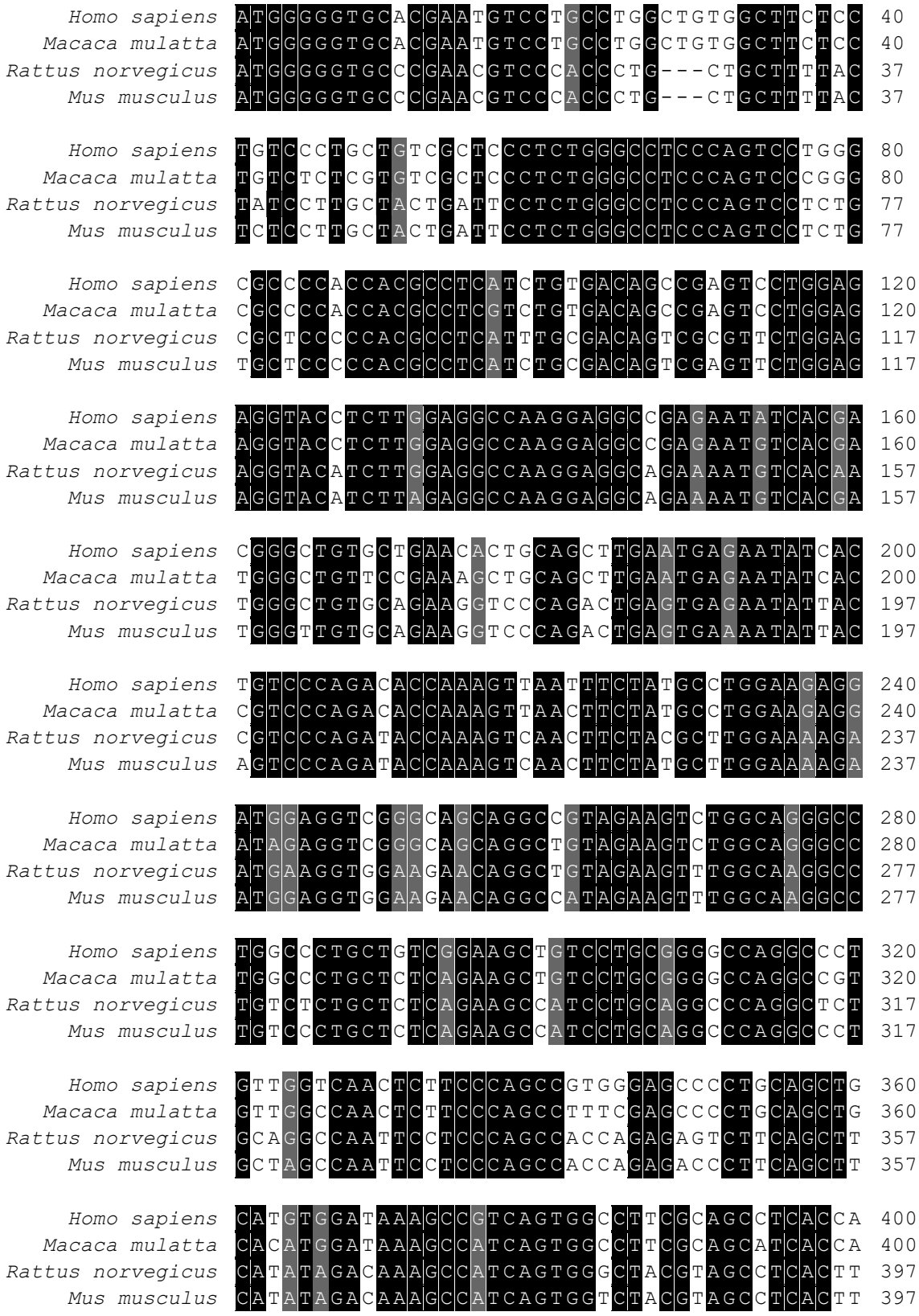
1.3 Evolutionary conservation of EPO

EPO demonstrates biological cross-reactivity amongst mammals,[84–87] suggesting it is evolutionarily conserved. The human *EPO* gene shows 91% similarity to monkey *EPO* and approximately 75% similarity to mouse and rat *Epo* (Figure 1.3). The protein also shows a high degree of conservation, with the human EPO protein having 90% similarity to monkey EPO and 82% to rat and 80% to mouse.[88] The conservation also extends to the *cis*-acting regulatory elements that regulate *EPO* expression, including enhancers.[89–92] The high degree of conservation has facilitated the therapeutic translation of animal model work and applied cell and molecular sciences to substantial human benefit.

1.4 Regulation of Erythropoietin

1.4.1 Molecular response to hypoxia

EPO is transcriptionally upregulated principally by the partial pressure of oxygen in tissues that are predominately sensitive to changes termed tissue hypoxia most commonly caused by a low level of oxygen, such as seen at high altitudes or in certain disease pathophysiology. Hypoxia activates cellular pathways that increase erythropoiesis, angiogenesis, and promote iron bioavailability.[93] The cellular response to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Macaca mulatta ATGGGGGTGCACGAATGTCCTGCCTGGCTGTGGCTTCTCC 40
Rattus norvegicus ATGGGGGTGCCCGAACGTCCCACCCTG---CTGCTTTTAC 37
Mus musculus ATGGGGGTGCCCGAACGTCCCACCCTG---CTGCTTTTAC 37
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Macaca mulatta TGTCTCTCGTGTCGCTCCCTCTGGGCCTCCCAGTCCCGGG 80
Rattus norvegicus TATCCTTGCTACTGATTCCTCTGGGCCTCCCAGTCCTCTG 77
Mus musculus TCTCCTTGCTACTGATTCCTCTGGGCCTCCCAGTCCTCTG 77
Homo sapiens CGCCCCACCACGCCTCATCTGTGACAGCCGAGTCCTGGAG 120
Macaca mulatta CGCCCCACCACGCCTCGTCTGTGACAGCCGAGTCCTGGAG 120
Rattus norvegicus CGCTCCCCCACGCCTCATTTGCGACAGTCGCGTTCTGGAG 117
Mus musculus TGCTCCCCCACGCCTCATCTGCGACAGTCGAGTTCTGGAG 117
Homo sapiens AGGTACCTCTTGGAGGCCAAGGAGGCCGAGAATATCACGA 160
Macaca mulatta AGGTACCTCTTGGAGGCCAAGGAGGCCGAGAATGTCACGA 160
Rattus norvegicus AGGTACATCTTGGAGGCCAAGGAGGCAGAAAATGTCACAA 157
Mus musculus AGGTACATCTTAGAGGCCAAGGAGGCAGAAAATGTCACGA 157
Homo sapiens CGGGCTGTGCTGAACACTGCAGCTTGAATGAGAATATCAC 200
Macaca mulatta TGGGCTGTTCCGAAAGCTGCAGCTTGAATGAGAATATCAC 200
Rattus norvegicus TGGGCTGTGCAGAAGGTCCCAGACTGAGTGAGAATATTAC 197
Mus musculus TGGGTTGTGCAGAAGGTCCCAGACTGAGTGAAAATATTAC 197
Homo sapiens TGTCCCAGACACCAAAGTTAATTTCTATGCCTGGAAGAGG 240
Macaca mulatta CGTCCCAGACACCAAAGTTAACTTCTATGCCTGGAAGAGG 240
Rattus norvegicus CGTCCCAGATACCAAAGTCAACTTCTACGCTTGGAAAAGA 237
Mus musculus AGTCCCAGATACCAAAGTCAACTTCTATGCTTGGAAAAGA 237
Homo sapiens ATGGAGGTCGGGCAGCAGGCCGTAGAAGTCTGGCAGGGCC 280
Macaca mulatta ATAGAGGTCGGGCAGCAGGCTGTAGAAGTCTGGCAGGGCC 280
Rattus norvegicus ATGAAGGTGGAAGAACAGGCTGTAGAAGTTTGGCAAGGCC 277
Mus musculus ATGGAGGTGGAAGAACAGGCCATAGAAGTTTGGCAAGGCC 277
Homo sapiens TGGCCCTGCTGTCGGAAGCTGTCCTGCGGGGCCAGGCCCT 320
Macaca mulatta TGGCCCTGCTCTCAGAAGCTGTCCTGCGGGCCAGGCCGT 320
Rattus norvegicus TGTCTCTGCTCTCAGAAGCCATCCTGCAGGCCCAGGCTCT 317
Mus musculus TGTCCCTGCTCTCAGAAGCCATCCTGCAGGCCCAGGCCCT 317
Homo sapiens GTTGGTCAACTCTTCCCAGCCGTGGGAGCCCCTGCAGCTG 360
Macaca mulatta GTTGGCCAACTCTTCCCAGCCTTTCGAGCCCCTGCAGCTG 360
Rattus norvegicus GCAGGCCAATTCCTCCCAGCCACCAGAGAGTCTTCAGCTT 357
Mus musculus GCTAGCCAATTCCTCCCAGCCACCAGAGACCCTTCAGCTT 357
Homo sapiens CATGTGGATAAAGCCGTCAGTGGCCTTCGCAGCCTCACCA 400
Macaca mulatta CACATGGATAAAGCCATCAGTGGCCTTCGCAGCATCACCA 400
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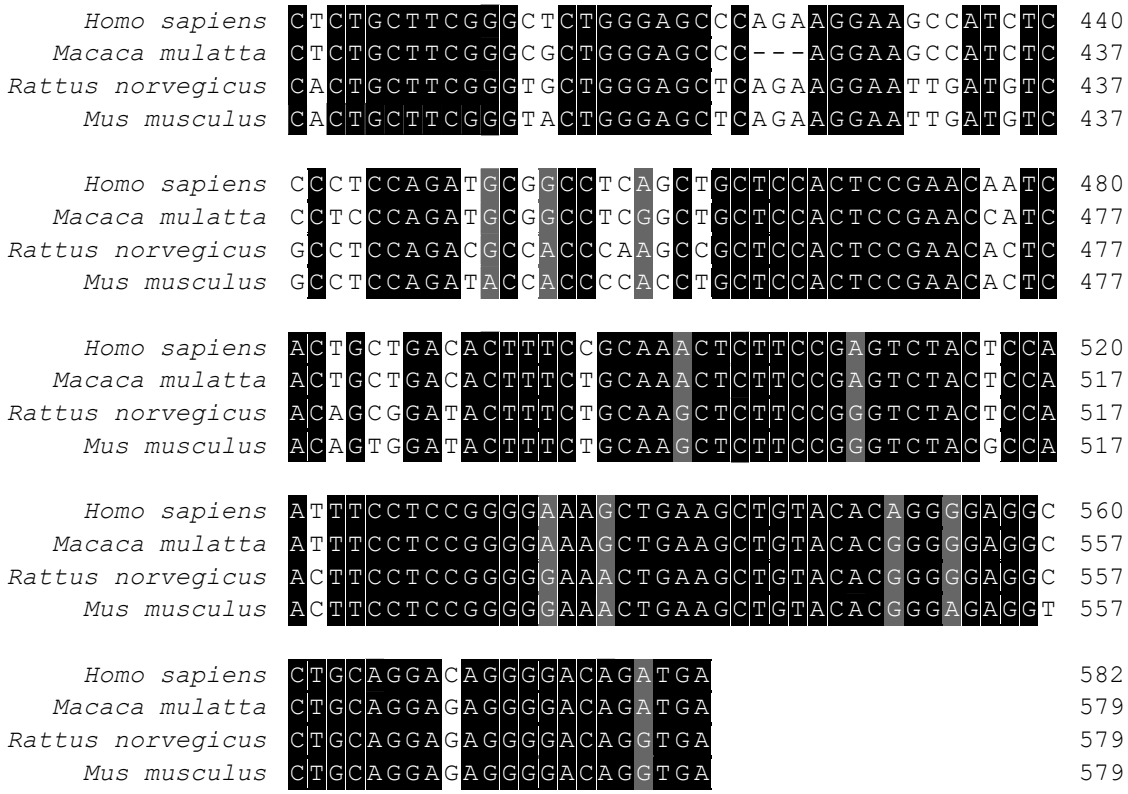

Figure 1.3- EPO mRNA is conserved from mouse to human.
Multiple alignment based on cDNA sequences from human (Homo sapiens, NM_000799.4), Rhesus monkey (Macaca mulatta, NM_001042736.1), rat (Rattus norevegicus, NC_051347.1), and mouse (Mus musculus, NC_041756.1). cDNA sequences were obtained from GenBank. Alignment was generated using T_Coffee Multiple Sequence Alignment Server (tcoffee.crg.eu) and visualized in Multiple Alignment Show (www.bioinformatics.org/SMS/multi_align.html). Nucleotides highlighted in black are conserved across all species and grey is conserved in most of the species compared to humans based on the alignment of the codons.

hypoxia is regulated by the prolyl hydroxylase (PHD) and hypoxia-inducible factors (HIFs). PHD is a non-heme, iron-containing dioxygenase that requires 2-oxglutarate (2-OG), iron and oxygen (O_2) for its catalytic activity.[94] PHD has three isoforms, PHD1, PHD2 and PHD3 that show differences in tissue expression and function.[95] A knockout mouse of *Phd2* is embryonically lethal between E12.5 and E14.5 days due to cardiac and placental defects. Knockout models of *Phd1* and *Phd3* were viable and show tolerance to ischemia albeit with exercise intolerance and sympathetic nervus system (SNS) mediated reductions in resting blood pressure phenotype, respectively.[96]

HIF has three isoforms, HIF1, HIF2 and HIF3, that are composed of an α and β subunit. The HIF1-β subunit is also known as hydrocarbon receptor nuclear translocator (ARNT), which has three paralogs.[93] The different roles of each of the ARNT paralogs remains to be elucidated.[97] HIF-1β is constitutively expressed and dimerizes with one of the α-subunits, HIF-1α, HIF-2α or HIF3-α, to form HIF-1, HIF-2 or HIF-3, respectively. The HIF-α subunits show tissue specificity, which contributes to the different roles of the HIFs. HIF-1α is ubiquitously expressed and responsible for vascular and metabolic response to hypoxia.[95] HIF-2α expression is more restrictive, primarily being expressed in the brain, heart, lung, kidney, liver, pancreas and intestine.[98,99] HIF-2α plays a major role in regulating erythropoiesis and iron metabolism in response to hypoxia.[95] HIF-1α responds quickly to hypoxia exposure and is more transient. HIF-2α responds slower to hypoxia compared to HIF-1α but remains stabilized for longer hypoxic exposures.[100] The role of HIF-3α has not been well established, but has been proposed to be involved in the negative regulation of HIF-1α and HIF-2α through competition for HIF-1β binding.[101] Early literature focused on the role of HIF1 regulating *EPO*, however, it was later

determined that HIF2 was principally responsible for *EPO* induction.[102]

Under normoxic conditions, PHD hydroxylates HIF-α at two proline residues (P402 and P564 in HIF-2α).[103] The hydroxylation of HIF-α promotes the ubiquitination at one or more lysine residues (K532, K538 and/or K547) by E3-ubiquitin ligase (elongin B, elogin C, cullin 2 and ringbox 1) mediated by von Hippel-Lindeau (pVHL). The ubiquitination of the HIF-α subunit targets it for degradation by the 26S proteasome (Figure 1.4).[95] Additionally, factor inhibiting HIF (FIH), another O_2 dependant protein, hydroxylates asparagine (N851) in the C-terminus of HIF-α. Hydroxylation by FIH prevents binding of the transcriptional co-activator CREB-EP300.[104]

Under hypoxic conditions, the decrease in O_2 inactivates PHD and FIH hydroxylation of HIF-α. Without the hydroxylation, HIF-α is not ubiquitinated resulting in its stabilization. The stabilized HIF-α heterodimerizes with HIF-1β to form HIF complex and translocates to the nucleus, therein binding to the hypoxia response elements (HRE) (Figure 1.4). HREs are conserved sequences composed of the nucleotides [A/G] CGTG that are found in the promoters of many genes that respond to hypoxia, including *EPO, HMOX1, VEGF* and *GLUT1*.[105]

HREs are found in a multitude of genes that are involved in erythropoiesis (ex/ *EPO*), angiogenesis (ex/ *VEGF*) and iron metabolism (ex/ *TfR* and *HMOX-1*) that lead to increased oxygen capacity.[105,106] Iron is necessary for proper erythropoiesis, as well as many other functions, and can be obtained from dietary iron or from recycled cellular iron. In response to hypoxia, the HIFs increase heme oxygenase 1 (HMOX-1) that, in macrophages, recover heme from phagocytized erythrocytes in the spleen and liver.[106,107] The HIFs also increase transferrin (*TfR*) that transports the recycled and absorbed iron in

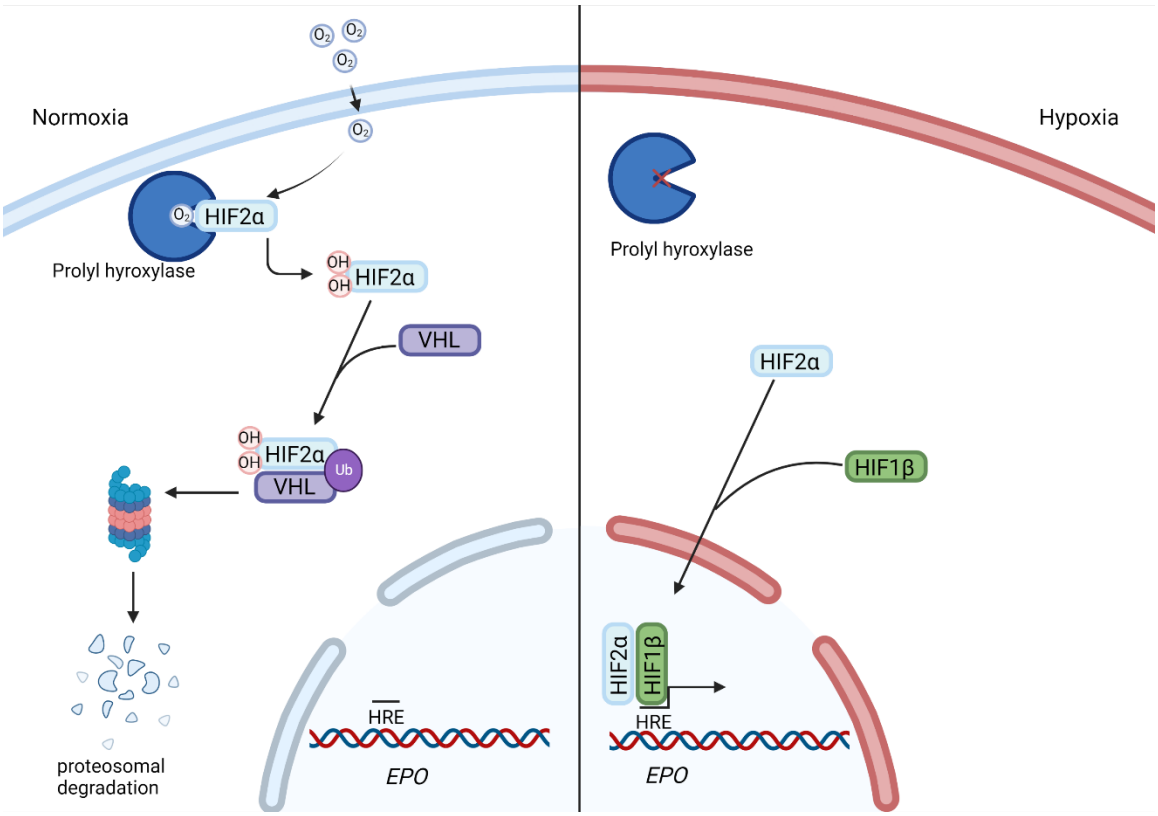

Figure 1.4- *EPO* transcription is regulated through HIF2.
Under normoxia (left, blue) the oxygen sensing enzyme prolyl hydroxylase (PHD) binds and hydroxylates Hypoxia Inducible Factor 2 α (HIF2α), allowing for von Hippel Lindau (VHL) to ubiquitinate HIF2α, resulting in the proteasomal degradation of HIF2α. Under hypoxia (right, red), oxygen is not present to bind to PHD preventing the hydroxylation of HIF2α, thus preventing the proteasomal degradation. HIF1β is then able bind to HIF2α and translocates to the nucleus, where the HIF complex binds to hypoxia response elements (HREs) resulting in the transcription of *EPO*. Created with BioRender.com

the blood to bone marrow to be used in erythrocytosis.[106,108] HIFs decrease hepcidin,[109] promoting the release of recycled iron from macrophages[110] and increases iron absorption in the small intestine. Together, this results in an increase in iron bioavailability to enable erythropoiesis.[106]

1.4.2 Erythropoietin gene regulation

The human *EPO* gene is located on chromosome 7q22.1 and consists of five exons and four introns.[111] The *EPO* gene has two enhancer sequences that contain HREs, the kidney inducible element (KIE) and liver inducible element (LIE), located upstream and downstream, respectively. The KIE is located between 9.5 and 14 kilobase (kb) 5' of *EPO* and the LIE is located within 0.7 kb 3' of the EPO gene.[112] In the 5' promoter region of *EPO* there are GATA-2 and NF-κB binding sites. GATA-2 and NF-κB act as suppressors of *EPO* and their binding is reduced during hypoxia (Figure 1.5),[113] though NF-κB can also synergize with hypoxia to drive gene expression *in vitro* and *in vivo* when hypoxia and inflammation are concomitant,[114,115] but this could vary by gene or cell type.[116] NF-κB can also be activated in the EPO signaling cascade (see Section 1.5.3). This suggests that the role of NF-κB may be context dependant or act in concert with other factors to regulate *EPO* expression. Between the HRE and inhibitory sequences, *EPO* transcription is upregulated in response to hypoxia by up to 100-fold *in vitro*[117] and approximately 1000-fold *in vivo*.[118] However, EPO levels have been implicated in circadian rhythm[119] as well as responding to nutrient deprivation without changes to oxygen.[120] Therefore, there may be additional regulatory mechanisms of *EPO* that are as yet unknown.

In addition to transcriptional regulation, *EPO* has also been revealed to possess

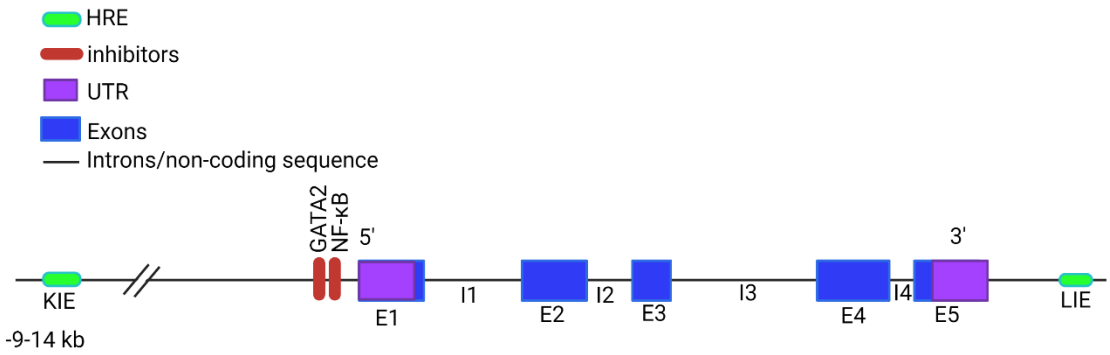

Figure 1.5- Human EPO gene and its regulatory elements.
Two HRE sequences (green) are found upstream (KIE) and downstream (LIE) that act as enhancers of *EPO*. Two inhibitory binding sequences (red, GATA and NF-κB) are located in the promoter region. EPO has 5 exons (E) with the E1 and E5 containing 5'UTR and 3'UTR, respectively. Non-coding regions are shown as black lines with the introns denoted by I. Created with BioRender.com

some post-transcriptional regulation. The peak steady state of mRNA is approximately 8 hours after induction of hypoxia in Hep3B cells (hepatocellular cells), after which mRNA levels start to decrease.[118] However, EPO protein levels continue to increase for 24 hours suggesting at least partial post-transcriptional regulation of *EPO*.[121] Erythropoietin mRNA-binding protein (ERBP) binds to a sequence located in the 3' UTR of *EPO*. ERBP aids in stabilizing the mRNA and increases its half-life.[122,123] Thus far, ERBP has only been identified in Hep3B cells and its prevalence in other cell types in unknown or working in concert that remain to be defined.

1.5 Physiology of Erythropoietin

1.5.1 Structure of Erythropoietin

Human EPO is a 30.4 kiloDalton (kDa) glycosylated hematopoietic cytokine. EPO belongs to a family of helical cytokines that include growth hormone, prolactin, interleukins 2-7, G-CSF, oncostatin M, leukemia inhibitory factor and ciliary neurotrophic factor.[124–127] The immature polypeptide is 193 amino acids, which includes the 28 amino acid signal peptide (Figure 1.6).[128] The mature EPO peptide consists of 165 amino acids with three *N*-linked glycans (Asn24, Asn38, and Asn83) and one *O*-linked glycan (Ser126)(Figure 1.6 and Figure 1.7).[129] Glycosylation of EPO accounts for approximately 40% of it molecular mass and is critical for its stability and solubility.[130]

EPO has four-antiparallel amphipathic α-helical bundles (A, B, C and D) and a short α-helix segment (B') that is critical for the binding to the EPO receptor (EPOR). A disulfide bond between Cys7 and Cys161 links helices A and D and packs them against the B and C helices (Figure 1.7).[131] This configuration results in a hydrophobic core and

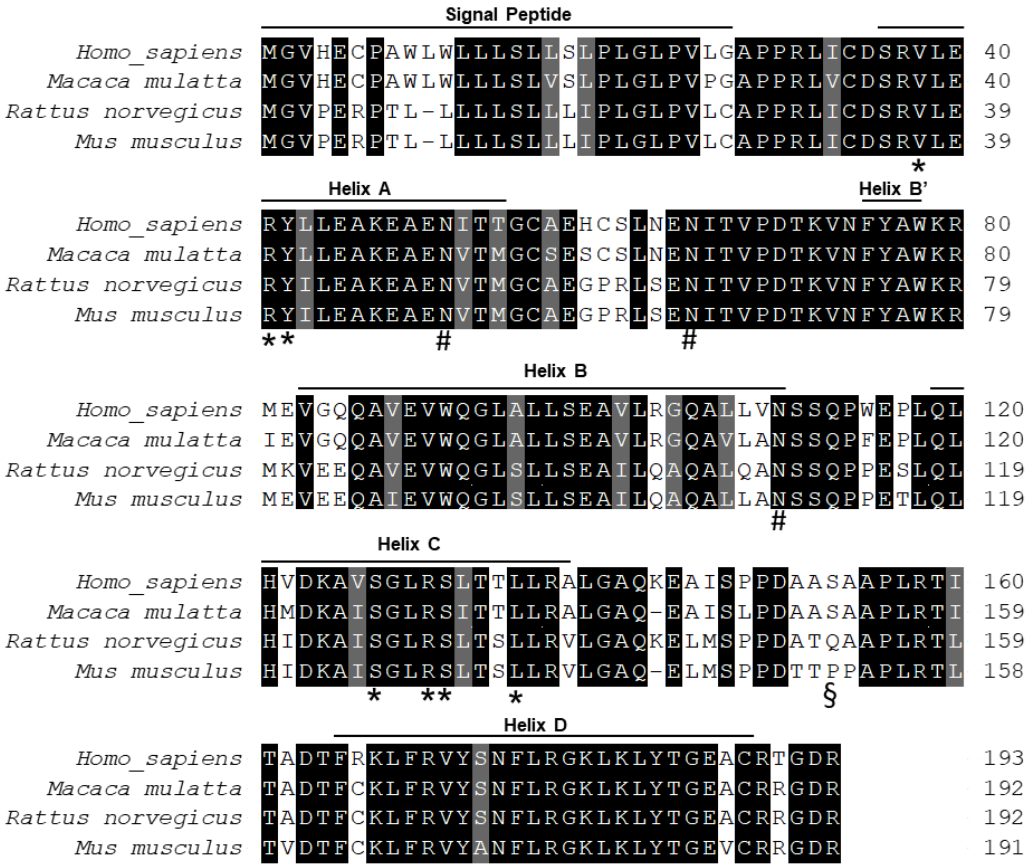

Figure 1.6- Protein sequence is conserved from mouse to human.
Multiple alignment based on predicted peptide predicted by cDNA sequences from human (Homo sapiens, NM_000799.4), Rhesus monkey (Macaca mulatta, NM_001042736.1), rat (Rattus norevegicus, NC_051347.1), and mouse (Mus musculus,NC_041756.1). Peptide sequences were obtained from GenBank. Alignment was generated using T_Coffee Multiple Sequence Alignment Server (tcoffee.crg.eu) and visualized in Multiple Alignment Show (www.bioinformatics.org/SMS/multi_align.html). Amino acids highlighted in black are conserved across all species and grey is conserved in most of the species compared to humans. The region of the protein or signal peptide are indicated by the bars above the alignment. * denotes sites involved in low affinity binding site, # indicates *N*-linked glycosylation sites and § indicates the *O*-linked glycosylation site.

Figure 1.7- Structure of EPO protein with glycosylation sites.
EPO protein consists of four α-helical bundles (A, B, C and D) and a small helix (B'). Disulfide bonds link helixes A and D and pack them against the B and C helices to make a hydrophobic core. EPO has three *N*-linked glycosylation sites (Asn24, Asn 38 and Asn83) and one *O*-linked glycosylation site (Ser126). GlcNAc=*N*-acetylglucosamine, Neu5Ac=*N*-acetylneuraminic acid (sialic acid), GalNAc=*N*-acetylgalactose. Structure generated using Protein Homology/analogy Recognition Engine (Phyre) v 2.0 and visualized using Mol*3D Viewer at protein data bank (PDB, rscb.org/3d-view). Glycan structures added using BioRender.com

is a shared confirmation to other helical cytokines.[127] Using site-directed mutagenesis Wen *et al.*[88] identified two patches of surface amino acids that affected binding to the EPOR. Residues at the helix D:AB loop interface are part of a high-affinity receptor binding site. A low-affinity binding site comprises of residue Val11, Arg14, Tyr15, Ser100, Arg103, Ser104 and Leu108. These residues are conserved amongst mammals (Figure 1.6).

1.5.2 The erythropoietin receptor (EPOR)

EPOR is a 66 kDa glycoprotein that belongs to the type-I cytokine receptor family.[132] The *EPOR* gene is composed of eight exons and seven introns. The extracellular domain is encoded by exons 1-5, the signal transmembrane domain is encoded by exon six and the intracellular domain is encoded by exons seven and eight.[133,134] The *EPOR* gene is transcriptionally regulated by GATA-1[135] and stem-cell leukemia (SCL) protein.[136] The final protein is translocated to the plasma membrane.[137,138]

EPOR has been shown to have multiple splice forms, including a splice variant in which intron five is translated into protein.[133,134] The insertion of intron five causes a frameshift with a stop codon in exon seven, thus making a truncated variant of EPOR. The truncated EPOR is capable of binding EPO.[134] However, the exact role of the truncated EPOR and if it differs from canonical; whether it is localized to plasma membrane or shed is unknown.

1.5.3 EPO signaling and the erythropoietic response

EPO binds to the dimer of EPOR and induces conformational changes to bring

constitutively associated Janus family tyrosine kinases 2 (JAK2) into close proximity to stimulate activation by transphosphorylation.[132] This results in JAK2 phosphorylating residues in the cytoplasmic domain of EPOR to induce several downstream signalling cascades including signal transducers and activators of transcription (STAT) family of proteins,[139,140] phosphatidylinositol 3-kinase (PI3K),[139] Src homology region 2 domain-containing phosphatase-1 (SHP1),[141] and/or mitogen-activated protein kinase (MAPK)[142] (Figure 1.8).

The erythropoietic effects of EPO occur by promoting the survival of erythroid progenitor cells. The EPOR is found on the surface of Burst-Forming Unit-Erythroid (BFU-E), Colony-Forming Unit-Erythroid (CFU-E), proerythroblasts and erythroblasts. Although EPOR is present on BFU-E, EPO and EPOR are not essential for the proliferation and differentiation of CFU-E. However, EPO and EPOR are important in the survival, proliferation and terminal differentiation of CFU-E to proerythroblasts and their progeny (Figure 1.9b).[143] EPO acts synergistically in the proliferation and differentiation of erythrocyte precursors with stem cell factor (SCF), GM-CSF, IL-3, IL4, IL-9 and IGF-1.[144–146] At the highest density before becoming proerythroblasts, cells contain 100 to 1000 EPOR molecules. Although EPOR density is lower than most receptors, the affinity of EPO for EPOR is high, with a dissociation constant (Kd) of approximately 100 pM.[147] As proerythroblasts differentiate into reticulocytes and further into mature erythrocytes they stop expressing EPOR and are no longer responsive to EPO.[143]

1.5.4 Other EPO receptors

The classical EPOR homodimer is not the only EPO receptor combination. EPOR

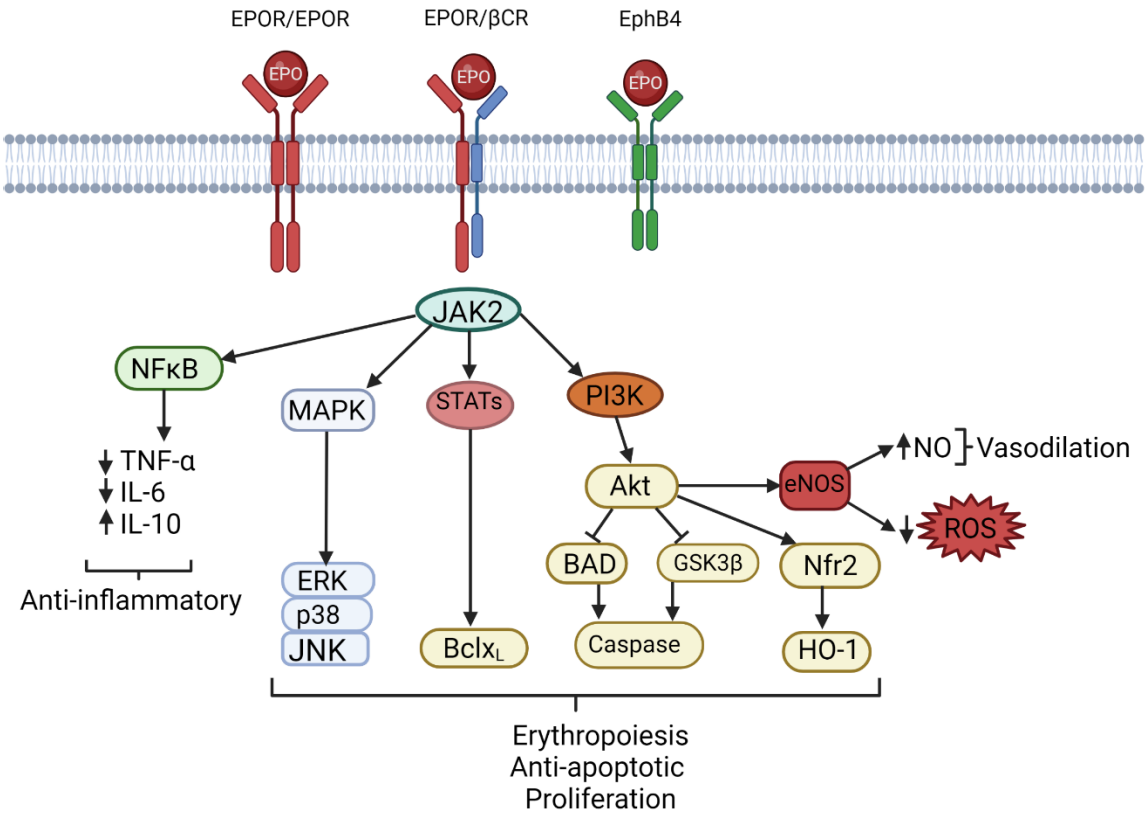

Figure 1.8- EPO initiates signaling through binding to either homodimer of the EPO receptor (EPOR) of heterodimers of EPOR and Beta common receptor (β-CR). EPO has also been shown to bind EphB4 in cancer to activate the JAK2-STAT pathway. Binding of EPO results in conformational changes that activate the JAK2-STAT survival pathway. Along with the activation of MAPK and PI3K pathway are anti-apoptotic, proliferation, and erythropoietic. Other signaling cascades include the activation of NFκB for anti-inflammatory response and eNOS to increase vasodilation and reduce reactive oxygen species (ROS). Created with BioRender.com

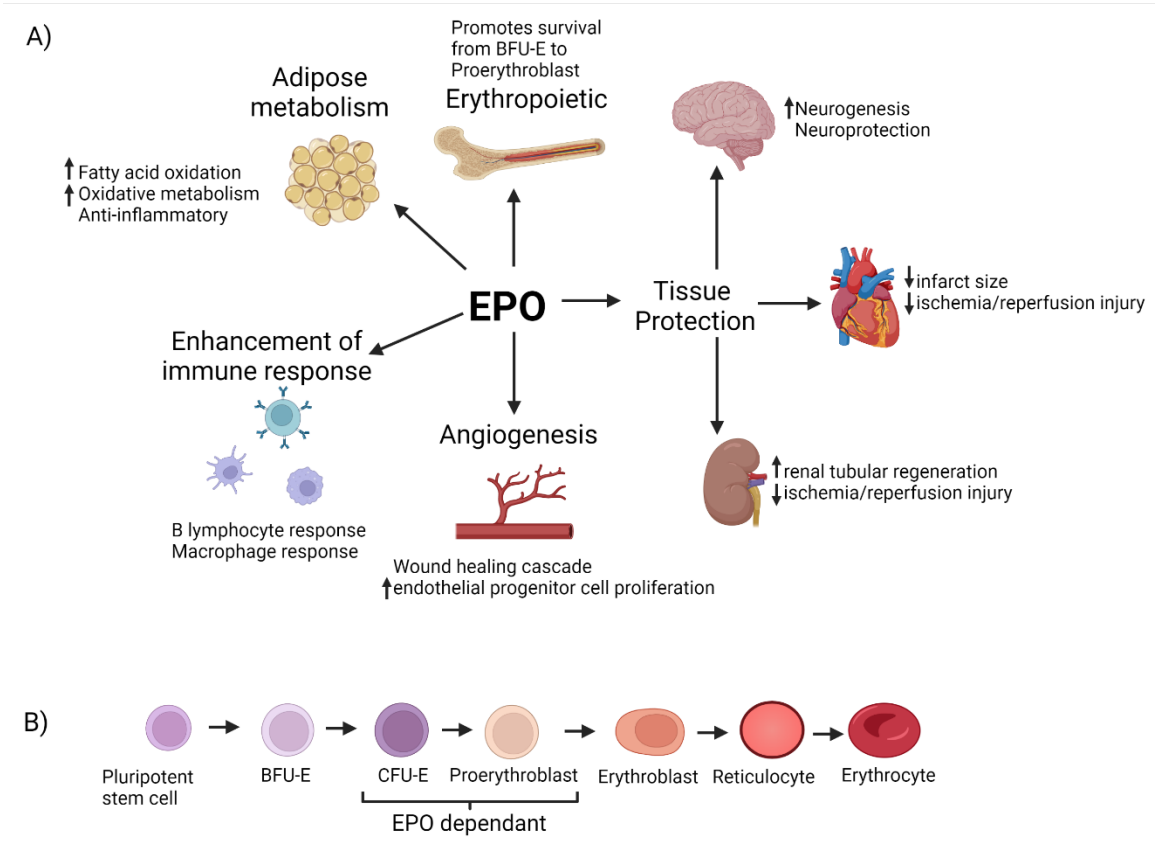

Figure 1.9- The pleiotropic effect of EPO on multiple systems in the body.
A) Some of the functions of EPO of various systems. B) The maturation of erythrocytes with the stages that are dependent on EPO to promote is survival indicated. Created with BioRender.com

has also been shown to form heterodimers with β-common receptor (β-CR).[148–152] EPO has been found to bind to the EPOR:β-CR through its B-helix.[153] The heterodimer of EPOR:β-CR is also referred to as the tissue protective receptor (TPR) due to a purported role in the cytoprotective function of EPO.[154] The presence of β-CR has been found to be important in obtaining the cytoprotective effects of recombinant EPO (rEPO). In a β-CR knockout mouse model[151] and in β-CR knockdown (KD) endothelial cell models[152,155] there was no cytoprotective phenotype observed with rEPO administration. Similar to EPOR, β-CR expression is also upregulated in damaged tissue.[156] Activation of the EPOR:β-CR has significant overlap with the pathways activated for erythropoietic functions. These pathways include the activation of JAK2 that activate STAT3 and STAT5, the PI3K pathway (leading to stabilized mitochondria and increase nitric oxide), and the mitogen-activated protein kinase (MAPK), which inhibits GSK3β to decrease inflammation.[154] The EPOR and β-CR are typically localized to intercellular compartments in quiescent cells, but rapidly translocate to the cell surface in the presence of hypoxic and proinflammatory cytokines. Compared to the EPOR:EPOR, EPOR:β-CR has a lower binding affinity for EPO.[154]

The ephrin type-B receptor B4 (EPHB4) has also been found to be a receptor for EPO in cancer cells. EPHB4 plays a role in vascular development.[157,158] A knockdown of EPHB4 in MCF-7 cells reduced viability by 77%.[159] EPO binding to EPHB4 results in the phosphorylation of Src and activation of the STAT3 pathway. EPHB4 has been associated with tumour progression, which leads to a decreased survival in cancer patients undergoing rhEPO therapy.[160] The role of EPO and EpHB4 outside of cancer has not been investigated, and therefore its relevance in physiology is still unknown.

1.5.5. Non-erythropoietic functions of EPO

EPO is a hormone with many purported functions (Figure 1.9a). EPO has been shown to be protective against ischemic injuries in the brain,[161] retina,[162] spinal cord,[163] heart,[164] skin,[165] and intestine.[166] EPO is cytoprotective in multiple tissues through both anti-apoptotic and anti-inflammatory pathways.[83] The cytoprotective functions of EPO are purported to be through the EPOR:β-CR heterodimers. Activation of the EPOR:β-CR activates many anti-apoptotic pathways, including the STAT proteins, PI3K and MAPK. In addition, EPO activates NFκB signaling to regulate survival and/or anti-inflammatory pathways and may play an important feedback regulatory role in cell survival signaling.[154,167,168]

In the brain, astrocytes and neurons are reported to produce EPO that acts on neurons in a paracrine manner.[117] EPO is important in neurodevelopment and neuroprotection from hypoxia and ischemia.[102] EPO has been shown to mimic ischemic preconditioning by protecting both neuronal and cardiac cells from ischemic injury and from cytotoxic drugs.[169] In ischemic stroke models, rhEPO was shown to decrease infarct size and apoptosis in the surrounding tissue.[170,171] EPO has also been shown to stimulate production of neuronal stem cells through the NFκB pathway.[172]

In the heart, EPO has also been shown to decrease infarct size and myocardial cell apoptosis in acute myocardial infarction (AMI) models. EPO was able to protect against ischemia reperfusion injury, improve left ventricular contractility and promoted ventricular remodeling.[173,174] In chronic heart failure (CHF), rhEPO was found to improve ventricular performance and exercise capacity, while decreasing hospitalizations.[175–177] In a trial using subcutaneous rhEPO in patients with heart failure

with preserved ejection fraction (HFpEF), a form of diastolic heart failure, and anemia (a common co-morbidity of HFpEF) showed improved exercise capacity and decreased end diastolic volume and decreased diastolic blood pressure.[178] Additionally, models of renal injury also show EPO to be renoprotective.[83]

EPO can regulate the immune response, including through B-lymphocytes,[179] dendritic cells,[180] and machrophages.[181] However, EPO does not affect neutrophils.[182] Through the EPOR:β-CR, EPO is purported to augment immune response, whilst the effects on sterile inflammation or autoimmunity or immunoregulatory effects remain to be described fully.[154] Peng *et al*.[154] provides a detailed review of the effects of EPO on the immune system.

EPO also has a role in metabolism and energy homeostasis. EPO and EPOR are expressed in adipocytes, particularly in white adipocyte tissue.[183] Mice that overexpress Epo have reduced body weight and white adipose tissue accumulation, improved glucose tolerance and reduced insulin resistance.[184] Additionally, mouse knockout of EpoR outside hematopoietic cells have increased body weight and white adipose accumulation.[183,185] EPO was also associated with regulation of mitochondrial biogenesis, cellular oxygen consumption and fatty acid metabolism in adipose tissue, including augmenting brown-beige adipose tissue formation and function.[185–187] In diet-induced obesity mouse models, Epo promoted an anti-inflammatory phenotype.[188] This shows EPO is a hormone that has important roles in many physiological processes, including immunometabolism and cytoprotection.

1.5.6 Pathophysiology of EPO

In healthy adults under normoxic conditions, plasma EPO ranges from 4-30 mU/mL (~25-200 ng/mL). In conditions that cause tissue hypoxia, such as certain forms of anemia, EPO can increase 100-fold.[189] High serum levels of EPO in patients following an AMI who undergo percutaneous coronary intervention is associated with a smaller infarction.[190,191] EPO can also be decreased in certain diseases, such as CKD and other inflammatory anemias. In CKD, a decrease in EPO is purported to cause anemia, which is also suspected in other inflammatory anemias.[192] Pro-inflammatory cytokines can interfere with EPO binding to the EPOR, thus preventing EPO signaling.[193–195] Together, this suggest that endogenous EPO production and agonism are both influenced by multiple pathophysiological states with high levels of inflammation or circulating cytokines.

Genetic variants in *EPO* have been associated with changes in EPO serum levels and diseases. In a Genome wide association study (GWAS) examining low levels of circulating EPO, an association to the promoter single nucleotide polymorphism (SNP) rs1617640 in European and African datasets was shown. The association of rs1617640 with EPO level was confirmed with an expression quantitative trait loci (eQTL) in liver from a European cohort.[196] Additionally, three SNPs in *EPO* (rs1617640, rs507392 and rs551238) have been associated with microvascular complications in diabetes mellites and anemia as well as other diseases (see Chapter 3 and Table 3.1 for more details). Given the pleiotropic functions of *EPO*, it is unsurprising that gene variations in *EPO* would have variable physiological effects. There may yet be other phenotypes that are linked to these genetic variants that have yet to be identified.

1.6 Pharmacology of Erythropoietin

1.6.1 Measurement of Erythropoietin

Since endogenous EPO and exogenous rhEPO are mixtures of isoforms that differ in bioactivities, EPO concentrations tend to be expressed in 'units' (U).[197,198] A U is defined as the same erythropoiesis stimulating response in rodents (typically fasted rats) as five micromoles (μmol) of cobaltous chloride.[199] The first standard for EPO (Standard A) was produced from sheep plasma, which became the first International Reference Preparation (IRP).[200] With the introduction of Standard B (2 IU/mg glycoprotein, implemented by National Institute for Biological Standards and Controls (NIBSC)) in 1972 from human urine 'International units' (IU) were introduced.[201] In 1992, the NIBSC on behalf of the World health organisation (WHO), developed the current standard using purified rDNA-derived human EPO (~130 000 IU/mg glycoprotein).[202] The use of IU is restricted to samples that have been determined by *in vivo* bioassays using the international EPO reference standard. All other samples should be expressed as U, and then only when referring to erythropoietic activity levels, and estimated U by molar or weight per volume protein quantity vary and can be used to estimate relative activity only.[199] The discrepancies in reporting EPO pharmacologically are a limiting feature to accurately describing or reconciling the literature in terms of pharmacokinetics and pharmacodynamics.

1.6.2 Erythropoietic stimulating agents and their use in anemia

rhEPO was first used in 1986 to treat anemia in CKD.[80] On June 1, 1989 Epoetin α, marketed as Epogen®/Procrit®, was the first erythropoiesis stimulating agent (ESA) to

be approved by the FDA to treat anemia in CKD. This approval was expanded on April 1, 1993 to include anemia from myelosuppressive chemotherapy.[81] In recent decades there have been more ESAs introduced that are based on the actions of EPO (Table 1.1). Epoetins are ESAs that are based on the endogenous EPO amino acid sequence. Epoetins can very slightly in amino acid sequence and/or in their glycosylation. Epoetins that have amino acid substitutions are denoted with prefixes.[199] For example, darbepoetin α has five amino acid substitutions (Ala30Asn, His32Thr, Pro87Val, Trp88Asn, and Pro90Thr) compared to EPO.[128] Differences in the glycans are denoted by Greek letters. For example, Epotein α has less tetra-acidic acid in its glycans compared to Epoetin β[197] (Table 1.1). Variations in glycosylation are one of the primary differences between the ESAs and can affect pharmacokinetics and/or pharmacodynamics.

The two most common cells lines used to produce rhEPO are CHO and baby hamster kidney (BHK), which produce glycans that differ from endogenous human EPO.[197,203] One primary difference is that most mammals can produce *N*-glycolylneuraminic acid (Neu5Gc), which humans do not produce.[204] Neu5Gc accounts for 1.3% of the glycan from cells produced in CHO cells.[205] Since humans do not produce Neu5Gc, it has been suggested that successive injections could produce an allergy-like symptom,[206] and the evaluation of sialic acids in rhEPO is mandatory due to the possible risk of immunogenicity, which could be of greater concern in immunocompromised, autoimmune conditions, or cancer patients receiving immunomodulatory therapies.[207,208]

The sialic acid content, particularly the tetra-sialyated tetra-antennary N-glycan, and the branching pattern of the N-linked glycans effects the pharmacodynamics, speed

Table 1.1- Erythropoeitic stimulating agents (ESAs).

ESA	Variation*	Cell source	Glycans				Half-life (h)		Clearance (mL*h-1*kg-1)
			% MW	# of *N*-linked	%tetra-acidic	# of sialic acid residues	IV	SC	
EPO	None	human	40	3	0		n/a	n/a	n/a
Epotein α	None	CHO	40	3	19	<14	4-11	19-25.3	8.1-8.6
Epotein β	None	CHO	40	3	46	<14	8.8-104	24	7.9
Epotein δ	None	HT-1080	40	3	44	<14	7-12	18-20	ND
Epotein ω	None	BHK	ND	3	21	ND	ND	ND	ND
Darboepotein α	A30N, H32T, P87V, W88N, P90T	CHO	52	5	ND	<22	18-25.3	48.8	2

ESA=erythropoietic stimulating agent, EPO=endogenous erythropoietin, CHO=Chinese hamster ovary, HT-1080=human fibrosarcoma, BHK=baby hamster kidney, % MW=percentage of molecular weight of EPO molecule, IV=intravenous, SC=sub-cutaneous, mL=milliliter, h=hour, kg=kilogram, ND=not determined

*Refers to amino acid substitution from endogenous EPO

of catabolism and biological activity.[197,209–212] The oligosaccharide structure of recombinant proteins, such as rhEPO, is dependent on the cell type, culture methods and expression methods.[197,198] The three most commonly used epoetins worldwide are α, β and ω. The degree of tetra-sialyated carbohydrates is one of the main differences between the epoetins. Epoetin β has a larger molecular weight, a wider spectrum of isoforms, higher proportion of more basic isoforms, a lower number of sialylated glycans but a higher proportion of tetra-sialylated glycans compared to Epoetin α.[198] In a study of 18 healthy young men, Epoetin β has a longer terminal elimination half-life, significantly delayed absorption after subcutaneous administration and more pronounced reticulocytosis compared to Epoetin α.[213] However, in a study of patients with CKD on haemodialysis or peritoneal dialysis there was no difference in biological half-life between Epoetin α and Epoetin β.[214] Current guidelines on anemia correction do not differentiate on the use of Epoetin α and β and may vary primarily on location, with Epoetin α being more common in North America and Epoetin β being more common in Europe.[215]

Whereas Epoetin α and β are produced using CHO cells, Epoetin ω is produced in BHK cells[216] resulting in only 60% of Epoetin ω being *O*-glycosylated at Ser126[217] and the *N*-glycosylation sites containing phosphorylated oligomannosidic side chains.[218] Epoetin ω is newer but has also been shown to be useful in correcting anemia, with one study suggesting a reduction in dose of approximately 20% compared to Epoetin α in the heamodialysis population.[215,219] However, more studies are needed to determine clinical difference between Epoetin ω from Epoetin α and β.

Epoetin δ is generated by gene activation in human fibrosarcoma cell line (HT-

1080),[215] which results in a glycosylation pattern similar to endogenous EPO.[208] However, most clinical trials found the number of adverse events for Epoetin δ to be similar compared to Epoetin α and β.[220] Epoetin δ was removed from the market in 2009, likely due to inadequate sales, or lack of clinical distinction in subclinical populations.[221]

In addition to the type of glycan, the degree of glycosylation is relevant. Darbepoetin α has five amino acid substitutions that result in an additional two *N*-glycosylation sites (for a total of five) that increase the tetra-sialylated glycans.[222] The increased glycosylation results in a longer half-life compared to epoetins (25.3 hours vs. 8.5 hours of Epoetin α).[223] The longer half-life results in less frequent dosing (once a week compared to three times a week of epoetins).[215] The epoetins are measured in IU, whereas darbepoetin α is measure in μg of protein, with a dose convergence of 200 IU per 1 μg.[215] However, at higher doses the dose conversion ratio of epoetin α to darbepoetin α increases,[224,225] suggesting darboepoetin α may be more potent than epoetins on a peptide mass basis.[215]

Many of the adverse effects of ESAs occur in all the epoetins and darboepoetin α. The most common adverse effects include fever, hypertension (particularly with high doses), splenomegaly, thrombosis, seizures, allergic reactions, and hypercalcemia.[226–228] A rare but serious adverse effect is red blood cell aplasia caused by production of antibodies against erythropoietin. These antibodies are thought to be produced in response to the differences in carbohydrate moieties,[226] which is what led to an investigation for more biologically similar ESAs and different types of ESAs.

There have been efforts to improve the efficacy of ESAs as well as decrease adverse effects. Some strategies have included additional PTMs to rhEPO, such as

pegylation (addition of polyethylene glycol to a glycosylated rhEPO (ex/ pegzyrepoetin α)). Other approaches have attempted to find agonists of the EPOR, such as EPO mimetic peptides (EMP)[229] or continuous erythropoiesis receptor activator (CERA).[215] Another strategy has been to target further upstream and inhibit PHD. Hypoxia inducible factor prolyl hydroxylase inhibitors (HIF-PHIs) inhibit PHD to stabilize HIFs and results in an increase in EPO. In addition to the increase in EPO, HIF-PHI increases many downstream signaling mechanisms of the HIFs including those that increase iron bioavailability.[94,95,230,231] Since iron bioavailability is generally decreased in anemia and ESAs do not increase iron bioavailability, many patients with anemia are treated with both ESAs and iron.[232]

ESAs have improved the treatment of anemia, including anemia in cancer. Although ESAs have been shown to increase hemoglobin (Hb) in cancer populations, there is risk for elevated overall mortality.[233] The erythropoietic effect of EPO increases Hb, but rhEPOs are also suspected to be cytoprotective, perhaps even immune permissive, to cancerous cells, resulting in tumour growth.[157] Much of the development of ESAs has focused on the erythropoietic effects of EPO, but all functions of EPO require consideration when treating patients with ESAs.

1.6.3 ESAs for treatment of other pathophysiologies

The pleiotropic functions of EPO have led to the investigation of ESAs to treat various pathologies outside of anemia. Due to the cytoprotective properties of EPO, ESAs have been investigated to protect tissue after an AMI or ischemic stroke. In mouse,[234] rat,[164,235–237] rabbit,[238] dog,[239] and pig[240] models, rhEPO demonstrated some level of cardioprotection. However, the results in human trials were not consistently

positive. There are some studies that show cardioprotection, while many do not.[191] Additionally, the dose required to elicit cytoprotective effects is higher than that which is erythropoietic.[241,242] These higher doses carry more risk of adverse effects, such as thrombotic effects. One clinical trial attempted to use a lower rhEPO dose in ST-segment elevation myocardial infarction (EPO-AMI-II trial), which did decrease the adverse effects but failed to elicit cytoprotective effects in ischemic stroke.[243] The prevalence of thrombotic events have led to investigations related to non-erythropoietic rhEPO that may avoid adverse effects.

Carbamylated EPO (CPEO) is generated using carbamylation to transform EPO's lysines to homcitrullines.[150] Asilo-EPO is a desialyated form of EPO, which results in a half-life of only 1.4 minutes.[244] CPEO and asilo-EPO have been demonstrated to be cytoprotective but not erythropoeitc.[153,244–246] These EPO derivatives have been used in animal models, but they have not been translated to humans. Their clinical use is limited by their high cost, structural instability and potential for immunogenic antibody formation.[247] The translation of preclinical models of rhEPOs into clinical effectiveness could be improved by comprehensive characterisation of the various EPO function in different rhEPOs.

1.7 Objectives

1.7.1 Role of EPO SNPs in phenotype of a cardiovascular patient cohort

There are three common SNPs in *EPO*, rs1617640, rs507392, rs551238, that have been associated with a variety of outcomes including anemia and microvascular complications in diabetes (See Table 3.1). HFpEF has been associated with coronary

microvascular dysfunction.[248] EPO adipocyte knockout mice have been shown to have increased insulin resistance, such as seen in type 2 diabetes mellites (T2DM).[183,185] Treatment with rhEPO can potentially modulate lipoproteins (decreased triglycerides and cholesterol)[249] and increase the risk of hypertension.[250] Based on the potential role of physiological EPO in anemia, dyslipidemia, T2DM, HFpEF, and hypertension. We will investigate the potential association of the SNPs rs1617640, rs507392 and rs551238 genotype in patients at risk of anemia, dyslipidemia, hypertension, HFpEF, T2DM.

1.7.2 Role of variants in the mRNA on EPO regulation

EPO is transcriptionally regulated and does not have intracellular protein stores.[251] Intron retention has been identified as a mechanism to store mRNA in neurons.[54] We hypothesize that EPO utilizes intron retention as a form of post-transcriptional regulation in response to hypoxia and ischemia. We will investigate the role of intron retention in multiple cell lines from different tissues to examine intron retention in response to hypoxia or ischemia.

1.7.3 Variations in rhEPOs functional properties

rhEPOs have been used in the past decades to treat anemia and investigated for their ability to protect against ischemic injury.[191,242] The glycosylation of rhEPOs has been associated with their stability and function.[197,209–212] Given the different glycosylation patterns of rhEPOs, we hypothesize there may be some variation in their known cellular functions. We will utilize different rhEPOs to examine the erythropoietic potential in human hematopoietic stem cells and explore variation to cytoprotective potential or metabolic effects using a rat myoblast cell stress model.

Chapter 2- Material and Methods

2.1 Materials

Material used in this study are listed in Table 2.1.

Table 2.1- Reagents

Reagent name	Supplier
Cell Culture reagents	
0.25% Trypsin	Corning
Actinomycin D	Invitrogen
Aranesp ® (darbepoetin alfa)	AMGEN
Astrocyte Medium	Gibco
Collagen Type 1, rat tail	Sigma
Dexamethasone	Sigma
Doxetaxel	Sanofi-Aventis
Dulbecco's Modified Eagle Medium (DMEM)- High-glucose	Gibco
Dulbecco's phosphate-buffered saline (DPBS)	Corning
EndoGRO MV complete culture media	Sigma
EPREX ® (epoetin alfa)	Janssen
Fetal Bovine Serum (FBS)	Gibco
Forskolin, 98%	ThermoScientific
Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix	Gibco
Ham's F-12 Nutrient Mix	Gibco
Hemin	Sigma-Aldrich
Human EGF Recombinant Protein	Gibco
Hydrogen peroxide	Sigma
Minimum Essential Medium (MEM)	Gibco
PrestoBlue™ Cell Viability Reagent	Invitrogen
Recombinant Human EPO (rhEPO)	Peprotech
RPMI-1640 + 4.5% D-glucose	Gibco
Staurosporine	Sigma
StemPro™ Accutase™ Cell Dissociation Reagent	Gibco
Trypan Blue	Ameresco
Molecular Reagents	
Agarose	Anresco
Ambion TRIzol Reagent	Life Technologies
AmpliTaq Gold™ 360 Master Mix	Applied Biosystems
Applied Biosystems™ High Capacity Reverse Transcription Kit	ThermoScientific
BigDye XTerminator™ Purification Kit	Applied Biosystems

Table 2.1 cont.- Reagents

Reagent name	Supplier
BigDye™ Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
Chloroform	Fisher
DNA 7500 Kit	Agilent
DNAzol® reagent	GibcoBRL
dNTP set 100 mM Solutions	ThermoScientific
ExoSAP-IT™ PCR product cleanup reagent	Applied Biosystems
GeneRuler 100 bp Plus DNA Ladder, ready-to-use	Thermo
Isopropanol	VWR
Oligo$(dT)_{20}$ primer	ThermoScientific
OneTaq® Hot Start DNA polymerase	New England Biolabs
PerfeCTa SYBR Green SuperMix Low Rox	Quantabio
Platinum™ SYBR™ Green qPCR SuperMix-UDG	Invitrogen
QIAamp® DNA Blood Mini Kit	Qiagen
Qiagen RNeasy Mini Kit	Qiagen
QIAquick® Gel extraction kit	Qiagen
QIAzol Lysis Reagent	Qiagen
qScript cDNA Supermix	Quantabio
RNA 6000 Nano Kit	Agilent
RNase Free DNase Set	Qiagen
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Thermo
SuperScript® IV Reverse Transcriptase	Thermo
SYBR™ Safe DNA stain	Invitrogen
Protein Reagents	
Ferritin Human ELISA Kit	Invitrogen
Human EPO ELISA Kit	Invitrogen
Human Hepcidin Quantikine ELISA Kit	R&D systems
Methanol	VWR
NP-40	IGEPAL
Phosphatase Inhibitor	Calbiochem
Protease Inhibitor	Sigma
Sodium Fluoride	Sigma
Sodium Orthovandate	Calbiochem
Tetrasodium pyrophosphate	Sigma
Transferrin Human ELISA Kit	Invitrogen
Tris /Glycine/SDS buffer	Biorad
Tris/Glycine buffer	Biorad
Chemicals	
Acetic Acid	VWR
EDTA	Calbiochem
Tris	Anresco

2.2 Cell Culture

Cell lines and their growth media are listed in Table 2.2. Cells were grown according to supplier instructions in the recommended growth media (Table 2.2) at 37ºC and 5% carbon dioxide (CO_2) and passaged between 70%-80% confluency in T75-flask or 10 cm plates. For hCMEC/D3 and astrocytes flasks or plates were coated with collagen or Geltrex™, respectively, for one hour prior to use. Cells were rinsed with 1X Phosphate buffered saline (PBS) then detached from growth surface using 0.25% Trypsin (STEMPro Accutase™ was used for Astrocytes). Once cells detached, then media was added to the plate and cells were collected to a centrifuge tube and centrifuged at 500 x rcf for 5 minutes at room temperature (300 x rcf for Astrocytes). The supernatant was discarded and the cell pellet was resuspended in media and transferred to new plates or flask. Cells underwent at least one passage after cryogenic storage prior to experimentation.

Table 2.2- Cell lines and growth media

Cell line	Cell type	Species	Supplier	Media composition	Sex
HEK293	Kidney	Human	ATCC	DMEM HG+10 % FBS	Female
HEPG2	Liver	Human	ATCC	RPMI-40 +10% FBS	Male
HTB16	Neuroblastoma	Human	ATCC	MEM +10% FBS	Male
CHP212	Glioblastoma	Human	ATCC	1:1 MEM:F-12 +10% FBS	Male
hCMEC/D3	Blood Brain Barrier	Human	Millipore Sigma	EndoGRO complete culture media	Female
Astro	Astrocytes	Human	Gibco	Gibco™ Astrocyte Media	ND
H9c2	Cardiac Myoblast	Rat	ATCC	DMEM HG+ 10% FBS	Male

DMEM HG=Dulbecco's modified Eagles Media with high glucose, MEM=Minimum essential medium, RPMI=Roswell Park Memorial Institute, FBS= Fetal bovine serum, ATCC=American Type Culture Collection, ND=not determined

2.2.1 Hypoxia/Ischemia challenge *in vitro*

Cells were plated in 60 mm plates (with the exception of HTB-16, which were

grown in 10 cm plates) and grown in regular growth media to confluency in 37°C and 5% CO_2. Media was changed to either normal growth media and maintained in 37°C and 5% CO_2 (controls), normal growth media and placed in 37°C and 1% oxygen (hypoxia) or DMEM with nor glucose or FBS and grown at 37°C and 1% oxygen (ischemia). Cells were incubated for either 2, 4, 8, 16 or 24 hours. Cells were harvested for either RNA or protein, as needed alternating between a plate for RNA and a plate for protein. For the hypoxia and ischemia plates extractions had to be completed within 1 minute from removal from incubator with no more than 12 minutes passing from the initial opening of the incubator to ensure minimized reoxygenation from hypoxia molecular stability. Plates were kept cold on ice immediately after removal from hypoxia/ischemia to reduce enzymatic breakdown due to transient reoxygenation during extraction.

2.2.2 Modeling circadian rhythm *in vitro*

HTB16 cells were plated on 60 mm plates and grown to confluency plus two days in regular growth media. Two hours prior to time point 0 hours, the media was changed to media with no FBS, 100nM Dexamethasone, and 20 μM Forskolin.[252–254] Vehicle control had 0.01% ethanol added to the media with no FBS. Three plates were harvested for RNA at this media change as pre-treatment controls. After two hours (time point 0 hours) Dexamethasone and Forskolin media was changed to media with no FBS. Plates were harvested for RNA (Section 2.2.1) every four hours for the next 48 hours. Circadian rhythm was verified using real time PCR (rtPCR) using primers for *CLOCK, CRY2, NR1D1* and *PER2* (Table 2.3, Section 2.6.3).

2.2.3 Inhibition of transcription *in vitro*

Actinomycin D is an chemotherapeutic antibiotic that inhibits DNA-dependent

RNA polymerase resulting in transcription inhibition.[255] HTB16 cells were plated on 10 cm plates and grown to 70-80% confluency. Cells were rinsed with 1X PBS and media containing 5µg/mL of Actinomycin D (MEM with 10% FBS for controls and DMEM with no glucose or serum for ischemia) applied to the cells. Cells were incubated for 1 hour to ensure Actinomycin D had blocked transcription[256–258] before the ischemic cells were placed in hypoxia (1% O_2). Control cells were maintained at 5% CO_2. Cells were harvested for RNA as described in Section 2.2.1.

2.2.4 Cell survival assay

Cell viability was determined using resazurin reagent (PrestoBlue™). Cells were mixed to a density of 50,000 cells/mL (5000 cells/well), unless otherwise stated, and 100µL were plated per well on 96 well plates. Cells were left for 24 hours at 37ºC in 5% CO_2 to reattach to the surface. Media was replaced with media containing either a form of recombinant EPO (rEPO) (for proliferation analysis and survival assays) or xenotoxic agent (for IC_{50} analysis) and incubated for 24 hours at 37ºC in 5% CO_2. Proliferation and IC_{50} were measured after the 24-hour incubation. For survival assays, the xenotoxic agent was added to the media and incubated for an additional 24 hours at 37ºC in 5% CO_2. To measure cell viability 10 µL of Presto Blue was added to each well and incubated at 37ºC for 1 hour. Spectral absorbance was measured on a Synergy™ H4 Hybrid Reader (BioTek) at excitation wavelength of 560 nm and emission wavelengths of 590 nm. Absorbance values were normalized, less cell-free media background subtraction to the control to determine cell survival or proliferation. GraphPad Prism v9 for windows (GraphPad Software, San Diego, California USA, www.graphpad.com) was used for graphing and analysis. For proliferation analysis the log(agonist) vs. response-

variable slope (four parameters) was used to generate curves. For IC_{50} analysis log(inhibitor) vs. normalized response- variable slop was used. For the cytoprotective assays the survival was normalized to cells treated with the same does of rEPO but no cytotoxic agent. Two-way ANOVA was used to determine significant differences with $p<0.05$ being deemed significant.

2.3 Nucleic Acid Extractions

2.3.1 Genomic DNA extraction

Genomic DNA (gDNA) was isolated from buffy coat of blood samples collected as part of the impact of Obesity on Postoperative Outcomes following cardiac Surgery (OPOS) study. The OPOS cohort consists of participants aged 18-75 years old that underwent elective cardiac surgery at either New Brunswick Heart Center in Saint John, New Brunswick or Maritime Heart Centre in Halifax, Nova Scotia.[259] Buffy coat was removed from the blood sample and gDNA was isolated using either DNAzol® reagent (for tissues) or QIAamp® DNA blood mini (for whole blood buffy coat) kit following the manufacturer's protocol.

2.3.2 RNA Extraction

2.3.2.1 RNA extraction from tissue

Post-mortem brain tissue was obtained from the Maritime Brain Tissue Bank (MBTB). Post-mortem kidney and heart tissue were obtained from the National Institute of Health (NIH) NeuroBioBank. Research ethics approval for human tissue was provided by Horizon Health Network research ethic board (file # 100071). Trizol (1 mL) was added to approximately 50 mg of tissue in a MP analyzer tube with homogenizer beads.

Tubes were placed on ice for 10 minutes. Tissues were homogenized using the appropriate program on the MP TissueLyser for the tissue type. Samples were centrifuged at 12,000 x rcf for 10 minutes at 4ºC followed by the supernatant being transferred to a clean 1.5 mL tube. Samples were then incubated for 5 minutes at room temperature before adding 200 µL of chloroform and vortexed for 10-15 seconds and incubated for another 15 minutes at room temperature. Samples were then centrifuged at 12,000 x rcf for 15 minutes at 4ºC and the clear upper layer was transferred to a clean 1.5 mL tube. 500 µL of isopropanol was added and tubes were inverted 5-6 times. The samples were added to Qiagen RNAeasy columns followed by manufacturer's instructions, including DNAse treatment.

2.3.2.2 RNA extraction from cells

RNA was isolated from cell lines using the Qiagen RNAeasy kit. Cells were grown to confluency. Media was aspirated and cells rinsed with 1X PBS; 600 µL RLT buffer containing β-mercaptoethanol (10 µL/mL) was added to the cells and homogenized by pipetting and collected into a 1.5 mL tube. Then 600 µL of 70 % ethanol was added and inverted to mix and added to RNAeasy columns in 700 µL aliquots and centrifuged at 12,000 x rcf for 30 seconds and flow through discarded; 350 µL of RW1 buffer was added to the column and centrifuged at 12,000 x rcf for 30 seconds and flow through discarded. Qiagen DNase was added to RDD buffer (10 µL of DNAase to 70 µL RDD buffer); 80 µL of the DNAase mixture was added directly to the column membrane and incubated at room temperature for 15 minutes. Then 350 µL of RW1 buffer was added to the column and centrifuged at 12,000 x rcf for 1 minute and flow through discarded and column transferred to a new collection tube; 500 µL of RPE

buffer was added to the column and centrifuged at 12,000 x rcf for 1 minute and flow through discarded twice. Columns were centrifuged again at 12, 000 x rcf for 1 minute to dry the column. The column was transferred to a clean collection tube; 30 µL of nuclease-free water was added directly to the membrane and incubated for 1 minute then centrifuged at 12,000 x rcf for 1 minute to obtain the RNA.

2.4 Nucleic acid quantification

2.4.1 Bioanalyzer

RNA was quantified using capillary electrophoresis using the Agilent Bioanalyzer following manufacturer's instructions. Briefly, RNA was denatured at 70°C for 2 minutes prior to loading 1 µL of sample onto an Agilent RNA 6000 Nano chip. The chips were vortexed at 2400 rpm for 1 minute on the IKA- Model MS3 vortex mixer prior to loading on the Bioanalyzer 2100 system (Agilent).

2.4.2 Plate reader

Nucleic acid, primarily DNA, was quantified by spectroscopy on a BioTek Synergy ™ H4 Hybrid Microplate reader: 2µL of sample was added to the Take3 microvolume plate in duplicate; the quality and quantity of the nucleic acid was determined by the 260:280 nm ratio by the Gen5 software (BioTek) and the average concentration of the duplicates was used.

2.5 cDNA synthesis

RNA (2 µg) was converted to complementary DNA (cDNA) using either QuantBio qScript™ cDNA Supermix or Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit by manufacturer's recommendations. For Poly-A enriched

cDNA, 1 µg of cDNA was generated using SuperScript® IV Reverse Transcriptase and Oligo(dT)$_{20}$ primers by manufacturer's recommendations. A no reverse transcriptase (No-RT) control was made for each RNA extraction. cDNA synthesis was carried out on a Mastercyler Nexus Gradient Thermocycler (Eppendorf).

2.6 Polymerase Chain Reaction

2.6.1 Primer design

Primers were designed using Oligo 7 software.[260] Primers designed for gDNA sequencing included M13 sequences[261] to facilitate Sanger sequencing. For detection of intron retention, primers for *EPO* were designed to either include the intron, if present (IR-E primers), or to exclude the intron (IE primers) by having one of the primers spanning the SS. EPO2-3 primers spanned exons 2 and 3 and EPO4-5 primers spanned exon 4 and 5 to examine the two smallest introns in *EPO,* since smaller introns are more likely to be retained[43] (for primer design see Figure 2.1). Primer sequences are listed in Table 2.3.

2.6.2 End point PCR

2.6.2.1 End Point PCR for gDNA

End-point polymerase chain reaction (PCR) for the purposes of Sanger sequencing was performed on gDNA from OPOS participants using AmpliTaq Gold™ 360 Master Mix. PCR reactions consisted of 1 X AmpliTaq Gold™ 360 Master Mix, 0.3 µM of forward and reverse primers, 4 µg of Bovine Serum Albumin (BSA), 50 ng of gDNA and nuclease-free water up to 15 µL. Reactions were amplified on a Mastercyler

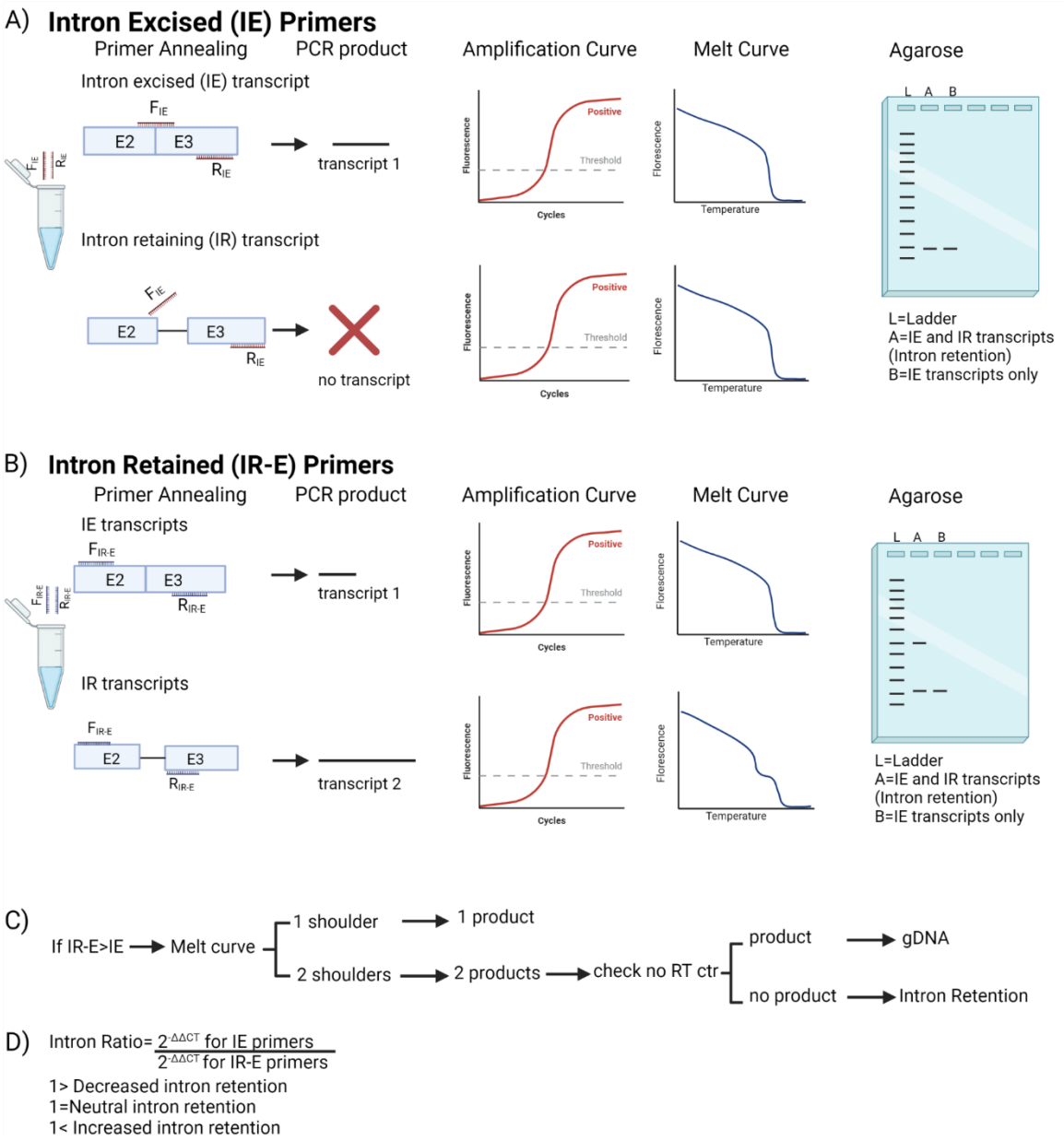

Figure 2.1- Primer design for detection of changes in intron retention using rtPCR. Two sets of primers, intron excised (IE) (A) and intron retained (IR-E) (B), are used in two separate reactions. A) The IE primers have one primer that spans the splice junction. When the intron has been excised the primers can anneal and produce a single product (no intron retention). When the intron is retained, the primer spanning the splice junction is unable to anneal and results in no product (intron retention). As intron retention only affects a small subset of the transcripts and the excised transcripts still make most transcripts the amplification curve will look the same if intron retention is present or not. In the IE primers, the melt curve and agarose gel will also look the same as they are only able to produce a product from the non-intron retaining transcripts. B) In the IR-E primers reaction, the primers are designed to anneal within the exons, which results in a

product in non-intron retaining transcripts and a larger (added intron) product in the intron retaining transcripts. When no intron retention is present the melt curve will show one peak and only one band on the agarose. When intron retention is present both the non-intron-retaining and intron-retaining transcripts are amplified. As we are using a single dye in rtPCR the amplification curve will look the same in both scenarios but there will be a slight change in CT value. However, the melt curve will have two products (non-intron retraining and intron retaining) and there will be two products on the agarose gel. C) The intron-retaining transcripts can also be confused for gDNA and therefore a no reverse transcriptase control must be used to ensure intron retention is not due to gDNA contamination. D) Using the $2^{-\Delta\Delta CT}$ of both primer sets we can measure the intron retention ratio to determine if intron retention is changing.

Nexus Gradient Thermocycler (Eppendorf) using the cycling conditions listed in Table 2.4.

PCR products were verified using 7500 Agilent DNA Chip on the Bioanalyzer 2100 system. PCR products were cleaned up using 2 µL of ExoSAP-IT™ in 5 µL of PCR product and incubated at 37ºC for 15 minutes. ExoSAP-IT™ was inactivated at 80ºC for 15 minutes. Cleaned PCR products were used for Sanger Sequencing (Section 2.8).

2.6.2.2 End point PCR for mRNA transcripts

End point PCR using cDNA was performed using *OneTaq*® Hot Start DNA polymerase. PCR mix contained 1 X *OneTaq*® standard reaction buffer, 200 µM each dNTP, 200 nM forward and reverse primer, 0.75 units *OneTaq*® Hot Start Polymerase and 200 ng of cDNA. Reactions were cycled on a Mastercycler Nexus Gradient Thermocycler (Eppendorf) using the following cycling conditions: initial denaturation at 94ºC for 30 seconds, 35 cycles of 94ºC for 15 seconds, 60ºC for 30 seconds and 68ºC for 45 seconds followed by a final extension at 68ºC for 5 minutes. PCR products were

Table 2.3- Primers

Target	Primer Name	Sequence 5'→3'
EPO rs507392	hEPO_1045F_M13	GTAAAACGACGGCCAGTGTGGCCC CAAACCATACCTG
	hEPO_1981R_M13	CAGGAAACAGCTATGACACTGACG GCTTTATCCACA
EPO rs551238	hEPO_2631F_M13	GTAAAACGACGGCCAGTCACCCTG CAAAATTTGATGCC
	hEPOdown677R_M13	CAGGAAACAGCTATGACAGTGACC CATGATTGCACC
EPO rs1617640	h_EPOpro1316F_M13	GTAAAACGACGGCCAGTAGTGCTG GGATTATAGGTGTCA
	h_EPOpro404_M13	CAGGAAACAGCTATGACAGAGATG CCTGGGTTGCTG
M13 sequencing	M13_F	GTAAAACGACGGCCAGT
	M13_R	CAGGAAACAGCTATGAC
EPO2-3_ IR-E	EPO2-3_IR_E-F	TCATCTGTGACAGCCGAGTCC
	EPO2-3_IR_E-R	ATTCTCATTCAAGCTGCAGTGTTCA
EPO2-3_IE	EPO2-3_IE-F	AATATCACGACGGGCTGTGCT
	EPO2-3_IE-R	ACCTCCATCCTCTTCCAGGCATA
EPO4-5_IR-E	EPO4-5_IR-E-F	CACCACTCTGCTTCGGGCTCT
	EPO4-5_IR-E-F	AGAGTTTGCGGAAAGTGTCAG
EPO4-5_IE	EPO4-5_IE-F	AGCCCAGAAGGAAGCCATCTCCC
	EPO4-5_IE-R	GGCCTCCCCTGTGTACAGCTTCA
CLOCK	h_CLOCK_F	AGTGGATTTGGCTTCAGACT[87]
	h_CLOCK_R	TTCAATGCCAAGTTCTCGTC[87]
PER2	h_PER2v2_F	GTATCCATTCATGCTGGGCT[87]
	h_PER2v2_R	TCGTTTGAACTGCGGTGAC[87]
CRY2	h_CRY2_F	TGCAGGTTGTACTCTGCTGC[87]
	h_CRY2_R	TGAAGAACTCAGCAAACGGG[87]
NR1D1	h_NR1D1F	CATGCAAAGCAGAACTCCG
	h_NR1D1R	GACTCACGATCAGGATCCGAA
*yWHAZ	yWHAZ-F	ACTTTTGGTACATTGTGGCTTCAA[262]
	yWHAZ-R	CCGCCAGGACAAACCAGTAT[262]
*B-actin	B-actin-F	ATGAAGATCAAGATCATTGCTCCTC [262]
	B-actin-R	ACATCTGCTGGAAGGTGGACA[262]
*HRPT1	HRPT1-F	TGACACTGGCAAAACAATGCA[262]
	HRPT1-R	GGTCCTTTTCACCAGCAAGCT[262]
*18S	18S-F	AGAAACGGCTACCACATCCA[262]
	18S-R	CACCAGACTTGCCCTCCA[262]

* Reference genes for rtPCR

Table 2.4- Cycling conditions for gDNA end point PCR.

Step	h_EPOpro1316F_M13, h_EPOpro404R_M13	hEPO_1045F_M13, hEPO_1981R_M13	hEPO_2631F_M13, hEPOdown677R_M13
Initial Denaturation	10 min @ 95ºC	10 min @ 95ºC	10 min @ 95ºC
Denaturation	30 sec @ 95ºC	30 sec @ 95ºC	30 sec @ 95ºC
Annealing	30 sec @ 58ºC	30 sec @ 58ºC	30 sec @ 58ºC
Extension	45 sec @ 72ºC	60 sec @ 72ºC	90 sec @ 72ºC
Cycles	35	35	35
Final Extension	10 min @ 72ºC	10 min @ 72ºC	10 min @ 72ºC

Min=minutes, sec=seconds @=at

analyzed using agarose gel electrophoresis (Section 2.7).

2.6.3 Real Time PCR

Real time PCR (rtPCR) was performed using either PerfeCTa® SYBR® Green SuperMix Low ROX or Platinum™ SYBR™ Green qPCR SuperMix-UDG and run on either an Applied biosystems ViiA7 or Applied biosystems QuantStudio™ 7 Flex system. rtPCR analysis was performed using qBase+ (Biogazelle, Zwijnaarde, Belgium - www.qbaseplus.com) using two house-keeping genes that were validated for each cell line using GeNorm analysis. Two-way ANOVA test were performed in in GraphPad Prism. A p-value of $p<0.05$ was deemed significant.

2.6.3.1 Calculating the intron retention ratio

The intron retention ratio was used to calculate changes in the level of intron retention using rtPCR. The $2^{-\Delta\Delta CT}$ was calculated for both the IR-E and IE primers and used in equation 1:

$$\text{Intron retention ratio} = \frac{2^{-\Delta\Delta CT}\ \text{IE primer}}{2^{-\Delta\Delta CT}\ \text{IR-E primer}} \qquad \text{Equation 1}$$

As the level of intron retention increases (IR-E increases compared to IE) the

intron retention ratio decreases (Figure 2.1). Equation 1 enabled the determination of changes in the degree of intron retention by rtPCR. A two-way ANOVA was used to determine significant changes with $p<0.05$ being deemed significant.

2.7 Agarose Gel Electrophoresis

Between 1 and 2 % (w/v) agarose was added to 1 X Tris-Acetate-EDTA (TAE) buffer (40mM Tris, 20 mM acetic acid, 1 mM EDTA) and heated until dissolved. After cooling, but prior to solidifying, 1: 10000 of SYBR® Safe® was added to the agarose mixture then poured into the cassette and allowed to solidify. Solid agarose gels were loaded into an electrophoresis tank with 1X TAE. Samples were mixed with 6 X Bromophenol blue/xylene cyanol and loaded into the wells. GeneRuler 100 bp plus ladder was added for sizing. 100-120 V electric field was applied until the tracking dye ran at least ¾ of the way down the gel or the bands achieved sufficient separation. Gels were imaged on under UV light the ChemiDoc™ MP Imaging System.

2.7.1 Band excision and purification

While visualizing agarose gels under UV light on a ChemiDoc™ MP Imaging system bands were excised and placed in Eppendorf tubes. Bands were purified from the agarose using a QIAquick® Gel extraction kit and DNA quantity measured with BioTek Synergy™ H4 Hybrid Microplate reader. Excised bands were used for Sanger sequencing as described in Section 2.8.

2.8 Sanger Sequencing

Sequencing reactions were prepared using BigDye™ Terminator v3.1 Cycle Sequencing Kit. Cleaned PCR product was added to a reaction of 1X Sequencing buffer, 1X BigDye™ MasterMix and 2μM primer. For gDNA sequencing of the OPOS cohort

M13 sequencing primers[261] were used in the sequencing reactions. For all other sequencing the primers of the PCR were used. Sequencing reactions were incubated at 96ºC for 1 minute and 25 cycles of 96ºC for 10 seconds, 50ºC for 5 seconds and 60ºC for 4 minutes.

BigDye™ Xterminator beads (45 µL SAM solution with 10 µL Xterminator solution) were added to the sequencing reaction and vortexed at 2000 rpm for 25 minutes. Samples were centrifuged for 2 minutes at 2000 x rcf and let rest for 2 minutes before another centrifugation at 2000 x rcf for 30 seconds and another 30 seconds rest to ensure beads are sedimented. 20µL of the sequencing reaction (without the beads) was transferred to a 96 well plate and loaded on the 3500 Genetic Analyzer (Applied Biosystems) for sequencing.

2.8.1 Sanger Sequencing Analysis

Sequences were analyzed using BioEdit Sequences Alignment Editor software.[263] Each sequence was reviewed using the chromatograph and edited as needed. The forward and reverse sequences were aligned using the ClustalW Multiple alignment tool to generate the consensus sequence for each product. Sequences were submitted to NCBI Blast to validate correct target amplification. For gDNA sequences from the OPOS cohort, sequences were compared to the NCBI reference sequence NG_021471.2 to identify variants. A variant is defined as any base calling that differed from the reference sequence.

2.8.2 SNP analysis

SNPStats (www.snpstats.net)[264] was used to determine allele and genotype

frequencies, test for Hardy-Weinberg equilibrium, asses SNP response using logistic regression in multiple inheritance models (co-dominant, dominant, recessive, over-dominant and log-additive), linkage disequilibrium statistics and haplotype analysis. Analysis used sex, age, and body mass index (BMI) as co-variants. A p-value < 0.05 was deemed significant.

Clinical test results were obtained from the BioBank that stored retrievable database of de-identified data abstracted from patient records. Comparison of clinical values with genotype was done in Excel using a student t-test to determine the p-value (<0.05 significant for combined and <0.1 significant for sex specific analysis). A Kolmogorov-Smirnov test in GraphPad was used to confirm normality. A ROUT test in GraphPad was used to determine outliers.

2.9 Enzyme-linked immunosorbent assay (ELISA)

Plasma samples collected as part of the OPOS study[259] were used for enzyme-linked immunosorbent assay (ELISA) for EPO (Human EPO ELISA kit), Transferrin (Transferrin Human ELISA Kit), Hepcidin (Human Hepcidin Quantikine ELISA kit) and Ferritin (Ferritin Human ELISA Kit). Plasma samples were diluted 1:2 for EPO, 1: 20, 000 for Transferrin, 1:5 for Hepcidin and 1:10 for Ferritin in the kits' diluent. ELISA were performed using the manufacturer's protocol and absorbance measured at 450 nm on the Synergy H4 Plate reader. ELISA data were compared to SNPs in GraphPad prism. Two-way ANOVA was used to determine the p-value. A p-value of <0.05 was significant. A ROUT test was used to determine outliers.

2.10 Immunoblotting

2.10.1 Protein Harvest and preparation for immunoblotting

Media was aspirated and the cells were washed with 1 X PBS; 60 µL of lysis buffer (20mM Tris, 5mM EDTA, 10 mM $NA_4P_2O_7$, 100 mM Sodium Fluoride, 1% NP-40, 2mM Sodium Orthovanadate, 2mM Protease inhibitor, 100 ug/mL Phosphatase inhibitor) was added to the plate and the cells were scraped and placed in a clean tube. Cell lysates were kept on ice for 30 minutes before sonicating at 20 kHz, 30% amplitude with the QSonicator. Protein concentration was determined using a Pierce® BCA protein assay kit. Protein samples (8-15µg) were boiled (95-100ºC) for 5 minutes in 4X Laemmli Buffer (62.5mM Tris-HCl, 20% glycerol, 4% SDS, bromophenol blue) with 0.1 M dithiothreitol (DTT).

2.10.2 Western Blotting

Boiled samples were separated in a 3.5% stacking gel and resolved in a 10% Mini-Protean sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or using a 4-20% Criterion™ TGX™ PreCast Gel (BioRad). Precision Plus Protein™ Standards Kaledioscope™ ladder (BioRad) was used as a size standard. Gels were run in 1X Tris/Glycine/SDS Electrophoresis Buffer (BioRad) until the dye front migrated to the bottom edge of the gel. Mini-Protein self-cast gels were run at 90V at room temperature. Criterion™ gels were run at 90 V for 30 minutes and 120V for 90 minutes on ice. Samples were transferred in 1 X Transfer Buffer (Biorad) with 20% methanol to a 0.2 µM nitrocellulose membrane (BioRad) at 100 V for 90 minutes at 4ºC. Membranes were rinsed in ddH_2O and total protein was stained with Pierce® Reversible Memcode Stain for 5 minutes and imaged using the ChemDoc™ MP Imaging System. Memcode staining was removed using Pierce® Stain Eraser. Membranes were blocked in 5% skim-

milk in 1 X Tris-Buffered-Saline-Tween 20 (TBS-T) for one hour at room temperature. Membranes were incubated in primary antibody (in 1% milk in TBS-T and 4% sodium Azide) (Table 2.5) overnight at 4°C. Membranes were rinsed and incubated in secondary horseradish peroxidase (HRP)-conjugated secondary antibody (in 5% milk in TBS-T) (Table 2.6). Immunoreactivity was detected using Clarity® Western Enhanced Chemiluminescence Substrate and imaged on a ChemiDoc™ MP Imaging System. Membranes were stripped on 0.5 M Tris-HCl/SDS buffer with 125 μL β-mercaptoethanol per 25 mL for 1 hour and reprobed.

Table 2.5- Primary Antibodies.

Antibody target	Supplier	Catalogue #	Dilution
HIF1α	R&D Systems	AF1935	1:400
HO-1	Millipore-Sigma	H-4535	1:1000
NFκB	CST	8242	1:1000
p-NFκB	CST	3033	1:1000
Erk1/2	CST	4696	1:1000
p-Erk1/2	CST	9101	1:1000
STAT3	SantaCruz	SC-8019	1:1000
Akt	Millipore-Sigma	05-591	1:1000
p-Akt	CST	9271	1:1000
eNOS	CST	32027	1:1000
p-eNOS (Ser 1177)	Invitrogen	MA514957	1:1000
PGC1-α	SantaCruz	13067	1:1000

CST=cell signaling technologies

Table 2.6- Secondary Antibodies

Antibody	Supplier	Catalogue #	Dilution	Targets
Donkey-anti-goat-IgG-HRP	SantaCruz	SC-2056	1:1000	HIF1α
Goat Anti-Mouse IgG-HRP	Biorad	1706516	1:1000	p-eNOS, Erk1/2, STAT3, Akt
Goat anti-rabbit IgG-HRP	Invitrogen	31460	1:1000	HO-1, NF-κB, p-NF-κB, p-Erk1/2, p-Akt, eNOS, PGC-1α

2.10.3 Coomassie Staining

Samples were prepared the same as boiled protein samples for Western blotting except only 1.5-2µg of samples were used. Samples were run on a Mini-Protean self-cast gel in 1X Tris/Glycine/SDS Electrophoresis buffer for 90 minutes at 90V at room temperature. Gel was rinsed with ddH_2O prior to immersion in the fixative solution (50% methanol, 10% glacial acetic acid) and incubated at room temperature shaking at 50 rpm for 30 minutes twice, then washed with ddH_2O again. Gel was incubated in 0.25% Coomassie buffer (50% methanol, 10% glacial acetic acid. 0.25% w/v Coomassie) shaking at 50 rpm overnight. The Coomassie buffer was rinsed off with ddH_2O before incubation in in fixative solution for 90 minutes to remove background staining. After 90 minutes the fixative solution was replaced and incubated for an additional 30 minutes and imaged on the ChemiDoc™ MP Imaging System.

2.10.4 Densitometry

ImageLab™ Software v5.0 (BioRad) was used for densitometric analysis. Relative integrated density was calculated as the density of the target protein normalized to the total protein density (respective membrane code-stained lane). A one-way ANOVA was used to assess significant changes in GraphPad Prism with a p-value of < 0.05 being deemed significant.

2.11 *In vitro* Colony-Forming Unit-Erythroid (CFU-E)

Colony-Forming Unit-Erythroid (CFU-E) assays were performed by STEMCELL Technologies. Briefly, EPO peptides were added to MethoCult™. Bone marrow (BM) mononuclear cells (MNCs) was added to the treated MethoCult™ before plating 1.1 mL in 35 mm dishes in triplicate. Cells were incubated for two weeks at 37ºC, in 5% CO_2 with ≥ 95% humidity and colonies were counted.[265] The highest number for CFU-E was

set as the percent of optimal growth (100%) and CFU-E was normalized to that value in GraphPad Prism. The EC_{50} was calculated using the log(agonist) vs. response -variable slope (four parameters) equation in GraphPad Prism.

2.12 Measuring mitochondrial respiration

We measured high-resolution mitochondrial respiration using a Oxygraph-2k (OROBOROS Instrument). H9c2 cells were grown in 60 mm plates to approximately 80% confluency. Either 24 hours (chronic) or 15 minutes (acute) prior to harvest for oxygraphy the cells were treated with 137 ng/mL epoetin α or media with no epoetin α. For harvest, cells were washed with 1 mL of 1X PBS and treated with StemPro Accutase® for 10-15 minutes (until cells detached) at room temperature. Cells were collected and centrifuged at 300 x rcf for 5 minutes at room temperature. Accutase was aspirated and cells were resuspended in MIR05 (0.5mM EGTA, 3mM $MgCl_2$, 60mM Lactobionic acid, 20mM Taurine, 10mM KH_2PO_4, 20mM HEPES, 110mM D-Sucrose and 1 g/L BSA). Cells were counted using Trypan Blue and diluted in MiR05 to 100 000 cells/mL and a total of 200 000 cells were added to the Oxygraph chamber.

Once the chamber stabilized 3 µL of digitonin was added to permeabilize the cell membrane. Subsequently the following compounds were added in order: 0.5mM Malate, 5mM Pyruvate, 0.1mM adenosine diphosphate (ADP), 0.5mM ADP, 5mM ADP, 0.5 µM carbonyl cyanide-p-trifluromethoxyphenylhydrazone (FCCP), 0.5 µM Rotenone, 10µM Succinate and 5µM Antimycin A. The chamber was allowed to stabilize in between each step. Upon completion the contents of the chamber were collected and centrifuged at 10 000 x rcf for 5 minutes at 4ºC and the supernatant discarded. The pellet was resuspended in lysis buffer and total protein quantified by BCA assay.

2.12.1 Oxygraph analysis

Data was normalized to the baseline after addition of Antimycin-A and protein concentration. Normalized values were used in GraphPad Prism to calculate two-way ANOVA for statistical significance with a p-value <0.05 being deemed significant.

Chapter 3- Erythropoietin variant in DNA

3. 1 Introduction

Genetic variants in *EPO* have been linked to a wide variety of disorders and *EPO* levels. These genetic variants include rare genetic variants and more common SNPs. Rare genetic variants include four pathogenic variants reported in association with erythrocytosis (c.-136G>A,[266] c.33delG,[267] c.20delC, and c.296A>G[268]) and a single pathogenic variant associated with Diamond-blackfin anemia (c.530G>A).[269] In a search of the ClinVar[270] database (ncbi.nlm.nih.gov/clinvar) in April of 2023 there have been additional *EPO* variants identified with six classified as variants of unknown significance (VUS) associated with inborn genetic diseases (c.16T>C, c.82G>A, c.175C>T, c.226T>C, c.521A>G, and c.559G>T). Two more VUSs were identified but no phenotype was provided (c.14-1G>C and c.286C>G). An additional 18 variants were classified as benign or likely benign. Many of the variants identified in ClinVar were found during clinical testing, which suggest that as sequencing technologies become more prevalent in clinical care the number of variants will continue to rise or be linked to outcomes clinically.

GWAS and eQTL analysis have shown the promoter SNP rs1617640 is associated with a lower level of EPO.[196] There are three SNPs, including rs1617640, in *EPO* that have been identified as risk factors for many disorders, such as microvascular complications in diabetes and anemia, two common comorbid conditions often associated with HFpEF, CKD, or obesity-related negative outcome.[271] Even in healthy donors these SNPs have been shown to correlate with EPO serum levels. The most studied SNP is

rs1617640 found in the promoter region (Table 3.1). It has been linked to diabetic microvascular complications, such as retinopathy and end-stage renal disease, in several studies in various populations.[277] However, studies by Balasubbu *et al.*[292] and Song *et al.*[289] did not find a significant association amongst the Indian or Brazilian populations, respectively.

Table 3.1- Erythropoietin SNP associations

SNP	Allele	Phenotype	References
rs1617640	G	Higher Hct in healthy blood donors	272
		Diabetic microvascular complications	273–281
		Decreased overall mortality in diabetes	282
		Higher Hb, Hct, RBC count and earlier onset PAD	283
		MDS and ALL	284
		Improved cognitive performance in Schizophrenia	285
	T	Anemia in T2DM	286
		Renal dysfunction following cardiac surgery	287
		Improved response to platinum-based chemotherapy in NSCLC	288
rs507392	C	Diabetic microvascular complications	273,275,276,289
		Decreased overall mortality in diabetes	282
rs551238	C	Higher Hct in healthy blood donors	272
		Diabetic microvascular complications	272,273,275–278,289,290
		Preterm infant brain injury	291

SNP=Single Nucleotide Polymorphism, Hct=hematocrit, Hb=hemoglobin, RBC=red blood cell count, PAD=peripheral artery disease, MDS=myelodysplatic syndrome, ALL=acute lymphoblastic leukemia, T2DM=type 2 diabetes mellites, NSCLC=non-small cell lung cancer

Cardiovascular disease (CVD) is one of the leading causes of death worldwide. There are multiple risk factors that can contribute to CVD, some have been linked to EPO using animal models or the use of ESAs.[82] Anemia is a common co-morbidity in many disorders including CVD.[293,294] Anemia in heart failure is associated with higher mortality,[295] severity and hospital admission.[296,297] Anemia is defined as hemoglobin of <13.5 g/dL in males and <12 g/dL in females.[297] In CKD a decrease in EPO is associated

with anemia.[192] In other forms of anemia EPO is found to be increased by up to 100-fold.[189] Previous studies have linked rs1617640 SNP in *EPO* to anemia in T2DM,[286] suggesting SNPs in *EPO* may be associated with anemia.

Dyslipidemia is also a risk factor for CVD that is characterized by abnormal serum levels of cholesterol, triglycerides and related lipoproteins.[298] In mice treated with rhEPO, triglycerides were decreased[299] and lipoprotein lipase (LPL), which is involved in lipogenesis, was elevated.[299,300] In humans, treatment with rhEPO is associated with lower triglycerides and cholesterol in end-stage renal failure.[249] However, the potential role of *EPO* in dyslipidemia has not previously been investigated.

T2DM presenting with resistance to insulin can result in hyperglycemia, elevated circulating glycation end-products and endothelial damage culminating in significant risk factors for CVD or CKD, with CVD being the main cause of death in T2DM patients.[301] Mouse models that overexpressed *Epo* had improved glucose tolerance and reduced insulin resistance.[184] Due to the metabolic effects of EPO, it has been suggested that rhEPO therapy could have benefits in T2DM.[302] One of the most commonly studied associations with *EPO* SNPs is diabetic microvascular complications (Table 3.1). These studies are typically conducted on groups with T2DM, and therefore a potential role for *EPO* SNPs as a risk factor for T2DM has not yet been investigated.

Hypertension is elevated blood pressure and another risk factor for CVD.[303] Hypertension is a relatively common adverse effect of rhEPO therapy, with a prevalence of between 20 and 30%.[250] This would suggest that EPO has an effect on blood pressure. However, the effect of *EPO* SNPs in association with hypertension has not been studied.

HFpEF is a complex CVD characterized by an ejection fraction of >50%,

diastolic dysfunction and cardiac stiffening, fibrosis, inflammation, and hypertrophy associated with microvascular rarefaction, obesity, renal dysfunction, anemia, insulin insensitivity or other metabolic and inflammatory risk factors variable with sex and age. An increase in left ventricular ejection fraction (LVEF) was found in multiple clinical trials using rhEPO in CVD.[304,305] Microvascular complications in diabetes are the most common disorder linked to *EPO* SNPs (Table 3.1). HFpEF has been linked to coronary microvascular dysfunction.[271] However, the potential role of *EPO* SNPs have not been investigated in relation to HFpEF.

EPO SNPs have previously been shown to be associated with EPO serum levels.[281] Anemia, dyslipidemia, T2DM, hypertension and HFpEF have disease characteristics that have been shown to be modifiable by rhEPO, but only anemia has previously been associated with *EPO* SNPs.[286] We hypothesize that three *EPO* SNPs (rs1617640, rs507292 and rs551238) correlate with EPO concentrations that are associated with either anemia, dyslipidemia, T2DM, hypertension and/or HFpEF in a clinical population requiring surgical interventions for CVD.

3.2 Results

3.2.1 OPOS sub-cohort

The OPOS sub-cohort consisted of 95 participants who were recruited as part of the OPOS study (358 participants). Within the sub-cohort we examined anemia, HFpEF, T2DM, dyslipidemia and hypertension. The demographics and clinical parameters of the OPOS cohort are shown in Table 3.2. Although many parameters are the same between controls and cases in each condition, there are notable differences. Some of those differences are expected since they are used to define the condition, such as a normal

Table 3.2- Demographics and clinical parameters of the OPOS cohort by disorder

	Total	Anemia			Dyslipidemia		
	(n=95)	Cases (n=31)	Ctrl (n=64)	p-value	Cases (n=66)	Ctrl (n=29)	p-value
Age (years)	63.5 ± 6.6	65.1 ± 5.8	62.7 ± 6.8	0.52	64.3 ± 6.3	61.7 ± 6.8	**0.031**
Males	65 (68%)	15 (48%)	50 (78%)	**0.004**	49 (74%)	16 (55%)	**0.045**
Females	30 (32%)	16 (52%)	14 (22%)		17 (26%)	13 (45%)	
BMI (kg/m^2)	31.8 ± 7.3	34.4 ± 9.4	30.5± 5.6	**0.007**	31.3 ± 6.0	32.7 ± 9.6	0.69
Normal	14 (15%)	4 (13%)	10 (16%)	0.18	9 (14%)	5 (17%)	0.86
Pre-obese	31 (33%)	8 (26%)	23 (36%)		22 (33%)	9 (31 %)	
Obese 1	22 (23%)	6 (19%)	16 (25%)		17 (26%)	5 (17%)	
Obese 2	13 (14%)	4 (13%)	9 (14%)		8 (12%)	5 (17%)	
Obese 3	15 (16%)	9 (29%)	6 (9%)		10 (15%)	5 (17%)	
NYHA							
1	18 (19%)	4 (14%)	14 (22%)	0.41	8 (12%)	10 (37%)	**0.005**
2	39 (42%)	11 (38%)	28 (44%)		31 (47%)	8 (30%)	
3	25 (27%)	11 (38%)	14 (22%)		16 (24%)	9 (33%)	
4	11 (12%)	3 (10%)	8 (13%)		11 (17%)	0 (0%)	
LVEF (%)	59.8 ± 11.2	60.9 ± 10.7	59.3 ± 11.4	0.98	58.0 ± 11.9	64.0 ± 7.8	0.06
LVEDP (mmHg)	18.0 ± 6.6	18.8 ± 6.4	17.7 ± 6.7	0.052	18.6 ± 6.7	16.6 ± 6.4	0.10
Hemoglobin (g/L)	134.8 ± 14.7	117.8 ± 11.5	142.6 ± 7.8	**<0.0001**	134.8 ± 14.6	135.0 ± 14.8	0.59
Hematocrit (L/L)	0.40 ± 0.04	0.35 ± 0.03	0.42 ± 0.02	**<0.0001**	0.40 ± 0.04	0.40 ± 0.04	0.46
HbA1c (%)	6.1 ± 1.0	6.2 ± 1.3	6.0 ± 0.9	0.68	6.2 ± 1.1	5.8 ± 0.8	0.28
Cholesterol (mmol/L)	4.1 ± 1.2	4.0 ± 1.1	4.1 ± 1.2	0.85	3.9 ± 1.1	4.5 ± 1.1	0.06
HDL (mmol/L)	1.2 ± 0.4	1.2 ± 0.4	1.2 ± 0.3	0.66	1.2 ± 0.3	1.4 ± 0.3	0.05
LDL (mmol/L)	2.1 ± 0.9	2.1 ± 0.9	2.1 ± 0.9	0.89	2.0 ± 0.8	2.5 ± 1.0	**0.027**
LDL/HDL	1.8 ± 0.8	1.7 ± 0.8	1.8 ± 0.7	0.63	1.7 ± 0.7	1.9 ± 0.7	0.38
Non-HDL (mmol/L)	2.9 ± 1.1	2.8 ± 1.0	2.9 ± 1.1	0.74	2.7 ±1.1	3.1 ± 1.0	0.16
Triglycerides (mmol/L)	1.6 ± 1.1	1.8 ± 1.2	1.6 ± 1.0	0.58	1.7 ± 1.2	1.3 ± 0.6	0.26
RBC (x10^12/L)	4.4 ± 0.4	4.0 ± 0.4	4.6 ± 0.3	**<0.0001**	4.5 ± 0.4	4.4 ± 0.4	0.81
WBC (x10^9/L)	7.4 ± 2.4	7.8 ± 3.2	7.2 ± 1.9	0.37	7.6 ± 2.6	6.8 ± 1.8	0.44
Platelets (x10^9/L)	205.5 ± 53.9	209.0 ± 72.2	203.9 ± 43.1	0.55	209.5 ± 57.1	195.8 ± 43.7	0.38
Neutro (x10^9/L)	4.9 ± 2.2	5.5 ± 3.3	4.7 ± 1.5	0.21	5.1 ± 2.3	4.5 ± 1.8	0.56
Lympho (x10^9/L)	1.7 ± 0.6	1.5 ± 0.7	1.7 ± 0.6	0.22	1.7 ± 0.6	1.6 ± 0.6	0.99
NLR	3.9 ± 5.1	6.1 ± 8.5	2.9 ± 1.2	0.10	3.8 ± 4.6	4.1 ± 6.0	0.47
Troponin (ng/L)	785 ±590	879 ± 824	741 ± 431	0.60	694 ± 428	999 ± 819	**0.030**
Creatine (mmol/L)	92.9 ± 71.1	113.3 ± 121.6	83.7 ± 19.4	0.21	97.8 ± 82.6	81.1 ± 22.9	0.25
Urea (mmol/L)	6.7 ± 2.5	7.2 ± 3.3	6.5 ± 2.1	0.054	7.0 ± 2.7	6.1 ± 2.0	0.06

Table 3.2 cont.- Demographics and clinical parameters of the OPOS cohort by disorder

	T2DM			HFpEF			Hypertension		
	Cases (n=33)	Ctrl (n=62)	p-value	Cases (n=77)	Ctrl (n=18)	p-value	Cases (n=70)	Ctrl (n=25)	p-value
Age (years)	64.6 ± 5.5	63.0 ± 7.1	0.17	63.9 ± 6.8	61.9 ± 5.5	0.25	64.2 ± 6.9	61.6 ± 5.3	**0.040**
Males	26 (81%)	36 (59%)	**0.040**	53 (69%)	12 (67%)	0.85	53 (76%)	12 (48%)	**0.010**
Females	6 (19%)	25 (41%)		24 (31%)	6 (33%)		17 (24%)	13 (52%)	
BMI (kg/m^2)	33.3 ± 6.0	30.9 ± 7.8	**0.010**	30.9 ± 6.2	35.5 ± 10.0	**0.015**	31.4 ± 6.3	32.8 ± 9.6	0.10
Normal	1 (3%)	13 (21%)	0.14	12 (16%)	2 (11%)	0.10	11 (16%)	3 (12%)	0.82
Pre-obese	10 (30%)	21 (34%)		29 (38%)	2 (11%)		22 (31%)	9 (36%)	
Obese 1	10 (30%)	12 (19%)		16 (21%)	6 (33%)		16 (23%)	6 (24%)	
Obese 2	5 (15%)	8 (13%)		8 (10%)	5 (38%)		11 (16%)	2 (8%)	
Obese 3	7 (21%)	8 (13%)		12 (16%)	3 (17 %)		10 (14%)	5 (20%)	
NYHA									
1	5 (15%)	13 (22%)	0.46	14 (18%)	4 (25%)	0.84	13 (19%)	5 (22%)	0.91
2	12 (36 %)	27 (45%)		32 (42%)	7 (44%)		29 (41%)	10 (43%)	
3	12 (36%)	13 (22%)		22 (29%)	3 (19%)		19 (27%)	6 (26%)	
4	4 (12%)	7 (12%)		9 (12%)	2 (13%)		9 (13%)	2 (9%)	
LVEF (%)	56.7 ± 11.3	61.5 ± 10.7	0.10	63.9 ±7.4	42.6 ± 7.6	**<0.0001**	60.5 ± 11.3	58.1 ± 10.7	0.25
LVEDP (mmHg)	18.8 ± 6.6	17.6 ± 6.6	0.35	17.4 ± 6.5	20.4 ± 6.8	0.11	18.7 ± 7.0	15.7 ± 4.8	0.10
Hemoglobin (g/L)	132.9 ± 14.9	135.9 ± 14.4	0.24	135.0 ± 14.6	134.3 ± 14.9	0.86	134.9 ± 15.2	134.7 ± 12.9	0.81
Hematocrit (L/L)	0.39 ± 0.04	0.40 ± 0.04	0.22	0.40 ± 0.04	0.40 ± 0.04	0.71	0.40 ± 0.04	0.40 ± 0.03	0.74
HbA1c (%)	7.0 ± 1.2	5.6 ± 0.3	**<0.0001**	6.0 ± 1.0	6.4 ± 0.9	0.19	6.1 ± 1.1	5.8 ± 0.8	0.41
Cholesterol (mmol/L)	3.6 ± 0.9	4.3 ± 1.2	**0.012**	4.1 ± 1.2	3.9 ± 1.1	0.62	3.9 ± 1.2	4.4 ± 1.1	0.52
HDL (mmol/L)	1.1 ± 0.4	1.3 ± 0.3	**0.045**	1.2 ± 0.4	1.2 ± 0.2	0.68	1.2 ± 0.4	1.3 ± 0.3	0.25
LDL (mmol/L)	1.7 ± 0.7	2.3 ± 1.0	**0.016**	2.1 ± 0.9	2.1 ± 1.1	0.84	2.0 ± 0.9	2.5 ± 1.0	0.51
LDL/HDL	1.6 ± 0.7	1.9 ± 0.7	0.59	1.8 ± 0.7	1.8 ± 0.9	0.70	1.7 ± 0.7	1.9 ± 0.8	0.91
Non-HDL (mmol/L)	2.5 ± 0.8	3.0 ± 1.1	0.06	2.9 ± 1.1	2.8 ± 1.1	0.82	2.8 ± 1.1	3.1 ± 1.0	0.79
Triglycerides (mmol/L)	1.9 ± 1.2	1.5 ± 0.9	0.70	1.6 ± 1.1	1.5 ± 0.7	0.53	1.7 ± 1.2	1.4 ± 0.6	0.58
RBC (x10^12/L)	4.4 ± 0.4	4.5 ± 0.4	0.58	4.5 ± 0.4	4.4 ± 0.5	0.63	4.4 ± 0.5	4.5 ± 0.3	0.52
WBC (x10^9/L)	8.0 ± 2.9	7.1 ± 2.0	0.26	7.3 ± 2.3	7.7 ± 2.9	0.56	7.4 ± 2.4	7.3 ± 2.5	0.45
Platelets (x10^9/L)	218.2 ± 68.9	198.5 ± 42.0	0.36	204.9 ± 56.6	208.1 ± 38.4	0.83	205.7 ± 56.5	204.9 ± 45.4	0.90
Neutro (x10^9/L)	5.5 ± 2.7	4.6 ± 1.8	0.15	4.9 ± 2.2	5.1 ± 2.5	0.75	5.0 ± 2.2	4.8 ± 2.2	0.62
Lympho (x10^9/L)	1.7 ± 0.7	1.7 ± 0.6	0.54	1.7 ± 0.7	1.8 ± 0.5	0.55	1.6 ± 0.7	1.7 ± 0.6	0.20
NLR	4.7 ± 6.1	3.5 ± 4.3	0.24	4.0 ± 5.6	3.1 ± 1.7	0.50	4.2 ± 5.7	3.1 ± 2.1	0.54
Troponin (ng/L)	754 ± 709	809 ± 521	0.42	838 ± 617	510 ± 301	0.057	794 ± 637	759 ± 421	0.50
Creatine (mmol/L)	110.8 ± 112.8	83.1 ± 23.6	0.25	85.7 ± 24.0	127.7 ± 158.5	**0.032**	89.0 ± 26.4	105.1 ± 134.6	0.21
Urea (mmol/L)	7.7 ± 3.1	6.2 ± 1.9	**0.0276**	6.6 ± 2.1	7.2 ± 3.8	0.47	7.0 ± 2.3	5.8 ± 2.8	0.08

NYHA=New York Heart Association, BMI=Body mass index, HDL=high-density lipoprotein, LDL=low-density lipoprotein, LVEF=Left ventricular ejection fraction, LVEDP=left ventricular end diastolic pressure, HFpEF=Heart failure with preserved ejection fraction, T2DM=type 2 diabetes mellitus, REB=red blood cells (erythrocytes), WBC=white blood cells (leukocytes), Neutro=Neutrophiles, Lympho= Lymphocytes, NLR=Neutrophiles Lymphocytes ratio. p-value for continuous variable (BMI, LVEF, LVEDP, Hemoglobin, Hematocrit, HbA1c, Cholesterol, HDL, LDL, Triglycerides, RBC, Troponin, Creatine, WBC, Neutrophiles, Lymphocytes, NLR, Urea) expressed as mean ±SD, p-value calculated using t-test. Categorical variable (Sex, BMI Class, and NYHA) expressed as number of cases (percentage of group), p-value calculated using Chi-square test. Significant p-value of <0.05 are bolded.

range LVEF in HFpEF, higher glycated hemoglobin (HbA1c) in T2DM, lower hemoglobin, hematocrit and erythrocytes in anemia and lower LDL in dyslipidemia. Males were more prevalent in T2DM, hypertension and dyslipidemia and females were more prevalent in anemia, with consideration to the sex-dependent normal ranges used for males/females of the study population. An increase in average BMI was found in T2DM and anemia, whereas HFpEF had a lower average BMI. Dyslipidemia was also associated with a higher New York Heart Association (NYHA) score.

3.2.2 Hardy-Weinberg equilibrium in the OPOS cohort

Sanger sequencing was used to determine the alleles for each rs1617640, rs507392 and rs551238 (Figure 3.1). We determined the allele frequencies of each allele for the three SNPs rs1617640, rs507392 and rs551238 using SNPstats. For rs1617640, A had an allele frequency of 0.57 and C had an allele frequency of 0.43. For rs507392, A had an allele frequency of 0.6 and G had an allele frequency of 0.4. For rs551238, T had an allele frequency of 0.63 and G had an allele frequency of 0.37. Based on these observed allele frequencies the expected genotypes were calculated and compared to observed genotypes to determine if our population was in Hardy-Weinberg equilibrium (Table 3.3). Our study population was not in Hardy-Weinberg equilibrium. The SNP rs1617640 has the most disorders we measured not in Hardy-Weinberg equilibrium.

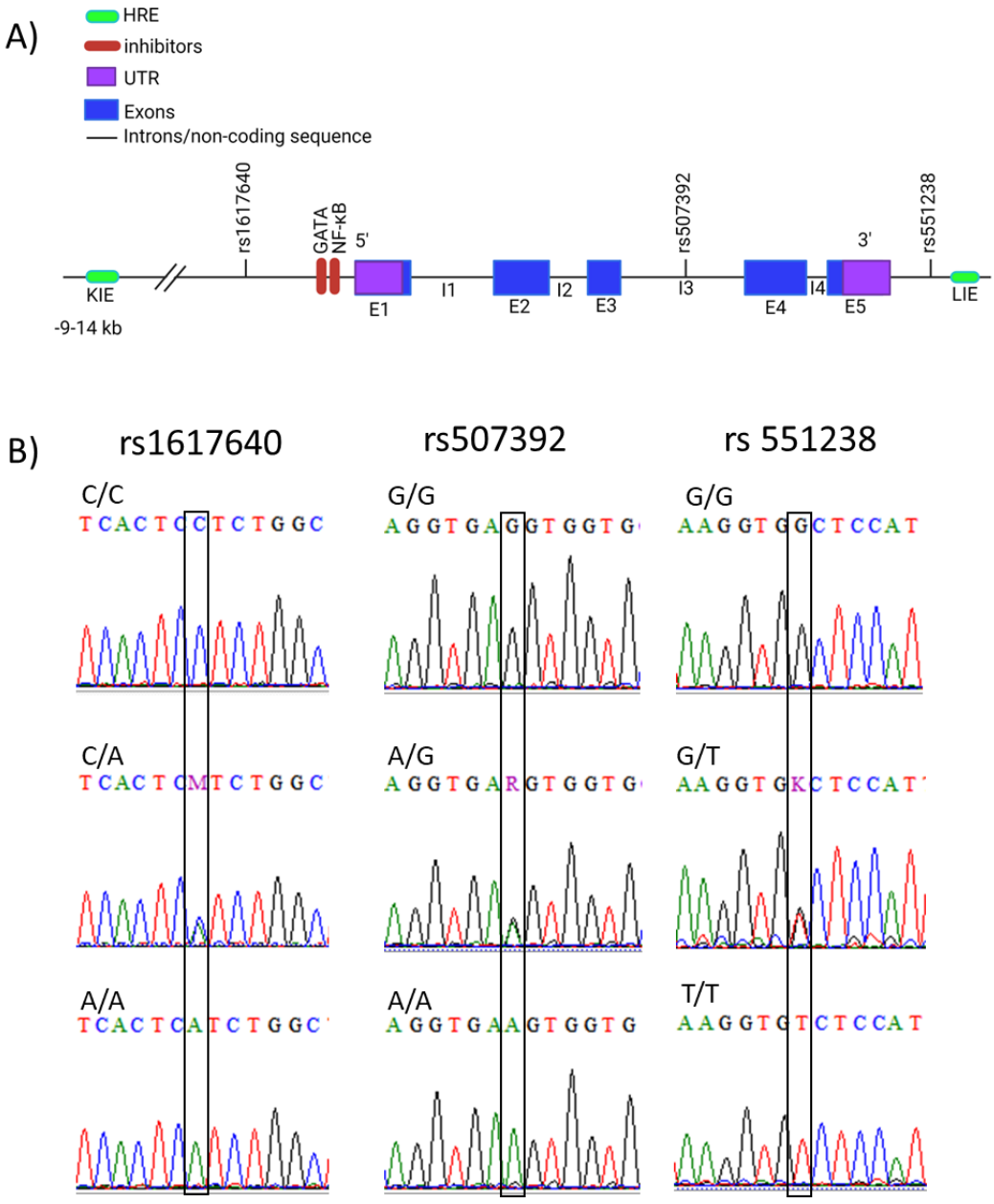

Figure 3.1- *EPO* polymorphisms identified using Sanger sequencing.
A) EPO gene and regulatory elements with SNPs locations by the SNP name. B) Representative chromatograms obtained by Sanger sequencing for each genotype of the three SNPs rs1617640, rs507392, and rs551238. The position of the SNP is identified by rectangles surrounding the base call.

Table 3.3- Hardy-Weinberg predicted vs. observed genotypes and allele frequencies.

	rs161640						rs507392						rs551238					
	A/A	A/C	C/C	A	C	p-value	A/A	A/G	G/G	A	G	p-value	T/T	T/G	G/G	T	G	p-value
Predicted	31	46	17	0.57	0.43	n/a	31	41	14	0.6	0.4	n/a	35	41	12	0.63	0.37	n/a
All subjects	38	32	24	108	80	**0.003**	36	31	19	103	69	**0.024**	40	31	17	111	65	**0.023**
Anemia	8	10	12	27	35	0.15	7	8	8	22	24	0.11	8	9	7	25	23	0.24
Ctrl anemia	30	21	12	81	45	**0.031**	29	23	11	81	45	0.21	32	22	10	86	42	0.08
Dyslipidemia	29	23	13	81	49	**0.060**	28	23	10	79	43	0.17	30	23	9	83	41	0.25
Ctrl Dyslipidemia	9	9	11	27	31	**0.062**	8	8	9	24	26	0.11	10	8	8	28	24	0.056
T2DM	11	15	6	37	27	1.00	11	13	5	35	23	0.71	13	13	5	39	23	0.70
Ctrl T2DM	27	17	18	71	53	**0.0006**	25	18	14	68	46	**0.12**	27	18	12	72	42	**0.021**
HFpEF	30	27	19	87	65	**0.019**	28	27	17	83	61	0.26	31	27	15	89	57	0.08
Ctrl HFpEF	8	5	5	21	15	0.14	8	4	2	20	8	0.054	9	4	2	22	8	0.23
Hypertension	28	24	18	80	60	**0.014**	27	25	15	79	55	0.07	29	24	14	82	52	**0.044**
Ctrl Hypertension	10	8	6	28	20	0.20	9	6	4	24	14	0.17	11	7	3	29	13	0.33

HFpEF=heart failure with preserved ejection fraction, T2DM=type 2 diabetes mellitus, Ctrl=control. Predicted genotypes are calculated using the Hardy-Weinberg equation based on observed allele frequencies (noted as proportion in predicted lines for allele). All other genotypes and alleles represent the observed for each group for each of the SNPs as calculated by SNPStats. P-values of <0.05 are deemed significant deviations from Hardy-Weinberg and are bolded.

3.2.3 Linkage disequilibrium

The three SNPs rs1617640, rs507392 and rs551238 are found within 4.2 kb of each other. Due to the proximity, linkage disequilibrium is likely to occur. We performed a linkage disequilibrium analysis using SNPstats and found there is a high degree of linkage disequilibrium between our three SNPs (Figure 3.2). The highest degree of linkage disequilibrium is between rs1617640 and rs507392, followed by rs507392 and rs551238. rs1617640 and rs551238 have the least degree of disequilibrium. These associations correlate with their positions along the *EPO* gene with those closest in proximity (Figure 3.1) showing the highest degree of disequilibrium.

3.2.4 rs1617640 correlations

The demographics and clinical parameters based on rs1617640 genotype are shown in Table 3.4. There was a significant association in BMI class between AA and CC genotypes, with a higher prevalence of classes Obese 2 and 3 in CC genotype. Hemoglobin was also significantly lower in CC compared to AA. Both total cholesterol and low-density lipoprotein (LDL) were higher amongst the CC genotype. Whereas the genotype AC has lower non-high-density lipoprotein (non-HDL). The CC genotype is associated with a higher neutrophil-to-lymphocyte ratio NLR (Figure 3.3). Taken together, this data suggests the rs1617640 SNP may potentially be associated with cholesterol metabolism, hemoglobin, and inflammation.

We next tested to see if rs1617640 correlated with either anemia (Table 3.5), dyslipidemia (Table 3.6), T2DM (Table 3.7), HFpEF (Table 3.8) or hypertension (Table 3.9). Each correlation was examined in five inheritance models (codominant, dominant,

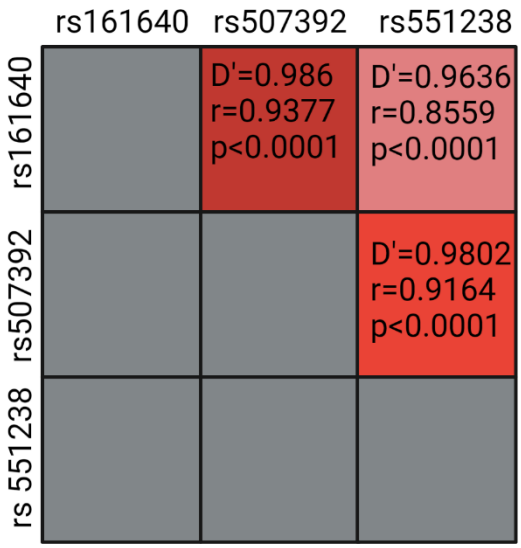

Figure 3.2- *EPO* SNPs show a high degree of linkage disequilibrium.
The linkage coefficient (D'), R-value and p-value are provided for each combination. Greyed boxes are duplicated combinations or between the same SNP and therefore not analyzed. Red boxes show combinations with darker red indicating greater degrees of linkage disequilibrium.

Table 3.4-rs1617640 demographics and clinical parameters.

	Genotype			p-value		
	AA (n=38)	AC (n=32)	CC (n=24)	AA-AC	AA-CC	AC-CC
Age (years)	64.1 ± 6.1	63.4 ± 6.8	62.6 ± 7.0	0.68	0.41	0.68
Males (%)	29 (76%)	20 (63%)	15 (63%)	0.20	0.24	0.99
Females	9 (24%)	12 (38%)	9 (38%)			
BMI (kg/m^2)	29.7 ± 5.3	31.8 ± 6.9	34.9 ± 9.3	0.16	**0.007**	0.16
Normal	7 (18%)	5 (16%)	2 (8%)	0.40	**0.008**	0.40
Pre-obese	14 (37%)	10 (31%)	7 (29%)			
Obese 1	12 (32%)	7 (22%)	2 (8%)			
Obese 2	3 (8%)	4 (13%)	6 (25%)			
Obese 3	2 (5%)	6 (19%)	7 (29%)			
NYHA						
1	8 (21%)	6 (19%)	4 (17%)	0.46	0.67	0.27
2	17 (45%)	9 (39%)	12 (52%)			
3	8 (21%)	11 (35%)	6 (26%)			
4	5 (13%)	5 (16%)	1 (4%)			
LVEF (%)	58.8 ± 11.8	61.4 ± 10.2	59.2 ± 11.3	0.32	0.88	0.45
LVEDP (mmHg)	17.0 ± 6.7	17.6 ± 7.0	20.5 ± 5.3	0.72	0.056	0.13
Hemoglobin (g/L)	137.3 ± 9.1	135.3 ± 18.6	129.6 ± 15.0	0.56	**0.017**	0.24
Hematocrit (L/L)	0.40 ± 0.03	0.40 ± 0.05	0.39 ± 0.04	0.77	0.08	0.32
HbA1c (%)	6.2 ± 1.2	5.9 ± 0.8	5.9 ± 0.7	0.25	0.31	0.92
Cholesterol(mmol/L)	4.2 ± 1.3	3.7 ± 1.1	4.4 ± 1.0	0.06	0.59	**0.019**
HDL (mmol/L)	1.2 ± 0.4	1.2 ± 0.4	1.3 ± 0.3	0.79	0.11	0.09
LDL (mmol/L)	2.2 ± 0.9	1.8 ± 0.9	2.4 ± 0.9	0.10	0.33	**0.018**
LDL/HDL	1.9 ± 0.8	1.6 ± 0.7	1.9 ± 0.8	0.13	0.80	0.11
Non-HDL (mmol/L)	3.0 ±1.2	2.5 ± 0.9	3.0 ± 1.0	**0.046**	0.99	**0.045**
TG (mmol/L)	1.9 ± 1.4	1.5 ± 0.7	1.3 ± 0.5	0.14	0.07	0.40
RBC (x10^12/L)	4.5 ± 0.3	4.4 ± 0.5	4.4 ± 0.4	0.82	0.32	0.60
WBC (x10^9/L)	7.1 ±2.1	7.4 ± 2.8	7.9 ±2.3	0.72	0.22	0.49
Platelets (x10^9/L)	207.0 ± 46.4	208.3 ± 64.6	199.8 ± 50.2	0.92	0.58	0.61
Neutro (x10^9/L)	4.6 ± 1.6	4.9 ± 2.7	5.5 ± 2.4	0.49	0.07	0.40
Lympho (x10^9/L)	1.7 ± 0.6	1.6 ± 0.6	1.6 ± 0.8	0.51	0.30	0.61
NLR	2.8 ± 1.0	3.5 ± 3.6	6.3 ± 8.7	0.22	**0.018**	0.12
Troponin (ng/L)	756.8 ± 521.6	796.7 ± 665.4	815.7 ± 599.2	0.79	0.70	0.91
Creatine (mmol/L)	85.2 ± 17.4	103.6 ± 117.8	92.4 ± 31.0	0.35	0.25	0.66
Urea (mmol/L)	6.3 ± 1.9	7.0 ± 2.7	7.1 ± 3.0	0.24	0.21	0.86

NYHA=New York Heart Association, BMI=Body mass index, HDL=high-density lipoprotein, LDL=low-density lipoprotein, LVEF=Left ventricular ejection fraction, LVEDP=left ventricular end diastolic pressure, HFpEF=Heart failure with preserved ejection fraction, T2DM=type 2 diabetes mellitus, TG=triglycerides, RBC=red blood cells, WBC=white blood cells (leukocytes), Neutro=Neutrophiles, Lympho= Lymphocytes, NLR=Neutrophiles Lymphocytes ratio. p-value for continuous variable (BMI, LVEF, LVEDP, Hemoglobin, Hematocrit, HbA1c, Cholesterol, HDL, LDL, Triglycerides, RBC, Troponin, Creatine, WBC, Neutrophiles, Lymphocytes, NLR, Urea) expressed as mean ±SD, p-value calculated using t-test. Categorical variable (Sex, BMI Class, and NYHA) expressed as number of cases (percentage of group), p-value calculated using Chi-square test. Significant p-value of <0.05 are bolded.

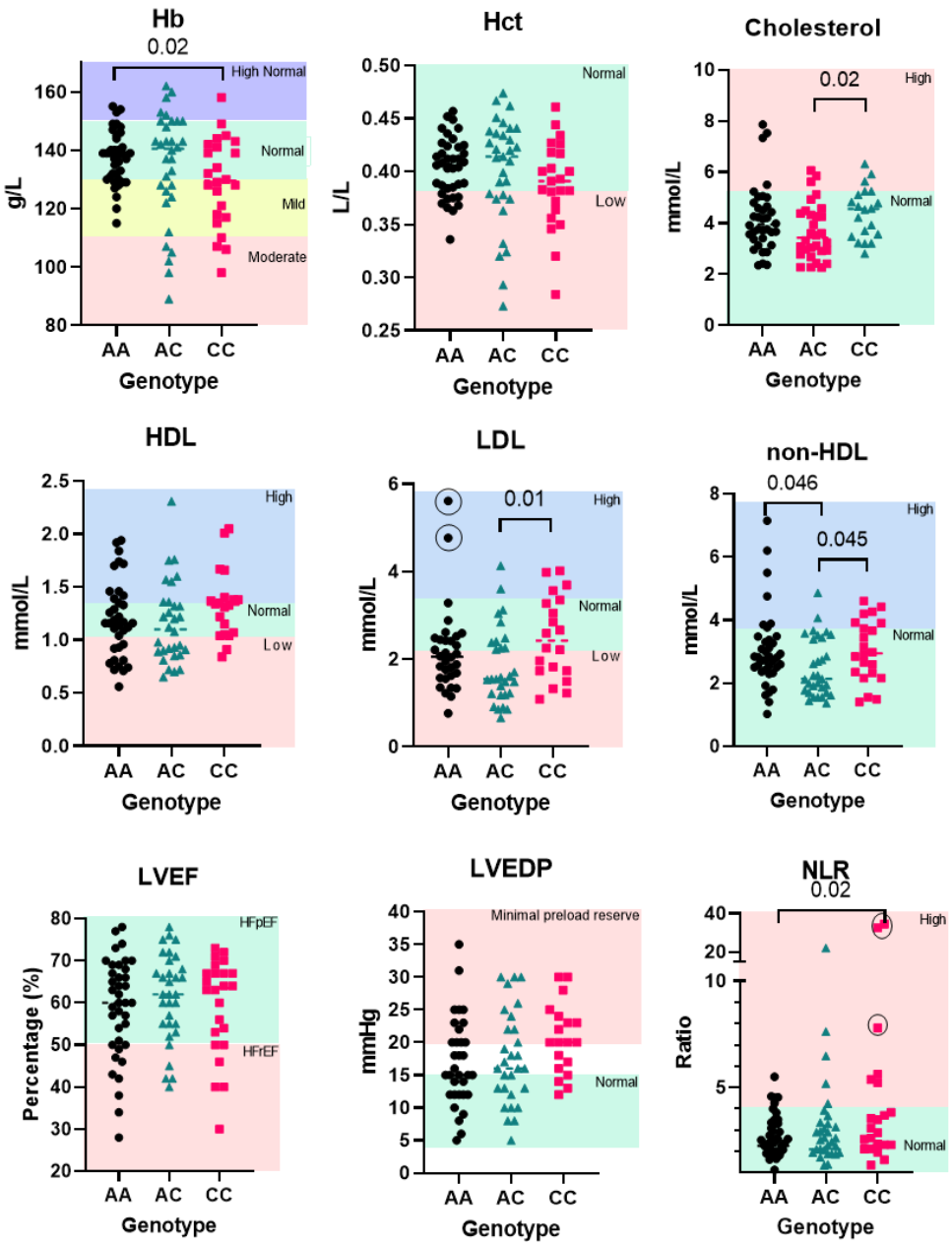

Figure 3.3- rs1617640 genotype correlation with clinical parameters.
The clinical parameter is indicated above each graph with normal and abnormal ranges indicated within the graph by background colour. Outliers removed from statistical analysis (by ROUT test or outside limits of detection of the assay) are indicated by circles. Hb=hemoglobin, Hct=hematocrit, HDL=high-density lipoprotein, LDL=low-density lipoprotein, LVEF=left ventricular ejection fraction, LVEDP=left ventricular end diastolic pressure, and NLR=neutrophile to lymphocyte ratio. Statistical significance determined by student t-test.

Table 3.5- rs1617640 association with anemia under different inheritance models.

Model	Genotype	Case	Control	OR (95% CI)	P-value
Codominant	A/A	8 (25.8%)	30 (47.6%)	1.00	0.12
	A/C	11 (35.5%)	21 (33.3%)	1.77 (0.53-5.83)	
	C/C	12 (38.7%)	12 (19.1%)	3.87 (1.05-14.32)	
Dominant	A/A	8 (25.8%)	30 (47.6%)	2.41 (0.82-7.07)	0.1
	AC-C/C	23 (74.2%)	33 (52.4%)		
Recessive	A/A-A/C	19 (61.3%)	51 (81.0%)	2.83 (0.93-8.60)	0.065
	C/C	12 (38.7%)	12 (19.1%)		
Overdominant	A/A-C/C	20 (64.5%)	42 (66.7%)	0.96 (0.35-2.60)	0.93
	A/C	11 (35.5%)	21 (33.3%)		
Log additive	A	27 (43.5%)	81 (64.3%)	1.96 (1.02-3.79)	**0.04**
	C	35 (56.5%)	45 (35.7%)		

OR=odds ratio

Table 3.6- rs1617640 association with dyslipidemia under different inheritance models.

Model	Genotype	Case	Control	OR (95% CI)	P-value
Codominant	A/A	29 (44.6%)	9 (31.0%)	1.00	0.28
	A/C	23 (35.4%)	9 (31.0%)	0.90 (0.29-2.80)	
	C/C	13 (20%)	11 (37.9%)	0.39 (0.11-01.35)	
Dominant	A/A	29 (44.6%)	9 (31.0%)	1.00	0.4
	AC-C/C	36 (55.4%)	20 (69.0%	0.65 (0.24-1.78)	
Recessive	A/A-A/C	52 (80.0%)	18 (62.1%)	1.00	0.11
	C/C	13 (20.0%)	11 (37.9%)	0.41 (0.14-1.23)	
Overdominant	A/A-C/C	42 (64.6%)	20 (69.0%)	1.00	0.56
	A/C	23 (35.4%)	9 (31.0%)	1.34 (0.50-3.60)	
Log additive	A	81 (62.3%)	27 (46.6%)	0.64 (0.34-1.19)	0.15
	C	49 (37.7%)	31 (53.4%)		

OR=odds ratio

Table 3.7- rs1617640 association with T2DM under different inheritance models.

Model	Genotype	Case	Control	OR (95% CI)	P-value
Codominant	A/A	11 (34.4%)	27 (43.5%)	1.00	0.083
	A/C	15 (46.9%)	17 (27.4%)	2.39 (0.79-7.28)	
	C/C	6 (18.8%)	18 (29.0%)	0.62 (0.16-2.36)	
Dominant	A/A	11 (34.4%)	27 (43.5%)	1.00	0.46
	AC-C/C	21 (65.6%)	35 (56.5%)	1.46 (0.53-4.01)	
Recessive	A/A-A/C	26 (81.2%)	44 (71%)	1.00	0.11
	C/C	6 (18.8%)	18 (29%)	0.39 (0.12-1.29)	
Overdominant	A/A-C/C	17 (53.1%)	45 (72.6%)	1.00	**0.035**
	A/C	15 (46.9%)	17 (27.4%)	2.88 (1.06-7.79)	
Log additive	A	37 (57.8%)	71 (57.3%)	0.88 (0.47-1.63)	0.67
	C	27 (42.2%)	53 (42.7%)		

OR=odds ratio, T2DM=Type 2 diabetes mellitus

Table 3.8- rs1617640 association with HFpEF under different inheritance models.

Model	Genotype	Case	Control	OR (95% CI)	P-value
Codominant	A/A	30 (39.5%)	8 (44.4%)	1.00	0.67
	A/C	27 (35.5%)	5 (27.8%)	1.84 (0.47-7.17)	
	C/C	19 (25.0%)	5 (27.8%)	1.49 (0.33-6.80)	
Dominant	A/A	30 (39.5%)	8 (44.4%)	1.00	0.39
	AC-C/C	46 (60.5%)	10 (55.6%)	1.70 (0.50-5.74)	
Recessive	A/A-A/C	57 (75.0%)	13 (72.2%)	1.00	0.91
	C/C	19 (25.0%)	5 (27.8%)	1.07 (0.28-4.06)	
Overdominant	A/A-C/C	49 (64.5%)	13 (72.2%)	1.00	0.46
	A/C	27 (35.5%)	4 (27.8%)	1.55 (0.47-5.17)	
Log additive	A	87 (57.2%)	21 (58.3%)	1.26 (0.58-2.73)	0.55
	C	65 (42.8%)	15 (41.7%)		

OR=odds ratio, HFpEF=Heart failure with preserved ejection fraction

Table 3.9- rs1617640 association with hypertension under different inheritance models.

Model	Genotype	Case	Control	OR (95% CI)	P-value
Codominant	A/A	28 (40.0%)	10 (41.7%)	1.00	0.8
	A/C	24 (34.2%)	8 (33.3%)	1.41 (0.43-4.64)	
	C/C	18 (25.7%)	6 (25.0%)	1.49 (0.37-6.04)	
Dominant	A/A	28 (40.0%)	10 (41.7%)	1.00	0.51
	AC-C/C	42 (60.0%)	14 (58.3%)	1.44 (0.49-4.26)	
Recessive	A/A-A/C	52 (74.3%)	18 (75.0%)	1.00	0.73
	C/C	18 (25.7%)	6 (25.0%)	1.24 (0.36-4.29)	
Overdominant	A/A-C/C	46 (65.7%)	16 (66.7%)	1.00	0.73
	A/C	24 (34.3%)	8 (33.3%)	1.21 (0.42-3.48)	
Log additive	A	80 (57.1%)	28 (58.3%)	1.24 (0.62-2.48)	0.54
	C	60 (42.9%)	20 (41.7%)		

OR=odds ratio

recessive, overdominant and log additive) in a regression analysis adjusted for sex, age, and BMI. In anemia the C allele was associative with a log additive model, suggesting that more C alleles are a risk factor for anemia. For T2DM, the over-dominant model showed a significant correlation, suggesting that heterozygotes may be at a higher risk of T2DM. There were no correlations between rs1617640 and dyslipidemia, HFpEF or hypertension. Taken together, this suggests that the rs1617640 genotype may be a risk factor in anemia (C allele) and T2DM (heterozygotes).

To explain the observed variation in clinical parameters and phenotype we determined EPO plasma concentrations, along with transferrin, ferritin and hepcidin using an ELISA assay. EPO was significantly reduced in the CC genotype compared to AA and AC (Figure 3.4). This suggests the C allele circulates less EPO compared to the A allele. An alteration in EPO production by the C allele would likely be the cause of the clinical phenotypes we observed.

3.2.5 rs507392 correlations

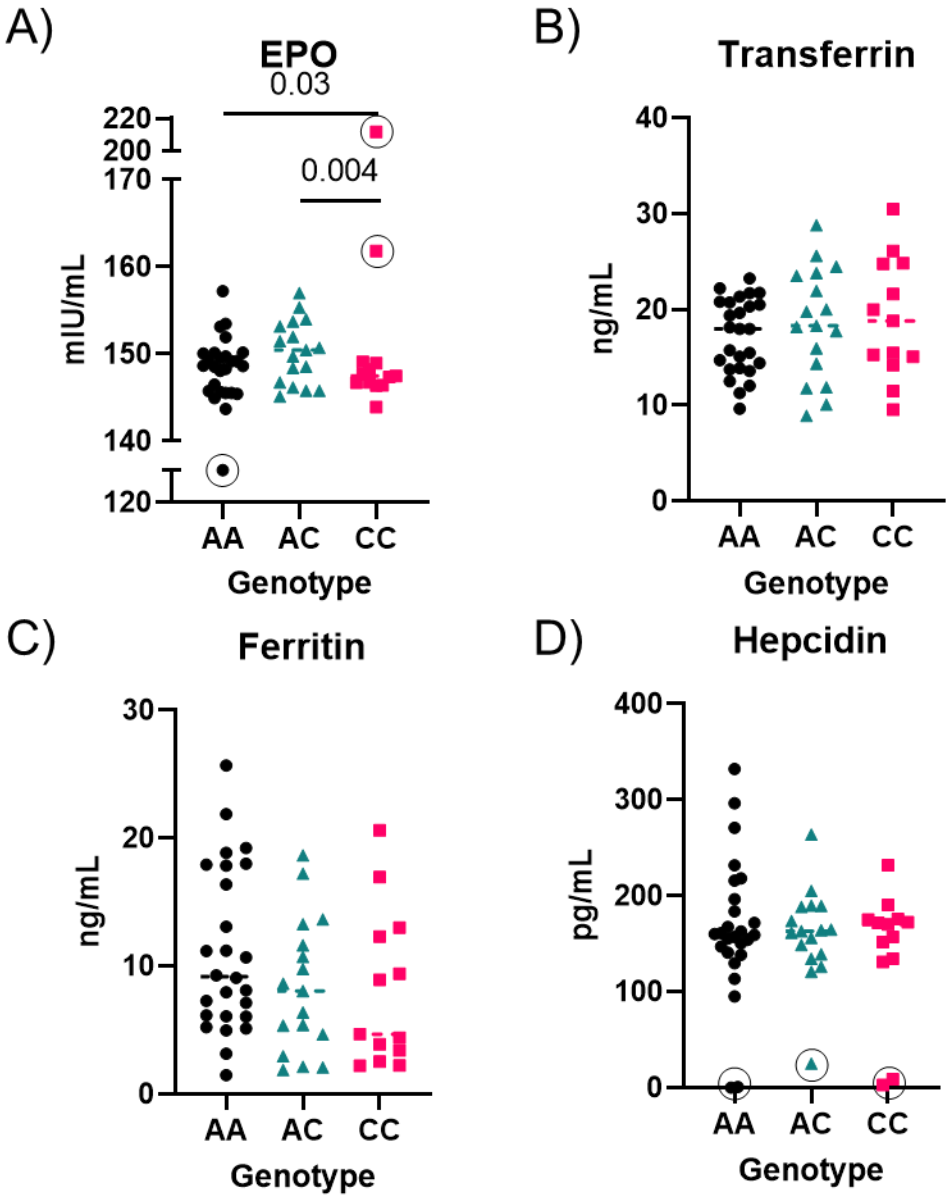

Figure 3.4- rs1617640 associations with levels of A) EPO, B) Transferrin, C) Ferritin, and D) Hepcidin.
Outliers removed from statistical analysis (by ROUT test or outside limits of detection of the assay) are indicated by circles. Statistical significance was determined by Two-way ANOVA with a p-value <0.05 being significant.

The demographics and clinical parameters compared to the genotype of rs507392 are shown in Table 3.10. A GG genotype correlated to higher obese category compared to AA, but no significant difference between age, sex, or NYHA classification. There were no significant changes in hemoglobin observed in rs1617640 but there were significant changes in cholesterol, LDL, and non-HDL lipoproteins similar to those seen in rs1617640. We did not observe a significant change in the NLR as seen in rs1617640 (Figure 3.5). This suggest that rs507392 potentially affects lipoprotein metabolism.

We next examined if rs507392 correlated with the clinical phenotypes of anemia (Table 3.11), dyslipidemia (Table 3.12), T2DM (Table 3.13), HFpEF (Table 3.14) or hypertension (Table 3.15). For each phenotype we examined five inheritance models (codominant, dominant, recessive, overdominant and log additive) in a regression analysis adjusting for sex, age, and BMI using SNPstats. rs507392 is significant in a recessive model with dyslipidemia, suggesting a GG genotype is potentially protective against dyslipidemia. There were no significant correlations with anemia, T2DM, HFpEF, or hypertension.

To examine potential mechanisms for the dyslipidemia associated with rs507392 we examined serum levels of EPO, transferrin, ferritin and hepcidin using ELISA (Figure 3.6). There was a significant decrease in EPO in the GG phenotype compared to either AA or AG. This suggests that a decrease in EPO caused by the GG genotype may be a protective for dyslipidemia.

3.2.6 rs551238 correlations

Demographics and clinical parameters associated with genotypes of rs551238 are

Table 3.10- rs507392 demographics and clinical parameters

	Genotype			p-value		
	AA (n=36)	AG (n=31)	GG (n=19)	AA-AG	AA-GG	AG-GG
Age (years)	63.8 ± 6.1	63.4 ± 7.3	63.9 ± 6.9	0.89	0.86	0.80
Males	29 (81%)	21 (68%)	7 (37%)	0.22	0.15	0.73
Females	7 (19%)	10 (32%)	12 (63%)			
BMI (kg/m^2)	29.4 ± 5.1	31.1 ± 6.3	32.1 ± 6.6	0.23	0.10	0.60
Normal	7 (19%)	5 (16%)	2 (11%)	0.29	**0.036**	0.71
Pre-obese	13 (36%)	11 (35%)	7 (37%)			
Obese 1	12 (33%)	6 (19%)	2 (11%)			
Obese 2	3 (8%)	4 (13%)	5 (26%)			
Obese 3	1 (3%)	5 (16%)	3 (16%)			
NYHA						
1	8 (22%)	6 (19%)	4 (21%)	0.68	0.79	0.63
2	15 (42%)	10 (32%)	9 (47%)			
3	8 (22%)	11 (35%)	5 (26%)			
4	5 (14%)	4 (13%)	1 (5%)			
LVEF (%)	59.0 ± 12.0	61.4 ± 10.3	60.5 ± 11.4	0.38	0.66	0.76
LVEDP (mmHg)	17.1 ± 6.7	17.7 ± 7.1	20.4 ± 4.9	0.75	0.09	0.18
Hemoglobin (g/L)	138.0 ± 8.8	137.1 ± 16.8	133.1 ± 13.1	0.78	0.11	0.39
Hematocrit (L/L)	0.41 ± 0.03	0.41 ± 0.05	0.40 ± 0.03	0.96	0.32	0.51
HbA1c (%)	6.2 ± 1.2	5.9 ± 0.8	5.9 ± 0.7	0.20	0.25	0.92
Cholesterol (mmol/L)	4.2 ± 1.3	3.7 ± 1.0	4.6 ± 0.9	0.07	0.33	**0.005**
HDL (mmol/L)	1.2 ± 0.4	1.2 ± 0.4	1.3 ± 0.3	0.86	0.14	0.11
LDL (mmol/L)	2.2 ± 0.9	1.8 ± 0.8	2.6 ± 0.9	0.11	0.16	**0.005**
LDL/HDL	1.9 ± 0.8	1.6 ± 0.7	2.0 ± 0.7	0.15	0.51	0.051
Non-HDL (mmol/L)	3.1 ± 1.2	2.5 ± 0.9	3.2 ± 0.9	**0.046**	0.63	**0.011**
TG (mmol/L)	1.9 ± 1.4	1.4 ± 0.7	1.4 ± 0.6	0.057	0.11	0.98
RBC (x10^12/L)	4.5 ± 0.3	4.5 ± 0.5	4.4 ± 0.4	0.92	0.54	0.63
WBC (x10^9/L)	7.2 ± 2.1	7.1 ± 2.4	7.9 ±2.3	0.88	0.25	0.25
Platlets (x10^9/L)	208.0 ± 47.5	206.2 ± 63.2	202.4 ± 52.1	0.89	0.69	0.83
Neutrophiles (x10^9/L)	4.6 ± 1.7	4.7 ± 2.3	5.5 ± 2.4	0.85	0.09	0.22
Lymphocytes (x10^9/L)	1.8 ± 0.6	1.7 ± 0.6	1.6 ± 0.8	0.56	0.48	0.78
NLR	2.8 ± 1.0	3.3 ± 3.6	6.3 ± 9.4	0.36	**0.031**	0.12
Troponin (ng/L)	782.2 ± 523.4	795.3 ± 663.0	859.6 ± 631.2	0.93	0.64	0.74
Creatine (mmol/L)	86.1 ± 17.4	81.8 ± 25.2	91.6 ± 30.1	0.42	0.40	0.23
Urea (mmol/L)	6.4 ± 1.9	6.4 ± 1.7	7.2 ± 2.9	0.99	0.23	0.23

NYHA=New York Heart Association, BMI=Body mass index, HDL=high-density lipoprotein, LDL=low-density lipoprotein, LVEF Left ventricular ejection fraction, LVEDP=left ventricular end diastolic pressure, HFpEF=Heart failure with preserved ejection fraction, T2DM=type 2 diabetes mellitus, TG=triglycerides, RBC=red blood cells, WBC=white blood cells (leukocytes), Neutro=Neutrophiles, Lympho= Lymphocytes, NLR=Neutrophiles Lymphocytes ratio. p-value for continuous variable (BMI, LVEF, LVEDP, Hemoglobin, Hematocrit, HbA1c, Cholesterol, HDL, LDL, Triglycerides, RBC, Troponin, Creatine, WBC, Neutrophiles, Lymphocytes, NLR, Urea) expressed as mean ±SD, p-value calculated using student t-test. Categorical variable (Sex, BMI Class, and NYHA expressed as number of cases (percentage of group), p-value calculated using Chi-square test. Significant p-value of <0.05 are bolded.

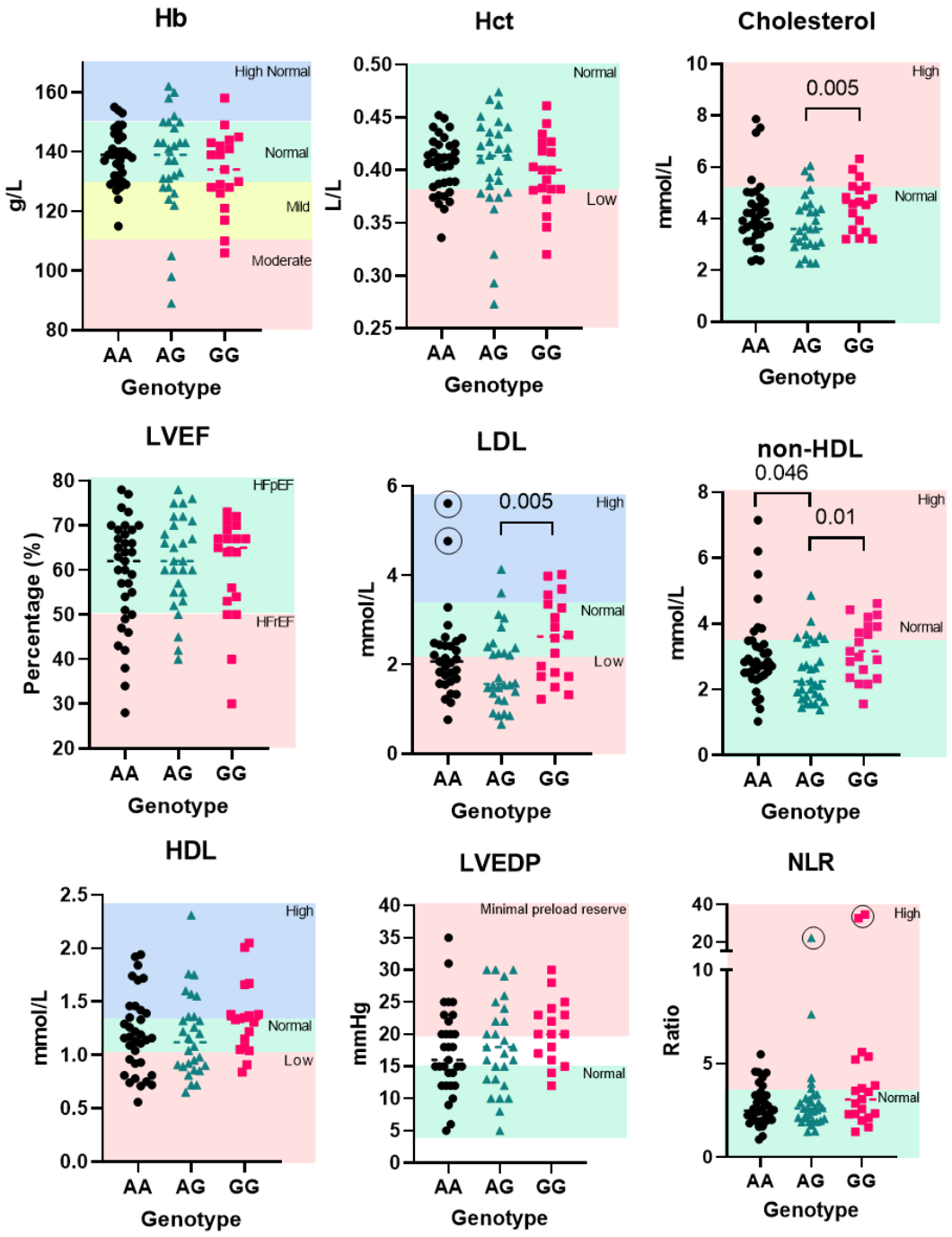

Figure 3.5- rs507392 genotype correlations with clinical parameters.
The clinical parameter is indicated above each graph with normal and abnormal ranges indicated within the graph by background colour. Outliers removed from statistical analysis (by ROUT test or outside limits of detection of the assay) are indicated by circles. Hb=hemoglobin, Hct=hematocrit, HDL=high-density lipoprotein, LDL=low-density lipoprotein, LVEF=left ventricular ejection fraction, LVEDP=left ventricular end diastolic pressure, and NLR=neutrophile to lymphocyte ratio. Statistical significance determined by student t-test.

Table 3.11- rs507392 association with anemia under different inheritance models

Model	Genotype	Case	Control	OR (95% CI)	P-value
Codominant	A/A	7 (30.4%)	29 (46.0%)	1.00	0.27
	A/G	8 (34.8%)	23 (36.5%)	1.34 (0.37-4.84)	
	G/G	8 (34.8%)	11 (17.5%)	3.02 (0.75-12.11)	
Dominant	A/A	7 (30.4%)	29 (46.0%)	1.00	0.29
	A/G-G-G	16 (69.6%)	34 (54.0%)	1.85 (0.59-5.81)	
Recessive	A/A-A-G	15 (65.2%)	52 (82.5%)	1.00	0.12
	G/G	8 (34.8%)	11 (17.5%)	2.58 (0.78-8.55)	
Overdominant	A/A-G/G	15 (65.2%)	40 (63.5%)	1.00	0.73
	A/G	8 (34.8%)	23 (36.5%)	0.83 (0.28-2.47)	
Log additive	A	22 (47.8%)	81 (64.3%)	1.73 (0.86-3.46)	0.12
	G	24 (52.2%)	45 (35.7%)		

OR=odds ratio

Table 3.12- rs507392 association with dyslipidemia under different inheritance models

Model	Genotype	Case	Control	OR (95% CI)	P-value
Codominant	A/A	28 (45.9%)	8 (32.0%)	1.00	0.13
	A/G	23 (37.7%)	8 (32.0%)	0.80 (0.24-2.65)	
	G/G	10 (16.4%)	9 (36.0%)	0.26 (0.07-1.03)	
Dominant	A/A	28 (45.9%)	8 (32.0%)	1.00	0.25
	A/G-G-G	33 (54.1%)	17 (68.0%)	0.54 (0.18-1.55)	
Recessive	A/A-A-G	51 (83.6%)	16 (64.0%)	1.00	**0.048**
	G/G	10 (16.4%)	9 (36.0%)	0.29 (0.09-1.00)	
Overdominant	A/A-G/G	38 (62.3%)	17 (68.0%)	1.00	0.6
	A/G	23 (37.7%)	8 (32.0%)	1.32 (0.46-3.80)	
Log additive	A	79 (64.8%)	24 (48.0%)	0.53 (0.27-1.05)	0.066
	G	43 (35.2%)	26 (52.0%)		

OR=odds ratio

Table 3.13- rs507392 association with T2DM under different inheritance models

Model	Genotype	Case	Control	OR (95% CI)	P-value
Codominant	A/A	11 (37.9%)	25 (43.9%)	1.00	0.41
	A/G	13 (44.8%)	18 (31.6%)	1.53 (0.48-4.91)	
	G/G	5 (17.2%	14 (24.6%)	0.60 (0.14-2.56)	
Dominant	A/A	11 (37.9%)	25 (43.9%)	1.00	0.84
	A/G-G-G	18 (62.1%)	32 (56.1%)	1.12 (0.38-3.27)	
Recessive	A/A-A-G	24 (82.8%)	43 (75.4%)	1.00	0.26
	G/G	5(17.2%)	14 (24.6%)	0.48 (0.13-1.77)	
Overdominant	A/A-G/G	16 (55.2%)	39 (68.4%)	1.00	0.25
	A/G	13 (44.8%)	18 (31.6%)	1.84 (0.65-5.25)	
Log additive	A	35 (60.3%)	68 (59.6%)	0.84 (0.42-1.68)	0.63
	G	23 (39.7%)	46 (40.4%)		

OR=odds ratio, T2DM=Type 2 diabetes mellites

Table 3.14- rs507392 association with HFpEF under different inheritance models.

Model	Genotype	Case	Control	OR (95% CI)	P-value
Codominant	A/A	28 (38.9%)	8 (57.1%)	1.00	0.36
	A/G	27 (37.5%)	4 (28.6%)	2.14 (0.50-9.11)	
	G/G	17 (23.6%)	2 (14.3%)	3.30 (0.50-21.92)	
Dominant	A/A	28 (38.9%)	8 (57.1%)	1.00	0.17
	A/G-G-G	44 (61.1%)	6 (42.9%)	2.47 (0.65-9.33)	
Recessive	A/A-A-G	55 (76.4%)	12 (85.7%)	1.00	0.33
	G/G	17 (23.6%)	2 (14.3%)	2.27 (0.39-13.07)	
Overdominant	A/A-G/G	45 (62.5%)	10 (71.4%)	1.00	0.57
	A/G	27 (37.5%)	4 (28.6%)	1.46 (0.39-5.47)	
Log additive	A	83 (57.6%)	20 (71.4%)	1.88 (0.75-4.70)	0.16
	G	61 (42.4%)	8 (28.6%)		

OR=odds ratio, HFpEF=Heart failure with preserved ejection fraction

Table 3.15- rs507392 association with hypertension under different inheritance models.

Model	Genotype	Case	Control	OR (95% CI)	P-value
Codominant	A/A	27 (40.3%)	9 (47.4%)	1.00	0.73
	A/G	25 (37.3%)	6 (31.6%)	1.62 (0.45-5.85)	
	G/G	15 (22.4%)	4 (21.1%)	1.55 (0.33-7.37)	
Dominant	A/A	27 (40.3%)	9 (47.4%)	1.00	0.43
	A/G-G-G	40 (59.7%)	10 (52.6%)	1.60 (0.50-5.14)	
Recessive	A/A-A-G	52 (77.6%)	15 (79.0%)	1.00	0.79
	G/G	15 (22.4%)	4 (21.1%)	1.21 (0.29-4.94)	
Overdominant	A/A-G/G	42 (62.7%)	13 (68.4%)	1.00	0.58
	A/G	25 (37.3%)	6 (31.6%)	1.39 (0.4304.45)	
Log additive	A	79 (59.0%)	24 (63.2%)	1.29 (0.60-2.81)	0.51
	G	55 (41.0%)	14 (36.8%)		

OR=odds ratio

shown in Table 3.16. There was a significant difference between TG and GG for total cholesterol and for non-HDL lipoproteins between TT-GG, but otherwise many of the associations seen with rs1617640 and rs507392 were not seen in rs551238 (Figure 3.7). This suggest that rs551238 is not as highly correlated with lipoprotein metabolism.

We examined if rs551238 correlated with the clinical phenotypes of anemia (Table 3.17), dyslipidemia (Table 3.18), T2DM (Table 3.19), HFpEF (Table 3.20) or hypertension (Table 3.21). For each phenotype we examined five inheritance models (codominant, dominant, recessive, overdominant and log additive) in a regression analysis adjusting for sex, age and BMI using SNPstats. rs551238 did not correlate with any of the disease phenotypes we tested. This suggest that rs551238 is not associated with anemia, dyslipidemia, T2DM, HFpEF of hypertension in this subpopulation.

To examine if rs551238 affected serum levels of EPO, transferrin, ferritin or hepcidin they were measured by ELISA and correlated with genotype. rs551238 did not show a significant change in EPO, transferrin, ferritin or hepcidin (Figure 3.8). Taken

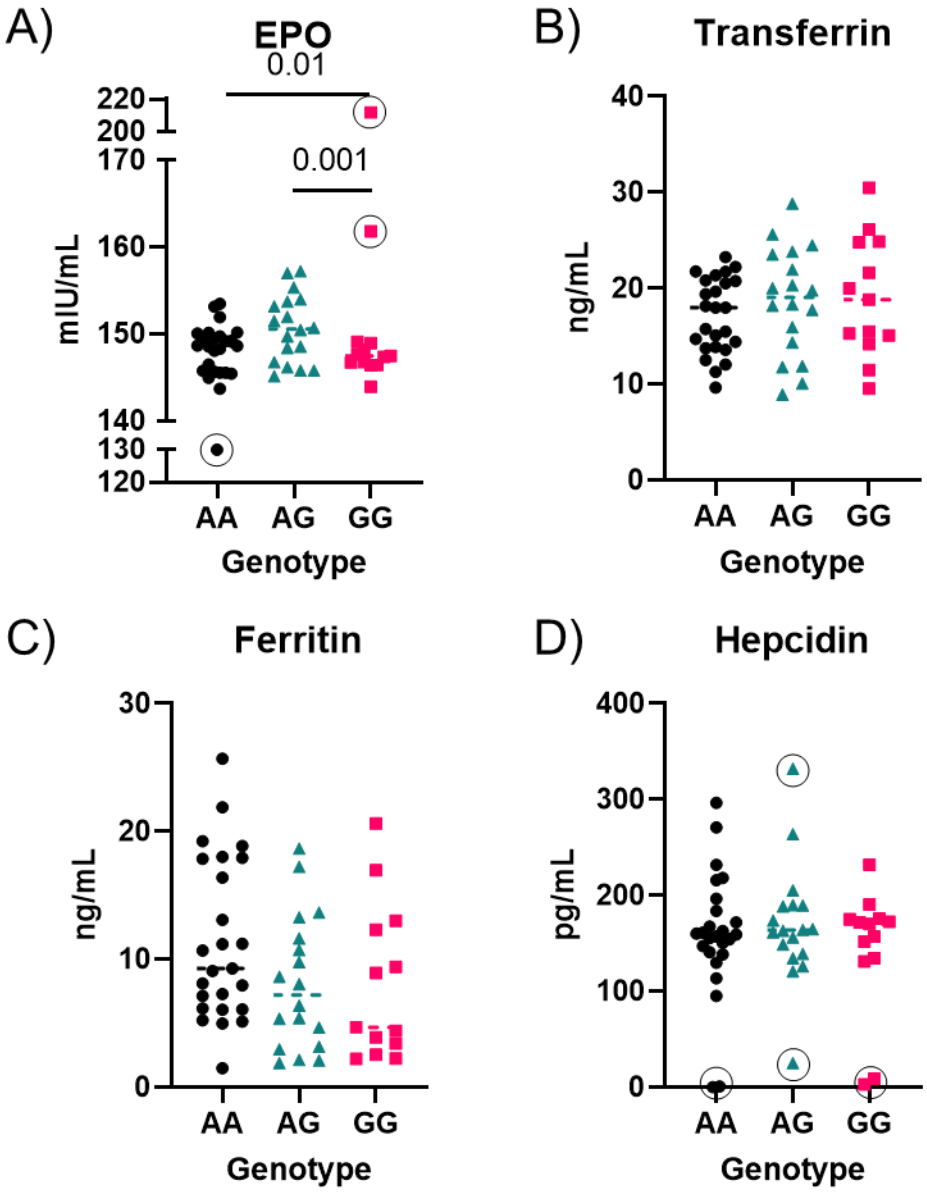

Figure 3.6- rs507392 genotype association with levels of A) EPO, B) Transferrin, C) Ferritin, and D) Hepcidin.
Outliers removed from statistical analysis (by ROUT test or outside limits of detection of the assay) are indicated by circles. Statistical significance was determined by Two-way ANOVA with a p-value of <0.05 being significant.

Table 3.16- rs551238 demographics and clinical parameters

	Genotypes			p-value		
	TT	TG	GG	TT-TG	TT-GG	TG-GG
Age (years)	63.9 ± 6.0	63.4 ± 7.1	63.6 ± 7.3	0.74	0.89	0.90
Males	32 (80%)	20 (65%)	11 (65%)	0.14	0.21	0.98
Females	8 (20%)	11 (35%)	6 (35%)			
BMI (kg/m^2)	30.0 ± 6.0	31.3 ± 6.6	32.9 ± 6.5	0.38	0.11	0.44
Normal	7 (18%)	5 (16%)	2 (12%)	0.19	0.06	0.19
Pre-obese	14 (35%)	11 (35%)	5 (29%)			
Obese 1	14 (35%)	5 (16%)	2 (12%)			
Obese 2	3 (8%)	4 (13%)	5 (29%)			
Obese 3	2 (5%)	6 (19%)	3 (18%)			
NYHA						
1	9 (23%)	7 (23%)	2 (12%)	0.88	0.83	0.83
2	16 (41%)	10 (32%)	8 (47%)			
3	9 (23%)	9 (29%)	6 (35%)			
4	5 (13%)	5 (16%)	1 (6%)			
LVEF (%)	59.7 ± 11.8	61.7 ± 10.1	58.9 ± 11.5	0.45	0.82	0.39
LVEDP (mmHg)	16.8 ± 6.5	18.1 ± 7.1	19.9 ± 5.1	0.42	0.11	0.42
Hemoglobin (g/L)	139.1 ± 9.4	136.0 ± 15.5	131.9 ± 15.6	0.31	0.04	0.40
Hematocrit (L/L)	0.41 ± 0.03	0.40 ± 0.04	0.39 ± 0.04	0.48	0.11	0.46
HbA1c (%)	6.3 ± 1.3	5.9 ± 0.8	5.9 ± 0.8	0.17	0.26	0.97
Cholesterol (mmol/L)	4.2 ± 1.2	3.7 ± 1.1	4.4 ± 1.0	0.06	0.57	**0.031**
HDL (mmol/L)	1.2 ± 0.4	1.2 ± 0.4	1.3 ± 0.3	0.77	0.13	0.09
LDL (mmol/L)	2.2 ± 0.9	1.9 ± 0.9	2.4 ± 0.9	0.14	0.45	0.06
LDL/HDL	1.9 ± 0.8	1.6 ± 0.6	1.9 ± 0.8	0.12	0.97	0.21
Non-HDL (mmol/L)	3.1 ± 1.2	2.5 ± 0.9	3.1 ± 1.0	**0.045**	0.97	0.06
TG(mmol/L)	1.9 ± 1.4	1.4 ± 0.7	1.4 ± 0.5	0.07	0.18	0.88
RBC (x10^12/L)	4.5 ± 0.3	4.5 ± 0.5	4.4 ± 0.5	0.83	0.16	0.35
WBC (x10^9/L)	7.2 ± 2.1	7.1 ± 2.4	8.0 ± 2.5	0.89	0.23	0.25
Platelets (x10^9/L)	206.6 ± 46.0	211.9 ± 63.5	188.6 ± 47.4	0.68	0.19	0.20
Neutrophiles (x10^9/L)	4.6 ± 1.6	4.7 ±2.3	5.6 ± 2.6	0.76	0.08	0.23
Lymphocytes (x10^9/L)	1.8 ± 0.6	1.6 ± 0.6	1.6 ± 0.8	0.32	0.35	0.83
NLR	2.7 ± 1.0	3.5 ± 3.6	6.7 ± 9.9	0.22	**0.018**	0.11
Troponin (ng/L)	769.9 ± 505.5	858.2 ± 704.0	822.6 ± 563.5	0.56	0.73	0.86
Creatine (mmol/L)	85.5 ± 17.0	80.8 ± 24.8	94.6 ± 31.3	0.35	0.17	0.10
Urea (mmol/L)	6.4 ± 1.9	6.3 ± 1.9	7.5 ± 3.0	0.81	0.12	0.12

NYHA=New York Heart Association, BMI=Body mass index, HDL=high-density lipoprotein, LDL=low-density lipoprotein, LVEF=Left ventricular ejection fraction, LVEDP=left ventricular end diastolic pressure, HFpEF=Heart failure with preserved ejection fraction, T2DM=type 2 diabetes mellitus, TG=triglycerides, RBC=red blood cells, WBC=white blood cells (leukocytes), Neutro=Neutrophiles, Lympho= Lymphocytes, NLR=Neutrophiles Lymphocytes ratio. p-value for continuous variable (BMI, LVEF, LVEDP, Hemoglobin, Hematocrit, HbA1c, Cholesterol, HDL, LDL, Triglycerides, RBC, Troponin, Creatine, WBC, Neutrophiles, Lymphocytes, NLR, Urea) expressed as mean ±SD, p-value calculated using t-test. Categorical variable (Sex, BMI Class, and NYHA) expressed as number of cases (percentage of group), p-value calculated using Chi-square test. Significant p-value of <0.05 are bolded.

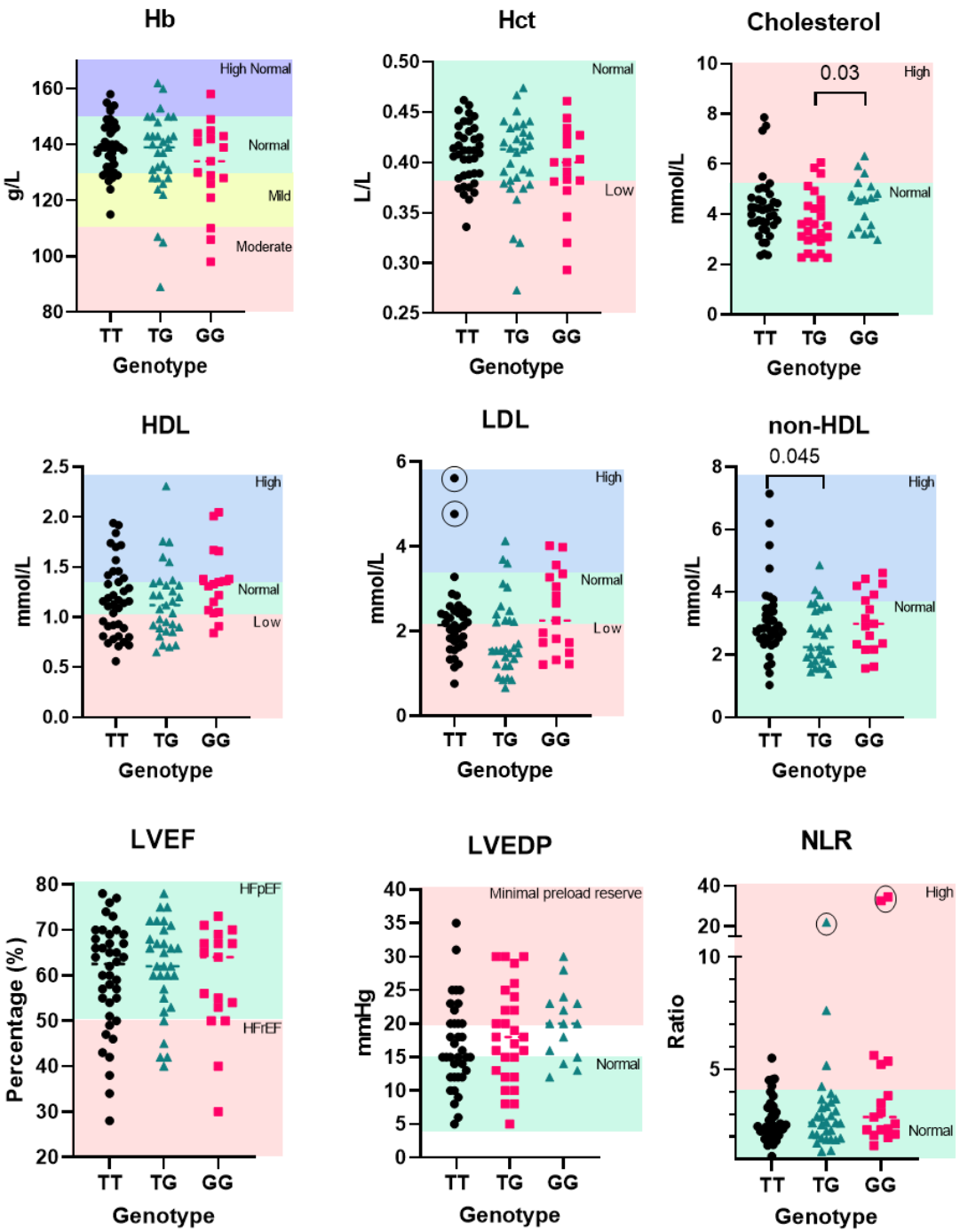

Figure 3.7- rs551238 genotype correlations with clinical parameters.
The clinical parameter is indicated above each graph with normal and abnormal ranges indicated within the graph by background colour. Outliers removed from statistical analysis (by ROUT test or outside limits of detection) are indicated by circles. Hb=hemoglobin, Hct=hematocrit, HDL=high-density lipoprotein, LDL=low-density lipoprotein, LVEF=left ventricular ejection fraction, LVEDP=left ventricular end diastolic pressure, and NLR=neutrophile to lymphocyte ratio. Statistical significance determined by student t-test.

Table 3.17- rs551238 association with anemia under different inheritance models

Model	Genotype	Cases	Control	OR (95% CI)	P-value
Codominant	T/T	8 (33.3%)	32 (50.0%)	1.00	0.36
	T/G	9 (37.5%)	22 (34.3%)	1.38 (0.40-4.74)	
	G/G	7 (29.2%)	10 (15.6%)	2.72 (0.68-10.83)	
Dominant	T/T	8 (33.3%)	32 (50%)	1.00	0.3
	T/G-G/G	16 (66.7%)	32 (50%)	1.78 (0.59-5.37)	
Recessive	T/T-T/G	17 (70.8%)	54 (84.4%)	1.00	0.18
	G/G	7 (29.2%)	10 (15.6%)	2,32 (0.68-7.97)	
Overdominant	T/T-G/G	15 (62.5%)	42 (65.6%)	1.00	0.91
	T/G	9 (37.5%)	22 (34.4%)	0.94 (0.31-2.80)	
Log additive	T	25 (52.1%)	86 (67.2%)	1.63 (0.81-3.25)	0.17
	G	23 (47.9%)	42 (32.8%)		

OR=odds ratio

Table 3.18- rs551238 association with dyslipidemia under different inheritance models

Model	Genotype	Case	Control	OR (95% CI)	P-value
Codominant	T/T	30 (38.5%)	10 (38.5%)	1.00	0.28
	T/G	23 (37.2%)	8 (30.8%)	1.08 (0.34-3.46)	
	G/G	9 (14.5%)	8 (30.8%)	0.38 (0.10-1.48)	
Dominant	T/T	30 (48.4%)	10 (38.5%)	1.00	0.59
	T/G-G/G	32 (51.6%)	16 (61.5%)	0.75 (0.27-2.12)	
Recessive	T/T-T/G	53 (85.5%)	18 (69.2%)	1.00	0.11
	G/G	9 (14.5%)	8 (30.8%)	0.26 (0.11-1.27)	
Overdominant	T/T-G/G	39 (62.9%)	18 (69.2%)	1.00	0.45
	T/G	23 (37.1%)	8 (30.8%)	1.50 (0.52-4.32)	
Log additive	T	83 (66.9%)	28 (53.8%)	0.66 (0.33-1.30)	0.23
	G	41 (33.1%)	24 (46.2%)		

OR=odds ratio

Table 3.19- rs551238 association with T2DM under different inheritance models

Model	Genotype	T2DM	Control	OR (95% CI)	P-value
Codominant	T/T	13 (41.9%)	27 (47.4%)	1.00	0.5
	T/G	13 (41.9%)	18 (31.6%)	1.56 (0.50-4.90)	
	G/G	5 (16.1%)	12 (21.1%)	0.69 (0.16-2.92)	
Dominant	T/T	13 (41.9%)	27 (47.4%)	1.00	0.74
	T/G-G/G	18 (58.1%)	30 (52.6%)	1.20 (0.42-3.40)	
Recessive	T/T-T/G	26 (83.9%)	45 (79%)	1.00	0.38
	G/G	5 (16.1%)	12 (21.1%)	0.55 (0.15-2.08)	
Overdominant	T/T-G/G	18 (58.1%)	39 (68.4%)	1.00	0.29
	T/G	13 (41.9%)	18 (31.6%)	1.76 (0.62-5.02)	
Log additive	T	39 (62.9%)	72 (63.2%)	0.92 (0.46-1.82)	0.8
	G	23 (37.1%)	42 (36.8%)		

OR=odds ratio, T2DM=Type 2 diabetes mellitus

Table 3.20- rs551238 association with HFpEF under different inheritance models

Model	Genotype	Case	Control	OR (95% CI)	P-value
Codominat	T/T	31 (42.5%)	9 (60.0%)	1.00	0.27
	T/G	27 (37.0%)	4 (26.7%)	2.44 (0.57-10.40)	
	G/G	15 (20.6%)	2 (13.3%)	2.61 (0.38-17.93)	
Dominant	T/T	31 (42.5%)	9 (60.0%)	1.00	0.12
	T/G-G/G	42 (57.5%)	6 (40.0%)	2.80 (0.74-10.62)	
Recessive	T/T-T/G	58 (79.5%)	12 (85.7%)	1.00	0.3
	G/G	15 (20.6%)	2 (14.3%)	2.03 (0.34-12.13)	
Overdominant	T/T-G/G	47 (63.5%)	11 (73.3%)	1.00	0.48
	T/G	27 (36.5%)	4 (25.7%)	1.59 (0.43-5.90)	
Log additive	T	89 (61.0%)	22 (73.3%)	1.62 (0.65-4.08)	0.11
	G	57 (39.0%)	8 (26.7%)		

OR=odds ratio, HFpEF=Heart failure with preserved ejection fraction

Table 3.21- rs551238 association with hypertension under different inheritance models

Model	Genotype	Hypertension	Control	OR (95% CI)	P-value
Codominant	T/T	29 (43.3%)	11 (52.4%)	1.00	0.54
	T/G	24 (35.8%)	7 (33.3%)	1.63 (0.48-5.48)	
	G/G	14 (20.9%)	3 (14.3%)	2.33 (0.45-12.16)	
Dominant	T/T	29 (43.3%)	11 (52.4%)	1.00	0.3
	T/G-G/G	38 (56.7%)	10 (47.6%)	1.80 (0.58-5.59)	
Recessive	T/T-T/G	53 (79.1%)	18 (85.7%)	1.00	0.43
	G/G	14 (20.9%)	3 (14.3%)	1.81 (0.39-8.30)	
Overdominant	T/T-G/G	43 (64.2%)	14 (66.7%)	1.00	0.68
	T/G	24 (35.8%)	7 (33.3%)	1.27 (0.41-3.88)	
Log additive	T	82 (61.2%)	29 (69.0%)	1.55 (0.70-3.40)	0.27
	G	52 (38.8%)	13 (31.0%)		

OR=odds ratio

together, rs551238 does not affect EPO concentrations and does not correlate with anemia, dyslipidemia, T2DM, HFpEF or hypertension in this subpopulation.

3.2.7 Haplotype correlations

The haplotype examines alleles in all three SNPs in combination (i.e. A allele from rs1617640, A allele from rs507392 and T allele from rs551238 are written as AAT). Since our SNPs are in linkage disequilibrium, haplotype information may provide additional information over the individual SNPs. We used haplotype analysis to examine links of haplotype with the clinical outcomes. The predominant haplotypes of AAT and CGG did not correlate with any clinical phenotype. However, one of the rare haplotypes of CGT, which occurred in approximately 3% of our sample did correlate with dyslipidemia. The OR of 0.05 suggests the CGT is protective against dyslipidemia (Table 3.22). However, given the low prevalence of this haplotype this finding requires validation in a larger cohort.

3.2.8 Sex differences for SNP correlations

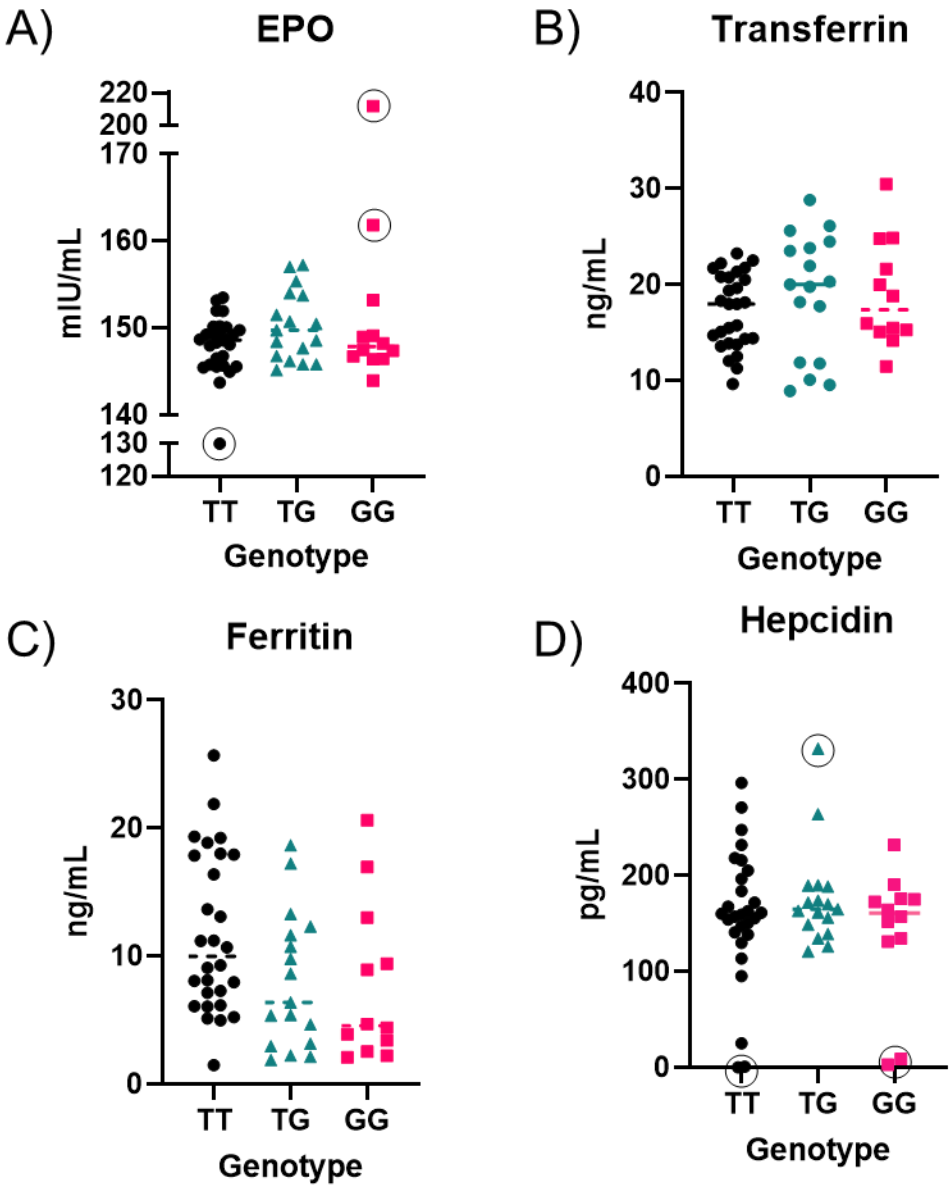

Figure 3.8- rs551238 genotype association with levels of A) EPO, B) Transferrin, C) Ferritin, and D) Hepcidin.
Outliers removed from statistical analysis (by ROUT test or outside limits of detection of assay) are indicated by circles. Statistical significance was determined by Two-way ANOVA with a p-value of <0.05 being significant.

Table 3.22- Haplotype associations with clinical phenotypes.

Haplotype	Anemia			Dyslipidemia			T2DM		
	Freq.	OR (95% CI)	p-value	Freq.	OR (95% CI)	p-value	Freq.	OR (95% CI)	p-value
AAT	0.5674	1.00	n/a	0.567	1.00	n/a	0.567	1.00	n/a
CGG	0.3837	1.98 (1.01-3.91)	0.051	0.384	0.72 (0.36-1.44)	0.36	0.384	0.99 (0.51-1.91)	0.97
CGT	0.0311	3.05 (0.38-24.72)	0.3	0.029	0.05 (0.00-0.51)	**0.014**	0.030	0.26 (0.02-2.82)	0.27
GHA p-value		0.19			**0.031**			0.8	

Table 3.22 cont.- Haplotype associations with clinical phenotypes.

Haplotype	HFpEF			Hypertension		
	Freq.	OR (95% CI)	p-value	Freq.	OR (95% CI)	p-value
AAT	0.5674	1.00	n/a	0.567	1.00	n/a
CGG	0.3826	1.51 (0.64-3.56)	0.35	0.383	1.45 (0.69-3.05)	0.33
CGT	0.292	0.68 (0.02-27.15)	0.84	0.329	0.39 (0.05-2.99)	0.37
GHA p-value		0.34			0.36	

Freq=frequency, GHA=global haplotype association, T2DM=Type 2 Diabetes mellitus, HFpEF=Heart Failure with preserved ejection fraction

Sex differences have been documented in CVD and many of the CVD risk factors that we have examined herein. The prevalence of dyslipidemia, T2DM, and hypertension are higher in males[306], whereas the prevalence of anemia[307] and HFpEF[308] is higher in females. Given the sex difference in the clinical phenotypes we are examining, we analyzed the differences in our SNPs within each sex to determine if sex differences could affect our results. The rs507392 AG and GG genotypes and rs551238 TG genotype associated with hypertension in females, but not in males (Table 3.23). This suggests in hypertension that the rs507392 and rs551238 could be a risk factors in females, but not in males.

3.2.9 Other variants detected in our population

During the analysis of the sequences obtained for rs1617640, rs507392, and rs551238 other variants were identified (Table 3.24). Many of these variants were only detected in a single individual as a heterozygote. There are some variants that are found in a few individuals with most of these also being heterozygotes. A majority of these have not previously been reported and therefore their significance is unknown. There were three variants that were found in a significant subset of our population. These include a four-base pair deletion (2167?-2170?del). This was found to be homozygous in 76 cases and suspected to be heterozygous in four. This variant is found in a repeat region in intron 3 and therefore the breakpoint may be inexact. 3154T>G was found in 83 individuals, with 77 of these being homozygotes. Lastly, 3806T>C was found in 33 individuals, with 31 of these being heterozygotes.

3.3 Discussion

The SNP rs1617640 genotype CC and rs507392 genotype GG were associated

Table 3.23- Sex differences in SNPs correlations with phenotypes

		Anemia				Dyslipidemia				T2DM			
S	Geno.	Cases	Ctrl.	OR (95% CI)	Int.	Cases	Ctrl.	OR (95% CI)	Int.	Cases	Ctrl.	OR (95% CI)	Int.
						rs1617640							
F	A/A	4	5	1.00	0.99	5	4	1.00	0.5	1	8	1.00	0.8
	A/C	6	6	1.69 (0.25-11.25)		8	4	2.19 (0.33-14.68)		4	8	4.83 (0.37-63.55)	
	C/C	6	3	4.03 (0.45-35.91)		4	5	0.82 (0.11-6.28)		1	8	0.80 (0.03-18.12)	
M	A/A	4	25	1.00		24	5	1.00		10	19	1.00	
	A/C	5	15	1.82 (0.39-8.43)		15	5	0.55 (0.13-2.33)		11	9	1.96 (0.56-6.88)	
	C/C	6	9	3.80 (0.79-18.35)		9	6	0.27 (0.06-1.27)		5	10	0.61 (0.14-2.64)	
						rs507392							
F	A/A	3	4	1.00	0.65	4	3	1.00	0.65	1	6	1.00	0.97
	A/G	3	7	0.73 (0.08-6.54)		7	3	1.88 (0.21-16.79)		2	8	1.13 (0.06-21.74)	
	G/G	4	3	3.55 (0.31-40.81)		3	4	0.43 (0.04-5.13)		1	6	0.45 (0.01-14.55)	
M	A/G	4	25	1.00		24	5	1.00		10	19	1.00	
	G/G	5	16	1.89 (0.41-8.82)		16	5	0.55 (0.13-2.33)		11	10	1.61 (0.46-5.67)	
	C/C	4	8	2.78 (0.51-15.09)		7	5	0.21 (0.04-1.11)		4	8	0.63 (0.13-3.02)	
						rs551238							
F	T/T	4	4	1.00	0.57	4	4	1.00	0.42	1	7	1.00	0.9
	T/G	4	7	0.59 (0.08-4.36)		8	3	3.22 (0.40-25.64)		3	8	2.79 (0.18-43.94)	
	G/G	3	3	1.80 (0.17-19.69)		2	4	0.55 (0.05-6.61)		1	5	1.14 (0.04-32.91)	
M	T/T	4	28	1.00		26	6	1.00		12	20	1.00	
	T/G	5	15	2.26 (0.48-10.58)		15	5	0.62 (0.15-2.52)		10	10	1.40 (0.40-4.92)	
	G/G	4	7	3.40 (0.61-18.82)		7	4	0.32 (0.06-1.72)		4	7	0.63 (0.13-3.10)	

Table 3.23 cont.- Sex differences in SNPs correlations with phenotypes

				HFpEF				Hypertension	
S	Geno.	Cases	Ctrl.	OR (95% CI)	Int.	Cases	Ctrl.	OR (95% CI)	Int.
				rs1617640					
F	A/A	7	2	1.00	0.48	3	5	1.00	0.1
	A/C	9	3	1.01 (0.11-9.48)		8	2	9.38 (0.90-98.15)	
	C/C	8	1	2.54 (0.15-43.18)		4	2	6.48 (0.47-89.67)	
M	A/A	23	6	1.00		24	5	1.00	
	A/C	18	2	2.94 (0.48-18.14)		16	4	0.50 (0.10-2.55)	
	C/C	11	4	1.13 (0.13-4.48)		12	2	0.54 (0.07-4.09)	
				rs507392					
F	A/A	5	2	1.00	0.48	2	5	1.00	**0.03**
	A/G	9	1	3.62 (0.20-66.26)		8	2	**18.48 (1.50-227.27)**	
	G/G	7	0	n/a		5	2	**15.43 (1.02-233.40)**	
M	A/G	23	6	1.00		25	4	1.00	
	G/G	18	3	1.90 (0.37-9.73)		17	4	0.51 (0.10-2.63)	
	C/C	10	2	2.01 (0.27-15.06)		10	2	0.45 (0.06-3.49)	
				rs551238					
F	T/T	5	3	1.00	0.26	2	6	1.00	**0.02**
	T/G	10	1	7.30 (0.48-111.05)		9	2	**19.16 (1.84-199.49)**	
	G/G	6	0	n/a		4	2	11.33 (0.84-153.49)	
M	T/T	26	6	1.00		27	5	1.00	
	T/G	17	3	1.67 (0.32-8.67)		15	5	0.47 (0.11-2.06)	
	G/G	9	2	1.78 (0.23-13.69)		10	1	1.11 (0.10-12.27)	

S=sex, F=female, M=male, Geno.=genotype, OR=odds ratio, Ctrl.=controls, int=interaction p-value, T2DM=Type 2 diabetes mellites, HFpEF=Heart failure with preserved ejection fraction

Table 3.24- Other variants identified by Sanger sequencing

Variants	Total	Anemia	Dyslipidemia	T2DM	HFpEF	Hypertension
-681C>T	1	0	0	0	1	0
-591C>T	12	4	8	7	11	9
-500G>T	1	1	1	1	0	1
-494T>G	1	1	1	1	0	1
-454T>C	1	1	0	0	0	0
-433T>A	1	1	0	0	0	0
-428G>A	1	1	0	0	1	1
-422G>A	2	1	1	0	1	1
673A>G	1	1	1	1	1	1
1775A>G	2	0	2	2	1	2
1789insA	3	1	2	2	3	3
1797insA	1	0	0	0	0	1
1817G>A	4	1	4	3	4	4
1826G>A	1	0	1	1	1	1
2159C>T	6	3	3	1	6	4
2163C>T	6	3	3	1	6	4
2167?-2170?del[1]	76	19	56	24	63	57
2167?-2170?del[2]	4	1	3	3	4	4
2237G>A	1	0	1	1	1	1
2374C>A	1	0	1	0	0	0
2376insC	1	1	1	0	1	1
2384C>A	1	0	1	0	1	1
2397C>T	1	0	1	1	0	1
2404G>A	1	1	1	1	1	1
3107C>T	3	1	2	1	3	2
3132insG	1	1	1	0	1	1
3136G>A	1	0	1	0	0	1
3149C>G	1	0	0	0	1	1
3150T>A	1	0	1	0	0	1
3154T>G	77	20	55	26	62	56
3154T>G	6	1	3	4	6	5
3194G>C	1	0	1	1	1	1
3289T>A	1	0	1	1	1	1
3300T>A	1	0	1	1	1	1
3324G>A	1	0	1	1	1	1
3363insT	1	0	1	0	0	1
3434C>T	4	1	3	0	3	3
3573C>A	1	0	1	1	1	1
3606insG	1	0	1	1	1	1
3786C>T	1	0	1	0	0	1
3793insC	1	0	1	0	0	1
3798C>T	1	1	0	0	1	0
3806T>C	2	0	2	0	2	2
3806T>C	31	9	23	12	26	25
3833insA	1	0	1	0	0	1
3747insA	2	0	1	1	2	2
3892G>A	2	0	1	0	1	1
3892insC	1	0	1	0	0	1
3948T>G	1	0	1	0	1	1
3976G>T	1	1	0	1	1	1

ins=insertion, del=deletion, T2DM=type 2 diabetes mellites, HFpEF=heart failure with preserved ejection fraction

[1]-deletion is heterozygous

$^{2-}$deletion is homozygous

with a lower appreciable EPO plasma level. These also correlated with comparably higher cholesterol, LDL and non-HDL lipoprotein. The rs1617640 CC also was associated with lower Hb and anemia in a log additive model; T2DM in an overdominant model. rs507392 GG genotype showed association with dyslipidemia in a recessive model. Although hypertension did not show significance in association with the SNPs, rs507392 and rs551238 were associated with hypertension amongst females.

Much of the literature for rs1617640 report the alleles based on the minus strand, whereas rs507392 and rs551238 are reported based on the positive strand. Herein, we examine the positive strand for all three SNPs and therefore the C allele herein, relates the G allele in many studies and the A allele corresponds T allele in the literature. Chiou *et al*.[286] found that the T allele (A allele herein) associated with anemia in a T2DM population. However, in our study the C allele (G allele) associated with anemia and a decreased hemoglobin. The Chiou *et al*. study was in a Chinese population with T2DM and also have a lower G allele frequency compared to the C allele in our study (0.19[286] vs.0.43, respectively). In studies that examine the EPO level the G allele tends to be associated with higher EPO levels[275,281], whereas in our study it was associated with lower EPO levels. Fan *et al*.[275] also found the rs507392 CC (GG herein) and rs551238 CC rs551238 CC (GG herein) were associated with higher EPO protein in a Chinese population of T2DM. As with the rs1617640, we found the inverse in rs551238 with GG correlating with lower EPO and no significant correlation with rs507392. This may indicate a difference between the population. Within our population we did find a higher

prevalence of the minor alleles compared to other studies. In many other studies the minor allele for each of the SNPs are under 0.3.[272,275] In our population we found the minor allele frequencies to range from 0.37 to 0.43, showing our population cohort has a higher proportion of the minor alleles. However, this difference is likely due to ethnicity differences. Many of the previous studies were in Asian populations whereas New Brunswick has predominantly European ancestry. The Ensembl database supports a lower minor allele frequency of the *EPO* SNPs in Asian compared to European populations.[309] Additionally, many of these previous studies were within a population cohort of diabetes. Our population consists of CVD patients and therefore there may be other clinical parameters we did not investigate that would also be a factor in these differences. Therefore, there may be potential population differences in these *EPO* SNPs and further studies would need to investigate these variations.

Iron bioavailability is an important contributor in two of the most common forms of anemia, iron deficiency anemia and anemia of inflammation. Anemia of inflammation is the form anemia the most associated with chronic diseases, such as CVD.[310] The rs1617640 C allele that associated with anemia, also showed an increase in the NLR. A higher NLR ratio is associated with an active inflammatory processes.[311] This suggests the C allele for rs1617640 is associated with inflammation and a decrease in Hb. It has been purported that EPO can have an indirect anti-oxidant effect by utilizing iron in erythrocytosis resulting in less bioavailable iron.[83] Patients with anemia of inflammation commonly have reduced serum iron and normal to elevated iron stores.[312] In blood, iron is bound to transferrin[313] and in macrophages and hepatocytes it is bound to ferritin.[314] Hepcidin is a hormone that reduces iron bioavailability by decreasing the release of

recycled iron.[310] The HIFs reduce hepcidin, while increasing transferrin and ferritin to increase iron bioavailability.[106] To examine if the *EPO* SNPs altered iron bioavailability we also measure hepcidin, transferrin and ferritin, but did not find any significant differences. This leads us to conclude that the SNPs in *EPO* are not exerting their effects through iron bioavailability, but we cannot at this time rule out other effects in heme metabolism.

Although rhEPO has been shown to modulate triglycerides, cholesterol and other lipoproteins,[249,299,300] *EPO* SNPs have not previously been investigated for their association to dyslipidemia. In a mouse model of CKD treatment with rhEPO, triglycerides, cholesterol and LDL were decreased, but not HDL.[299] The GG genotype of rs507392 correlated with lower EPO concentrations and had an increase in cholesterol, LDL and non-HDL, which corresponds with the results of Li *et al*.[299] These variations are also expected in dyslipidemia, which the GG genotype correlated with a protection against dyslipidemia. This contradiction may be due to unexamined parameters. Those diagnosed with dyslipidemia are likely to be prescribed statins that would lower their lipoproteins.[315] Therefore, the lipoproteins of those with dyslipidemia may be decreased below baseline in our data set due to medications. However, we did not examine use of medications, such as statins, which should be included in future studies. Although rs1617640 did not reach significance for dyslipidemia, there was some variation in cholesterol, LDL and non-HDL lipoproteins. The only difference seen in rs551238 was in cholesterol and non-HDL. Given the disequilibrium between these SNPs, these variations may be primarily associated with rs507392. Together, our results show that *EPO* SNPs influence lipid metabolism and rs507392 is associated with dyslipidemia.

This is the first time this association is being made and adds to Table 3.1 (see Table 3.25) where dyslipidemia is now established as another risk factor to be associated with variation in *EPO*.

Table 3.25- Erythropoietin SNP associations (revised).

SNP	Allele	Phenotype	References
rs1617640	G	Higher Hct in healthy blood donors	272
		Diabetic microvascular complications	273–281
		Decreased overall mortality in diabetes	282
		Higher Hb, Hct, RBC count and earlier onset PAD	283
		MDS and ALL	284
		Improved cognitive performance in Schizophrenia	285
	T	Anemia in T2DM	286
		Renal dysfunction following cardiac surgery	287
		Improved response to platinum-based chemotherapy in NSCLC	288
rs507392	C	Diabetic microvascular complications	273,275,276,289
		Decreased overall mortality in diabetes	282
		Dyslipidemia	Northrup et al. in prep
rs551238	C	Higher Hct in healthy blood donors	272
		Diabetic microvascular complications	272,273,275–278,289,290
		Preterm infant brain injury	291

Hct=hematocrit, Hb=hemoglobin, RBC=red blood cell count, PAD=peripheral artery disease, MDS=myelodysplatic syndrome, ALL=acute lymphoblastic leukemia, T2DM=type 2 diabetes mellites, NSCLC=non-small cell lung cancer

In addition to each individual SNP, we conducted analysis of the haplotype combining each of the SNPs. The GCC haplotype has been associated with diabetic retinopathy[273] and less mortality in T2DM.[282] In our study the CGG haplotype did not reach significance to any phenotype. However, the p-value for anemia was just outside the significant range (0.051). Potentially a larger sample size, which could also allow for an ANCOVA to remove other influences may yet show an association with anemia. The CGT haplotype was found to be protective against dyslipidemia. However, this was a rare haplotype found in less than 3% of our population cohort and may risk type 1 error.

Therefore, this finding should be investigated further in a larger cohort and interpreted cautiously.

Many of the clinical phenotypes we examined show sex differences in their prevalence, response to treatment, or clinical outcomes and therefore examination of sex difference in our population was imperative but limited to potential sampling bias as fewer female patients are operated upon in this cohort population. Although hypertension did not show an association with the SNPs with the sexes combined, the presence of the G allele in rs507392 and the TG genotype in rs551238 appeared to be a risk factor in females but not males. Hypertension is more prevalent in males[306] and our population did contain more males than females, which would explain why this potential association was masked in our initial analysis. The relationship between estrogen and the renin-angiotensin-aldosterone system (RAAS) is thought to be one of the main reasons for the sex differences seen in hypertension. The RAAS has also been found to be modulated by EPO. In Wistar rats rhEPO increases renin[316] and in isolated rat kidneys rhEPO promotes angiotensin II (AngII) production.[317] Also, at least in the oviduct and endometrium, estrogen (17β (E2)) can promote the expression of *EPO*.[154,318] Given the relationship between EPO and the RAAS system by estrogen, this could be a potential mechanism for why G allele of rs507392 and TG genotype of rs551238 are risk factors for hypertension in females only. Although this finding does require validation in a larger cohort, preferably with more females and direct assessment of sex hormones, this may be significant since hypertension tends to be a more a salient risk factor for CVD in females compared to males.[303]

In addition to our SNPs, we identified novel variants that have not been

previously identified. Most of these variants occurred as heterozygotes in a single individual. However, there were four that occurred in multiple individuals and are potential polymorphisms. Herein, we did not examine these polymorphisms for clinical or pathophysiological association but report them for future studies in that direction. Genetic variation in *EPO* has previously been linked to isolated families. More recently there have been more variants reported to ClinVar, which is likely due to the increase in sequencing. In clinical care, sequencing has become more prevalent with the integration of next-generation sequencing technologies into standard of care. In others disorders, this has led to an increase in variants being detected.[319] Surprisingly, *EPO* has long been overlooked on many arrays and panels, which may account for the low prevalence of *EPO* variants in the literature. Herein, we sequenced a small section of non-coding element of EPO and therefore we are unable to perform a ratio of non-synonymous to synonymous substitutions (dN/dS) to determine potential natural selection acting on *EPO*. However, since our *EPO* SNPs are not in Hardy-Weinberg equilibrium a dN/dS analysis could indicate selection acting on these SNPs. As the sequencing of *EPO* increases, we anticipate there will yet be further variants detected and potentially allow for dN/dS analysis.

Our study is not without limitations. One major limitation is the small sample size and depth of patient file analyses, which does not allow us to account for many covariates, and could affect our results, such as ethnicity, other comorbidities, medical management or medications. As we selected a small subset of the OPOS cohort sampling bias could be present in our sub-cohort. Our small sample size also results in some of our groups comprising only a few individuals. Or in the case of female HFpEF controls for

two of our SNPs, none. In that regard this study serves as a pilot to identify additional associations, prioritized further analysis for further investigation and priority screening. Our study did identify dyslipidemia as a novel disorder and therefore will direct further sequencing to include more dyslipidemia and potentially investigate the use/efficacy of statins as a covariant. Additionally, hypertension was identified in females but not males with rs507392 G allele and rs551238 TG genotype being risk factors. As we had relatively few females, our future sequencing will attempt to bias recruitment to include more females with hypertension to investigate such potential sex-differences further. Also, we were unable to account for renal function within this cohort at this time. Since decreased renal function is associated with lower EPO,[80] this should be a variable that is included in a larger sample and might be collected prospectively with greater attention. We used a ROUT test to determine outliers which assumes normal distribution. Many of our variables examined did show normal distribution as determined by Kolmogorov-Simirnov test. However, some variables such as Hb should have variation between males and female and thus should not be normally distributed. The normal distribution we see may be due to the small female cohort. Outliers could be caused by biological/population variations or by technical effects (i.e. matrix effects), which we are unable to distinguish at this time. Although our samples size is a limitation, we have been able to identify variables that will be beneficial to include in future studies with clinical relevance for screening or precision medical management.

For the first time we show a correlation with the GG genotype for rs507392 to dyslipidemia. The genotype also correlates with changes in the lipid profiles. The rs1617640 C allele was correlated in anemia, but also showed changes in lipid

metabolism and inflammation. T2DM associated in an over-dominance model with heterozygotes (TC) with rs1617640. Although there was no overall correlation with HFpEF or hypertension, the rs507392 and rs551238 SNPs correlated with hypertension in females only. Together, our results suggest SNPs in *EPO* are involved in Hb, lipid metabolism, inflammation and potentially a female specific effect on blood pressure.

Chapter 4- Erythropoietin variants in RNA

4.1 Introduction

Intron retention is a form of alternative splicing that regulates gene expression and bioavailability for translation.[34,35] Splicing removes introns from the pre-mRNA to form mature mRNA with 5' caps and poly (A) tails.[14,15] Intron-retaining transcripts retain one or more introns in their mature mRNA with a 5'cap and poly(A) tail. Although intron retention is a common form of alternative splicing in plants, fungi, and viruses, until recently intron-retaining transcripts were considered improper splicing that would result in decay of the transcript.[34,35] However, recent transcriptomics studies describe intron retention could occur in up to 80% of human genes intentionally and suggest a significant role in post-transcriptional regulation.[36,37]

The fate of intron-retaining transcripts varies. Intron-retaining transcripts can either remain within the nucleus (commonly referred to as intron detained) or be exported to the cytoplasm. The intron-detained transcripts act as a reserve of mRNA until a stimulus results in splicing to remove the final introns for extranuclear transition and translation to the canonical protein.[34,35,320] Alternatively, intron-retained transcripts are exported to the cytoplasm, where the transcript may similarly be processed to remove introns, be susceptible to nonsense-mediated decay or directly translated to form a non-canonical protein isoform (Figure 1.2).[49–53] The variable fates identified for intron-retaining transcripts suggest intentional biological functions and that intron retention regulation are important mechanisms to control gene expression and function.

Intron retention is more prevalent in brain and immune cells, although it is not

limited to these cell types. Intron-retention is more apparent in certain developmental stages of the brain of drosophila, mouse, and human.[35,36,38,39] Hematopoietic cell lines, including erythroblasts,[321] megakaryocyte progenitors,[322] granulocytes[40] and CD4+ cells,[323] have also demonstrated salient intron retention. In addition to differential intron retention in varied tissues, it has been linked to various pathologies or stress stimuli. An increase in intron retention has been linked to Alzheimer's disease and some hematological malignancies.[39,324] A decrease in intron retention has been linked to amphitropic lateral sclerosis (ALS).[325] Taken together, this suggests intron retention is an important mechanism in physiology and pathophysiology.

Intron retention increases in response to hypoxia, as Memon *et al*. [55] found an increase in intron retention in HTC116 (human colorectal carcinoma) cells in hypoxic conditions. This correlated with a decrease in protein translation, which they suggest is a mechanism to reserve transcripts for translation until the cell has more energy to invest in translation. Introns were identified as important in nutrient deprivation in yeast.[56] As such, there is potential to use intron retention as a mechanism to regulate gene expression as a means of adapting to energy demands elsewhere in the cell.

EPO is a glycoprotein and hematopoietic cytokine that is primarily responsible for erythropoiesis, but also involved in angiogenesis and cytoprotection. Unlike many other hormones, EPO does not have an intercellular protein reserve.[251] *EPO* is transcriptionally regulated by HIF1/2 and is upregulated in response to hypoxia, often in concert with other stimuli associated with nutrient sensing or inflammation.[114,326] There have been alternative splice forms of *EPO* identified in both human and mouse.[327] However, the role of intron retention as a form of alternative splicing in *EPO* has not been previously

investigated. Herein we investigate intron retention as a potential mechanism for post-transcriptional regulation of *EPO*.

4.2 Results

4.2.1 *EPO* Introns 2 and 4 are present in RNAseq datasets

To determine which introns were likely to be retained we examined RNAseq datasets submitted to the National Center for Biotechnology Information (NCBI) Gene server (https://www.ncbi.nlm.nih.gov/gene/2056) for EPO. Intron 2 and intron 4 show reads in the RNAseq, whereas intron 1 and intron 3 do not show full coverage (Figure 4.1). This indicates that introns 2 and 4 are most likely to be retained. These two introns are also the smallest introns in EPO which also favors their retention.

4.2.2 Intron retention is present in human tissues

We first wanted to examine if intron-retaining transcripts were present in human tissues. Post-mortem brain tissues were obtained from the Maritime Brain Tissue Bank (MBTB). Post-mortem kidney and heart tissues were obtained from the National Institute of Health (NIH) Neurobiobank. Using IR-E primers for EPO2-3 and EPO4-5 we were able to detect intron-retaining and intron-excised transcripts in brain, kidney, and heart. The band for intron-retaining transcripts is more dominant in brain compared to kidney, to the degree where the intron-excised is outcompeted for amplification. The intron-retained transcripts are found in all brain samples (Figure 4.2). In both kidney and heart the intron-retaining transcripts are only found in some samples, and there is variation between *EPO*2-3 (Figure 4.2a) and *EPO*4-5 (EPO4.2b). Sanger sequencing of intron-retaining, and intron-excised transcripts confirmed the identity of the transcripts in

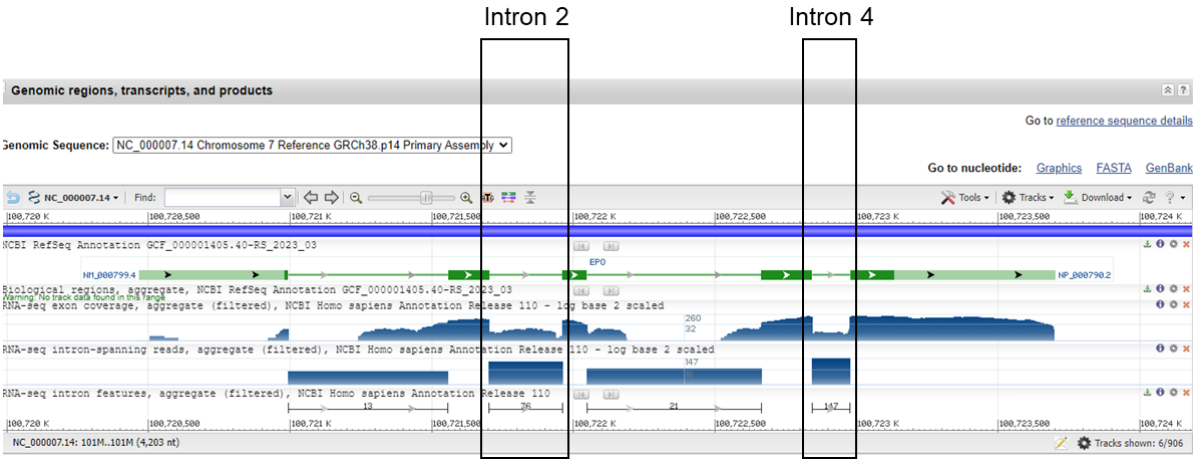

Figure 4.1- *EPO*'s introns 2 and 4 are featured in RNAseq datasets.
Summary page of National Center for Biotechnology Information (NCBI) Gene server or human *EPO* shows that introns 2 and 4 (in boxes) have been identified in previous RNAseq datasets.

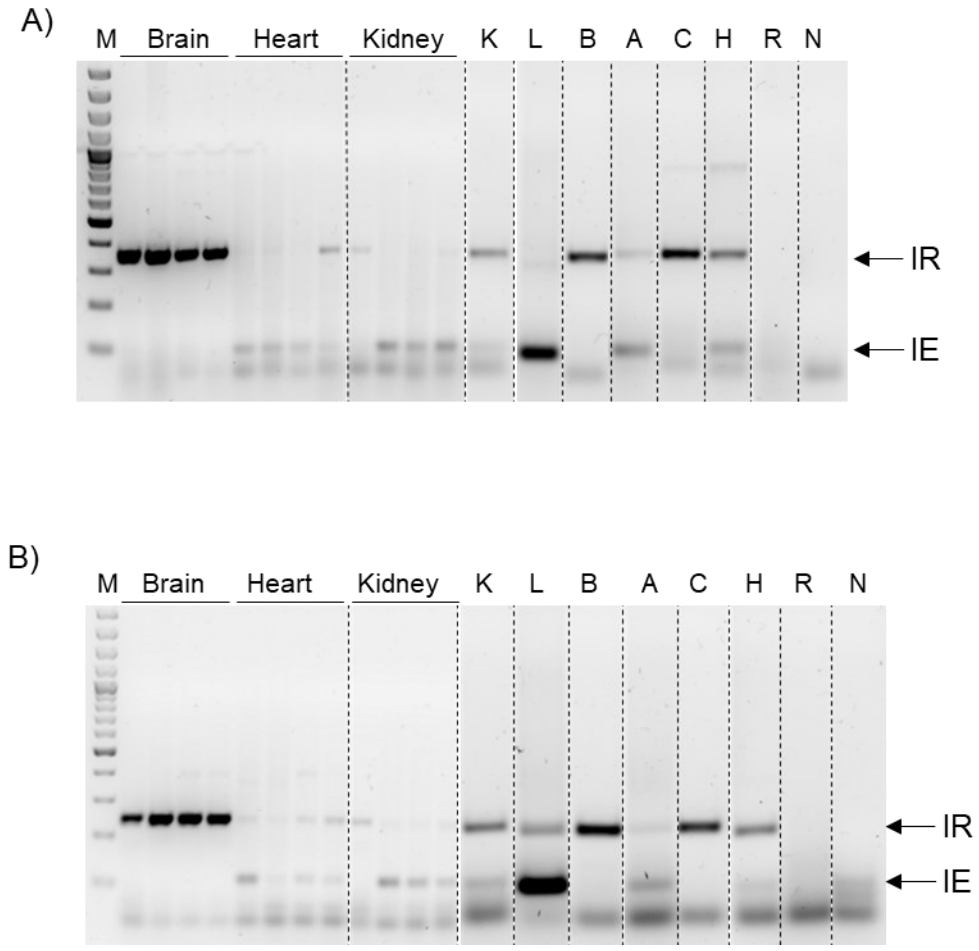

Figure 4.2- Intron retaining transcripts are detected in normal human tissues and cell lines.

A) End-point PCR using EPO2-3 IR-E primers and B) EPO4-5IR-E primers showing intron-retaining transcripts (IR) and intron excised (IE). Brain tissue (brain) consistently has IR transcript with no IE, which suggests IR is more dominant in the brain. For both kidney tissue (kidney) and heart tissue (heart) IR is found in only select tissues whereas the IE is found in all samples. We see similar results with IR being present in most cell lines, with brain cell lines (lanes B, C and H), with the exception of Astrocytes (A), having more dominant IR bands. Liver cells (L) have only IE in EPO2-3 and a small amount of EPO4-5 IR with IR being the dominant band. The kidney cells (K) show a high degree of IR transcripts, in contrast to their response to ischemia. M= GeneRuler 100 bp plus DNA ladder, K=HEK293(kidney), L=HEPG2 (liver), B=hCMEC/D3 (blood brain barrier), A=Astrocyte, C=CHP212 (Neuroblastoma), H=HTB16 (Glioblastoma), R= No reverse transcriptase control, N=no template control.

both *EPO*2-3 and *EPO*4-5 to be *EPO* intron-retained transcript and *EPO* intron-excised transcripts. These data suggest that intron retention is organ and context dependent as a means of regulating mRNA processing and stability.

4.2.3 Validation of *in vitro* hypoxia/ischemia responses

To investigate *EPO* in a cell model we used hypoxia (1% O_2) and simulated ischemia (nutrient-reduced media + 1% O_2) to induce *EPO* transcription. HIF1-α is ubiquitously expressed and stabilized in response to hypoxia, which allows it to heterodimerize with HIF1β and translocate to the nucleus to act as a transcription factor to genes with HREs, including *GLUT1* and *VEGF*.[105] To validate that hypoxic signaling was maintained during cell harvesting we performed immunoblotting for HIF1-α, which was increased in the cell models of both hypoxia and ischemia, confirming the cell model was hypoxic/ischemic after 24 hours of hypoxia and ischemia (Figure 4.3a). HIF1-α was increased at 2, 4, 8, and 16 hours of hypoxia (Figure 4.3c) and ischemia (Figure 4.3d), confirming hypoxia/ischemia signaling was increased by 2 hours and retained for at least 24 hours *in vitro*.

To investigate *EPO* expression, we used an rtPCR approach. The collection of protein to confirm hypoxia is not practical given the number of samples needed and the different experimental approaches used. To address this and yet ensure internal reference to hypoxia was sustained, an mRNA marker of hypoxia was confirmed. After 24 hours of hypoxia and ischemia *GLUT1* and *VEGF* expression were increased in hypoxia, and *GLUT1* was increased in ischemia, but no significant increase in mRNA was seen in *HIF1-α* (Figure 4.3b). *GLUT1* (Figure 4.3e) and *VEGF* (Figure 4.3f) are both increased in hypoxia at 2, 4, 8, and 16 hours and in ischemia at 4, 8, and 16 hours, consistent with

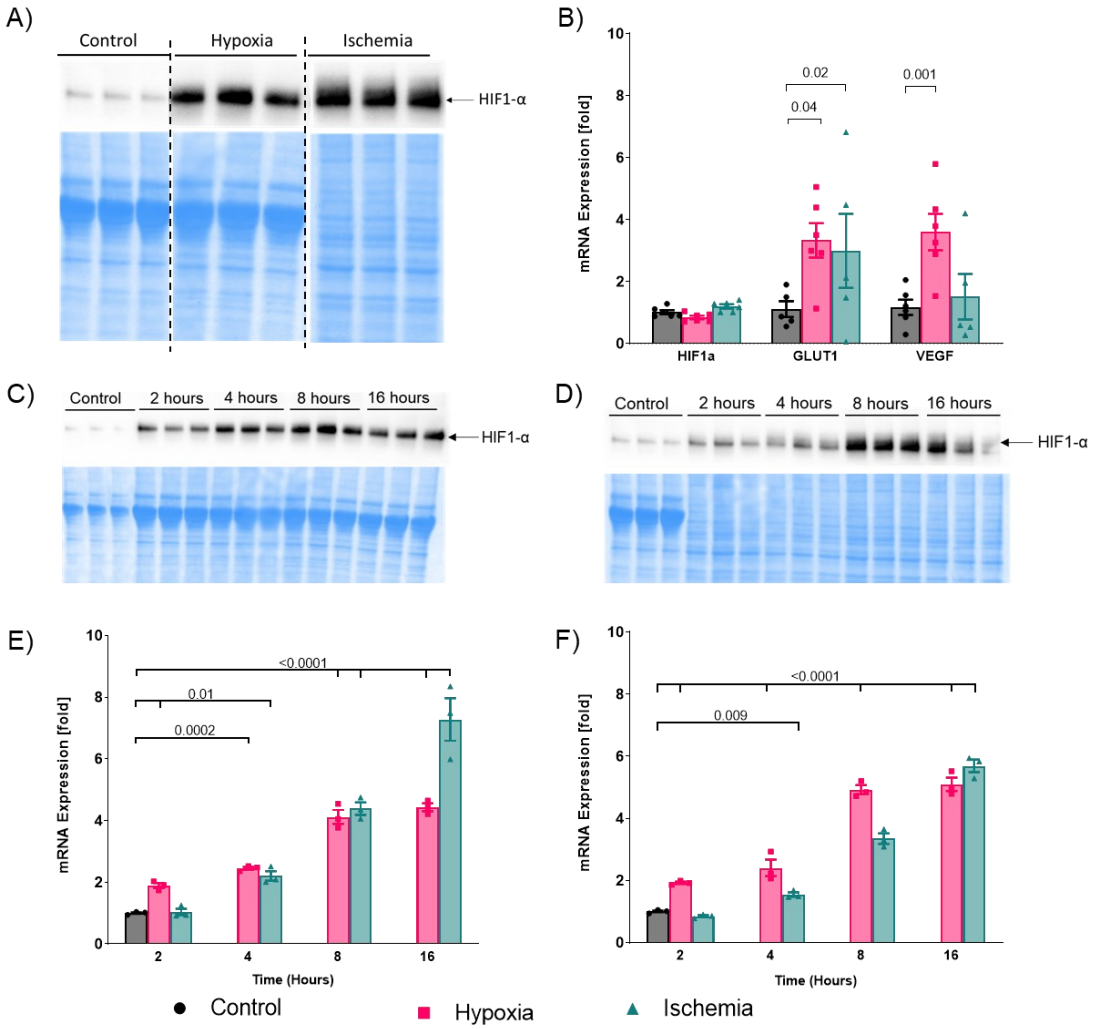

Figure 4.3- Hypoxia and ischemia signaling are preserved in protein and mRNA. A) HIF1α protein, an indicator of hypoxia, was increased after 24 hours of hypoxia (1% O_2) and ischemia (1% O_2, no glucose) in glioblastoma cells (HTB-16). B) *GLUT1* mRNA was increased in both hypoxia and ischemia after 24 hours and *VEGF* was increased in hypoxia. *HIF1α* mRNA was unchanged. HIF1α protein was increased at 2, 4, 8, and 16 hours in hypoxia (C) and ischemia (D) in HTB-16. *GLUT1* (E) and *VEGF* (F) mRNA were increased at 2, 4, 8, and 16 hours of hypoxia, with ischemia being increased at 4, 8, and 16 hours. Significance determined using two-way ANOVA and $p<0.05$ were significant. Error bars represent standard error of the mean (SEM).

protein stabilization of HIF1-α. Similar data was obtained for other cell lines used (data not shown). These data demonstrate the utility of *VEGF* and *GLUT1* mRNA expression to verify *in vitro* cellular hypoxia and ischemia by rtPCR.

4.2.4 Intron retention is a mechanism of post-transcriptional regulation of *EPO* in brain cells

After 24 hours of ischemia *EPO* expression was increased in all but the blood brain barrier and liver cells (Figure 4.4). The intron retention ratio decreases in most brain cells lines, with the exception of astrocytes (Figure 4.5c-f). Liver cells showed an increase in intron retention ratio and there was no change in kidney cells (Figure 4.5a-b), suggesting intron retention could be a tissue-specific mechanism of *EPO* regulation in response to ischemia. The melt curve for the IE primer (Figure 4.5g) confirms a single product, whereas the melt curve for IR-E primers shows two products (Figure 4.5h). Since there was no product in the no RT control (data not shown), and intron retention transcripts have been sequence validated, we conclude the second product is the intron retaining transcript. The low transcript ratio suggests that intron retention is accumulating in response to ischemia in the brain.

4.2.5 Intron retention shows a temporal effect

To investigate potential temporal effects of intron retention we examined intron retention after 2, 4, 8, and 16 hours of ischemia or hypoxia. We observed temporal variations in the overall expression of *EPO* (Figure 4.6) and the transcript ratio (Figure 4.7 a-f). The temporal effect is also seen when comparing the $2^{-\Delta\Delta CT}$ changes between the primer pairs (Figure 4.7 g). The IE primers increase at a slower rate compared to the IR-E suggesting that intron retention is increasing over time.

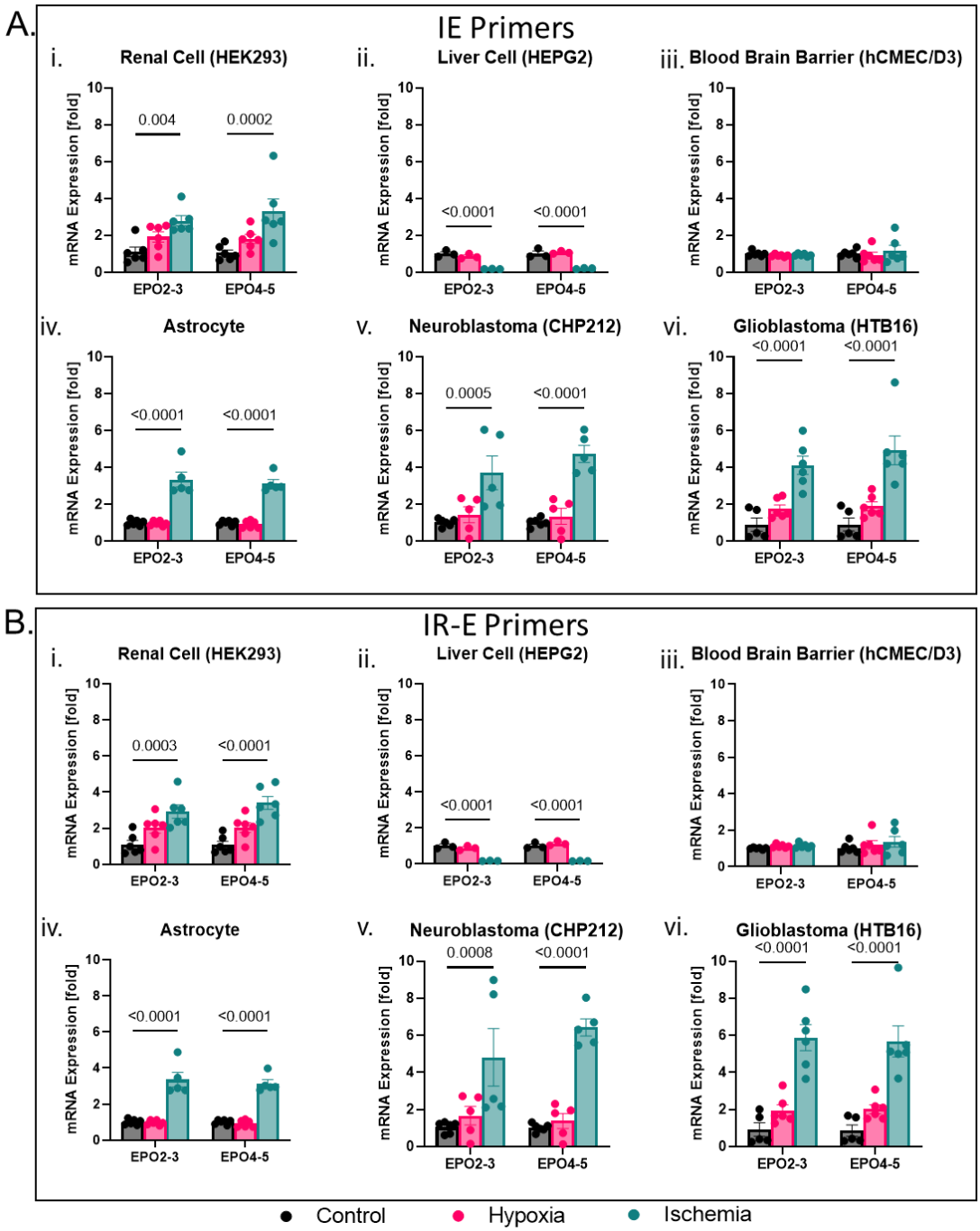

Figure 4.4- EPO expression in cell models after 24 hours of hypoxia and ischemia. Box A shows the mRNA expression using intron excised (IE) primers showing the fold change of the $2^{-\Delta\Delta CT}$ from controls for i) HEK293, ii) HEPG2, iii) hCMEC/D3, iv) astrocyte, v) CHP212, vi) HTB16. Box B shows the mRNA expression using the IR-E primers showing the fold change of the $2^{-\Delta\Delta CT}$ from controls (black) for i) HEK293, ii) HEPG2, iii) hCMEC/D3, iv) astrocyte, v) CHP212, vi) HTB16EPO 2-3 refers to primers spanning exons 2-3 and EPO4-5 primers spanning exons 4-5. Error bars represent SEM. p-value calculated using Two-way ANOVA.

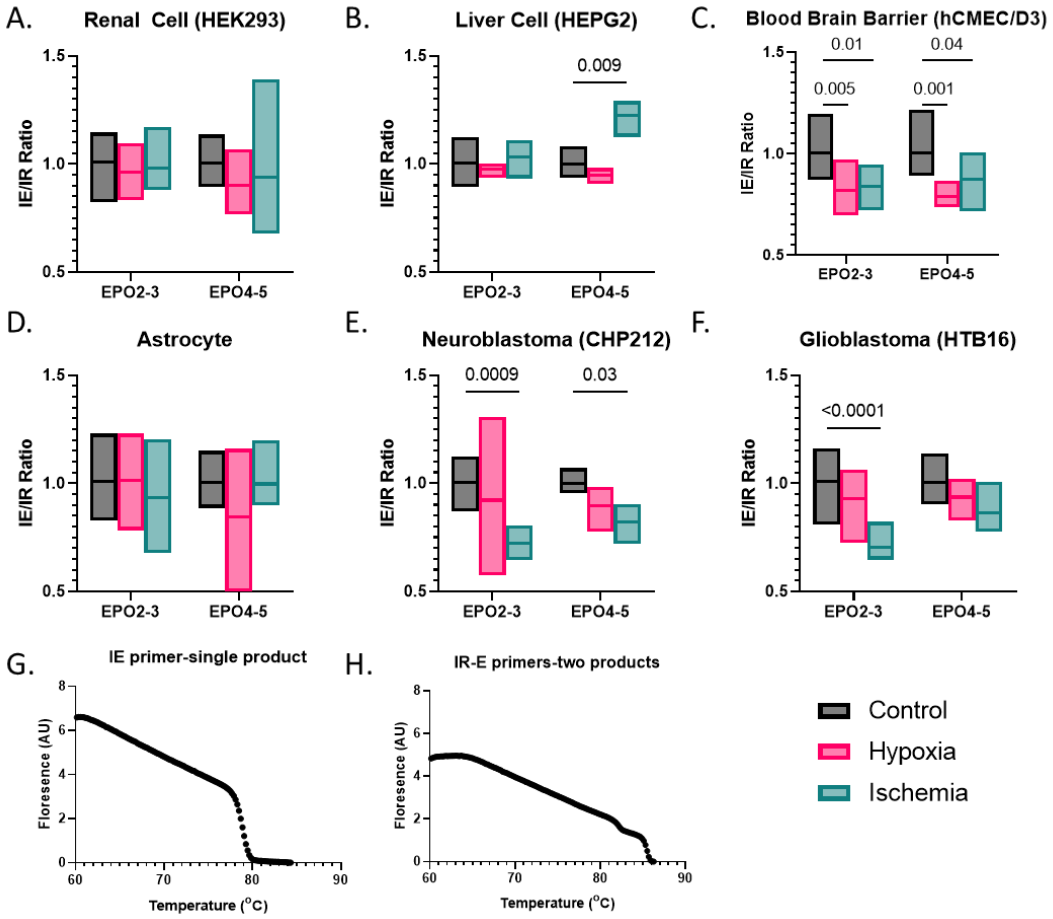

Figure 4.5 - Intron retention increases in most brain cell lines in response to ischemia after 24 hours.

Renal (A) cells showed no change in intron retention ratio and liver (B) showed an increase in intron retention ratio in EPO4-5 in response to ischemia. The BBB (C), CHP212 (E), and HTB16 (F) all showed a decrease in intron retention ratio, corresponding to an increase in intron retention, suggesting Astrocytes (D) being the only brain cell line not to demonstrate a change in intron retention ratio. The presence of a single product is shown in the IE primers from BBB (G) and two products in the IR-E primers from BBB (H).

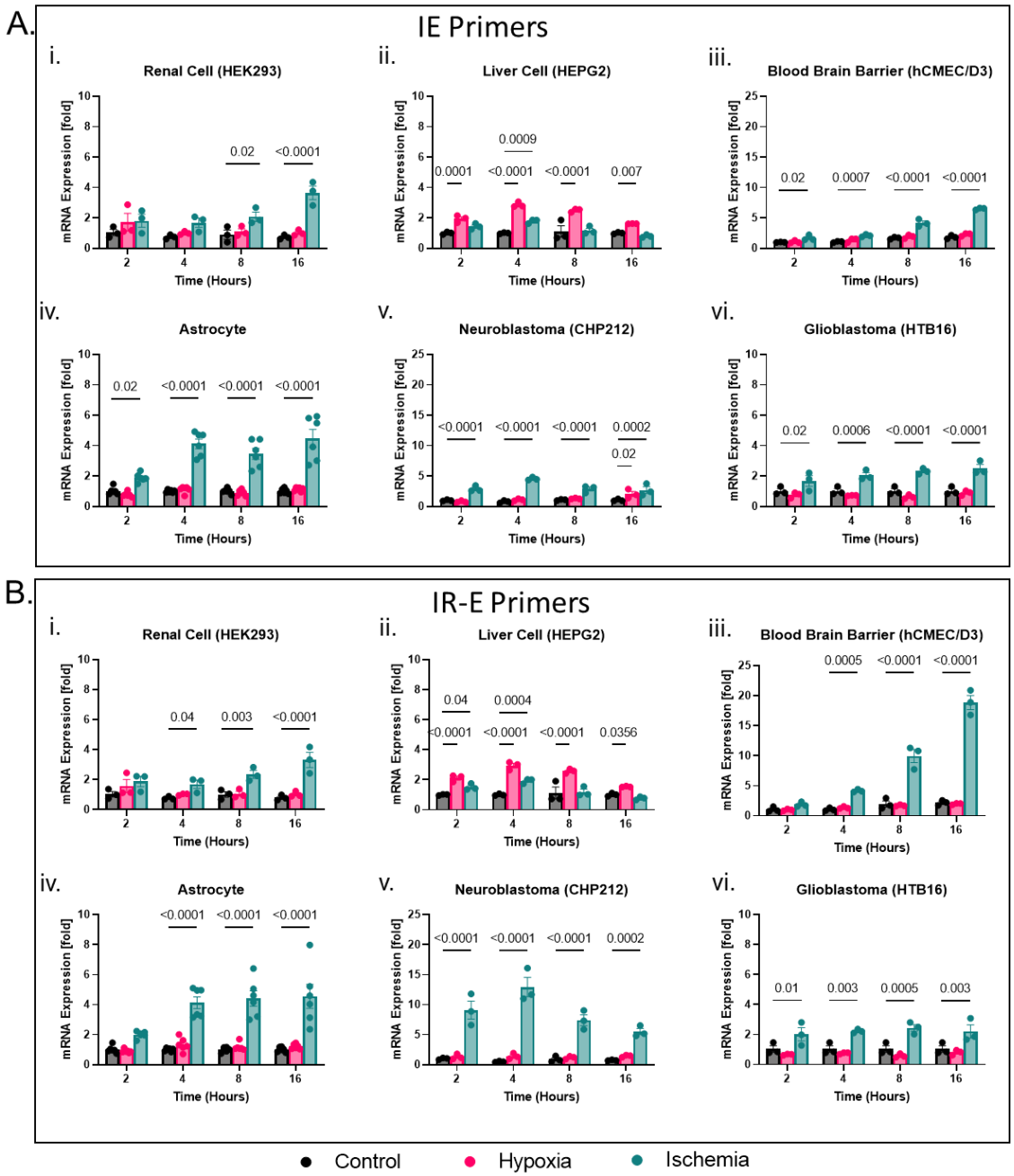

Figure 4.6- *EPO* expression shows temporal changes between 2 and 16 hours of hypoxia and ischemia in EPO4-5.

Box A shows the mRNA expression using IE primers fold change of the $2^{-\Delta\Delta CT}$ from controls for i) HEK293, ii) HEPG2, iii) hCMEC/D3, iv) astrocyte, v) CHP212, vi) HTB16 at time points of 2, 4, 8 and 16 hours of hypoxia and ischemia. Box B shows the mRNA expression using the IR-E primers fold change of the $2^{-\Delta\Delta CT}$ from controls (black) for i) HEK293, ii) HEPG2, iii) hCMEC/D3, iv) astrocyte, v) CHP212, vi) HTB16 at time points of 2, 4, 8, and 16 hours. Error bars represent SEM. Two-way ANOVA used to determine p-value.

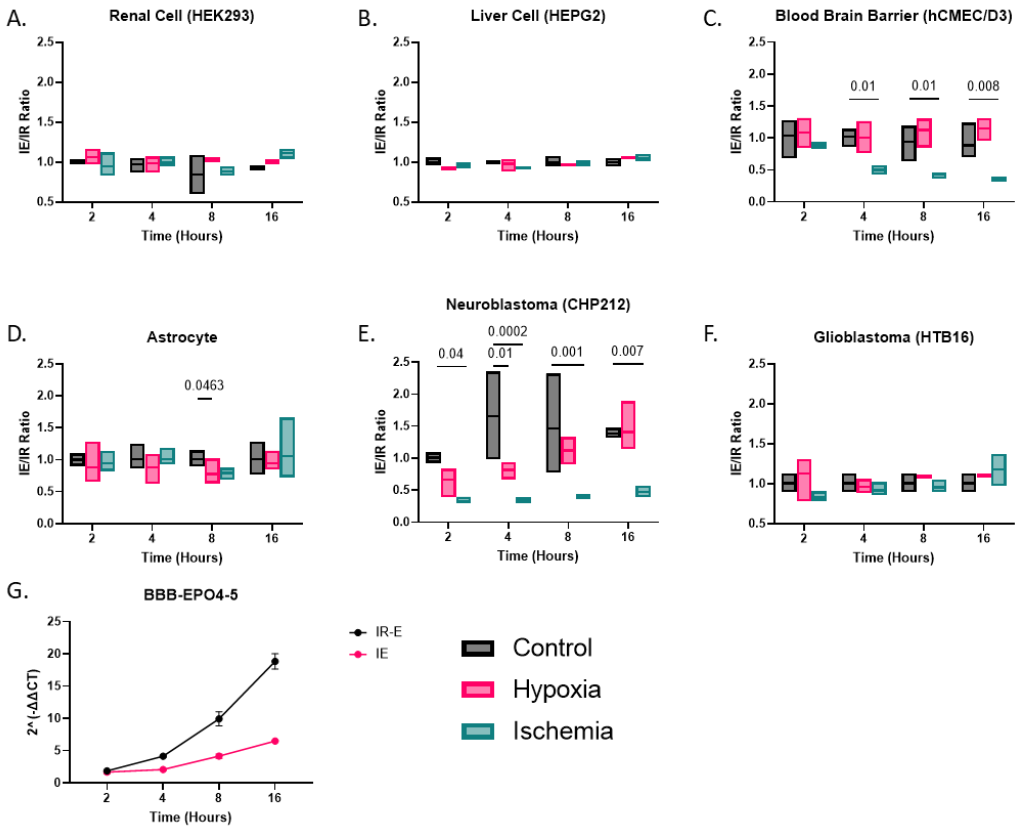

Figure 4.7- Intron retention ratio is decreased in a temporal manner in most brain cells in the EPO4-5 primers.

Renal (A) liver (B) cells show no change in intron retention ratio in EPO4-5 in response to ischemia. The BBB (C), CHP212 (E) show a decrease in the intron retention ratio suggesting an increase in intron retention with Astrocytes (D) having no change in the intron retention ratio. Changes in intron retention ratio increased over time in HTB16 (F) but did not reach the significance seen at 24 hours. G) The $2^{-\Delta\Delta CT}$ of the EPO4-5 IE and IR-E primers showing IR-E changing at a faster rate than IE primer from 2-16 hours. P-values were calculated using Two-way ANOVA.

4.2.6 Intron retention is not an artifact of pre-mRNA

Introns could be present as immature pre-mRNA prior to splicing and processing of the 5'cap and poly-A tail to form mature mRNA.[14,15] To verify the transcript ratio was not due to the presence of newly formed pre-mRNA, we used Oligo-dT primers to generate cDNA from HTB-16 cells after 24 hours of ischemia. The use of oligo-dT generates a cDNA library only of mRNA that has a poly-A tail (i.e. mature mRNA). The transcript ratio is maintained when using cDNA generated with oligo-dT (Figure 4.8), indicating the intron retention ratio is not immature mRNA contamination.

Actinomycin D (ActD) is an antibiotic that blocks transcription by intercalating with the DNA and blocking RNA polymerase elongation.[256–258] We used ActD to block *de novo* transcription in HTB-16 cells prior to ischemia. We confirmed with both the IR-E and IR primers that ActD blocked transcription in response to ischemia after 24 hours (Figure 4.8 a and b). Both controls (normoxia) and ischemic cells treated with ActD resulted in an increase in the transcript ratio compared to vehicle controls (Figure 4.8c). This indicates that intron retention is decreasing in ActD treated cells, suggesting intron-retaining transcripts are being utilized (converted to intron-excised and potentially translated) in response to ischemia when new transcripts are not being generated. Taken together our results show that intron retention is an intentional mature mRNA product that is accessible for post-transcriptional processing and translation when transcription is otherwise repressed at the site of initiation of mRNA production.

4.2.6 Intron retention shows circadian rhythmicity

Serum levels of EPO have been shown to have circadian rhythm.[119] To

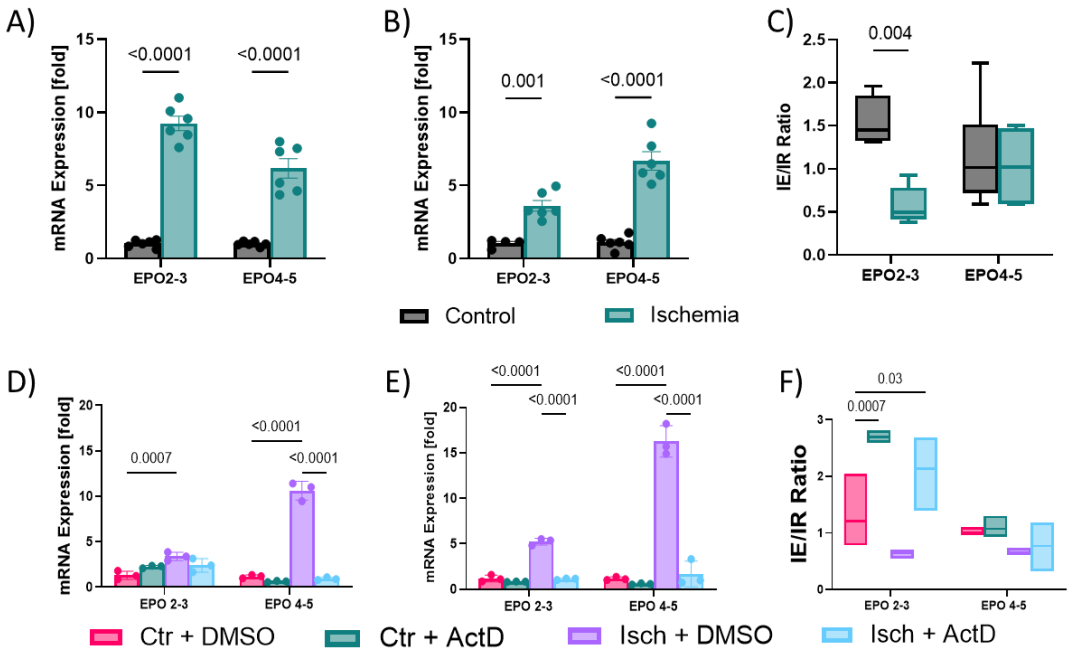

Figure 4.8. Changes in intron retention ratio is not an artifact of pre-mRNA. The IE primers (A) and IR-E primers (B) both show an increase in the expression of EPO, as previously seen, when a poly(A) enrichment cDNA synthesis protocol was used. The intron retention ratio in EPO2-3 (C) also is maintained indicating the intron retention ratio differences seen in ischemia are not caused by immature mRNA. Actinomycin D diminishes the increase in *EPO* expression in response to ischemia and decreases intron retention after 24 hours of ischemia in HTB-16. HTB-16 cells were treated with 5µg/mL Actinomycin D (or DMSO as vehicle) for one hour before being placed in ischemia. For both IE primers (D) and IR-E primers (E) treatment with Actinomycin D diminished the increase in *EPO* transcription in response to ischemia. The intron retention ratio (F) increased in both control and ischemia treated with Actinomycin D. Ctr=control, Isch=ischemia, DMSO=dimethyl sulfoxide, ActD=Actinomycin D. Two-way ANOVA was used with <0.05 being significant. Error bars represent SEM.

investigate if intron retention may also respond to circadian rhythm, we synchronized the circadian rhythm in HTB-16 using dexamethasone and forskolin. We measured mRNA of circadian rhythm genes *NR1D1*, *PER2*, *CRY2* and *CLOCK* to confirm circadian synchronisation (Figure 4.9 Box A). We did not observe circadian rhythmicity in the *EPO* mRNA expression but did observe a degree of rhythmicity in the transcription ratio (Figure 4.9 Box B). These results suggest that intron retention may also have a role in the circadian rhythm of EPO.

4.3 Discussion

The transcript ratio decreases in most brain cell models in response to ischemia, but not in kidney or liver cell types. The changes in the transcript ratio show a temporal effect with an increase in intron retention over time in most brain cell lines. The transcript ratio also shows a degree of circadian rhythmicity. The transcript ratio changes are not due to the formation of new immature mRNA. Taken together this demonstrates that intron retention is a physiological mechanism of *EPO* post-transcriptional regulation in brain cell types.

An overall increase in intron retention has previously been linked to hypoxia in colon cancer cells,[55] and breast cancer cells.[328] In our cell models, we did not observe a significant change by hypoxia but did with ischemia. Hypoxia is a component of ischemia, but ischemia also involves the lack of nutrients with an accrual of metabolites, suggesting a role of nutrient-hypoxia regulation of intron retention in the brain. Although brain hypoxia and ischemia are sometimes expressed interchangeably or synonymously as stimuli, they do not result in the same pathophysiology or molecular mechanisms of regulation. A study by Miyamoto and Auer[329] showed in Wistar rats that ischemic coma

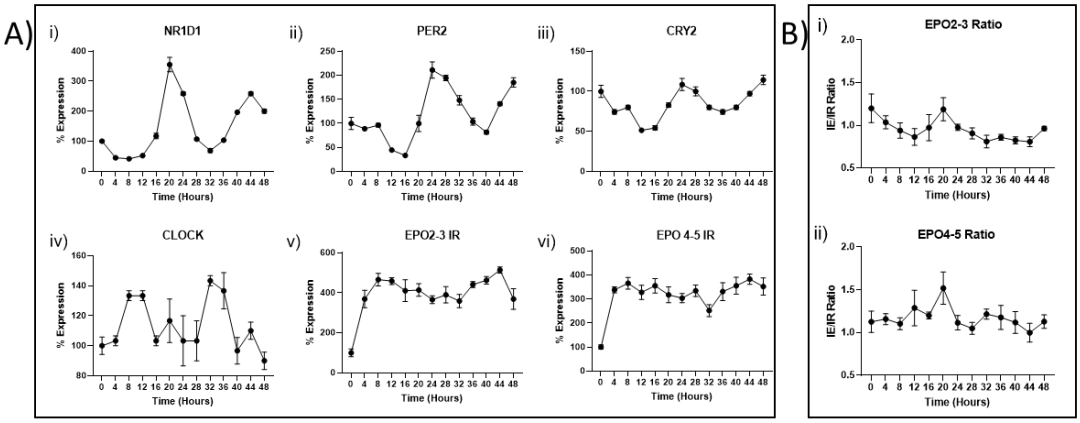

Figure 4.9 - Intron retention ratio in *EPO* shows a circadian rhythm.
Circadian rhythm was established *in vitro* using dexamethasone and forskolin and confirmed using circadian genes *NR1D1* (Box A i), *PER2* (Box A ii), *CRY2* (Box A iii), and *CLOCK* (Box A iv). EPO2-3 IR-E (Box A v) and EPO4-5 IR-E (Box A vi) did not demonstrate a circadian rhythm. Y-axis in box A is the percentage of the $2^{-\Delta\Delta CT}$ of time 0 hours. The intron retention ratio based on the IR and IR-E primers did demonstrate circadian rhythm in EPO2-3 (Box B i) and EPO4-5 (Box B ii). Y-axis in box B is the intron retention ratio. Error bars represent SEM.

caused brain necrosis, whereas hypoxic coma did not. Our results also suggest *EPO*, and in particular the intron-retained *EPO*, responds differently to ischemia than hypoxia. Many disease states, such as anemia, are salient risk factors for fulsome heart or brain ischemia[293,294] or directly contribute to tissue hypoxia. Therefore, intron retention in *EPO* may play a role in pathophysiology of chronic disease of the brain contributing to or sensitive to conditions of anemia or ischemia.

An increasing number of pathophysiologies have been linked with differential intron retention, including neurodegenerative disorders such as Alzheimer's disease.[39,330] Recombinant human EPO (rhEPO) has also been proposed as a treatment for Alzheimer's disease as it has been shown to decrease amyloid cytotoxicity and improve cognition in animal models.[331–333] The benefits of exogenous rhEPO could indicate that endogenous EPO levels may have an effect on the pathophysiology of neurodegenerative disorders. The increase in intron retention seen in Alzheimer's disease may also increase intron retention in *EPO* thus decreasing overall EPO levels for metabolic regulation and cytoprotection. As intron retention has recently only begun to be investigated in pathophysiologies there are likely many clinical situations where intron retention becomes maladaptive or causative to the underlying disease phenotype. The role of intron retention in *EPO* requires further studies in physiological, preclinical models, or clinical settings to reveal this molecular mechanism's relevance.

We found that intron retention was increased in response to ischemia, which we hypothesize is decreasing the amount of protein EPO compared to *EPO* mRNA produced. However, IE *EPO* mRNA is also increased, so likely EPO itself would still overall be upregulated during ischemia. Gunga *et al.*[120] tested the effects of fluid and

food restriction in 29 men undergoing survival training and found that during fluid and food deprivation, serum EPO was lowered but increased above the normal range during the recovery phase. This suggests there is a mechanism by which EPO is suppressed under stress and increased independent of hypoxia. Intron retention may be one such mechanism, as it has been shown to be a mechanism to decrease translation in response to cell stress.[55] Introns have been found to be important to starvation responses in yeast. Parenteau *et al*.[56] deleted introns from budding yeast and found they were less resilient to starvation compared to intron containing yeast. In our cell model, we postulate the increase in intron-retained transcripts is to hold in ready reserve some *EPO* transcripts for translation after the ischemia (HIF trough), whereas the intron-excised transcripts are generated for immediate response to hypoxic insult (HIF peak). The intron-excised *EPO* would be available to protect the tissue during ischemia, whereas the intron-retaining *EPO* transcripts would be translated thereafter during the recovery phase when the cell has more energy to invest in translation or to paracrine/autocrine actions of EPO on its cognate receptor(s) (Figure 4.10a). In a physiological state this might occur during the reperfusion phase, as a means of protection from oxidative ischemia-reperfusion injury. This may also be a potential mechanism to protect the brain tissue during this time as well in the case of stroke or else contribute as a mechanism mediating remote ischemic pre/post-conditioning. However, intron-retaining transcripts have been found to have varied fates, including forming different isoforms of protein, such as was identified with EPO's cognate receptor.[52] Additionally, it has been shown that EPO has alternative splice forms in the brain,[327] and therefore intron retention could have a role in production of alternative splice forms. As in many of our cell models, the canonical transcript is also

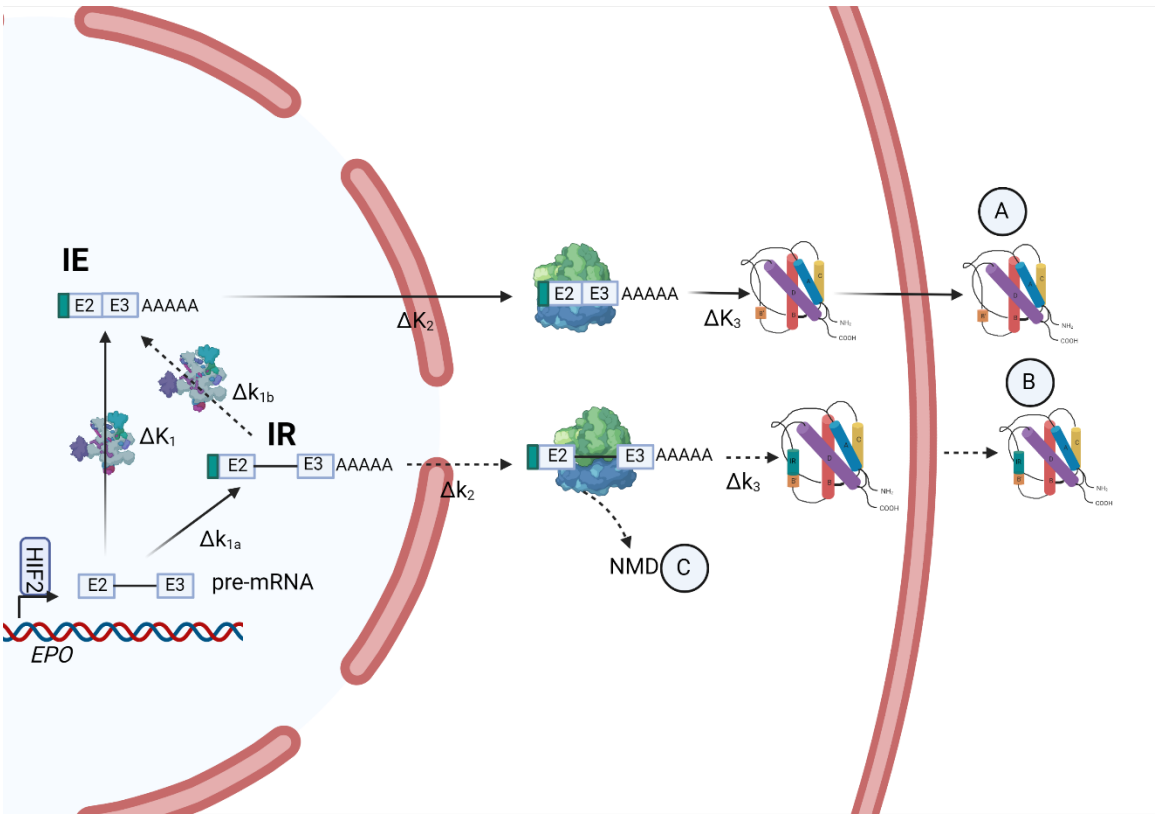

Figure 4.10 - Potential mechanism of intron retention and fates of intron retaining transcripts in *EPO*.
Transcription of *EPO* is stimulated by HIF2 to generate the pre-mRNA. Most transcripts will undergo full splicing (ΔK_1, IE) to generate EPO protein (ΔK_2) which is secreted. In ischemia, a subset of mature mRNA will retain one or more introns (Δk_{1a}, IR). The intron retaining transcripts can either be detained within the nucleus until a stimulus causes splicing (Δk_{1b}), followed by export of spliced mRNA to nucleus (Δk_{1b}), translated to canonical EPO (ΔK_3) and secreted (A). Alternatively, the intron retaining transcript can be exported to the cytoplasm (Δk_2) where it can either be translated to a novel isoform (Δk_3) and is secreted (B) or undergo nonsense mediated decay (NMD) (C). Image created using Biorender.com.

increased in response to hypoxia, so we cannot track which EPO molecules are generated from either intron-excised or intron-retained transcripts at this time. The exact fate of the intron-retaining transcripts is outside of the scope of this study, but future studies could investigate differential fates, intracellular compartmentation, or translated products.

We observed intron retention responding to ischemia in brain cells, but not in other cell types tested. This was correlated with the intron-retaining transcripts being more dominant in brain tissues compared to kidney but could also be the brain cells are more sensitive and energy conservative by nature. This indicates a degree of tissue specificity to intron retention for *EPO*. Many forms of alternative splice forms show tissue specificity. Bonnas *et al*.[327] identified an *EPO* splice variant that is missing exon 3 (hEPOΔ3) that is found in the brain but not liver. Intron retention has also been shown to be more prevalent in the brain generally compared to other tissues,[35] but it is not limited to the brain, which may rely on such mechanisms due to metabolic sensitivity and low tolerance to substrate limitations. Our data suggest that intron retention of brain *EPO* is a post-translational regulatory mechanism responsive to ischemia. This correlates with the intron retaining band that is more dominant in the brain compared to the kidney or heart. Kidney cells did not change their transcript ratio in our cell model but still possess intron-retaining transcripts. This could indicate that either a different cell type in the kidney is regulated by intron retention or the stimulus for intron retention may not be ischemia in kidney cells. Even within our different brain cell types, we observed variations to intron retention patterns, with astrocytes showing no transcript ratio changes. This may indicate that along with tissue specificity, even the different cells within a tissue utilize intron retention in varied ways. Further studies are needed to understand the tissue and cell

specific mechanisms that control intron retention.

EPO has been shown to have circadian rhythmicity, with the lowest concentration in the morning.[119] Circadian rhythm regulates many behavioural and physiological processes in a rhythmic oscillation based on the 24 hour day. Disruptions in circadian rhythm has been linked to CVD,[334] metabolic disorders,[335] and cancer.[336] At the cellular level, BMAL and CLOCK are higher during the daylight phase (i.e., morning). BMAL/CLOCK translocate to the nucleus and bind to E-box elements in the promoter region of the Period genes (*PER1, PER2*, and *PER3*) and cryptochrome genes (*CRY1* and *CRY2*). PER and CRY are upregulated during the dark (i.e. night) phase and inhibit BMAL and CLOCK in a negative feedback loop. Additional feedback loops occur with REV-ERBα/β (*NR1D1* and *NR1D2*) repressing BMAL gene (*ARNTL*) and retinoic acid-related orphan receptor (ROR)α/β/γ (*RORA, RORB* and *RORC*) that activates *ARNTL*.[337,338] In our cell model we used *NR1D1, PER2, CRY2* and *CLOCK* to verify we established circadian rhythmicity. The "dark" regulators *NR1D1, PER2* and *CRY2* were highest between 20 and 24 hours, whereas the "day" regulator *CLOCK* was highest between 8 and 12 hours, which confirmed we had circadian-like synchronization in our cell models. Hypoxia has been reported to disrupt circadian rhythm, particularly when mice are kept in constant darkness.[339,340] BMAL/CLOCK are reported to interact bidirectionally with the HIFs that regulate EPO in hypoxia. Additionally, there is an E-box in the *EPO* promoter (-36 to -31 bp from TSS). HEK293 cells that overexpressed CLOCK/BMAL increased *EPO* expression in a luciferase reporter assay. This increase in *EPO* expression could be eliminated when the E-box was mutated in the promoter.[341] Although we did see variation in the expression of *EPO*, it did not show circadian

rhythmicity. However, when we examined the intron retention ratio there was evidence of a circadian rhythm with peaks at 20 hours (similar to *NR1D1*). A transcriptomics study of genes with circadian oscillations in *Arabidopsis thaliana* showed that intron retention was a major type of alternative splicing.[342] This indicates that intron retention may be an important post-transcriptional regulatory mechanism to control circadian rhythm. Since the intron retention ratio in *EPO* shows some rhythmicity, it could be a potential post-translational regulatory mechanism that contributes to the circadian rhythm of EPO. This warrants further investigation to elucidate the role of intron retention and circadian rhythm and their potential function in physiology and pathophysiology and requires validation *in vivo* where circadian rhythm can be accounted for fully.

Our study does have its limitations. We are measuring the intron retention ratio using an indirect measurement of the inclusion of the intron, which can decrease the sensitivity. However, since *EPO* is a low expression gene, direct targeting of the intron for our primers would likely fall below the limit of detection using rtPCR. Potentially targeting the intron with a more sensitive method, such as droplet digital PCR or RNAscope, could directly target and assess the intron in *EPO*. Droplet digital PCR (ddPCR) would allow for a more sensitive measurement as well as an absolute assessment of intron retention, compared to the qPCR's relative assessment.[343] However, this would increase the cost and could be impractical for a discovery-based study, such as this seeking to establish the first line evidence for intron retention in *EPO*. We are also unable to account for other genes that may be located within intron, such as snoRNAs. A long read RNAseq, such as PacBio, could be used to confirm the intron retention is not due to other gene present in the introns. In our circadian-like rhythm *in vitro* we are

unable to combine circadian synchronicity with ischemia since we must open the incubator to harvest every 4 hours, thus resulting in re-oxygenation and abolishing ischemia signaling. Establishing the intron retention ratio in a mouse model where we could manipulate circadian rhythm and potentially investigate its role in pathophysiology, such as anemia AMI, or stroke, would allow for a better understanding of the role if intron retention in *EPO* on its circadian rhythm.

Herein, we demonstrate that at least two of the four introns in *EPO* undergo intron retention as a post-transcriptional regulation mechanism in response to ischemia in the brain. Although intron 2 and intron 4 show some similarities in intron retention, they can vary slightly between cell types, suggesting the roles of these introns may also slightly differ. The change in the intron retention ratio has temporal, tissue-specific effects, indicating a potential role in physiology. Further study is needed to elucidate the fate of the intron retaining transcripts and their potential physiological roles. Since intron retention has begun to be linked to some pathophysiology,[39] further investigation is needed to establish if intron retention in *EPO* may also impact pathophysiology.

Chapter 5- Erythropoietin Variants in Protein

5.1 Introduction

EPO is a hematopoietic cytokine best known for its erythropoietic effects but also reported to elicit cytoprotective and metabolic functions in various cells and stress causing contexts (Figure 1.9). Most ESAs are based on the amino acid sequence of the endogenous human EPO. ESAs were originally approved to treat anemia in CKD by virtue of their erythropoietic effects,[80] and have since been expanded to treat other forms of anemia, such as in cancers despite the risk for adverse effects in reducing efficacy of chemotherapeutic agents.[81] The cytoprotective properties of EPO also led to investigations using rhEPO in ischemic injuries, such as AMI and stroke.[191,243] Although the erythropoietic effects are well established,[344] the results from studies examining the cytoprotective properties have been inconsistent, and it remains uncertain why there is such variability, though it might possibly be the results of variation in ESA formulation and dosing translationally from model to human, in addition to physiological variability.[191,243]

The erythropoietic potential of ESAs has been well established with multiple clinical trials that have shown increased Hb in patients treated with ESAs.[344] Currently there is insufficient evidence to recommend one form of ESA over another based on erythropoietic potential. Other considerations such as dosing and access are considerations in the clinical setting.[344] However, ESAs do carry risks that are dose dependant. A meta-analysis compared risk of nonfatal thrombosis and worsening hypertension in studies that targeted high vs low Hb targets. Those targeting higher Hb,

which typically requires higher doses of ESAs, were associated with elevated risk of adverse events.[345] Although ESAs are beneficial to improving Hb/Hct, there remains uncertainty about whether it resolves the comorbid risks associated with anemia fully without the additional risk of adverse events. Indeed when used to treat anemia in cancer it may not reduce mortality, because the mitogeneic and cytoprotective effects promote tumour growth and resiliency.[157,233]

In *in vitro* assays rhEPO has been found to be cytoprotective against hydrogen peroxide,[346,347] hypoxia, anoxia,[348] staurosporine[346] and chemotherapeutic agents including cisplatin[349–351] and paclitaxel.[351] This could be helpful in reducing cardiotoxicity of chemotherapy to mitigate the negative effects associated with cancer anemia. Yet, other studies using hydrogen peroxide, hypoxia and staurosporine have not found rhEPO to be cytoprotective.[346] The cell types, formulation/source of rhEPO, dose, duration, and timing of the interventions vary in these studies, which may account for this lack of consensus. The amount of EPOR and β-CR can also vary between cell types, which can affect the ability of EPO to act on the cells.[346] Glycosylation patterns can also vary between rhEPOs, with the intended or unintended effects by pharmacodynamics and pharmacokinetics.[197,209–212] Although no significant difference between ESAs for erythropoietic functions have been observed clinically (and were often established *a priori* as primary comparative outcomes for non-inferiority),[344] this has not been as well studied for cytoprotection. The dose of rhEPO that tend to elicit cytoprotection are higher than for needed for erythropoiesis.[242,352]

The inconsistency seen amongst *in vitro* models has also been observed *in vivo*. rhEPO has been found to be cardioprotective in many animal models, including mouse,[234]

rat,[164,235–237] rabbit,[238] and dog.[239] Yet, rhEPO was also not cardioprotective in a sheep model and was inconsistent in pigs.[191] The inconsistency was carried forward into clinical trials with some trials showing improved LVEF and infarct size,[304,305] others showing no significant improvement but rather higher risk of adverse effects.[191] As with the *in vitro* models, the *in vivo* and clinical trials doses of rhEPO varied and did not rely upon any patient tailoring nor were informed by a theranostic readout, such as platelet reactivity, monocyte receptor density, or titration to set point of Hb/Hct. One study that attempted to decrease the dose of rhEPO below the risk threshold was able to mitigate adverse effects but then showed no improvement in stroke outcome.[243] This has also led to the investigations of EPO derivatives that are more selective for the varied functions of EPO.

The glycosylation pattern of ESAs varies based on the cell type used and purification protocols used.[197,198] *N*-linked glycosylation has been shown to be necessary for the erythropoietic functions with EPO derivatives (CPEO and asilo-EPO) that lack N-linked glycan having no erythropoietic functions.[150,153,244,245] Since CPEO and asilo-EPO are cytoprotective the *N*-linked glycosylation likely play a less significant role in EPO's cytoprotective functions. Given the importance of the *N*-glycans, darbepoetin α (Aranesp®) was developed by five amino acid substitutions that result in an additional two N-linked glycosylation sites being incorporated. The additional glycosylation increases the half-life, but also does decreased the binding affinity to the EPOR.[353] Darbepoetin α was shown to be cytoprotective against prostaglandin induced apoptosis, but not against hydrogen peroxide, suggesting that receptor affinity and signaling and type of stress vary in whether EPO is cytoprotective or not. Against staurosporine some studies did find cytoprotection using other ESAs, although there did not appear to be

cytoprotection with darbepoetin α.[346]

Epoetin α (Eprex®) and darboepoetin α (Aranesp®) differ in the degree of glycosylation. A rhEPO from Peprotech is similar to epoetin α but may contain slight variation in the glycoforms. Mouse recombinant Epo (rmEpo) is based on the mouse Epo, which shares the three *N*-glycans but the *O*-glycan site is not conserved (Figure 1.6). Herein, we test the erythropoietic and cytoprotective functions of these rEPOs to compare their relative biological functions.

5.2 Results

5.2.1 Variation in rhEPOs and mrEpos

Glycosylation accounts for approximately 40% of the molecular mass of EPO and is important in its stability and function.[197,198] Changes to the glycosylation pattern will result in variation of apparent molecular mass. To see the differences in the molecular mass of the recombinant EPOs we ran a Coomassie gel (Figure 5.1d). Epoetin α (Eprex®) (Figure 5.1a,d), Peprotech (commercially available rhEPO from CHO cells) (Figure 5.1a,d) and rmEpo (based on mouse sequence with similar *N*-linked glycosylation as rhEPO)(Figure 5.1c,d) have similar molecular weights at ~37 kDa (ranging from 30 to 40 kDa). Peprotech and rmEpo have broader bands, that may indicate greater variations in glycoforms within the mixtures compared to epoetin α. The band at ~70 kDa in Peprotech is albumin from the BSA added to stabilize the suspended protein. The larger molecular mass shown in darbepoetin α (Aranesp®)(~50kDa) (Figure 5.1b,d) reflects the higher degree of glycosylation as it contains five *N*-glycosylation sites compared to three in the other rEPOs.[128]

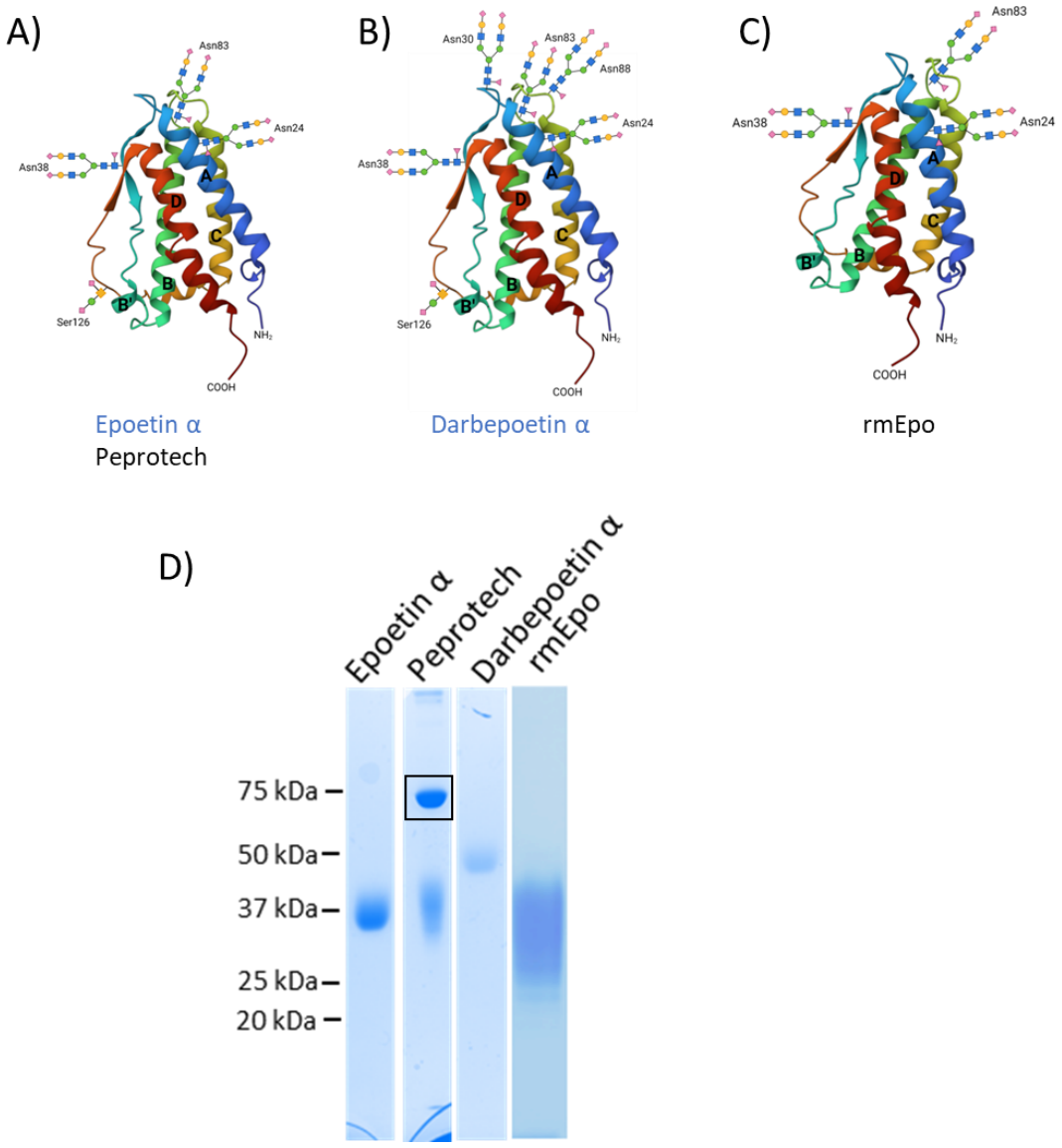

Figure 5.1- Coomassie staining of commercial rhEPOs and rmEpo.
Molecular structures of a) Epoetin α and Peprotech, b) darbepoetin α, and rmEpo. Pharmaceutical grade ESAs are written in blue and commercially available rEPOs are indicated in black. D) Coommasie gel that lane 1 contains epoetin α, lane 2 is Peprotech, lane 3 darbepoetin α and lane 4 rmEpo. The molecular weight is indicated to the left. The ~70kDa band seen in the Peprotech is albumin (not EPO) that was added when resuspending the protein for stability.

5.2.3 Erythropoietic potential of mouse rEPOs

ESAs are commonly used to treat anemia due to EPO's erythropoietic properties.[215] To compare a human and a mouse recombinant EPO, we performed a CFU-E assay with epoetin α (human) and rmEpo (mouse). Epoetin α and rmEpo has similar erythropoietic activity, with an EC_{50} of 0.3 ng/mL and 0.8 ng/mL, respectively and achieved EC_{max} at approximately 1.65 ng/mL. The small variation in response in the CFU-E between epoetin α and rmEpo was not significant, as expected based on the similar N-linked glycosylation.

5.2.4 Proliferative effect of rhEPOs

We first wanted to determine an appropriate seeding density for H9c2 myoblast in a 96 well plate for proliferative and cytoprotection assays. We seeded cells at densities of 5,000 to 15,000 cells for either 24 or 48 hours (Figure 5.3). PrestoBlue was used as an indicator of relative number of viable cells, with a higher absorbance indicating more metabolically active cells present, which we interpreted as either more or more viable cells depending on the timing of growth or insult. Between 5,000 and 10,000 cells showed a linear relationship with absorbance and number of cells seeded, whereas the curve plateaus above 10,000 cells per well. 5,000 and 7,500 cells also showed a difference between 24 hour and 48 hours indicating that cells were still proliferating at these densities. 10,000 to 15,000 cells showed a marked decrease in variation between 24 and 48 hours suggesting the cells were confluent by 24 hours at these densities. Therefore, we concluded the seeding density should be between 5,000 and 10,000 cells per well.

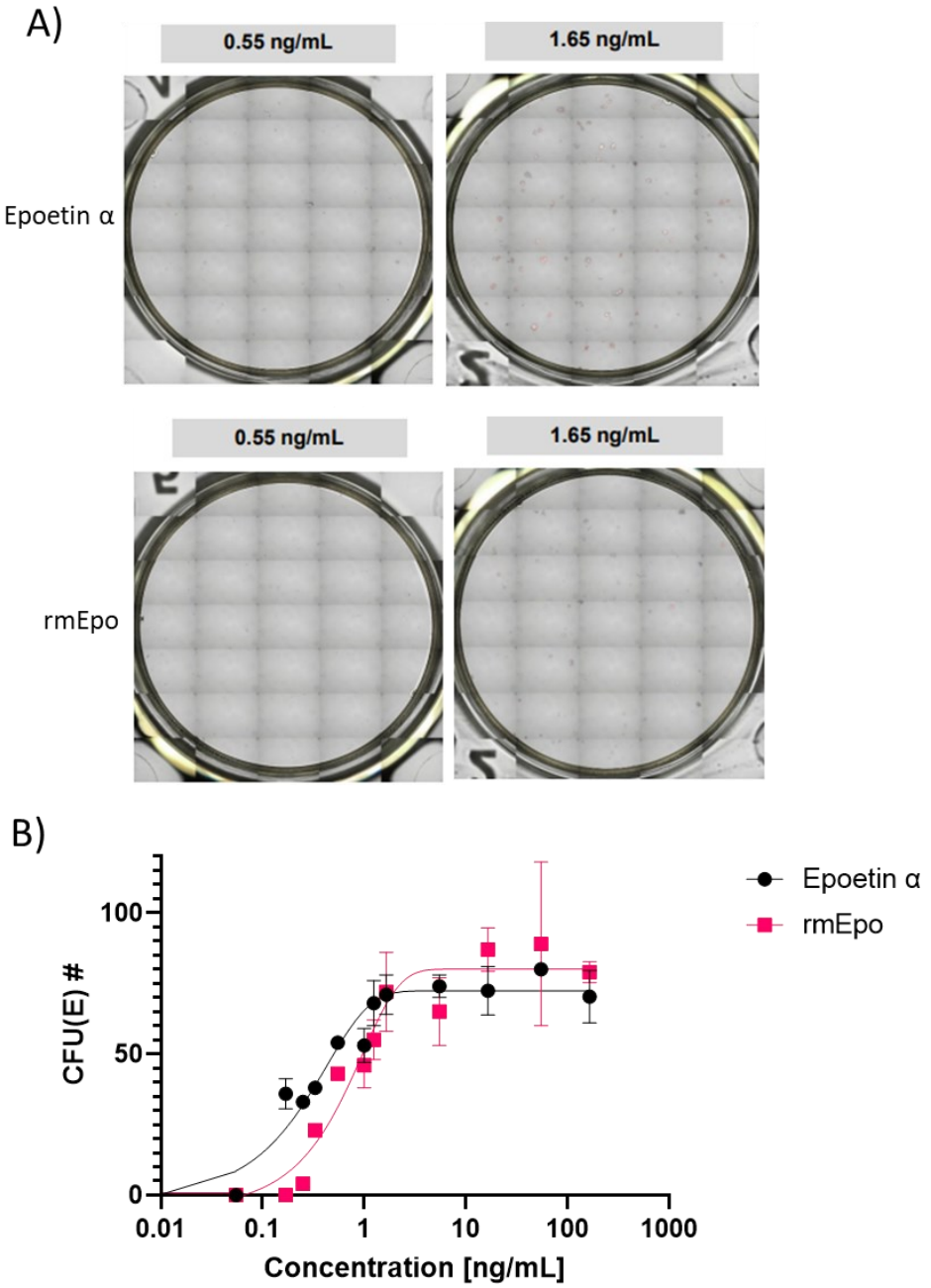

Figure 5.2- Epoeitin α and rmEPO show comparative erythropoietic potential.
A) Examples of CFU-E plates at 0.55 ng/mL (left) and 1.65 ng/mL (right) with epoetin α on the top and rmEpo on the bottom. B) The dose of Epoetin α or rmEPO in ng/mL is on the x-axis and the CFU(E) are on the y-axis. Non-linear regression line generated using log (agonist) vs. response-variable slope (four parameters) equation in GraphPad. Error bars show the SEM. n=3

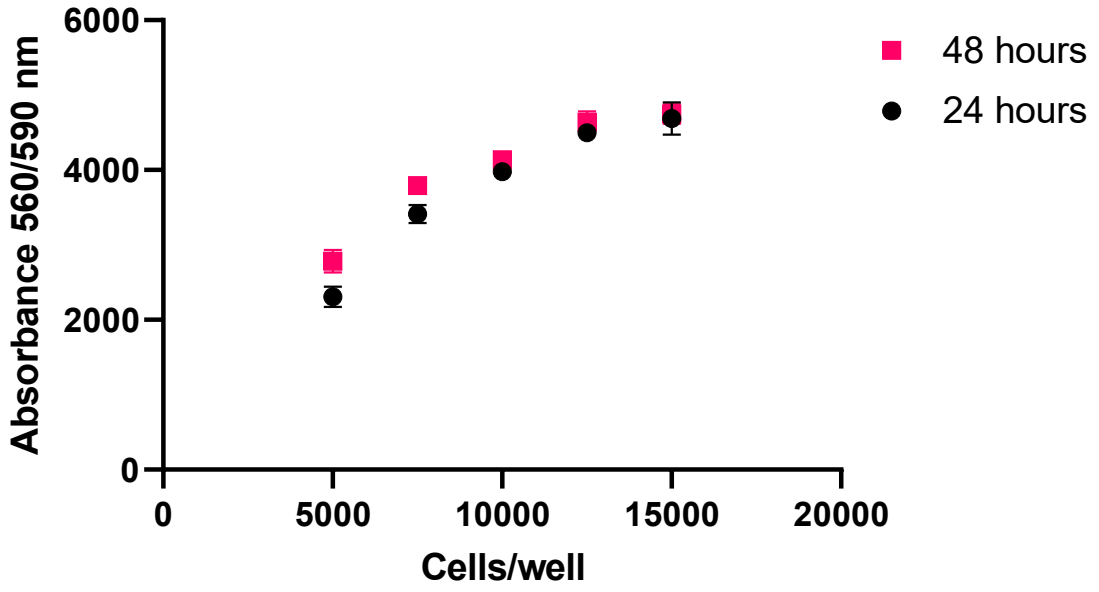

Figure 5.3- Absorbance versus cell density in H9c2 myoblasts.
Cells were seeded at varying densities and grown for either 24 or 48 hours. Presto Blue was added 1 hour before reading the absorbance at 560/590 nm. Error bars represent the SEM. n=6

To establish the effects of the rhEPO on H9c2 myoblast we plated cells at either 5,000 or 10,000 cells per well. Cells were treated with either Epoetin α, Peprotech-rhEPO or darbepoetin α for 24 hours and used PrestoBlue to determine the relative number of viable cells. The absorbance was normalized to non-treated cells (set as 100%). We found that epoetin α has the highest degree of proliferation (determined as a higher increase in % absorbance) followed by Peprotech and lastly darbepoetin α at 10,000 cells per well (Figure 5.4 A-C, top row). Both epoetin α and Peprotech reached their E_{max} by 55 ng/mL. The same dose elicits only a small increase in the darbepoetin α treated cells. With as starting number of 5,000 cells/well 55 ng/mL also elicits an increase in epoetin α and Peprotech but to a lesser extent than that seen at 10,000 cells/well. However, in epoetin α we see the E_{max} by 5.5 ng/mL, which then showed a flattening of the curve. The largest difference between 5,000 and 10,000 cells was seen with darbepoetin α. At 5,000 cells darbepoetin α did not show any appreciable proliferation up to 137 ng/mL but then increased at doses up to 550 ng/mL. When we compared the proliferative effects based on the % absorbance of all rEPO at 137 ng/mL at 5000 cells (Figure 5.4 G) amongst the human ESAs, epoetin α had the highest degree of proliferation, followed by Peprotech and darbepoetin α. Interestingly, rmEpo had the most effect on the degree of proliferation compared to epoietin α, suggesting there may be speciation or secondary structural effects. Taken together, this data suggests that these rEPOs can have a proliferative effect but that this capacity varies considerably with the dose, cell/receptor density and ESA formulation, which may partly explain the disparity in the literature due the lack of ligand-receptor pharmacodynamic accountability.

5.2.5 Metabolic effects of EPO

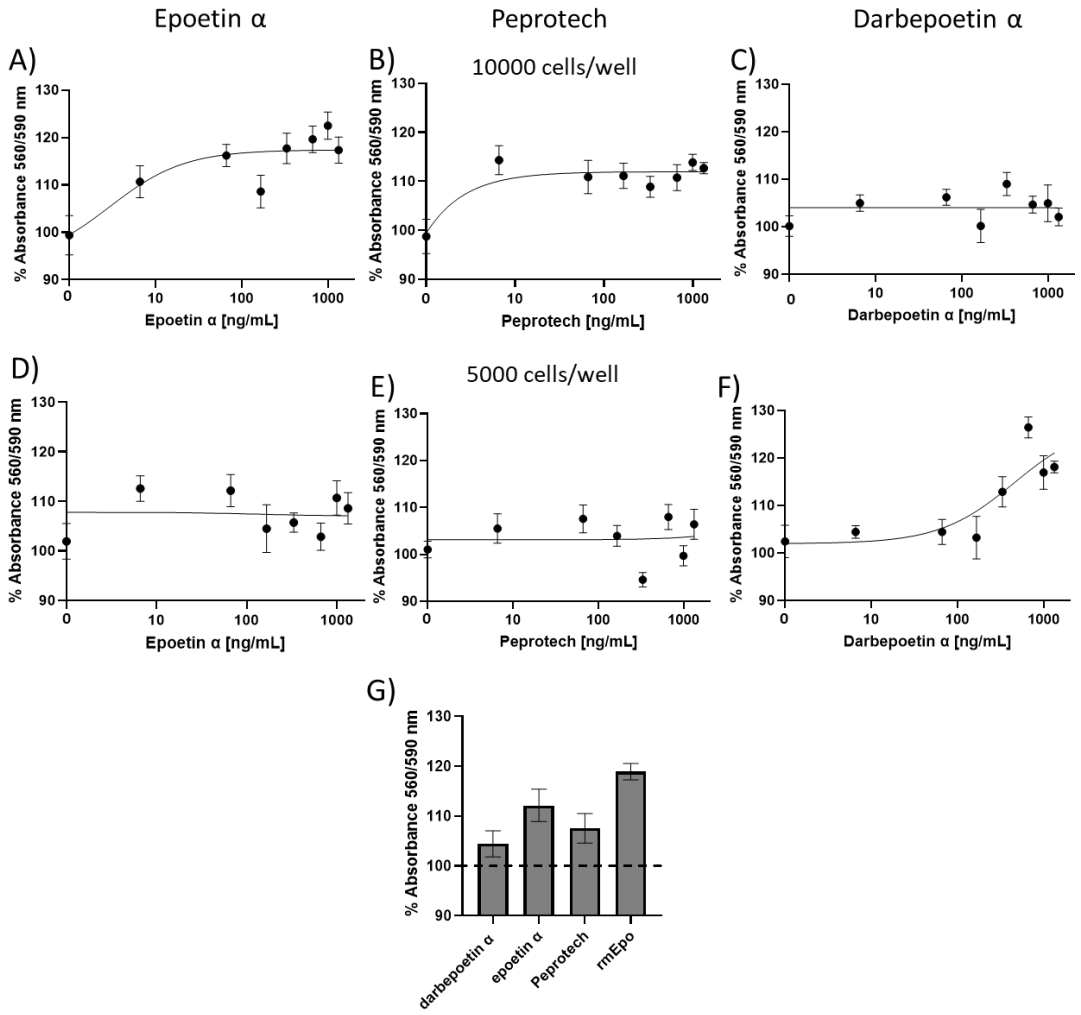

Figure 5.4- Proliferation of H9c2 myoblasts in response to rEPO.
Cells were seeded at 10,000 cells/well (top row, A-C)) or 5000 cells/well (middle row, D-F) 24 hours before treatment with Epoetin α (left column, A and D)), Peprotech (middle column, B and E)), or darbepoeitin α (right column, C and F)) for 24 hours. G is the proliferative effect of the rEPO, including rmEPO at 137 ng/mL on 5000 cells per well. Presto blue was added to cells 1 hour before absorbance was read at 560/590 nm. After subtraction of no cell control well, the % Absorbance was normalized to no EPO treatment (100%). Error bars represent SEM. Normalized absorbance versus dose of rhEPOs. n=6

EPO has also been shown to increase the mitochondrial respiration.[354] To examine if rhEPO changes mitochondrial respiration we used Oxygraph-2k on H9c2 cells that were treated with epoetin α for 15 minutes or 24 hours for acute and chronic measurements, respectively. There was no significant change in respiration after acute epoetin α exposure (Figure 5.5 a). After chronic exposure (24 hours) there was a significant increase in mitochondrial respiration in epoetin α treated cells compared to controls (Figure 5.5 b). The malate and the pyruvate prime the cells for oxidative phosphorylation. The first does of 0.1 mM ADP (D0.1) is a physiological amount of ADP. The addition up to 0.5 mM followed by 5 mM show the maximal limit of oxidative phosphorylation. The addition of FCCP uncouples mitochondrial respiration allowing us to examine the electron transport capacity.[355] There was a significant increase at these stages in the oxygraphy after 24 hours. Rotenone is a complex I inhibitor, which prevents oxidative phosphorylation through complex I.[356] Succinate is the substrate for Complex II, and therefore its addition allows us to examine Complex II respiration.[357] The addition of succinate did increase after chronic EPO exposure but did not reach significance. Taken together this suggests that after 24 hours of epoetin α treatment H9c2 have a higher mitochondrial respiration.

5.2.6 Cytoprotective effects of rhEPOs

EPO has been investigated for its cytoprotective properties in the treatment of ischemic injuries, such as stroke and AMI.[191,243] To investigate the cytoprotection of rEPO we first did dose-response curves of various pro-oxidative or xenogenic agents. We tested hydrogen peroxide (H_2O_2), doxataxel (DTX), hemin and staurosporine (Figure 5.6) to determine the IC_{50}. Doxataxel only reached approximately 20% cell death and

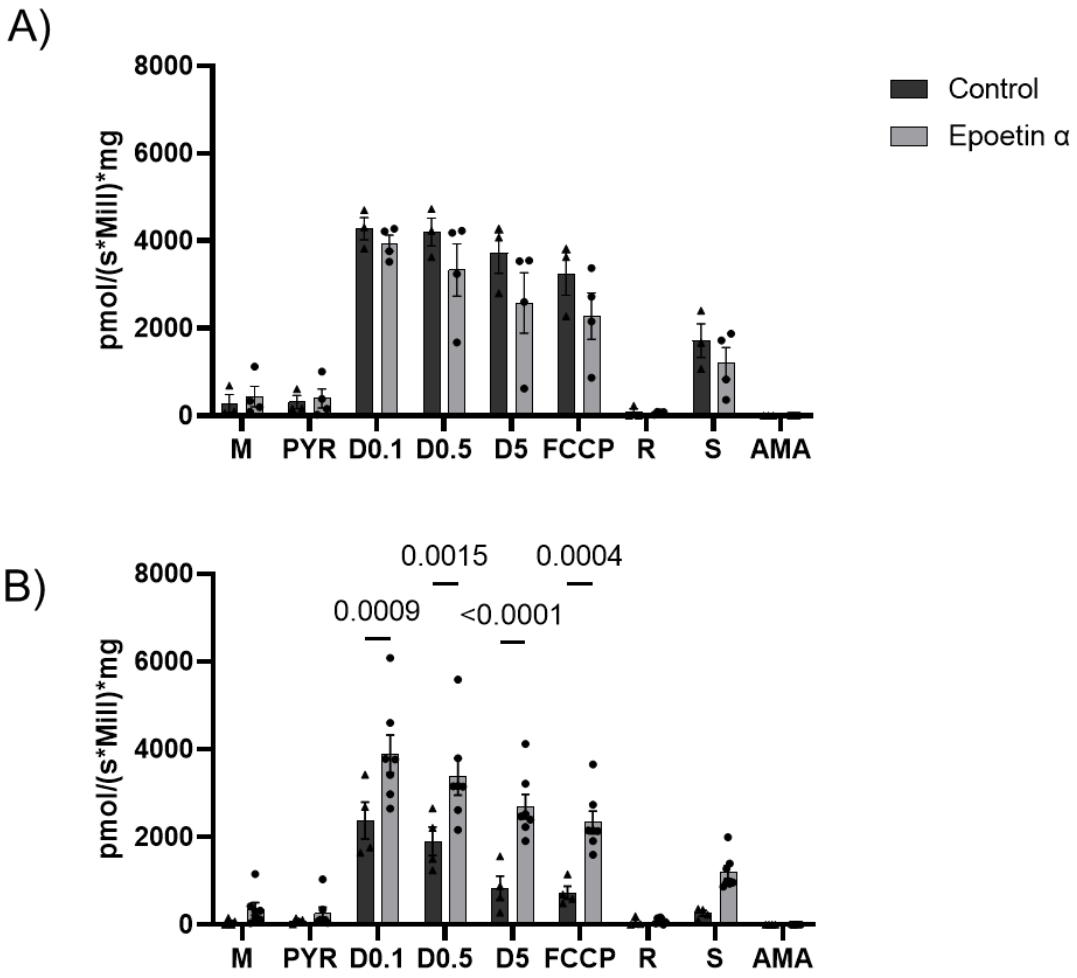

Figure 5.5- Epoetin α shows chronic but not acute changes to mitochondrial respiration.

A) There was no significant changes in mitochondrial respiration measured by Oxygraph-2k oroboros after 15 minutes of epoetin α treatment. B) After 24-hour treatment with 137 ng/mL epoetin α H9c2 cells showed a significant increase in mitochondrial respiration. M= 0.5 mM malate, PYR= 5mM pyruvate, D0.1= 0.1 mM Adenine Diphosphate (ADP), D0.5= 0.5mM ADP, D5=5mM ADP, FCCP=0.5µM carbonyl cyanide-p-trifluromethoxyphenylhydrazone (FCCP), R=0.5µM Rotenone, S=10 µM Succinate and AMA=5µM Antimycin A. Two-way ANOVA with a p-value of >0.5 deemed significant. Error bars represent SEM.

therefore an IC_{50} could not be calculated. Hydrogen peroxide had an IC_{50} of 140 μM, hemin had an IC_{50} of 176 μM and staurosporine had and IC_{50} of 8.2 nM. Both hydrogen peroxide and hemin reached 100% cell death at 200 μM and 300 μM, respectively. Staurosporine only reached approximately 75% cell death by 25 nM and did not respond further at increasing doses. Since hydrogen peroxide can induce both necrotic and apoptotic cell death[358] and hemin[359] and staurosporine[360] are generally associated with oxidative apoptosis, we decided to use these three agents in survival assays.

H9c2 cells were seeded at 5,000 cells per well and allowed to adhere for 24 hours. Cells were then treated with the rhEPOs for 24 hours before the addition of each xenogenic agent. The rhEPOs ranged in doses from 5.5 ng/mL up to 550 ng/mL, which was the range of doses that elicited a proliferative response in both 10,000 and 5,000 cells and were in agreement with prior literature ranges for cytoprotection. The IC_{50} was used at a constant dose across all EPO concentrations. The use of hydrogen peroxide was cytoprotective at 55 ng/mL and 137 ng/mL in epoetin α (Figure 5.7a), 5.5 ng/mL in Peprotech (Figure 5.7b) and 55 ng/mL and 550 ng/mL in darbepoetin α (Figure 5.7c). We also tested the rmEpo at 137 ng/mL, but it did not show any cytoprotection (Figure 5.5 d). The greatest degree of cytoprotection was seen at 137 ng/mL of epoetin α. Taken together, this data suggests epoetin α offers the greatest degree of cytoprotection, while darbepoetin α offers the least cytoprotection for the rhEPOs.

To determine cytoprotection against apoptotic agents we used staurosporine (Figure 5.8) and hemin (Figure 5.9). None of the rhEPOs showed cytoprotection against either hemin or staurosporine. This would suggest that EPO is not protective against oxidative apoptotic agents. Taken together with the hydrogen peroxide, there appears to

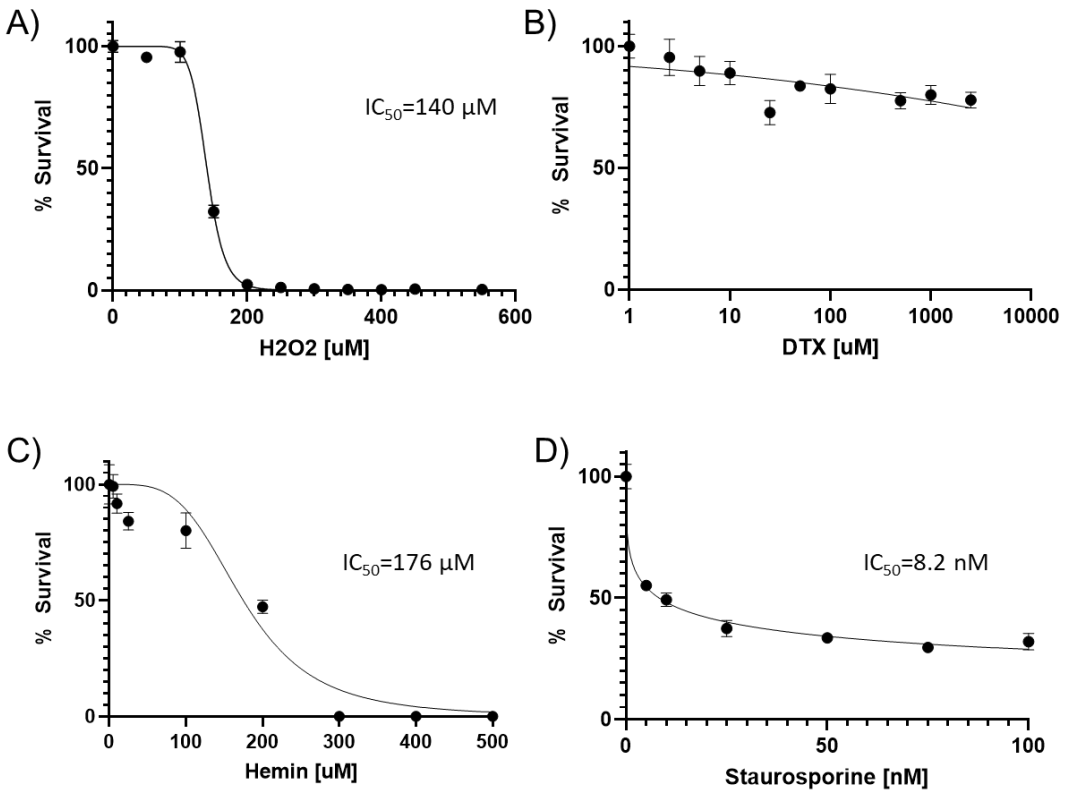

Figure 5.6- Survival of H9c2 cells against various toxins.
A) Hydrogen peroxide (H_2O_2), B) Doxataxel (DTX), C) Hemin, and D) Staurosporine. Y-axis shows the percent of survival as compared to no treatment control cells based on the absorbance at 560/590nm. X-axis shows the concentration of each cytotoxin. The IC_{50} is indicated in each graph with the exception of DTX as an IC_{50} could not be calculated. Error bars represent SEM. n=6

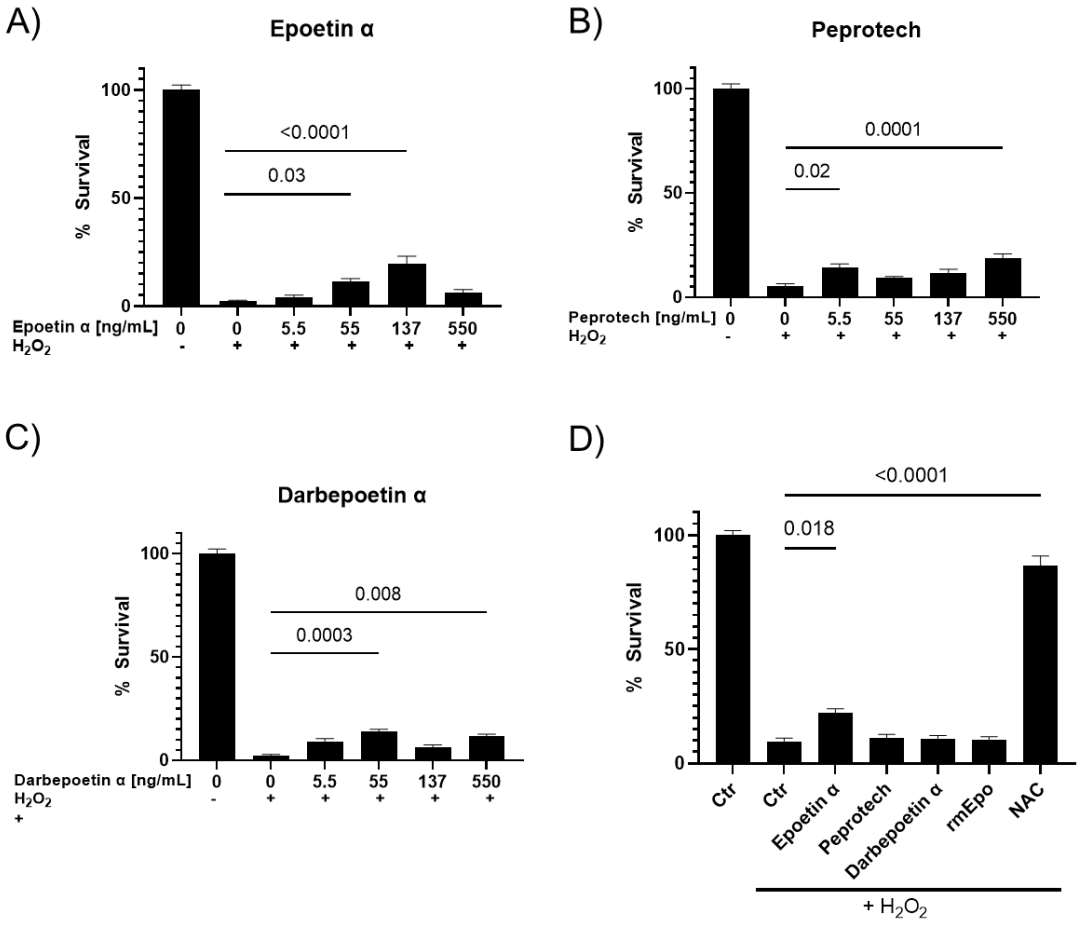

Figure 5.7- rEPOs show different degrees of cytoprotection against H_2O_2.
H9c2 myoblast were plated at a density of 5000 cells/well 24 hours before a 24-hour prophylactic treatment with EPO. After 24 hours in EPO 140µM of H_2O_2 was applied for 24 hours. A) Epoetin α is cytoprotective at 55 and 137 ng/mL. B) rhEPO is cytoprotective at 5.5 ng/mL and 550 ng/mL and C) Dabepoeitn α is cytoprotective at 55 ng/mL and 550 ng/mL. D) a comparison of rhEPO and rmEpo at 137 ng/mL with the hydrogen peroxide treat indicated by bar +H2O2. 400µM N-acetylcysteine (NAC) is a positive control for cryoprotection. Absorbance values were subtracted from no cell controls and normalized to no treatment controls (100%). P-values are determined by Two-way ANOVA. Error bars represent SEM. n=6

be a small degree of cytoprotection against necrotic cell death but less so apoptotic.

To investigate potential mechanisms for the cytoprotection observed we treated H9c2 cells with 137 ng/mL of rhEPOs for 24 hours before harvesting the protein. We used Western blot to examine some of the downstream targets of EPO to see which pathways were activated. Although we did see an increase in some proteins, such as Hmox-1 and Akt, most did not reach significance (Figure 5.10). Yet there was a significant increase in darbepoetin α in phosphorylated Erk1/2 and total Erk1/2 (ration did not reach significance). The apparent lack of sustained response in many of these pathways that are typically thought to be involved in the cytoprotective effect of EPO may explain the lack of cytoprotection against hemin or staurosporine and the minimal amount of cytoprotection against hydrogen peroxide in the time and doses tested.

5.3 Discussion

Epoetin α, as a representative of rhEPO, had a similar erythropoietic effect compared to rmEpo. However, rmEpo did not elicit cytoprotective effects (despite having shown the most proliferative effects), whereas rhEPOs offered varying degrees of protection against hydrogen peroxide in H9c2 cells. Epoetin α had a greater degree of cytoprotection followed by Peprotech and lastly darbepoetin α. The rhEPOs were cytoprotective against hydrogen peroxide but not hemin or staurosporine. No significant increases in downstream EPO signaling were detected, except possibly by Erk1/2 and phos-Erk1/2. There was a significant increase in mitochondrial respiration after chronic epoetin α exposure. Taken together, we show that rEPOs with similar effects for erythropoietic potential vary in their mitogenic or cytoprotective effects.

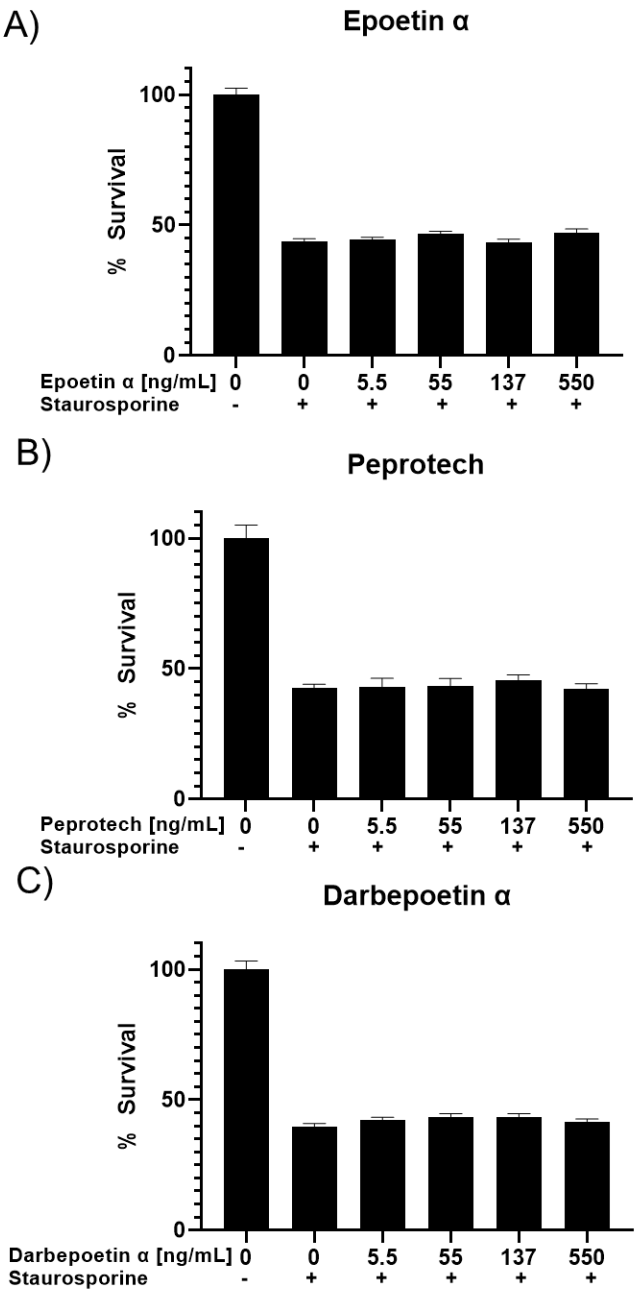

Figure 5.8- rhEPOs are not cytoprotective against staurosporine.
H9c2 myloblast were plated at a density of 5000 cells/well 24 hours before a 24-hour prophylactic treatment with rhEPO. After 24 hours in rhEPO 10nM of Staurosporine was applied for 24 hours. A) Epoetin α B) Peprotech C) Darbepoetin α. None of the EPOs were able to show any significant cytoprotection. Absorbance values were subtracted from no cell controls and normalized to no treatment controls (100%). P-values are determined by Two-way ANOVA. Error bars represent SEM. n=6

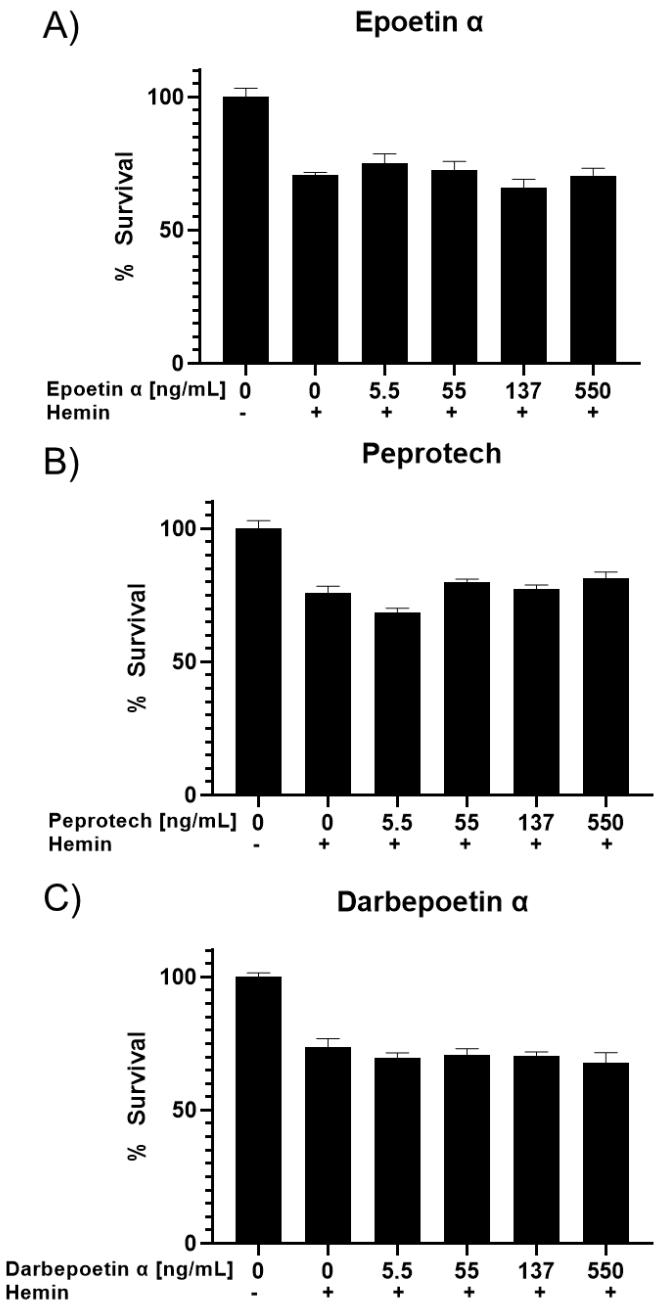

Figure 5.9- rhEPOs are not cytoprotective against hemin.
H9c2 myloblast were plated at a density of 5000 cells/well 24 hours before a 24-hour prophylactic treatment with rhEPO. After 24 hours in rhEPO 170μM of hemin as applied for 24 hours. A) Epoetin α B) Peprotech C) Darbepoetin α. Absorbance values were subtracted from no cell controls and normalized to no treatment controls (100%). P-values are determined by Two-way ANOVA. Error bars represent SEM. n=6

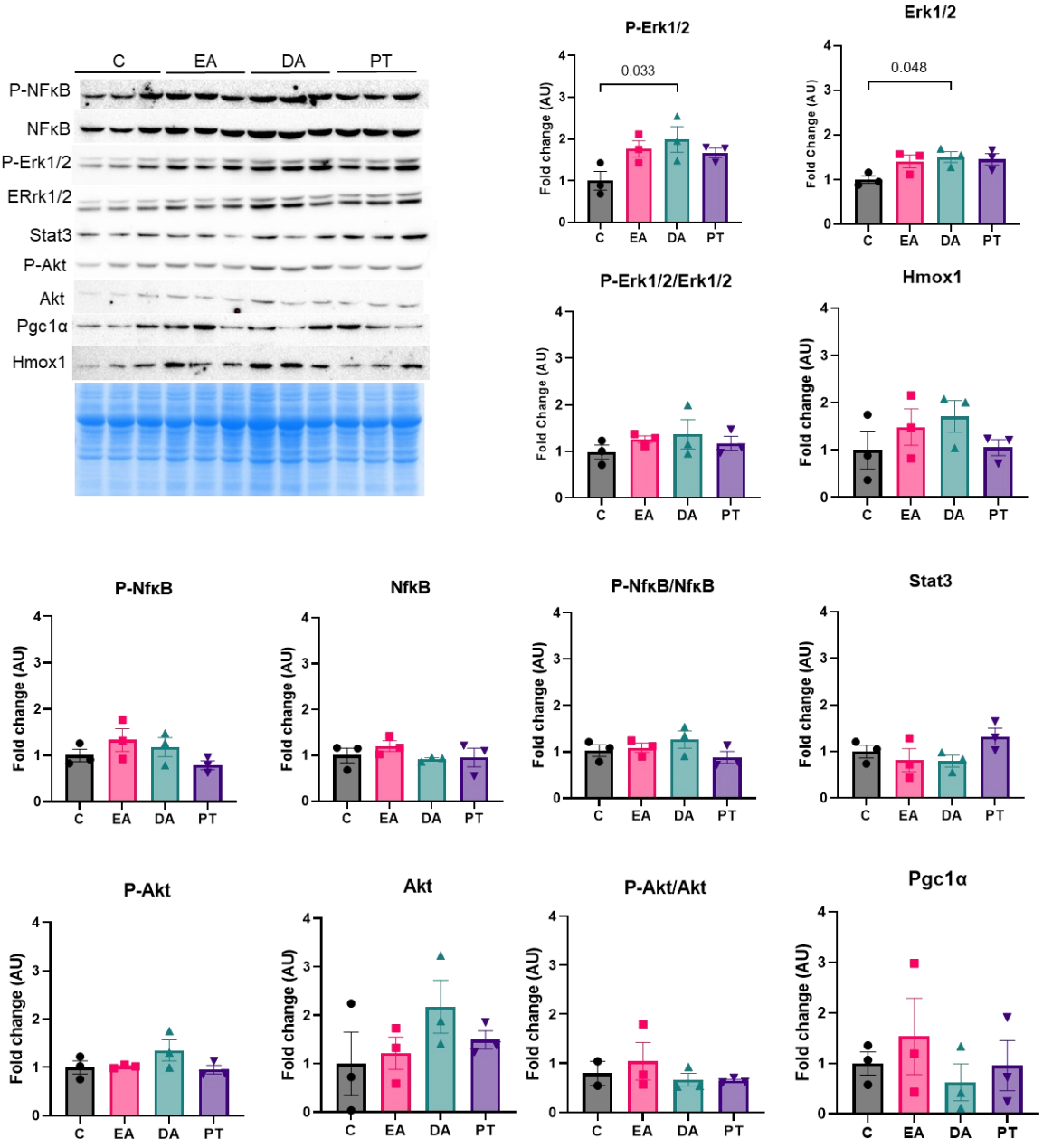

Figure 5.10- Protein changes in response to rhEPOs.
H9c2 cells were treated with 137 ng/mL of each EPO for 24 hours before cell harvest. C=control, EA=epoetin α, DA=darbepoetin α, PT=peprotech. Protein densities were normalized to total protein (Memcode) and significance calculated by One-way ANOVA with a $p<0.05$ being significant. Error bars represent SEM. n=3

One of the main uses clinically of ESAs is to treat anemia due to EPO's erythropoietic function.[344] We found that both epoetin α (a rhEPO) and rmEpo had similar erythropoietic potential in a CFU-E assay. Biological cross-reactivity has been documented due to the high degree of evolutionary conservation between vertebrates in EPOs.[84–87] Additionally, the site of *N*-glycosylation are conserved, which have been shown to be important for the erythropoietic activity.[361] We did not test CFU-E by darbepoetin α or Peprotech herein and therefore are using the results of epoetin α to represent rhEPO. Peprotech's data sheet reports their rhEPO has a similar EC_{50} and EC_{max} as we observed with epoetin α. Jamal *et al*.[353] compared epoetin α to darbepoetin α and found both to have erythropoietic potential, but darbepoetin α's EC_{50} was 3.78 IU/mL compared to epoetin α's EC_{50} of 0.32 IU/mL. However, there was no significant difference in their EC_{max}. The increased number of *N*-glycosylation sites on darbepoetin α does increase the half-life but can decrease receptor binding,[353] which may be the causes of the higher determined EC_{50}.

Mitochondrial respiration was increased in response to chronic exposure to epoetin α. This is in agreement with previous studies that have found rhEPO increases mitochondrial respiration.[354] However, PrestoBlue, which we use to measure viability, also measures metabolic activity.[362] As such, the change in metabolic function may impact our proliferative analysis interpretations. This is less likely to affect our cytoprotective assay as we normalize the viability to cells treated with the same appropriate dose without the xenogeneic agent. As we were more concerned with the cytoprotective functions, we did not investigate further to resolve specificity of mitogenic effects, which were marginal regardless. However, a more sensitive assay to

proliferation, such as measurement of DNA synthesis by EdU flow cytometry or direct proliferation using carboxyfluorescien succinimidyl ester (CFSE) dye in flow cytometery, could be used in future to resolve mitogenic variation.

Although epoetin α and rmEpo had similar erythropoietic potential, rmEpo did not exhibit cytoprotective function at the same dose of epoetin α, darbepoetin α or Peprotech. rmEpo did have a higher degree of mitogenic potential, but unlike the rhEPO this did not translate to an increase in cytoprotection. The cytoprotective functions are reportedly not as dependent on the *N*-glycosylation sites, which are conserved between humans and mouse. Two EPO derivatives that lack *N*-linked glycosylation (CPEO and asilo-EPO) are cytoprotective but lack erythropoietic function.[150,153,245,363] The *O*-glycosylation site is not conserved between mouse and human (Figure 1.6). Although *O*-glycosylation has been shown to play a significant role in the function in other proteins, it has been much less studied in EPO compared to the *N*-glycosylation.[361] One study that examined both the *N* and *O*-linked glycosylation found the *N*-linked were necessary for erythropoiesis, but the *O*-glycosylation was not.[361] The effects of *O*-glycosylation on the cytoprotection have not been studied. Therefore, understanding the role of the *O*-glycosylation may be another avenue in understanding the cytoprotective effects of EPO.

Both the rhEPO used and the seeding density affected the mitogenic effect observed. Epoetin α showed a dose-response up to 55 ng/mL at 10,000 cells but reached E_{max} by 5.5 ng/mL at 5,000 cells. Darbepoetin α saw very little mitogenic effect at 10,000 cells but showed some at higher doses with 5,000 cells seeded. The increased glycosylation of darbepoetin α compared to epoetin α results in a longer half-life (25.3 hours vs. 8.5 hours, respectively).[223] This difference in response by cell number may be

due to receptor bioavailability. The doubling time for H9c2 cells is approximately 24 hours.[364] We postulate that epoetin α saturates the receptors in 10,000 cells/well at 55 ng/mL and 5.5 ng/mL in 5,000 cells. As cells proliferate the epoetin α may have degraded or been internalized and cannot act on the newly generating cells (or new receptors). With darbepoetin α, the increased *N*-glycosylation can decrease EPOR binding (though with unknown effects on β-CR), decreasing its proliferative effect. However, as more cells proliferate the higher doses of darbepoetin α are still likely persistent in media and thus being persistently present would be able to act on the receptors of the newly formed cells to potentially cause a greater degree of proliferation. This could also indicate a preconditioning of the cells to increase EPOR:β-CR could also increase rhEPO effects. Detailed analyses of agonist-receptor interactions, agonist bioavailability, and receptor expression/compartmentation optimization for pharmacological assays in future will be necessary to resolve these findings. However, we can conclude that erythropoietic-bioequivalence is neither proportional nor transferable to an apparent mitogenic or proliferative effect.

Cytoprotection using rhEPO *in vitro* has been reported in different cell types with different xenogenic agents but with widely inconsistent results.[346] There are multiple forms of cell death that occur due to activation of various pathways, with variably time dependencies to reverse or arrest death activation cascades although there is overlap between the various pathways.[365] In our study we examined the cytoprotective effects of rhEPO in response to two forms of generally presumed cell death; necrosis and apoptosis. Necrosis is typically considered uncontrolled cell death that is induced by external injury, such as oxidative stress or toxic agents.[365,366] Apoptosis is “programmed cell death” that

occurs physiologically and pathophysiologically, such as in response to hypoxia or inflammation.[365,366] Hydrogen peroxide is elevated in inflammatory states,[347] and can trigger both necrosis and apoptosis.[358] EPO has been purported to be cytoprotective through both anti-oxidant and anti-apoptotic down-stream signaling,[83] which may be cytoprotective against necrosis and apoptosis, respectively. In cardiac myocytes, a majority of studies showed a modest increase in viability against hydrogen peroxide,[346,367,368] which is consistent with our results. However, another two studies found no increase in viability.[346,369] In one study using DLD-1 cells (colon cancer cell line) the use of rhEPOs was even suggested to increase the cytotoxicity of hydrogen peroxide.[370] Against staurosporine, an apoptotic agent,[371] three of six studies showed modest reduction in apoptosis, whereas the others did not.[346,372] In our study, none of the rhEPO were protective against staurosporine nor hemin induced apoptosis. Sinclair *et al.*[346] suggest one reason for the lack reproducibility in EPO cytoprotection is due to EPOR being undetectable in many non-erythroid cell types. In neonatal rat cardiomyocytes they were unable to elicit downstream EPO signaling using 10 U/mL of rhEPO (estimated to be 66 ng/mL).[346] Herein, at 137 ng/mL (approximating 25 IU/mL) we elicited a small increase in Erk1/2 and phoso-Erk1/2 with no change to the ratio. This may suggest that H9c2 myoblasts do not have or have low expression of the EPOR:β-CR. However, we did not examine EPOR or β-CR as we did not anticipate this potential limitation *a priori* given the demonstrated of effects in H9c2 cells[367,368] and to do so at this time is outside the scope of our intended comparative evaluation but will be considered in further investigations.

Another factor that may potentially limit the reproducibility of *in vitro* rhEPO

experiments is the lack of other growth factors and cytokines that would be present endogenously or appear in serum supplementation. It has previously been shown that factors, such as SCF, are necessary for EPO's erythropoietic function.[373] Indeed, our CFU-E assays are performed using SCF background in the media formulation. In cervical cancer cell lines, it has been shown that EPO alone resulted in transient ERK1/2 activation, whereas the combination of EPO and SCF had a stronger and more sustained activation.[374] This suggests that SCF could be a potentiation factor of EPO, and coordinated paracrine signaling *in vivo* may lead to differentiated effects. The activation of Erk1/2 may also explain the increase in cytoprotection by darbepoetin α. In our study darbepoetin α had the lowest degree of cytoprotection and yet was mitogenic at 5000 cells at higher doses, which we suspect is due to its longer half life. Darbepoetin α was the only rhEPO to reach significance in Erk1/2 and phos-Erk1/2. Again, this may be due to its long half-life and being extended presence in media to activate Erk. This may also suggest that variable timing may elicit different results. Therefore, future studies could examine testing additional compound combinations with EPO and potentially different time points to improve rhEPO's effectiveness. However, the intended need for lasting protection rather than transient signalling interference suggests a limitation in translational benefit clinically.

As a molecular mechanism screening and pilot study to examine the various facets of rhEPO by *in vitro* models it has several limitations. One major limitation is we did not examine EPOR and β-CR in our study. Sinclair *et al*.[346] has suggested the low amount of EPOR may be the cause of limited reproducibility with rhEPO. EPOR:βCR are required for cytoprotective effects of EPO.[151,152,154,155] Therefore, if proliferating

H9c2 do not express EPOR:βCR, or else internalize in response to stress or agonism, then it will not respond to rhEPO and is therefore not a good model. Even by exploring this expression by mRNA, the EPOR:βCR also must be localized to the plasma membrane, which typically happens in hypoxia and in the presence of proinflammatory cytokines.[154] Therefore, along with the addition of SCF, preconditioning to localize the EPOR:βCR to the plasma membrane may be necessary to resolve any impact in the response to rEPOs. Also, testing different time points after rhEPO therapy may be necessary to see the full effects in signalling. We opted to use 24 hours based on previous studies, but this may not have been the optimal time, though the absence of Hmox1 suggest that there is no lasting phase-2 detoxification response to protect cells from accumulative oxidative stress either.

We have established that rhEPO and rmEpo have similar erythropoietic potential, but do not have proportional mitogen or cytoprotective profiles. The rmEpo was not cytoprotective but the rhEPOs were cytoprotective against hydrogen peroxide, but none we protective against hemin or staurosporine. This suggests the rhEPO, but not rmEpo, may be slightly protective against early necrotic/necroptotic cell death. Given that the rhEPO did not appear to activate downstream EPO anti-apoptotic signaling pathways, the cytoprotection observed may be due to EPO's ability to elicit some less well known associated anti-oxidant effects, or else alter the nature of cell metabolism alternatively. This demonstrates that the type of rhEPO may be important in the cytoprotective effects and requires further studies to elucidate these differences.

Chapter 6- Discussion

6.1 Summary of results

The aim of this thesis was to investigate the biological differences of variations in EPO at the various levels of regulation, including genomic, mRNA, and protein. The salient contributions achieved to the field include: 1) SNPs in *EPO* are associated with risk-factors and co-morbidities related to CVD, 2) intron retention is a form of post-transcriptional regulation of EPO in the brain, and 3) variation in glycosylation of rEPOs can result in variation in the functions of EPO in a stress context-dependent manner.

Variations in *EPO* in the gDNA, in the form of SNPs, are associated with risk factors and comorbidities for CVD (Chapter 3). Specifically, SNPs in *EPO* change the amount of EPO in the plasma, with the CC genotype for rs1617640 and the GG genotype of rs507392 associating with decreased plasma EPO. The CC genotype in rs1617640 also associated with lower Hb, higher cholesterol, non-HDL, LDL and NLR, suggesting a role in erythropoiesis, lipoprotein metabolism and inflammation. The C allele in rs1617640 associated with anemia in a log-additive model of inheritance, further supporting a potential role in erythropoiesis and potentially inflammation. The GG genotype in rs507392 was associated with increased cholesterol, non-HDL and LDL, suggesting a role in lipoprotein metabolism. The role in lipoprotein metabolism is further supported by an association in a recessive model of inheritance to dyslipidemia. In females only, the G alleles in both rs507392 and rs551238 associated with an elevated risk of hypertension. Together, this shows that variations in the gDNA alter the regulation of *EPO* leading to altered CVD co-morbidity risk.

Variations at the mRNA level of *EPO* can also alter the regulation of EPO. Intron retention in *EPO* mRNA increases in brain cell lines in response to ischemia, suggesting a tissue specific mechanism of post-transcriptional regulation (Chapter 4). The intron retention was shown to not be a result of immature mRNA, demonstrating intron retention is a deliberate regulatory mechanism. In addition to response to ischemia, intron retention in *EPO* mRNA also shows circadian rhythmicity. Together, this suggests that intron retention is a tissue specific mechanism to respond to ischemia and assist in EPO's circadian rhythmicity.

Variation in EPO's PTM, particularly glycosylation patterns, can alter the function of rEPOs (Chapter 5). Epoetin α and rmEpo had similar erythropoietic potential but differ in their cytoprotective potential. Epoetin α shows the greatest degree of cytoprotection against hydrogen peroxide, compared to a smaller degree by Peprotech-rhEPO and darbepoetin α, with no appreciable cytoprotection from rmEpo. However, there was no cytoprotection from any of the rEPOs against hemin or staurosporine, suggesting cytoprotection only against necrosis (i.e. hydrogen peroxide). Only Erk1/2 was elevated, with many of the kinases expected to respond to EPO showing no significant increases. However, chronic exposure (24 hours) to epoetin α did increase mitochondrial respiration, suggesting the cytoprotection may be indirect through metabolic processes. Collectively, our results show that genetic variations in the gDNA, mRNA and protein level influence the regulation and function of EPO.

6.2 Molecular variation of EPO by pathophysiological and anatomical drivers

EPO is a pleiotropic hormone that is expressed by, and acts on, many tissues.[375] The kidney has historically been seen as the primary producer of EPO in adults,[75]

although many other tissues including brain, uterus and heart have been found to express EPO.[376] It is speculated that the EPO produced by the kidney accounts for the majority of the circulating EPO and is responsible for EPO's erythropoietic function.[377] EPO produced by other organs may also contribute to the circulating EPO, but to a lesser extent, although this may be stimulus dependant. However, the production of EPO by many other tissues is thought to act in a paracrine or autocrine fashion to exert its effects on the tissue producing the EPO.[241] Therefore, there may be mechanism(s) for the regulation of EPO that are tissue specific. Indeed in the liver, which is the primary site of EPO production in the fetus before switching to the kidney in the third trimester,[378] hepatocyte nuclear factor 4 alpha (HNF-4α) in addition to HIF2 is required for *EPO* transcription. HIF2 and HNF-4α bind to the LIE to promote transcription.[379,380] Additionally in the uterus, *EPO* mRNA has been found to be under the transcriptional control of both hypoxia and 17β-estradiol (E_2).[376] In both the liver and uterus, HIFs are still required for *EPO* regulation and HNF-4α and E_2 act with HIF to upregulate *EPO*.[376,379,380] In our cell models, brain cells responded more to ischemia, in both the increase mRNA and intron retention, than hypoxia alone. In two neuroblastoma cell lines (Kelly and SH-SY5Y), Stolze *et al*.[381] reported an increase in *EPO* mRNA in response to anoxia (no oxygen) and a significantly lower response to hypoxia (3% O_2), while HEPG2 cells responded more to hypoxia than anoxia. This is similar to what we observed with brain cell lines responding more to ischemia than hypoxia, with HEPG2 (liver cells) responding more to hypoxia than ischemia. We used hypoxia to model hypoxic hypoxia (decreased O_2 availability) and ischemia to model anemic/stagnant hypoxia (impaired O_2 transport or delivery/insufficient blood flow).[382] The brain consumes more glucose

compared to its proportion of the bodyweight (consuming 20% of glucose for ~2% of body weight).[383] The high energy demand of the brain may make it more sensitive to anemic/stagnant hypoxia than hypoxic-hypoxia due to nutrient demand. The more potent response in the brain cells to ischemia in our cell model would support additional co-factors that are nutrient sensing in the brain cell lines that assist in regulating *EPO* transcription. Although HNF-4α is needed for *EPO* in the liver it is also a transcription factor in lipid and glucose metabolism,[384–389] and expressed in liver, kidney, intestine and pancreas,[389–391] but has not been found in brain.[381] Also E_2 is needed for *EPO* regulation in the uterus, but was not found to be involved in cerebrum *EPO* regulation.[376] As yet there has not been a brain specific co-factor that regulates *EPO* mRNA transcription but our results do suggest there may be one that has yet to be described.

In addition to the difference in *EPO* mRNA responding more to ischemia in the brain, intron retention also increased more in response to ischemia and then only in the brain. Intron retention is a form of alternative splicing that are more prevalent in the brain,[34,35,38,39] but the mechanisms governing its tissue specificity is unknown. Introns have been shown to be important for survival to nutrient deprivation in yeast.[56] Although intron retention was not examined in the study, the lack of intron in the gDNA would prevent intron retention. Therefore, intron retention being a mechanism to reserve mRNA during starvation, could explain their requirement for survival in yeast. Alzheimer's disease is linked to increased intron retention[39] and also linked to a decreased brain glucose metabolism.[392] This suggests there may also be mechanisms where nutrient deprivation regulates intron retention in a tissue specific manner in humans. At this time it is unknown if the intron retention in *EPO* will results in an mRNA reserve or a novel

isoform. We did perform preliminary analysis of potential transcripts generated by retention of intron 2 or intron 4 (Figure 6.1). The retention of intron 2 would result in small EPO that would not contain receptor binding sites and is therefore less likely to be a viable isoform. Only 40% of the intron 2 containing isoform (Figure 6.1 b) could be modeled since the portion that would be added by the intron does not resemble other known proteins. The unmodeled portion of the isoform may generate a unique isoform, but we are unable to verify this currently. However, retention of intron 4 produces a truncated EPO that still contains many of the receptor binding sites, suggesting it has the potential to be a novel isoform. It is possible the two introns have different biological roles and may explain some of the differences seen between the brain cells between the EPO2-3 and EPO4-5 primers. For example, EPO 2-3 showed more significant intron retention in glioblastoma compared to EPO4-5, whereas neuroblastoma had similar effects between both introns. This may suggest in addition to the tissue specificity, the cell type specificity may utilize intron retention for different functions. However, our understanding of the mechanisms governing tissue specific, or stimuli specific regulation of intron retention is very limited and therefore more studies are needed to better understand this phenomenon and its biological function.

Tissue differences may also affect the phenotypes observed in *EPO* SNPs. Although the CC genotype of rs1617640 and GG genotype of rs507392 both result in a reduced plasma EPO, they correlated with anemia and dyslipidemia, respectively. EPO from the kidney is primarily responsible for erythropoiesis that links the decrease in EPO to anemia. However, the liver is the primary site of lipoprotein metabolism,[393] where HNF-4α is also required for *EPO* regulation.[379,380] HNF-4α is also implicated in lipid

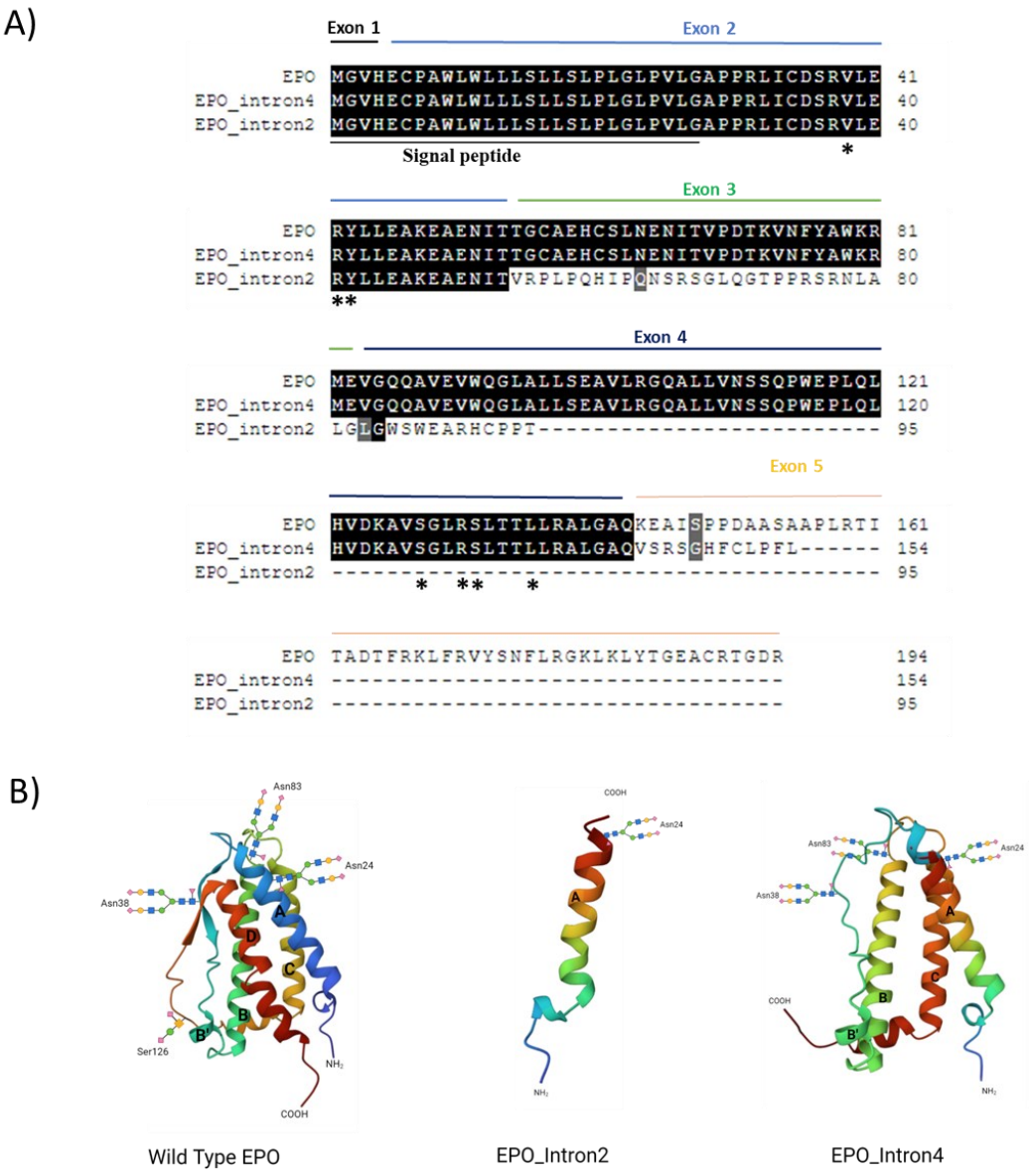

Figure 6.1- Predicted amino acid sequence and protein structure of intron-retaining transcripts.

A) Intron retaining transcripts compared to wild type. The exons are indicated above the alignment. Canonical amino acids up to Exon 4 are shown with a black background with the novel amino acids form the intron with a white background. Signal peptide is indicated below with a line and receptor binding sites are denoted with *. Alignment made in Multiple Alignment Show (www.bioinformatics.org/SMS/multi_align. html). B) Predicted protein structure based on intron retaining transcripts if generating novel isoforms. Structure generated using Protein Homology/analogy Recognition Engine (Phyre) v 2.0 and visualized using Mol*3D Viewer at protein data bank (PDB, rscb.org/3d-view). Glycan structures added using BioRender.com

metabolism, and therefore the GG genotype in rs507392 could have an effect on HNF-4α binding, potentially in linkage disequilibrium with the binding site, resulting in association with dyslipidemia. Additionally, the rs507392 SNP is located in an intron. Although not one of the introns investigated in Chapter 4, there may be some link to intron retention regulatory sequences. Our study (Chapter 3) was limited to investigating associations, and therefore the mechanisms were outside the scope of the study to establish causation (such as by mutagenic study or CRISPER techniques). A study by Tong *et al*.[281] examined the A and C allele for rs1617640 in a luciferase assay and found the C allele had an increased *EPO* expression. A eQTL study also suggested that the rs1617640 affected *EPO* expression.[196] Together these studies suggest a direct link with the rs1617640 SNP and *EPO* expression, however the exact mechanism governing the expression is unknown. It does not appear to be a direct binding site for transcription factors but is in proximity to transcription binding sites and it may act in a steric effect and transcription binding site. Similar studies have not been performed for the other SNPs and therefore more directed mechanistics studies to confirm their role in *EPO* expression would be beneficial. However, the link to dyslipidemia has not previously been reported and herein we provide evidence that warrants further investigation into EPO's role in lipid metabolism.

The temporal effects of EPO can also vary based on tissue. In a mouse model, kidney *Epo* mRNA peaked by 4 hours followed by a reduction. However, the cerebrum (i.e. brain) had a sustained *Epo* mRNA for up to the 24 hours.[376] It has been suggested that the sustained *Epo* mRNA in the brain may be necessary for the neuroprotection for the duration of the hypoxic exposure.[394] In our cell models the liver cells did increase

and then decrease in a temporal manner in response to hypoxia and ischemia, whereas the brain cells, with the exception of BBB, all increased and remained high throughout our 24 hour time period. The requirement for sustained EPO for neuroprotection may be a potential physiological reason for the intron retention specifically in the brain. As the brain is reperfused, intron-retaining transcripts could be used to generate EPO, thus maintaining EPO levels during the reperfusion to protect against ischemia-reperfusion injury. However, this is only one possible reason for the intron retention seen in the brain. As our data discovered these intron-retaining transcripts, future studies are needed to understand the role of intron retention in *EPO* more fully.

The tissue specific effects of EPO may also be due to receptor variations between tissues. The erythropoietic functions of EPO are elicited through the EPOR:EPOR homodimers on the erythroid progenitor cells.[132,143] In other tissues EPOR:β-CR heterodimers are thought to elicit many of the other EPO functions. Cytoprotective effects of EPO have been shown to require EPOR:βCR.[151] Receptors have also been suggested as a potential explanation for the irreproducible results on the cytoprotection of EPO *in vitro* and *in vivo*. Sinclair *et al*.[346] found the amount of EPOR was much less in non-hematopoietic tissues. However, Sinclair assumed all EPOR was a homodimer, which would not be the case outside the erythroid cells that would have EPOR:βCR heterodimer additionally or alternatively. Other studies have identified EPOR and βCR in neuronal mouse tissue,[395] adult mesenchymal cells,[396] umbilical cord blood,[152] liver, endothelial progenitor cells,[397] and in rat cardiomyocytes (H9c2).[367] In studies that identified the EPOR and β-CR, rEPO was shown to be cytoprotective.[151,152,367,396,397] Studies that did not observe cytoprotection did not directly examine the receptors, and

therefore the lack of, or the internalisation of the receptors, could be responsible for lack of the EPO response. Herein, we did not directly examine the receptors since H9c2 had previously been shown to express EPOR and β-CR.[367] However, as we did not directly measure the receptors and their localization our lack of EPO signaling could be due to the unavailability of the EPOR:βCR in our cell model. Previous studies that have found EPOR:βCR in H9c2 used differentiated cells,[367] whereas we used proliferating H9c2s, and the state of the cell may also change the receptors bioavailability. Future studies should include analysis of the receptors, along with their cellular localization to ensure the receptors are present and localized at the cell membrane.

The persistent *EPO* mRNA production in the brain has been speculated as needed to maintain he cytoprotection in the brain.[394] This would also require persistent activation of the receptors to elicit the cytoprotective effects. Herein, we used a cardio myoblast cell line, which has not been studied for its temporal production of *Epo* mRNA. However, we hypothesize that EPO is having a cytoprotective role in the heart and therefore may be similar to the persistent mRNA seen in brain. EPO signaling through ERK1/2 pathway with EPO alone was transient, but was sustained with the addition of SCF in cervical cancer cells.[374] In our study, darbepoetin α was the only rhEPO to elicit a response in Erk1/2 after 24 hours. Since darbepoetin α has a longer half-life, it may remain in our media longer to provide continuous activation of Erk1/2, similar to that of adding of SCF for co-receptor pathway activation. This may indicate timing of treatment as an avenue for future studies to determine if EPO signaling is time dependant and if addition of co-factors are dependent on the EPO response. Application of growth factor reduced media conditions (eg. FBS presence/type) with greater control of variables will

be necessary to resolve the cell signaling effects.

6.3 Molecular variation of EPO in therapeutics

ESAs have been used for the past few decades to treat different forms of anemia,[344] and recently investigated for use against ischemic injuries.[191,242] The erythropoietic function of rhEPO have been widely supported in many clinical trials. However, the results from pre-clinical and clinical studies on EPO's cytoprotective function have not been consistent. There are multiple factors that may be driving the irreproducibility, such as the cell type used (and potentially the receptor(s) expression), the type, dose and timing of rEPO or influence of potential cofactors (positive or negative cross signaling). Clinical trials examining EPO's cytoprotective role utilize ESAs that have been approved for use in anemia (pharmaceutical grade), whereas many pre-clinical trials utilize commercially available rhEPO or rmEpo (non-pharmaceutical grade). Although most ESAs and commercial rEPOs are generated using the endogenous EPO amino acid sequence, there can be variation in the glycoforms generated by the production process. We found small variations in size between ESAs and rEPOs used herein, which is likely due to variation in the glycoforms. Although we observed similar erythropoietic functions, there was variation in their cytoprotective potential, which may be attributable to the glycosylation. Much of the literature has focused on the significance of the *N*-linked glycosylation site of EPO since the *O*-linked glycosylation was not found to be significant in the erythropoietic potential, whereas the N-linked glycosylation was found to be necessary for erythropoiesis.[361] Although there is a high degree of conservation between mouse and human EPO and they demonstrate cross-reactivity/potency in erythropoiesis,[84–88] in our study and a previous study by Pathipati

and Ferriero[369] did not find rmEpo to be cytoprotective against H_2O_2. One region that differs between the mouse and human is in the region with *O*-linked glycosylation, with the site for *O*-linked glycosylation only present in human. Since the *O*-linked glycosylation was not found to be significant in the erythropoietic function[361] it has been assumed that it was not significant in EPO's other functions and therefore not typically examined in rEPOs. However, since there does appear to be a difference in the cytoprotective potential between human and mouse rEPOs, it suggests the *O*-linked glycosylation site may have significance in the cytoprotective function. There are other sites that differ between mouse and human that may also be responsible for this variation in function.

In addition to the type of rEPO varying between studies examining cytoprotection, the doses also vary between studies. The dosing of the rEPOs to elicit cytoprotection is generally higher than what is used to elicit erythropoiesis.[242,352] Comparing doses across studies is made difficult as different units are used for various rEPO. Many rhEPO use U, while epoetin use IU. There is a general standard for dose conversions between epoetin and darbepoetin α (1 μg~200 IU),[215] but conversion factors are not as clear from U to ng or IU. The use of different units of measurements may result in discrepancies in the doses required to elicit cytoprotection. It was for this reason we tested multiple doses in our study. As with other studies,[398] rhEPO showed cytoprotection up to a certain dose, but higher doses (550 ng/mL in our study) were not cytoprotective. This could suggest that the dose, both too high and too low, may result in a negative finding. The reason for this curve is unknown but may be due to overwhelming the receptors, which might then be internalized preventing a continuous

activation needed for cytoprotection.

There have also been a range of different cytotoxins used in studies examining cytoprotection with rEPOs. Not all cell death is equivalent and different cytotoxins can elicit different forms of cell death. Herein, we examine both necrosis, ferroptosis and apoptotic cell death and found EPO to be cytoprotective only against necrosis (i.e., hydrogen peroxide). However, other studies have found EPO to be protective against apoptotic and oxidative cell death.[151,367] The lack of EPO's anti-apoptotic signalling may be the reason behind the lack of anti-apoptotic cytoprotection, while pointing to a more anti-oxidant role in cytoprotection. This is supported by the increase in mitochondrial respiration. Therefore, the mechanism for cytoprotection may be another area of further investigation to improve reproducibility.

A better understanding of the properties that influence the cytoprotective function of EPO are important to improve the reproducibility and translation of EPO cytoprotective potential. Herein, we tested multiple rEPOs that demonstrated similarity in their erythropoietic potential but differed in the cytoprotective potential. Since we can see molecular weight differences between the rEPO, this indicates some variation in glycoforms that were more or less cytoprotective. Future studies should compare different glycoforms, such as epoietin ω that has variation in the *O*-glycan compared to other epoiteins.[217] If features are found in rhEPOs that effect the cytoprotection it may allow for development of ESAs that optimize these features, similar to the increase in N-linked glycosylation in darbepoieitin α to increase half-life.[223]

6.4 Molecular variation of EPO in national and global public health

EPO is a pleiotropic hormone that is involved in a multitude of functions and

SNPs in *EPO* have been associated with a variety of diseases (See Table 3.1). The SNPs rs1617640, rs507392 and rs551238 are generally thought to elicit effects by the amount of EPO produced. We found the CC genotype and GG genotype in rs1617640 and rs507392, respectively, did decrease the amount of EPO in the plasma and consequently associated with anemia and dyslipidemia, respectively. This likely has added to the diseases and co-morbidities associated with less EPO.

ESAs have been an important advancement in the treatment of anemia. However, there is variation between populations in their effectiveness.[399,400] In an observational study of ESA doses in CKD patients undergoing hemodialysis, black patients received 12.6% higher doses to achieve similar Hb as white patients.[400] Based on data from the 1000 genome project reported in the Ensembl database, the SNPs rs1617640, rs507392, and rs551238 do show variation in frequency across ethnicity. Generally Asian populations have a lower minor allele frequency compared to European.[309] Many of the previous studies investigated Asian populations and had similar allele frequencies as those reported in Ensembl, whereas the frequencies we report herein are more similar to European, which reflects the European ancestry predominant in New Brunswick. Although none of the SNPs we examined have been investigated across different ethnicities for function, a majority of prior work was assessed in Asian populations,[277] and other SNPs show variations of impact by ethnicity.[401] The variation in prevalence could indicate there may be variations in different populations on potential health implications. The association with *EPO* SNPs with responsiveness to ESAs has also not been examined. Since *EPO* SNPs have been associated with a decrease in EPO, higher doses of EPO may be needed to elicit the same response due to a lower starting point.

Our population in New Brunswick is relatively homogenous of European decent. We did not specially examine ethnicity and may struggle to obtain sufficient diversity in this population, which is a limitation of this study. Future studies should further examine the differences in these SNPs in different ethnic backgrounds and response to ESAs for precision medicine and clinical trial design that treat-to-effect. As mentioned previously, herein we did not investigate causality. Future studies to link the functionality of these SNPs to a clinical phenotype are needed to better understand the role of these SNPs to a clinical phenotype.

Intron retention has only recently been examined as a mechanism of post-transcriptional regulation in mammals. Already it has been linked to Alzheimer's disease,[39] ALS,[325] and various cancers,[34] which suggest a role in human pathophysiology. EPO has been shown to improve cognition,[285] and the increased intron retention in Alzheimer's disease could decrease available EPO in the brain, which may modulate the disease progression. Examination of *EPO* mRNA is common practice for the study of most tissue EPO since the glycosylation makes immunodetection difficult and the protein is not stored intracellularly. If measuring *EPO* mRNA in the brain, the intron retention may alter the predicted amount of EPO protein. The fate of the intron-retaining transcripts was outside the scope of this study, but future studies should explore the role of EPO protein level (such as with knock-in reporter cell lines or mouse strains). If intron retention is found to alter protein levels of EPO in the brain, this will need to be accommodated into study designs and limitation that otherwise might presume 1:1 ration of mRNA:protein for EPO, and where the feedback regulation or effects are described in terms of erythropoiesis, cytoprotection, or metabolism.

Intron retention has also been linked to a variety of cancers. Intron retention in cancer has been linked to aberrant proteins generated from the intron-retaining transcripts.[402,403] There is also evidence to suggest the intron-retaining transcripts may be able to evade nonsense-mediated decay (NMD) and result in partial inactivation of tumour suppressors, including *TP53, ARIDIA30 and VHL31*. In other genes and SNP may introduce a PTM into a retained intron that results in an increase in NMD, such as in *NBEAL2*.[403,404] Higher EPO levels of serum have been linked to myelodysplastic syndrome (MDS). In particular, higher EPO levels have been suggested as predictive marker in anemia of unknown etiology as an early indicator of MDS.[405] Although intron retention in *EPO* has not been investigated for a potential role in cancer, this may be an additional area to investigate for pathophysiological roles.

6.5 Future Directions

Herein we present three studies that investigate biological variations in EPO. These studies will serve as a basis for future studies to further investigate the genetic variations in EPO and their biological significance. Our association of *EPO* SNPs used a relatively small cohort, and should be expanded to include additional participants, particularly those with dyslipidemia and females with hypertension. In our study we did not include kidney function or medical management and/or medications. Future studies should include analysis of co-morbidities, medical management, and use of medication, such as ESAs or statins. Additionally, for the lab values we use one time point, which may not provide a full picture of the baseline lab values or medication effectiveness. Potentially a prospective study that incorporated more time points for lab values (potentially before introduction of medications) would be usefully in determining

baseline risk factors. The increased population size could also allow for more involved analysis such ANCOVA.

Herein, we showed intron retention in *EPO* increases in response to ischemia in the brain and its potential contribution to circadian rhythm of *EPO* expression. Although we speculate this is acting as a reserve of mRNA, we did not confirm the fate of mRNA. Future studies should investigate the role in intron retention in *EPO* on EPO protein levels. Herein we have investigated cell models, which limits our ability to manipulate circadian effects. An *in vivo* model could allow for manipulation of circadian rhythm and potentially in combination with hypoxia or ischemia. An *in vivo* model could also allow for investigation into pathophysiologies, such as anemia and stroke. Therefore, future studies should examine intron retention in *EPO* using *in vivo* models to delve into cell and system integrative physiology.

An improved understanding of the molecular basis governing rEPO effects *in vitro* could improve the translation to *in vivo* studies and inform clinical study design. This should include an analysis of the EPOR and β-CR in our cell model, including its localization. Cell models, like H9c2 myoblasts, are likely good models for study only if the receptors expression, density, and bioavailability are more certain and could be the basis to choose a more appropriate models to study. Further, it remains to be determined how much EPO signaling is interdependent on other cytokines, and whether pre-conditioning (for receptor localisation) or if potentiation factors, such as SCF, are required to elicit defined EPO signalling in cell or tissue-regulated manners. Additional time points may better capture EPO downstream signaling. By improving the EPO

downstream signaling *in vitro*, it could lead to improved *in vivo* translation that may eventually improve the use of ESAs as cytoprotectants in the appropriate clinical context.

6.6 Concluding remarks

For the first time we have demonstrated that the rs507392 SNP in *EPO* is associated with dyslipidemia and confirmed a role for rs1617640 in anemia. Also, for the first time we have shown that intron retention is a post-transcriptional regulatory mechanism of *EPO* in the brain. We lastly examined the various functions of rEPO to establish potential differences in their function. Together, our data show that variations at the gDNA, mRNA and protein level of EPO have implications in human health and potential therapeutics based on EPO. Our studies will serve as a basis for future studies to further elucidate the role and significance of these variations in fundamental biology, human health and the design and application of therapeutics.

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