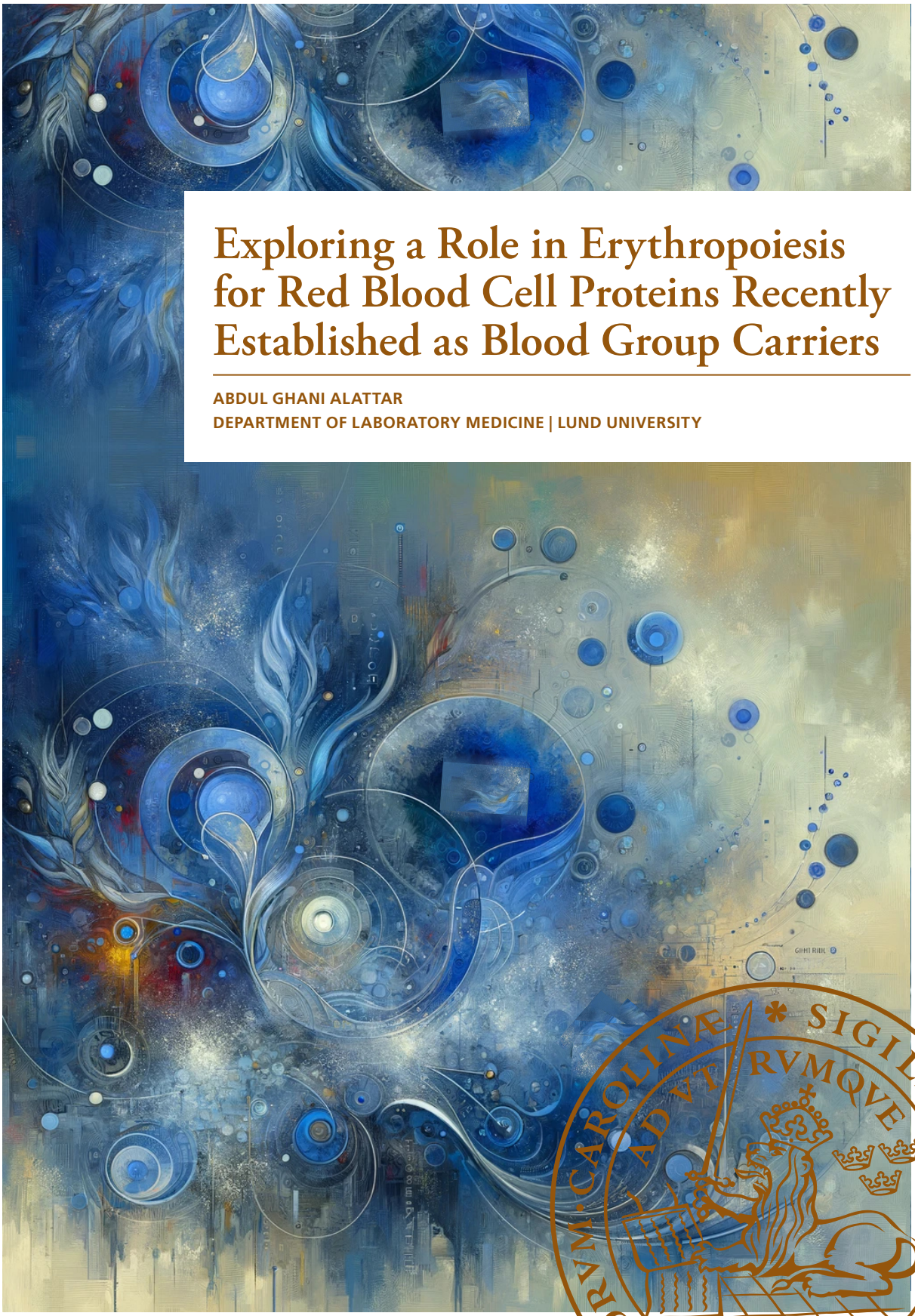

Exploring a Role in Erythropoiesis for Red Blood Cell Proteins Recently Established as Blood Group Carriers

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Exploring a Role in Erythropoiesis for Red Blood Cell Proteins Recently Established as Blood Group Carriers

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LUND
UNIVERSITY

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Abstract

Ensuring the availability of compatible blood for transfusion to patients in need is a crucial part of modern healthcare. Many different categories of patients benefit from these efforts but those requiring chronic transfusion support need special care to prevent adverse events associated with alloimmunization against blood groups beyond ABO and Rh. Thanks to the genetic revolution, the molecular bases of most clinically important blood groups have now been resolved. This makes blood group genotyping possible in clinical practice, which has helped to match blood donor and recipient to ensure a safe outcome. It has also enabled genetic screening for rare donors, antenatal blood group typing of cell-free fetal DNA in maternal plasma and synthesis of recombinant blood group proteins to use for neutralization/identification of blood group antibodies. However, all this is only possible when the molecular genetic basis of a blood group is known and this is when a blood group system can be acknowledged. In this thesis, studies are presented that formed the basis of two new blood group systems. In addition, it has been noted that some blood group molecules appear to regulate hematopoietic processes. Work presented here also aimed to start investigating a possible role in erythropoiesis for the proteins underlying these two blood group systems. Finally, we evaluated if a heme binding, antioxidative plasma and tissue protein can protect the hematopoietic system during treatment with a radioactive substance.

In **Paper I**, a wide array of molecular technologies was used to study all known cases of the rare and unresolved MAM− blood group phenotype that was originally reported over three decades ago. The genetic basis of MAM was elucidated together with an international team of investigators and revealed that EMP3 is responsible for MAM antigen expression. Surprisingly, stem cells from MAM− individuals were shown to give rise to ~5 times more RBCs than MAM+ when cultured *in vitro*. In **Paper II**, we used proteomics, co-immunoprecipitation and AlphaFold2 *in silico* predictions to find differences between MAM+ and MAM− RBCs, and to identify EMP3 binding partner candidates. CD44 and ENOA emerged as the main EMP3-interacting proteins among several other candidates, and a specific salt bridge predicted to be crucial for CD44-EMP3 interaction could be defined.

In **Paper III**, we established a new blood group system based on CD36, a protein previously known to play a role as target for antibodies made by individuals with CD36-deficiency type I. In this condition, CD36 is lacking on all cells. In pregnancy, a CD36+ fetus to a CD36− mother is known to be at risk for fetal and neonatal alloimmune thrombocytopenia (FNAIT) but severe fetal anemia may also result. Whilst CD36 is normally highly expressed in early erythroid progenitor cells, we encountered a CD36− donor when culturing erythroid cells in the laboratory. CD36 expression on reticulocytes and mature red blood cells was investigated in 20 regular blood donors. Low but significant levels were detected. In **Paper IV**, it was found that α1-microglobulin (A1M) could protect against hematopoietic toxicity following radioactive cancer treatment in a mouse model.

In summary, this Ph.D. project provided valuable data on new blood group systems and hematopoiesis, thereby extending our knowledge about the clinically important fields of hematology and transfusion medicine.

Key words: Blood group, MAM antigen, Nak^a antigen, *EMP3, CD36,* erythropoiesis, red blood cell, transfusion medicine

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To my parents,

Table of Contents

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Populärvetenskaplig sammanfattning

Blodgivning hjälper till att hålla våra vårdsystem igång - utan blod stannar sjukvården! Detta blod går till många olika patienter i behov av transfusion. Människor kan behöva en blodtransfusion för omedelbara behov som efter en olycka, kirurgi eller förlossning. Men det är ofta patienter med blodsjukdomar och cancerpatienter som behöver mest blod.

Varje persons blodceller är unika och skiljer sig från andras. Dock delar vi vissa likheter med våra familjemedlemmar eftersom många av skillnaderna faktiskt är ärftliga. Detta gäller till exempel våra blodgrupper. Den viktigaste blodgruppen som många känner till är ABO, som består av kolhydrater på ytan av de röda blodkropparna och på andra celler. Det finns fyra huvudtyper av ABO-blodgrupper: typ A, B, O och AB. Dessutom finns det hundratals andra blodgrupper som skiljer sig mellan människor. Den viktigaste efter ABO kallas Rh. Om du har RhD-proteinet, kallas du RhD-positiv och om du saknar det är du RhD-negativ.

För att en blodtransfusion ska fungera och vara säker, måste det donerade blodet matcha patientens blodgrupper. Om blodgruppen passar bra kommer patientens immunsystem att acceptera blodet och det kommer hjälpa patienten att bli frisk. Om inte, kan patienten skapa antikroppar mot de blodgrupper han eller hon inte känner igen. Därför kan patientens immunsystem avvisa det icke-matchande blodet och attackera det transfunderade blodet så att de röda blodkropparna förstörs, vilket kan leda till allvarliga konsekvenser. Utöver de två viktigaste blodgruppssystemen, ABO och Rh, som rutinmässigt kontrolleras, finns det 43 andra system som inte är så välkända för allmänheten och mindre ofta orsakar problem. Men när de inte passar, kan de också orsaka liknande komplikationer under graviditeter (om modern och barnet inte har samma blodgrupper) eller när en transfusion behövs. I denna bok beskriver jag upptäckten av två nya blodgruppssystem som inte fanns när jag började mina studier, MAM och CD36.

Att inte ha MAM-proteinet verkar främst orsaka komplikationer vid graviditet. I den första artikeln i denna studie samarbetade vi med forskare runt om i världen för att studera alla kända familjer som saknar MAM-blodgruppen. Alla hade förändringar i samma gen, som normalt ger upphov till ett protein som kallas EMP3. Det var inte känt att EMP3 skulle finnas på röda blodkroppar tidigare så det var en överraskning. På grund av genförändringarna saknar dessa sällsynta personer EMP3 på alla celler i kroppen, inklusive på deras röda blodkroppar och även deras blodplättar (små cellfragment som har immunfunktioner och hjälper till att stoppa blödningar). I laboratoriet noterade vi att stamceller från personer som är negativa för denna blodgrupp kan ge upphov till många fler röda blodkroppar när du odlar dem för att bli till blod, vilket är mycket intressant och kan hjälpa oss att odla fler röda blodkroppar på kortare tid för transfusioner i framtiden. Detta skulle vara särskilt

viktigt för MAM-negativa personer, eftersom det just nu bara finns en eller två blodgivare med denna blodgrupp i hela världen!

I den andra artikeln försökte vi ta reda på mer om EMP3 och hur det fungerar vid bildandet av röda blodkroppar och vilka proteiner det kan binda till för att utföra sina uppgifter.

Liksom i fallet med MAM-blodgruppen, kan personer som saknar ett protein som kallas CD36 utveckla komplikationer under graviditeter och vid transfusion (men i detta fall främst när blodplättar transfunderas, tror vi). CD36 blev ämnet för den tredje artikeln i denna bok. Den CD36-negativa blodgruppen är vanligare än att vara MAM-negativ men fortfarande sällsynt hos personer av europeiskt ursprung. Istället är den betydligt vanligare hos personer av afrikanskt, mellanöstligt och asiatiskt ursprung. Oavsett vad, är det viktigt att känna till ännu en blodgrupp och kontrollera den när det finns ett kliniskt problem med blodbrist eller låga blodplättar hos ett foster eller en nyfödd. Antikroppar mot CD36 kan också ge upphov till problem i rutinmässig blodgruppering och kan se ut som att patienten har bildat antikroppar mot andra, potentiellt farliga blodgrupper.

Röda blodkroppar för transfusion kan lagras upp till 42 dagar. Under denna tid kan kvaliteten på blodet påverkas och leda till skador på de röda blodkropparna. När innehållet läcker ut från skadade röda blodkroppar, kan hemoglobinet (proteinet som gör vårt blod rött) orsaka skada på andra vävnader i vår kropp. Detta kan hända efter transfusion men också om vi skadar våra egna blodceller så att de spricker, eller till och med om du får ett blåmärke efter att ha skadat dig. Det rödaktigt blåa märket du kan se genom huden är blod som kommit ut i vävnaderna av misstag. Detta orsakar inflammation eftersom hemoglobinet är mycket reaktivt.

Blodtransfusioner kan också ibland leda till komplikationer. Till exempel kan de röda blodkropparna skadas under lagringen eller för att blodgrupperna inte passar mottagarens immunförsvar. Detta kan leda till njurskador på grund av hemoglobinet. För att hjälpa mot detta utforskar vi sätt att skydda blodceller under transfusion och cellgiftsbehandling. I den fjärde och sista artikeln i denna studie har vårt arbete belyst ett potentiellt experimentellt läkemedel som kallas alfa-1-mikroglobulin, förkortat till A1M. Hoppet är att när det ges till patienter som påverkas av de ovan nämnda tillstånden, kan A1M-proteinet skydda vävnader och celler (inklusive blodcellerna själva) och leda till bättre terapiresultat.

Sammanfattningsvis har mina studier byggt på tidigare forskningsresultat för att kunna lägga till ett par små bitar till det stora pusslet av kunskap inom de kliniskt viktiga och intressanta områdena blodbildning och transfusionsmedicin.

Popular summary

Blood donation help keep our healthcare systems running. This blood goes to many different patients in need. People might require a blood transfusion for immediate needs like after an accident, surgery, or childbirth. But it is often patients with blood diseases and cancer patients who need the most blood.

Each person's blood cells are unique and different from those of others. However, we share some similarities with our family members since many of the differences are in fact inherited. This is for instance true about our blood groups. The most important blood group that many people know about is ABO, which is built up of carbohydrates on the red blood cell surface and on other cells. There are four main ABO blood groups: type A, B, O, and AB. In addition, there are hundreds of other blood groups which differ between people. The most important after ABO is called Rh. If you have the RhD protein, we call you RhD positive and if you lack it, you are RhD negative.

For a blood transfusion to work and be safe, the donated blood must match the patient's blood group. If the blood group is a good match, the patient's immune system will accept the blood and it will help the patient become healthy. If not, the patient may make antibodies against the blood groups he or she does not recognize. Therefore, the patient's immune system may reject the unmatched blood and attack the transfused blood so that the red blood cells are destroyed, which could lead to very dangerous consequences. Beyond the most common two blood group systems, ABO and Rh, that are routinely checked, there are 43 other systems, that are not so well known to the public and causes problem less frequently. But when they are unmatched, they can also cause similar complications during pregnancy (if the mother and baby are not blood-group-matched) or when a transfusion is needed. In this book, I describe the discovery of two new blood group systems that did not exist when I started my studies, MAM and CD36.

Not having the MAM protein may cause complications during pregnancy. In the first paper of this study, we collaborated with researchers around the world to study all known families lacking the MAM blood group. All had alterations in the same gene, which normally gives rise to a protein called EMP3, which was not known to be on red blood cells before so that was a surprise to us. Due to genetic changes, these rare people lack EMP3 in all cells in their body, including on their red blood cells and also their platelets (small cell fragments that have immune functions and help stop bleeding). In the lab, we noted that stem cells from people who are negative for this blood group can give rise to more red blood cells when you culture them to become blood, which is very interesting and might help us to grow more red blood cells in a shorter time for transfusions in the future. This would be particularly important for MAM-negative people, since there are only one or two blood donors with this blood group known worldwide!

In the second paper, we tried to find out more about EMP3 and how it works in red blood cell formation and which proteins it can bind to so that it can perform its tasks.

Like MAM, people who are negative for a protein called CD36 can develop complications in pregnancies and transfusion (but mainly when platelets are transfused, we think). CD36 became the topic of the third paper in this book. The CD36-negative blood group is more common than being MAM-negative but still rare in people of European origin. Instead, it is much more frequent in people of African, Middle Eastern and Asian populations. No matter what, it is important to know about yet another blood group and check for it when there is a clinical problem with anemia or low platelets in a fetus or newborn. Antibodies against CD36 can also give rise to problems in routine blood grouping and may look like the patient has made antibodies against other, potentially dangerous blood groups.

Red blood cells for transfusion can be stored for up to 42 days, during this time the quality of the blood might be affected and lead to damage of red blood cells. When the content leaks out from damaged red blood cells, hemoglobin (the protein that makes our blood red) may cause harm to other tissues in our body. This can happen after transfusion but also if we harm our own blood cells so that they burst, or even if you get a bruise after hurting yourself. The reddish-blue mark you can see under the skin is blood that came out in the tissues by accident. This causes inflammation because the hemoglobin is very reactive.

Blood transfusions can also sometimes lead to complications, like damage to red blood cells from being stored too long or from mismatching of blood groups. This can lead to kidney damage because of the hemoglobin. To help against this, we are exploring ways to protect blood cells during transfusion and chemotherapy. In the last paper in this study, our work has elucidated a potential experimental medicine called alpha-1-microglobulin, or A1M for short. The hope is that when it is given to patients affected by the conditions mentioned above, the A1M protein can protect tissues and cells (including the blood cells themselves) and lead to better therapy outcomes.

In summary, my studies have built on lots of previous research to add a couple of small pieces to the big jigsaw puzzle of knowledge within the clinically important and interesting fields of blood formation and transfusion medicine.

List of Publications

Paper I

Thornton N, Karamatic Crew V, Tilley L, Green, CA, Tay CL, Griffiths RE, Singleton BK., Spring F, Walser P, **Alattar AG**, Jones B, Laundy R, Storry JR, Möller M, Wall L, Charlewood R, Westhoff CM, Lomas-Francis C, Yahalom V, Feick U, Seltsam A, Mayer B, Olsson ML, Anstee DJ. Disruption of the tumour-associated EMP3 enhances erythroid proliferation and causes the MAM-negative phenotype. *Nature Communications*. 2020;11:3569. doi:10.1038/s41467-020-17060-4.

Paper II

Alattar AG, Ghosh S, Chouhan B, Pimkova B, Atkinson G, Flygare J, Hansson J, Storry JR, Olsson ML. In-depth proteomic analysis and structural modeling of protein-protein interactions identify known and novel molecular binding partners for EMP3 in erythroid cells. *Manuscript.*

Paper III

Alattar AG, Storry JR, Olsson ML. Evidence that CD36 is expressed on red blood cells and constitutes a novel blood group system of clinical importance. *Vox Sanguinis.* 2024; Feb 7. doi: 10.1111/vox.13595. On-line ahead of print.

Paper IV

Alattar AG, Kristiansson A, Karlsson H, Vallius S, Ahlstedt J, Forssell-Aronsson, E, Åkerström B, Strand SE, Flygare J, Gram M. Recombinant α_1-microglobulin (rA1M) protects against hematopoietic and renal toxicity, alone and in combination with amino acids, in a ^{177}Lu-DOTATATE mouse radiation model. *Biomolecules*. 2023;13:928. doi: 10.3390/biom13060928.

Author contributions for included papers

Paper I

N.T., V.K.C., L.T. and C.A.G. conceived, designed and coordinated the study. N.T., B.J., R.L. and C.A.G. performed serology experiments. L.T. performed whole exome sequencing and associated data analysis. L.T., V.K.C., B.K.S. and N.T. performed genetic analyses. V.K.C. and C.A.G. carried out primary and BEL-A2 cell cultures and with B.K.S. performed shRNA experiments. C.L.T. and B.K.S. designed and performed CRISPR/Cas9 experiments. R.E.G. and C.L.T. carried out confocal microscopy. C.A.G., C.L.T., V.K.C. and A.G.A. performed flow cytometry experiments. P.W. carried out protein modelling and molecular dynamics calculations and provided guidance for optimisation of serological testing with monoclonal antibodies. F.S., N.T. and J.R.S. designed and performed Western blotting. F.S. carried out immunoprecipitation experiments. N.T. designed and carried out MAIEA experiments. M.M. provided bioinformatic analysis of *EMP3* alleles. A.G.A. performed overexpression, platelet experiments and supplementary P10 cell culture. J.R.S. provided guidance and reagents for overexpression experiments. C.M.W. and C.L.F. identified and provided MAM-negative samples. M.L.O., L.W., R.C., V.Y., U.F., A.S. and B.M. detected and referred samples for antibody identification. L.W., M.L.O. and R.C. provided additional samples and clinical data. M.L.O. provided guidance for overexpression and supplementary P10 cell culture. D.J.A. and M.L.O. provided advice on experimental design, analysis and interpretation of data, manuscript preparation and critical review. N.T., V.K.C., L.T., C.A.G., J.R.S., M.L.O. and D.J.A. wrote the paper. All authors contributed to review of the final paper.

Paper II

A.G.A. and M.L.O. conceived the project; A.G.A., J.R.S., and M.L.O. planned the study and designed in vitro experiments; A.G.A., S.G., K.P. and J.H. designed mass spectrometry experiments; S.G. and J.H. provided proteomics expertise; B.C. and G.A. provided AlphaFold expertise; A.G.A., S.G., B.C. and K.P. performed experiments; A.G.A., S.G., B.C., J.R.S., K.P. and M.L.O. analyzed the data; J.F. and M.L.O supervised the first author; A.G.A., J.R.S. and M.L.O. wrote the manuscript with input from the other authors; all authors revised the final version of the manuscript.

Paper III

A.G.A., J.R.S. and M.L.O. conceived the study; A.G.A. and J.R.S. performed experiments; A.G.A. drafted the manuscript and created the figures. All authors analysed and interpreted the data and revised the manuscript. We would like to acknowledge the staff at the FACS Core Facility at the Lund Stem Cell Center for technical assistance.

Paper IV

Conceptualization, A.G.A., A.K., H.K., J.A., J.F. and M.G.; methodology, A.G.A., H.K., S.V. and J.A.; analysis, A.G.A., A.K., H.K., S.V., J.A., E.F.-A., B.Å., S.-E.S., J.F. and M.G.; investigation, A.G.A., A.K., H.K., S.V., J.A., B.Å., S.-E.S., J.F. and M.G.; data curation, A.G.A., A.K., J.F. and M.G.; writing original draft preparation, A.G.A., A.K., J.F. and M.G.; writing, review and editing, A.G.A., A.K., H.K., S.V., J.A., E.F.-A., B.Å., S.-E.S., J.F. and M.G.; visualization, A.G.A., A.K. and M.G.; supervision, J.F. and M.G.; project administration, J.F. and M.G. All authors have read and agreed to the published version of the manuscript.

Additional work

The following papers were published during the course of the PhD studies but are not included in the thesis:

Talkhoncheh MS, Baudet A, Ek F, Subramaniam A, Kao Y-R, Miharada N, Karlsson C, Oburoglu L, Rydström A, Žemaitis K, **Alattar AG**, Rak J, Pietras K, Olsson R, Will B, Larsson J. Ciclopirox ethanolamine preserves the immature state of human HSCs by mediating intracellular iron content. *Blood Advances*, 2023;7(24):7407-7417. doi: 10.1182/bloodadvances.2023009844.

Sjögren SE, Chen J, Mattebo A, **Alattar AG**, Karlsson H, Siva K, Soneji, S., Tedgård U, Chen JJ, Gram M, Flygare J. Targeting elevated heme levels to treat a mouse model for Diamond-Blackfan Anemia. *Experimental Hematology*, 2022;105:50-61. doi: 10.1016/j.exphem.2021.10.005.

Mattebo A, Sen T, Jassinskaja M, Pimková K, Prieto González-Albo I, **Alattar AG**, Ramakrishnan R, Lang S, Järås M, Hansson J, Soneji S, Singbrant S, van den Akker E, Flygare J. Yippee like 4 (Ypel4) is essential for normal mouse red blood cell membrane integrity. *Scientific Reports*, 2021:11(1):15898 (12 digital pages). doi: 10.1038/s41598-021-95291-1.

Kristiansson A, Bergwik J, **Alattar AG**, Flygare J, Gram M, Hansson SR, Olsson ML, Storry JR, Allhorn M, Åkerström B. Human radical scavenger α1-microglobulin protects against hemolysis in vitro and α1-microglobulin knockout mice exhibit a macrocytic anemia phenotype. *Free Radical Biology & Medicine*, 2021;162:149-159. doi: 10.1016/j.freeradbiomed.2020.02.018.

Siva K, Ek F, Chen J**, Ghani Alattar A**, Sigmundsson K, Olsson R, Wlodarski M, Lundbäck T, Flygare J. A Phenotypic screening assay identifies modulators of Diamond Blackfan Anemia. *SLAS Discovery*, 2019; 24(3):304-313. doi: 10.1177/2472555218823531.

Masoumi Z, Maes GE, Herten K, Cortés-Calabuig Á, **Alattar AG**, Hanson E, Erlandsson, L, Mezey E, Magnusson M, Vermeesch JR, Familari M, Hansson, SR. Preeclampsia is associated with sex-specific transcriptional and proteomic changes in fetal erythroid cells. *International Journal of Molecular Sciences*, 2019; 20(8):2038 (20 digital pages). doi: 10.3390/ijms20082038.

Abbreviations

A1M	α_1-microglobulin
AML	Acute myeloid leukemia
BasoE	Basophilic erythroblast
BasoE	Basophilic erythroblast
BFU-E	Burst forming unit erythroid
BM	Bone marrow
CDA	Congenital dyserythropoietic anemia
CFU-E	Colony forming unit erythroid
CHA	Congenital hemolytic anemia
CML	Chronic myeloid leukemia
CKD	Chronic kidney disease
cRBC	Cultured red blood cell
CS	Carnegie stage
CTL2	Choline Transporter-Like Protein 2
DAT	Direct antiglobulin test
DBA	Diamond Blackfan anemia
ECO	Enzyme conversion to group O
EMP3	Epithelial membrane protein 3
ENOA	Alpha-enolase 1
EPO	Erythropoietin
EPOR	Erythropoietin receptor
FA	Fanconi anemia
FNAIT	Fetal and neonatal alloimmune thrombocytopenia
GPA	Glycophorin A
GPIV	Glycoprotein of platelet surface
Gpx	Glutathione peroxidase
GvHD	Graft-versus-Host-Disease
Hb	Hemoglobin
HBA	α-globin
HDFN	Hemolytic disease of the fetus and newborn
hESC	Human embryonic stem cell
HLA	Human leukocyte antigen
HNA	Human neutrophil antigen
HO	Heme oxygenase
Hp	Haptoglobin
HPA	Human platelet antigen
Hpx	Hemopexin
HSC	Hematopoetic stem cell
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem and progenitor cell

HTR	Hemolytic transfusion reaction
IGF-1	Insulin-like growth factor
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-3	Interleukin-3
iPSCs	Induced pluripotent stem cells
ISBT	International Society Blood of Transfusion
KLF1	Krüppel-like factor 1
LMPs	Lymph-myeloid progenitors
MDS	Myelodysplastic syndrome
NETs	Neuroendocrine tumors
OrthoE	Orthochromatic erythroblast
PBMC	Peripheral blood mononuclear cell
PLTs	Platelets
PolyE	Polychromatic erythroblast
ProE	Proerythroblast
PRRT	Peptide receptor radionuclide therapy
PTP	Post transfusion purpura
rA1M	Recombinant A1M
RBC	Red blood cell
ROS	Reactive oxygen species
RPs	Ribosomal proteins
RUNX1	Runt-related transcription factor 1
SCD	Sickle cell disease
SCF	Stem cell factor
SNV	Single nucleotide variant
SOD	Superoxide dismutase
TA-GvHD	Transfusion-associated graft versus host disease
TACO	Transfusion-associated circulatory overload
TED	Terminal erythroid differentiation
TF	Transcription factor
TGF-ß	Transforming growth factor beta
TRALI	Transfusion-related acute lung injury
TSP-1	Thrombospondin-1
TTID	Transfusion-transmitted infectious diseases
WES	Whole-exome sequencing
WIRhE	Worldwide Initiative for Rh Disease Eradication

Introduction

Hematopoiesis/erythropoiesis

The hematopoietic system serves as an archetypal organ system, sustained by stem cells, characterized by its remarkable capacity for cellular turnover. It is estimated that this system generates an astonishing one trillion blood cells daily [1, 2]. Through extensive research, the journey from hematopoietic stem cells (HSCs) to fully mature and differentiated blood cells has been well-documented, involving multiple critical stages. Consider the marvel that, by the time you've read this sentence, your body will have produced around 10-15 million new red blood cells (RBCs). This fact becomes even more astonishing upon realizing that RBCs have a lifespan of about 120 days, which means we have to eliminate exactly as many cells as we produce not to burst with overproduction or go under due in anemia to underproduction. This means that the regulatory oversight of this process has to be extremely tight.

From infancy to adulthood, every blood cell originates from HSCs nestled within the bone marrow. This development is governed by a complex network of regulators, including enhancers and suppressors, which navigate the HSCs through numerous cell fate decisions and checkpoints along the hematopoietic lineage tree. This complex guidance system ensures their differentiation into mature blood cells. The outcome of this process results in the production of key blood components: megakaryocytes that generate platelets, erythrocytes that transport oxygen, granulocytes (neutrophilic, basophilic and eosinophilic) that combat microorganisms, and the critical elements of the immune system, including monocytes, macrophages, T cells, B cells, NK cells and the plasma cells and memory cells derived from B cells (Figure 1).

Erythropoiesis is a fundamental physiological process within hematopoiesis, coordinating the production and maturation of erythrocytes, also known as red blood cells (RBCs). The term “erythropoiesis” is derived from a combination of a Greek words, “erythro” coming from the “erythros” meaning red, and “poiesis” meaning making or production. Thus “erythropoiesis” translates to the production of erythrocytes, referring specifically to the complex process of RBC formation [3].

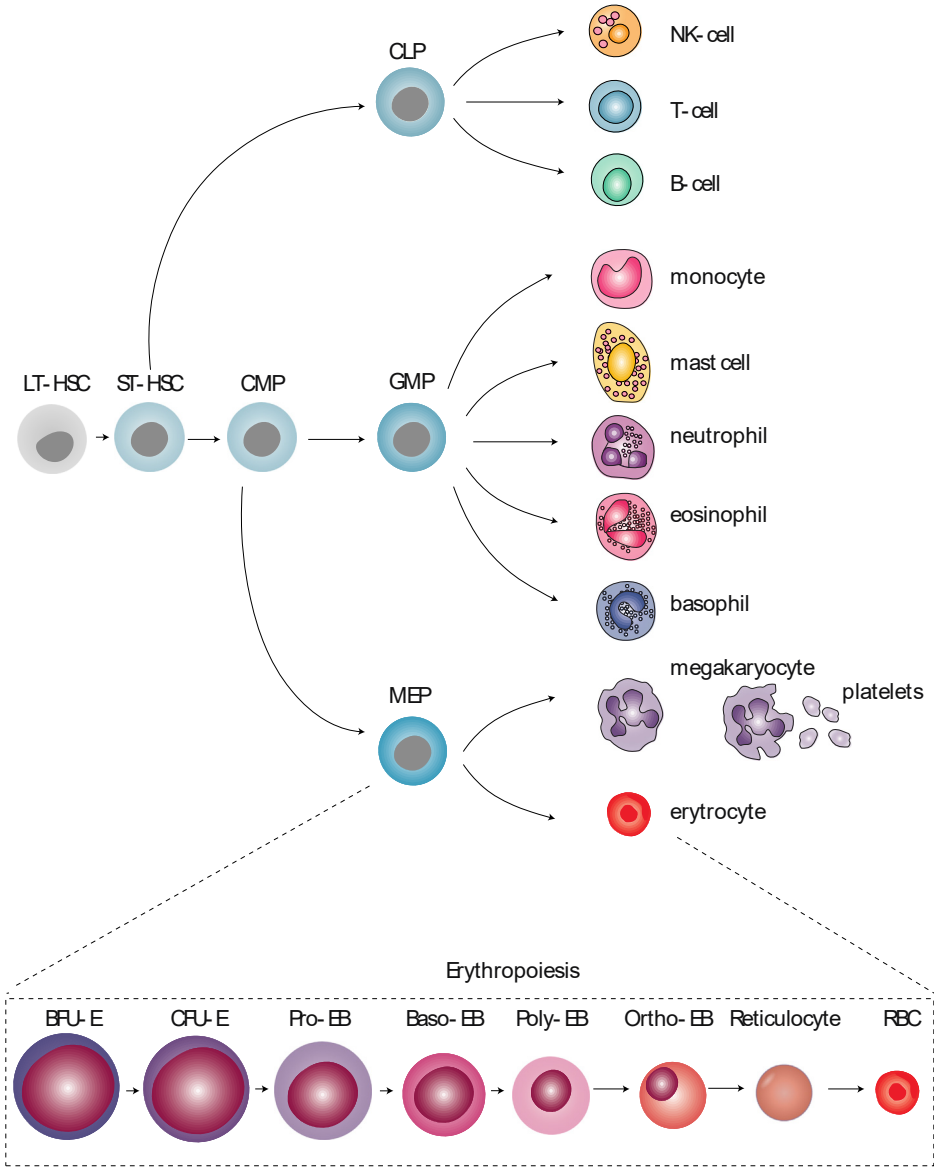

Figure 1. Hematopoiesis/erythropoiesis. An overview of hematopoiesis/erythropoiesis with the progenitor and precursor cells. Abbreviations: LT-HSC = long term-hematopoietic stem cell; ST-HSC = short term-hematopoietic stem cell ; CMP = common myeloid progenitor; GMP = granulocyte-macrophage progenitor; MEP = megakaryocyte-erythrocyte progenitor; CLP = common lymphoid progenitor; NK = natural killer; SL = small lymphocyte; T = T cell; B = B cell. The differentiation steps from the megakaryocyte erythroid progenitor (MEP) to the mature erythrocyte are shown. Abbreviations: BFU-E = Burst forming unit-erythroid; CFU-E = Colony forming unit-erythroid; ProE = pro-erythroblast; BasoE = basophilic erythroblast; PolyE = polychromatic erythroblast; OrthoE = orthochromatic erythroblast; RBC = red blood cell.

Developmental hematopoiesis/erythropoiesis

During embryonic development, HSCs that are essential for maintenance of adult hematopoiesis. The generated pool of HSCs home to bone marrow (BM) prenatally and reside there for the rest of life, making the BM the main site of hematopoiesis.

During embryogenesis, multiple temporally and partially overlapping waves of hematopoiesis emerge at different anatomical sites and give rise to distinct hematopoietic cell types with distinct functions. Developmental hematopoiesis can be divided into three waves: primitive, transient definitive (also known as pro-definitive or secondary primitive), and definitive (also known as HSC-forming) hematopoiesis [4, 5].

The first wave of hematopoiesis is initiated in the pre-circulation yolk sac at Carnegie stage 7-8 (CS7-8) of human development. Primitive hematopoiesis marks the formation of the first blood cells in extra-embryonic tissues. It predominantly produces nucleated primitive erythroblasts expressing embryonic globins and the cell surface marker glycophorin A (GPA), and occasionally CD45+ macrophages and megakaryocytes [6]. Embryonic globins have been shown to be linked with greater oxygen carrying capacity [7, 8]. The blood cells produced equip the embryo with the means for oxygen transport, and immune defense. The pre-circulating placenta serves as another extra-embryonic tissue that plays a role in primitive hematopoiesis by generating macrophages [9, 10]. As blood circulation commences, the yolk-sac-derived erythroblasts enter the bloodstream at CS10 and reach the placenta, where they interact with and are assisted by placental macrophages to enucleate (Figure 2).

The second wave of hematopoiesis originates in the yolk sac and possibly in the embryo proper at CS8-9, and produces tissue-resident macrophages, and lymphoid populations. Transient definitive hematopoiesis is characterized by the emergence of yolk-sac derived myeloid progenitors, followed by lymph-myeloid progenitors. These hematopoietic progenitors are HSC-independent progenitors, possess restricted multi-lineage differentiation potential and only differentiate upon transition to the liver [11, 12] (Figure 2).

The third wave of hematopoiesis is characterized by emergence of a cluster of hematopoietic cells in the aorta-gonad-mesonephros (AGM) region at CS14-16. Presence of a rare transplantable HSC in AGM (possibly only 1 per embryo) is confirmed by functional transplantation studies in immunodeficient mice [13, 14]. However, HSC identity of a population of hundreds of nascent HSC is evident in CS14-15 AGM and suggests that they are functionally more immature. As described so eloquently by Calvanese and Mikkola [15]: “These cells then undergo maturation, limited expansion and some give rise to terminally differentiated hematopoietic cells, as they migrate through yolk sac and placenta and seed the liver approximately at CS17. HSCs remain in the liver and become an active site of

hematopoiesis during the first and second trimester. HSCs then move to BM during the second trimester and remain there for the lifetime to support postnatal hematopoiesis" (Figure 2).

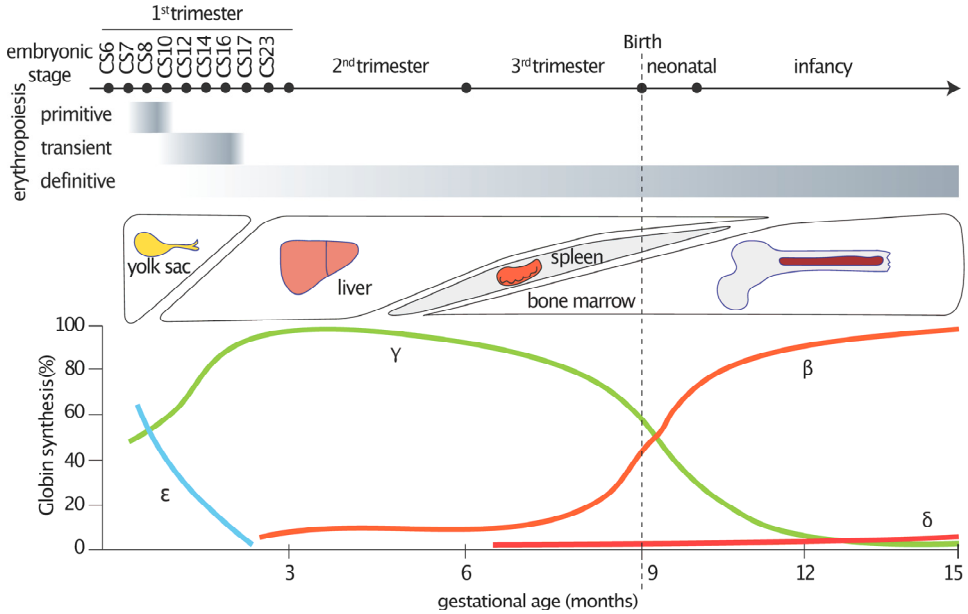

Figure 2. Developmental erythropoiesis. Schematic diagram represents developmental erythropoiesis waves and the site in humans highlighting the globin switches over the developmental sites. The figure representing the globin switch reproduced with modification based on a figure in hematology book [3].

Erythropoiesis

Erythropoiesis is a tightly regulated biological process [16]. In adults, this process occurs within the BM originating from multipotent hematopoietic stem and progenitor cells (HSPCs) [17]. These HSPCs differentiate into committed erythroid progenitors. The first committed erythroid progenitors are called the burst forming unit erythroid (BFU-E). The term "burst" reflects the capacity of the cells to proliferate extensively and give rise to a "burst" of small erythroid clusters containing thousands of erythroid cells, which is observed after 14 days of *in vitro* culture in semisolid media such as methylcellulose. BFU-E cells further progress into the next stage of differentiation, becoming colony forming unit erythroid (CFU-E) cells. These cells require up to seven days in culture and each colony contains approximately 50 cells. These progenitor cells differentiate into a series of morphologically distinct erythroid precursors, the proerythroblast (ProE),

basophilic erythroblast (BasoE), polychromatic erythroblast (PolyE) and orthochromatic erythroblast (OrthoE). This process is termed the terminal erythroid differentiation (TED). TED is a highly orchestrated process where each cell division represents a critical step of differentiation characterized by a general decrease in cell size, nuclear condensation, reduction in RNA content and subsequential accumulation of hemoglobin, preparing these cells for their ultimate function in oxygen transport [16]. These precursor cells are situated in a specialized microenvironment within the bone marrow surrounding macrophages, compartmentalized in so-called erythroblastic islands. These units, the recognition of which was first introduced by the French hematologist Marcel Bessis in 1958 [18], are composed of a central macrophage, often referred to as the "nurse" cell, that promotes erythroid maturation by facilitating direct cell-cell interaction (Figure 3). The macrophage enhances cellular survival and proliferation, as well as phagocytoses extruded nuclei (pyrenocytes) from OrthoEs. Pyrenocytes display very low ATP levels and begin to express phosphatidylserine on their surface [19]. The exposure of phosphatidylserine is a hallmark of early apoptosis acting as an "eat me" signal, thereby facilitating the nurse cell recognition and engulfment of these expelled nuclei [20]. Enucleated OrthoEs form reticulocytes, which are subsequently released into the blood stream. Within the circulation these reticulocytes undergo further maturation to become fully developed erythrocytes [21]. The transition from reticulocyte to erythrocyte involves significant plasma membrane reorganization leading to increased shear resistance, reduction of surface area and formation of the characteristic biconcave shape. The newly developed reticulocyte exhibits less mechanical stability compared to mature RBCs. Within 24 to 48 hours, reticulocytes separate from their bone marrow microenvironment and enter the bloodstream. During this process there is a extensive reorganization of cellular protein content and membrane architecture transitioning from the mechanically less stable membrane of young reticulocyte to the more resilient membrane of mature erythrocytes. This includes the loss of ribosomal RNA and the removal of organelles such as mitochondria, transitioning the cell to depend only on anaerobic glycolysis for energy production [22-24]. Notably, membrane vesiculation contributes to a 20% decrease in the surface area, highlighting a major reorganization of membrane components and cytoskeleton. These changes allow RBCs to navigate the narrowest capillaries efficiently while maximizing the surface area for gas exchange (Figure 3) [25].

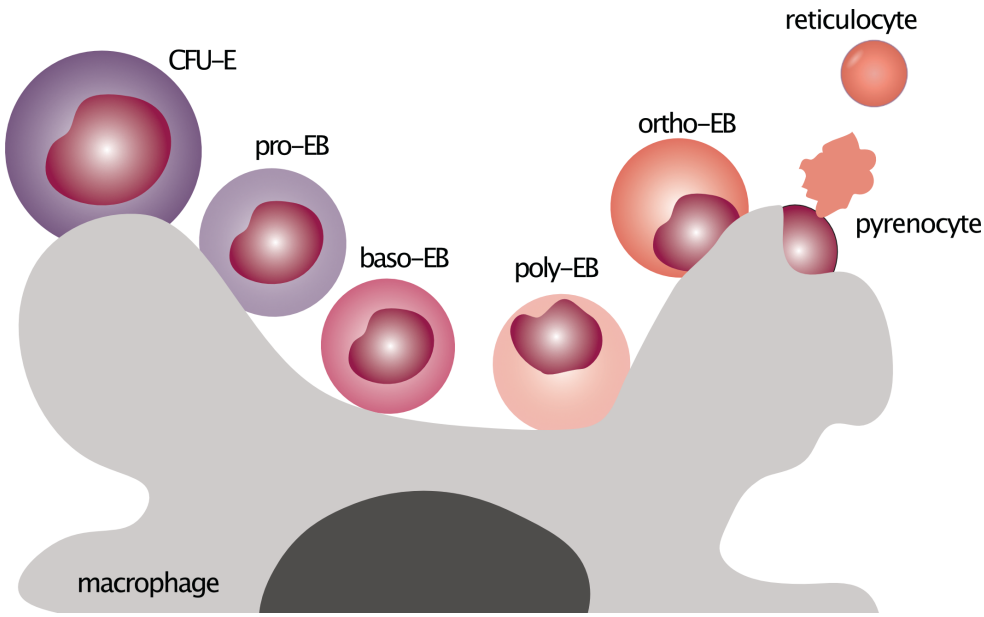

Figure 3. A schematic picture representing the process of erythropoiesis in the bone marrow with the support of macrophages in erythroblastic islands.

Extrinsic regulation of erythropoiesis

The differentiation, proliferation and survival of erythroid cells are governed by a sophisticated network of cytokine and signaling pathways regulating different stages of erythroid differentiation.

At the BFU-E stage interleukin-3 (IL-3) enhances the proliferation even though it is not erythroid-specific [26, 27]. Stem cell factor/KIT Ligand (SCF) promotes the proliferation and survival of the erythroid progenitors BFU-E, CFU-E and the erythroid precursors, ProE [28]. From the CFU-E stage onwards, erythropoietin (EPO) plays a pivotal role in erythroid differentiation, survival and proliferation despite its receptor EpoR being only weakly expressed initially and decreasing during TED [29]. HSPCs express the EpoR suggesting that EPO signaling could be involved in the erythroid commitment from HSPCs [30, 31]. Several other cytokines and soluble factors such as insulin, insulin-like growth factor (IGF-1), transforming growth factor beta (TGF-β) superfamily members, glucocorticoids, testosterone, iron and zinc, are reported to be involved in erythroid development [32, 33]. These cytokine growth factors are shown to activate a series of downstream signaling effectors. JAK/STAT, MAPK, and PI3K play important roles in the survival and differentiation of erythroid cells. The binding of EPO to its receptor EpoR triggers the activation of JAK2, facilitating the phosphorylation and nuclear translocation of STAT5, thereby influencing the erythroid gene expression landscape at large.

Overactivation of these signaling can lead to erythrocytosis, often linked to JAK2 because of germline or somatic mutations in *EPOR* [30].

Furthermore, the interaction between EPO and EpoR catalyzes signaling through various transduction pathways, including JAK/STAT5, PI3K/AKT/mTOR and MAPK.

Disruption within these pathways can adversely impact the fine balance of proliferation, maturation and survival of erythroid progenitors, and in the later stages of erythroid development. Understanding how erythropoiesis is regulated, and how these pathways contribute to diseases affecting RBCs at various developmental stages may identify potential therapeutic avenues for managing erythroid disorders [34].

Intrinsic regulation of erythropoiesis

In the early stages, transcription factors (TFs) such as GATA1 emerged as critical for erythroid lineage commitment, actively repressing GATA2 to promote erythroid maturation [35]. The dynamics between GATA1 and GATA2 is a hallmark of transcriptional control during erythropoiesis, with a significant reduction of GATA2 expression observed until the ProE stage, while GATA1 upregulation enhances the regulatory impact on TED [36].

Krüppel-like factor 1 (KLF1), another important erythroid TF, supports the early differentiation, promoting erythroid differentiation over megakaryocytic differentiation and plays a critical role in TED, regulating the cell cycle and chromatin condensation preparing the cells for enucleation [37-39]. Its disruption in mice and humans can cause severe anemia and results in a spectrum of erythroid phenotypes in humans, respectively [37, 40-42]. In the human setting, this includes abnormalities in RBC membrane proteins, sustained high levels of fetal hemoglobin (HbF) [43], hemolytic anemia [44], severe congenital dyserythropoietic anemia (CDA) [45], and may lead to hydrops fetalis as a result of severe anemia [46]. Additionally, TFs like TAL1, LMO2, LDB1 and GFI1B also contribute to erythropoiesis but their role is extended beyond the erythroid cells into broader hematopoiesis [47] and thereby beyond this thesis.

During erythropoiesis, GATA1 binds to a set of coactivators including TAL1, LMO2 and LDB1 and GFI1B to activate genes associated with erythroid differentiation [16]. On the other hand, GATA1 has the ability to bind to a set of co-repressors to subpress genes preventing erythroid maturation, such as friend of GATA protein 1/Zinc Finger Protein, FOG Family member 1 FOG-1/ZFPM1 and the nucleosome remodeling and deacetylase (NuRD) complex [16, 48]. Another repressor complex involves Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2) [49]. The fine-tuned balance between these TFs and associated co-

activator and co-repressor complexes facilitate the induction or downregulation of gene expression that is either essential or no longer needed, respectively, during TED [50, 51].

KLF1 and GATA1 represent two important examples regulating erythroid genes from the globins to blood group genes [52-54]. KLF1 plays a crucial role in the switch of different forms of globins during development from the embryonic (ζ and ε) to the fetal (α and γ), and finally to the adult stage (α and β) (Figure 2) [15, 52]. It does this by binding to the β globin gene promoter and activating the promoter of B-cell lymphoma/leukemia 11A (BCL11A), which is a protein that represses the fetal γ globin gene [52]. Genetic variation in *BCL11A* is shown to improve the ß-thalassemia and sickle cell disease phenotype through the persistence of HbF [55]. In line with this, further investigations showed that HbF is regulated by an erythroid specific repressor/enhancer of BCL11A [56-58]. In addition to that, variation in *KLF1* was reported to regulate the expression of several blood groups, including the In(Lu) phenotype, Colton (AQP1), Dombrock (ART4), Scianna (ERMAP), Ok (BSG), Duffy (ACKR1), Diego (SLC4A1) and LW (ICAM4) [43, 59, 60]. For this reason, KLF1 variants are summarized on the ISBT website under the RCIBGT Working Party pages. GATA1 is another example of not only regulating different aspects of erythropoiesis but also regulating blood group antigens. Recently, Wu et al. in a study towards deciphering the GATA1-driven regulation of blood group expression solved an old mystery, namely the mechanism underlying the Helgeson phenotype. This phenotype, characterized by the downregulation of CR1, is attributed to an abolished GATA1-binding motif in intron 4 of the *CR1* gene, thus elucidating the genetic basis of this phenotype [61].

A recent study explored the dynamic network of GATA1 interactions and found that it is correlated with stage-specific GATA1 binding, and orchestrates gene activity throughout the development of erythroid cells [62]. Interestingly, this study showed a decreased interaction signal in a metabolic enzyme called α-enolase (ENOA) involved in the synthesis of pyruvate. In this study, RNAseq data showed that lost GATA1 binding site is associate with genes that were downregulated during the transition from the erythroid progenitors (BFU-E/CFU-E) to the precursors (Pro-E) while it remains unchanged in the transition from HSPCs to erythroid progenitors [63]. In line with this data, ENO1 was reported as one of the unique proteins present in the erythroid progenitor cells [64]. This interesting finding suggests crosstalk between metabolic pathways and epigenetic regulation adding another layer of complexity on erythropoiesis, from HSPCs to RBCs.

Models to study erythropoiesis

Efficient erythropoiesis is fundamental for ensuring functional oxygen delivery to tissues throughout development and adulthood. This process is critically important not only for sustaining normal physiological functions but also in the context of various disease states. A detailed understanding of erythropoiesis at different stages of erythroid development is essential for diagnosing and treating different forms of anemias. In addition, anemia is often induced by cancer therapy treatments that primarily target the hematopoietic system [65]. Furthermore, transfusion-dependent patients, who do not respond to erythroid-stimulating agents like EPO, require regular blood transfusion [3, 66]. To advance our understanding of erythropoiesis in both healthy and diseased states a comprehensive approach employing various methodologies to evaluate this process at different developmental stages is required. Such an approach is essential towards identifying novel regulators that could potentially enhance RBC production *in vivo* and *in vitro* ensuring good yield and protection. These methodologies can be broadly categorized into *in vivo* and *in vitro* studies, each offering unique advantages and limitations. Both *in vivo* and *in vitro* approaches aim to deepen our understanding of erythropoiesis facilitating the development of more effective therapies.

In vivo studies of erythropoiesis

Common *in vivo* model organisms used to study erythropoiesis are mice and zebrafish. Despite the evolutionary distance from humans, zebrafish serve as a valuable model due to the conservation of many hematopoietic genes. The economic feasibility of large-scale genetic and drug screening in addition to the possibility of *in vivo* imaging of embryonic development uncovered numerous genes involved in erythropoiesis . Generation and analysis of thousands of mutants in these genes have enhanced our understating of the process [67, 68]. Notably, the study of a hypochromic zebrafish mutant has advanced our knowledge in iron metabolism, such as the identification of crucial molecules like SLC40A1/ferroportin 1 [69]. Murine hemopoietic cells share many morphological and molecular similarities with those of human and have provided a good model of erythropoiesis. This has permitted genetic manipulation in disorders such as sickle cell disease (SCD), β-thalassemia, Diamond Blackfan Anemia (DBA) and myelodysplastic syndrome (MDS). The ability of reverse genetic engineering techniques and a wide array of antibodies for HSPC enrichment has facilitated detailed *in vivo* and *in vitro* analysis. Modeling erythroid disorders in mice has enhanced our understanding of the pathogenesis of these disorders and enabled many preclinical studies [34].

Both mice and zebrafish offer invaluable insights into erythropoiesis, each comes with unique methodological strengths. Mice provide a closely related genetic and physiological model to humans, and some genetically modified models such as the

humanized mouse allow the analysis of human hematopoiesis [70-72]. Zebrafish offer not only a cost-effective model for scientific research due to their rapid reproductive cycle, but also offer advantages in genetic screening, and visualization of the developmental processes, which contribute to a better understanding of erythropoiesis [69, 73]. However, the specific limitations of each model necessitate careful consideration in their application to human erythropoiesis and related diseases [68]. Both mice and zebrafish exhibit distinct limitations as models for human diseases. Mice, due to their divergent transcriptional and epigenetic landscapes, may not always provide accurate reflections of human conditions. Zebrafish, on the other hand, are evolutionarily distant and possess RBCs that retain nuclei, marking another significant deviation from human biological traits [68]. Furthermore, the ethical considerations in using mice for research demand stringent welfare protocols to minimize distress and suffering. Similarly, the use of zebrafish also necessitates careful attention to their well-being in research settings.

In vitro studies of erythropoiesis

The benchmark for studying erythropoiesis involves primary culture of HSPCs, sourced from human donors or mice. In mice, bone marrow and fetal liver HSPCs are used [74, 75]. In humans, HSPCs can be collected from peripheral blood either following mobilization [76] or from peripheral blood mononuclear cells (PBMCs) obtained e.g. from blood donation waste bag leucocyte concentrates [77]. Bone marrow [78] and cord blood [79] are other common sources. This approach to cell culture is characterized by a multi-phase process aimed at achieving two main objectives: the expansion of the progenitor cells and their differentiation into precursor cells that eventually undergo enucleation. However, it is important to acknowledge that the rate of enucleation within these systems are generally low and exhibit significant variability.

Primary cell culture models provide invaluable insights into RBC biology, though they may not fully mirror the *in vivo* efficiency of cell expansion, differentiation and enucleation. This limitation can also be of an advantage to for example understanding the factors required for the enucleation process, which is yet to be fully clarified.

The use of human embryonic stem cells (ESCs) [80, 81], induced pluripotent stem cells (iPSCs) [82-88] and immortalized erythroid cell lines has provided a new possibility for modelling disorders of the erythroid lineage [89-91] . For each, these innovative strategies enabled the investigation of a specific disorder and offered a tailored approach to understanding the pathogenesis of the disorder, and to finding potential novel therapies for each disease. This approach can be taken even a step further aiming to find patient-specific treatments, a concept termed personalized medicine. Despite its potential, this approach has many challenges and limitations. However, it also presents a significant opportunity to advance our understanding of

RBC biology contributing to the development of novel treatments. Furthermore, this research area has the potential to revolutionize the field of transfusion medicine by generating RBCs that meet specific criteria for transfusion-dependent patients, e.g. those with a rare blood type and potentially the creation of a universal blood product opening the door to new therapeutic approaches for transfusion-dependent patients [90-92]. An added advantage of such a cultured product for transfusion purposes would be to transfuse mainly young RBCs or even reticulocytes, thereby potentially increasing the interval between hospital visits for transfusion-depending patient groups. Cell surface markers commonly utilized to study erythropoiesis *in vitro* are listed in Table 1 and the flow cytometry strategy used in this study is presented in Figure 4.

Table 1. An overview of cell surface markers used to study erythropoiesis, recently reviewd in [93].

Population	Cell surface marker	Source	Reference
BFU-E	$CD45^{+}CD235a^{-}CD123^{-}CD34^{+}CD36^{-}CD71^{lo}$	UCB	[79]
	EP1: $CD36^{+}CD45RA^{-}CD41a^{-}CD117^{+}CD123^{-}CD235a^{-}$ $CD71^{+}CD34^{+}CD105^{-}$	BM	[78]
CFU-E	$CD45^{+}CD235a^{-}CD123^{-}CD34^{-}CD36^{+}CD71^{hi}$	UBC	[79]
	Prog1: $CD34^{lo}CD36^{+}CD71^{med}CD235a^{-}Hb^{-}CD233^{-}CD49^{hi}$	UBC	[64]
	Prog2: $CD34^{-}CD36^{+}CD71^{hi}CD235a^{-}$Hb $CD233^{-}CD49^{hi}$		
	EP2: $CD36^{+}CD45RA^{-}CD41a^{-}CD117^{+}CD123^{-}CD235a^{-}$ $CD71^{+}CD34^{+}CD105^{lo}$	BM	[78]
	EP3: $CD36^{+}CD45RA^{-}CD41a^{-}CD117^{+}CD123^{-}CD235a^{-}$ $CD71^{+}CD34^{+/lo}CD105^{hi}$		
	EP4: $CD36^{+}CD45RA^{-}CD41a^{-}CD117^{+}CD123^{-}CD235a^{-}$ $CD71^{+}CD34^{+}CD105^{hi}$		
ProE	$CD45^{-}CD235a^{+}CD49d^{hi}CD233^{-}$	UBC	[94]
	$CD34^{-}CD36^{+}CD71^{hi}CD235a^{lo}CD233^{-}CD49^{hi}$	UBC	[64]
	$CD71^{+}Syto16^{+}CD105^{hi}CD235a^{lo}$	BM	[78]
	$CD71^{+}CD117^{+}CD105^{+}$	BM	[95]
	$CD36^{+}CD45^{lo/-}CD117^{+}CD105^{+}$		
BasoE	Early: $CD45^{-}CD235a^{+}CD49d^{hi}CD233^{lo}$	UBC	[94]
	Late: $CD45^{-}CD235a^{+}CD49d^{hi}CD233^{med}$		
	Early: $CD34^{-}CD36^{+}CD71^{hi}CD235a^{med}CD233^{lo}CD49^{hi}$	UBC	[64]
	Late: $CD34^{-}CD36^{+}CD71^{hi}CD235a^{hi}CD233^{med}CD49^{hi}$		
	Early: $CD71^{+}Syto16^{+}CD105^{hi}CD235a^{hi}$	BM	[78]
	Late: $CD71^{+}Syto16^{+}CD105^{med}CD235a^{hi}$		
	$CD71^{+}CD117^{-}CD105^{+}$	BM	[95]
	$CD36^{+}CD45^{lo/-}CD117^{-}CD105^{+}$		
PolyE	$CD45^{-}CD235a^{+}CD49d^{med}CD233^{med}$	UBC	[94]
	$CD34^{-}CD36^{+}CD71^{hi}CD235a^{hi}CD233^{med}CD49^{med}$	UCB	[64]
	$CD71^{+}Syto16^{+}CD105^{lo}CD235a^{hi}$	BM	[78]
	$CD71^{+}CD117^{-}CD105^{-}FSC^{med}$	BM	[95]
	$CD36^{+}CD45^{lo/-}CD117^{-}CD105^{-}FSC^{med}$		
OrthoE	$CD45^{-}CD235a^{+}CD49d^{lo}CD233^{hi}$	UBC	[94]
	$CD34^{-}CD36^{+}CD71^{med}CD235a^{hi}CD233^{hi}CD49^{lo}$	UBC	[64]
	$CD71^{+}Syto16^{+}CD105^{-}CD235a^{hi}$	BM	[78]
	$CD71^{+}CD117^{-}CD105^{-}FSC^{lo}$	BM	[95]
	$CD36^{+}CD45^{-}CD117^{lo/-}$CD105 FSC^{lo}		
Retic	$CD235a^{+}CD61^{-}$ Thiazole $Orange^{+}$	PB	[96]
RBC	$CD235a^{+}CD61^{-}$ Thiazole $Orange^{-}$	PB	[96]

Abbreviations: BFU-E = Burst forming unit-erythroid; CFU-E = colony forming unit-erythroid; ProE = pro-erythroblast; BasoE = basophilic erythroblast; PolyE = polychromatic erythroblast; OrthoE = orthochromatic erythroblast; Retic = reticulocyte, RBC = red blood cell; + = positive; – = negative; hi = high; med = medium; lo = low). Abbreviations for cell population as reported in the original publication: EP1-4= Erythroid progenitors 1-4.

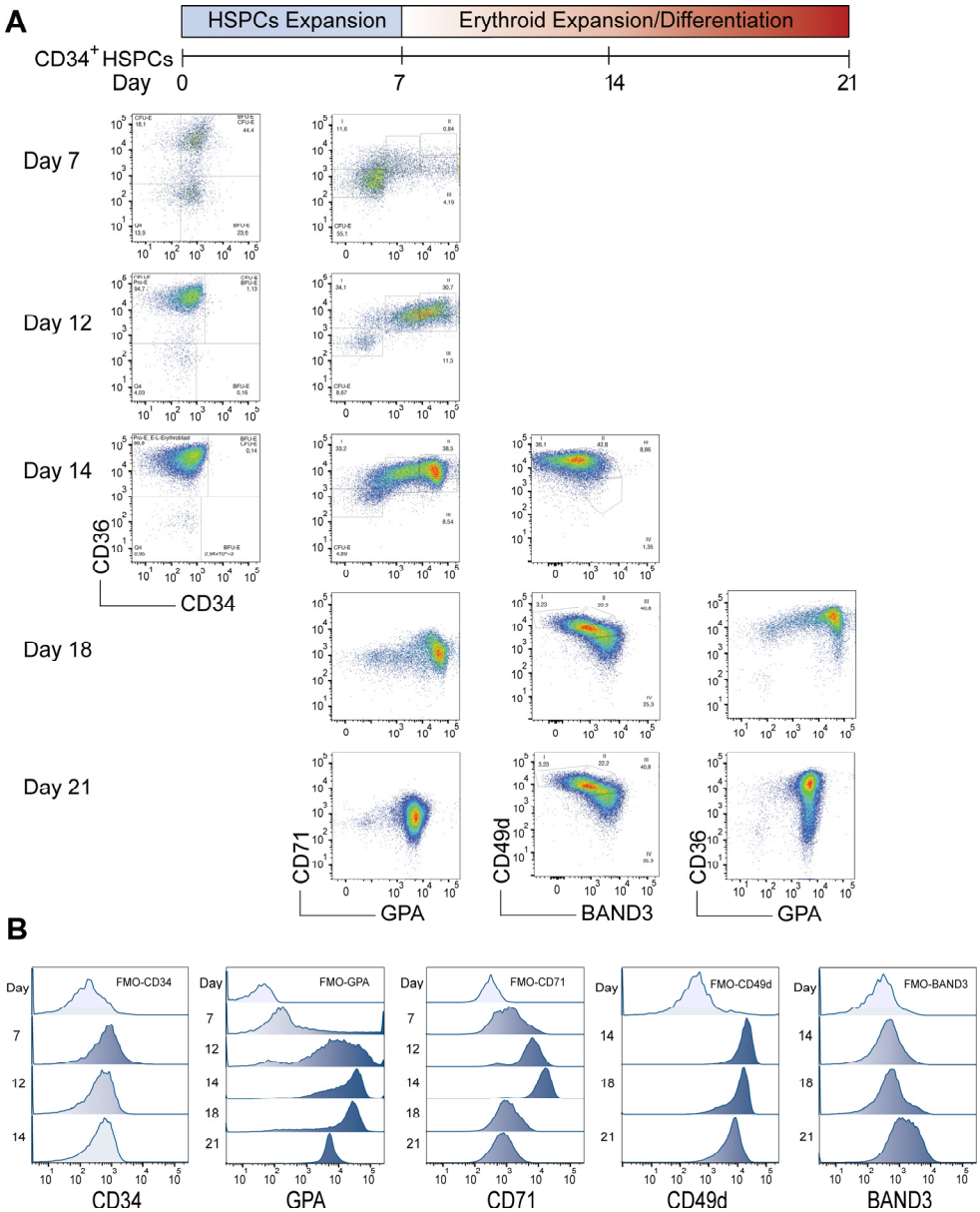

Figure 4. The flow cytometry strategy used for erythroid culture in this thesis**. A.** Cell culture protocol. **B.** Cell surface marker expression histogram overlays during the erythroid culture.

Anemia

Introduction

Anemia, typically characterized by a reduced number of circulating RBCs or decreased concentration of hemoglobin contributes significantly to global morbidity and mortality and can be divided into acquired and inherited disease [3]. The most predominant causes of acquired anemia include nutritional deficiencies (mainly iron deficiency but also vitamin B_9 or B_{12}), infection (e.g. malaria) and systemic etiologies (chronic kidney disease [97-99], inflammation [100-102] and cancer [103-105]). In addition, acute or chronic bleeding due to a large number of reasons can be added to this list.

Inherited anemias

Hemoglobinopathies constitute the most common forms of inherited anemias, with the most prominent diagnosis groups being sickle cell disease (SCD) and different forms of thalassemia. In addition, RBC enzyme deficiencies like pyruvate kinase deficiency and also various membrane disorders can lead to anemia [3]. As mentioned, inherited diseases of hemoglobin are collectively designated hemoglobinopathies and include SCD and thalassemia. The latter is the most common inherited disorder affecting millions of individuals worldwide, mostly children and results in the clinically significant forms of anemia. However, advancements in understanding the pathophysiology and breakthroughs in molecular technologies have enhanced the diagnostic capabilities, allowing for the identification of genetic defects, ensuring these forms of anemia are no longer missed. These advancements have helped in the development of new therapeutic approaches and drugs that are in clinical trials, currently or approved. Many inherited forms of anemia are rare, with problems affecting erythropoiesis at various stages and impacting many different components of the RBC (Figure 5). Anemias in this category are monogenic disorders resulting from inherited mutations. These disorders include rare conditions such as Diamond-Blackfan anemia (DBA), Fanconi anemia (FA) and congenital dyserythropoietic anemias (CDA).

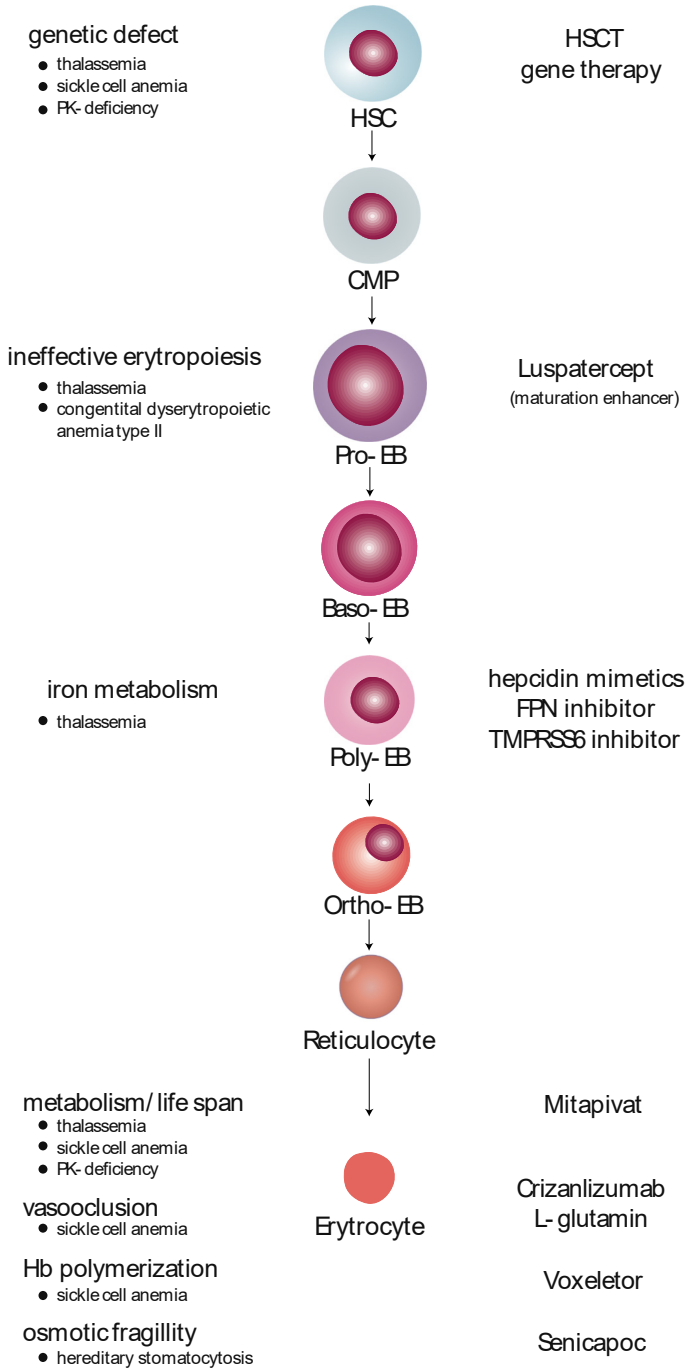

Figure 5. The information given in this figure was illustrated based on data reviewd in [66, 106, 107].

Defects of hemoglobin synthesis is a common feature for some of the most common anemia forms. β-thalassemia results from mutations that inhibit the synthesis of β-globin polypeptide [108]. Therefore, free α-globin molecules accumulate and create an insoluble aggregate [109]. Accumulation of these aggregates leads to the destruction of erythroid precursor cells in a process known as ineffective erythropoiesis. Excess α-globin interacts with various pathways that regulate protein quality control such as autophagy and ubiquitin-mediated proteolysis [109]. It has been shown that accumulation of α-globin (HBA) can lead to destabilization of GATA1 through binding of the excess α-globin to and sequestration of HSP70, a molecular chaperon within the cytoplasm [110]. This in turn affects gene expression and contribute to ineffective erythropoiesis, which causes several side effects such as metabolic abnormalities, bone tissue defects and organomegaly due to the marked increase in the proliferation of delayed and dying erythroid progenitors [108]. Additionally, the presence of free α-globin induces hemolysis and breakdown of mature RBCs in circulation. Therefore, β-thalassemia involves in diminishing the production of erythroid cells and accelerating their destruction.

Affected Populations: Thalassemia is prevalent in people from Mediterranean countries, the Middle East, Central Asia, India and Southeast Asia. The presence of thalassemia in these populations is likely an evolutionary consequence of the genetic adaptation in regions where malaria was or is prevalent, as thalassemia traits can confer some resistance to malaria [111].

In SCD, a single amino acid change in β-globin causes deoxygenated HBA tetramers to form stiff polymers inside RBCs, leading to their morphological changes into the characteristic sickle-like shape [112]. These changes in morphology affect the blood flow properties, along with secondary impacts on endothelium, plasma protein, and inflammatory cells, enhancing the sticking of sickled cells to each other and to the interior of small vessels. This leads to vaso-occlusion that causes pain, acute chest syndrome due to respiratory insufficiency and stroke, a condition defined as vaso-occlusion crisis (VOC) [113]. SCD predominantly affects people of African, Mediterranean, Middle Eastern, and Indian ancestries. Similar to thalassemia, the geographic distribution suggests a genetic adaptation to malaria, as carriers of the sickle cell trait have a protective advantage against malaria. The presence of Hemoglobin S (HbS) in individuals with sickle cell trait (carriers of one copy of the mutation rather than two) provides a selective advantage in malaria-endemic regions. This advantage arises because the malaria parasite reproduces less efficiently in sickle-shaped cells, reducing the severity of malaria infections. This protective effect is a classic example of balanced polymorphism, where the carrier state for a genetic disorder confers an advantage in certain environments, leading to the persistence of the mutation in the population.

Congenital hemolytic anemia

The congenital hemolytic anemias (CHA) represent a diverse collection of uncommon inherited disorders, can be either dominant, recessive or X-linked. CHAs exhibit a clinical spectrum that varies from mild, completely compensated anemia to chronic severe hemolysis that necessitates transfusion and splenectomy due to shortened RBC life span and the removal of RBCs from circulation, respectively. These conditions involve abnormalities in RBC membrane proteins, enzyme metabolism and defective erythropoiesis due to alterations affecting the erythroid progenitors [3].

In SCD, thalassemia and CHA, hemolysis of RBCs leads to the release of free hemoglobin into circulation that results in degradation of the vasodilator nitric oxide [113, 114]. In addition, free heme is released from Hb triggering neutrophil activation, inflammation and oxidative damage via various pathways [115].

A selection of current and future treatment options (some being evaluated in clinical trials) can be found in Figures 5 and 6 [107]. In the meantime, many of the patients with the disorders targeted by these treatment concepts are presently transfusion dependent which comes with the risk of side effects including iron overload and alloimmunization causing hemolytic adverse events and future challenges to find compatible blood.

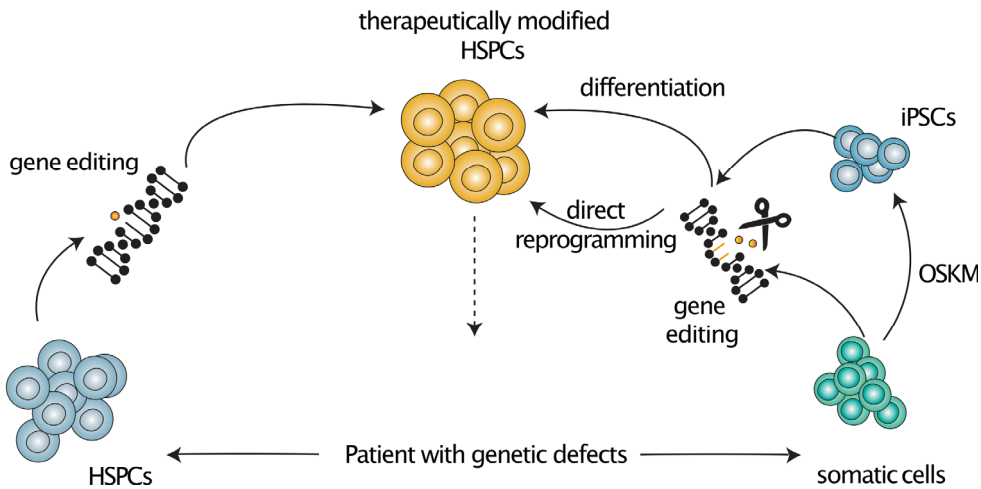

Figure 6. An overview of some of the many cellular therapy approaches developed for treatment of anemias of different backgrounds. Approaches to generating and implementing autologous blood cell therapies involve techniques for autologous transplantation and transfusion. These methods start by isolating either mature cells or progenitor cells, like those identified in peripheral blood mononuclear cells or somatic cells. Mononuclear cells can be directly differentiated into mature blood cells or directly reprogrammed into specific cell types, including hematopoietic HSCs. Reprogramming somatic cells into iPSCs using Yamanaka factors Oct3/4, Sox2, Klf4 and c-Myc (OSKM) [116] and gene ediding of these cells have the potential to differentiate into various mature blood cell types including HSPCs.

Transfusion Medicine

Historical background

The evolution of the field of transfusion medicine is a fascinating tale of scientific curiosity. It began with the groundbreaking work of an English physician William Harvey in 1928, who was the first to elucidate the concept of the human blood circulation, describing the motion of the heart and the principle that blood circulates in the body [117].

Despite the human xenotransfusion reported by Jean-Baptiste in 1667, where lambs' blood was transfused into humans, the practice faced significant opposition and was eventually banned by the Pope in the 1679 due to adverse reactions and ethical concerns [118].

In the early 19th century, the British obstetrician James Blundell conducted the first successful human-to-human blood transfusion on a woman suffering from postpartum hemorrhage, using her husband's blood [119]. Blundell performed a series of ten transfusions between 1825 to 1830, with five demonstrating clear benefits for patients [118]. In addition, Blundell's invention of specialized instruments for transfusion laid an important foundation of tools for the early attempts in the transfusion medicine field.

At the beginning of the 20th century, Karl Landsteiner observed that serum from one individual could cause agglutination of red blood cells in some other individuals, a phenomenon that was previously observed only between species [120]. Building on these observations, he classified human blood into three blood groups, A, B and C (C was later re-named O for the German "ohne" meaning "without"), based on their agglutination behaviors [121]. Shortly, after Landsteiner's discovery, his colleagues identified a fourth, rarer blood group, AB. Landsteiner's work revealed that blood transfusion within the same blood group does not cause RBC destruction, hence explaining why some transfusions failed while others succeeded and highlighting the critical role of blood group compatibility in transfusion safety. In 1930, Landsteiner was awarded the Nobel Prize in Medicine or Physiology for his groundbreaking discovery of the ABO blood group system. This knowledge was put into practice by Reuben Ottenberg in 1907, who performed the first blood transfusion based on blood typing and cross-matching. Moreover, Ottenberg's

observation of Mendelian inheritance of blood groups and recognition of the "universal" compatibility of blood group O donors for transfusions, has further contributed to reducing the risk of adverse reactions associated with blood transfusion and enabled more predictable and safe transfusions [118].

Another obstacle in blood transfusion was the coagulation of blood once collected. Consequently, transfusion had to be performed vein-to-vein via direct vascular connection between the donor and recipient. The discovery in 1914 that sodium citrate could act as an anticoagulant revolutionized blood storage and transfusion practices by facilitating the development of blood banks and making indirect transfusions feasible. This innovation marked the transition to modern transfusion medicine, enabling the widespread and routine use of blood transfusions in healthcare.

Transfusion medicine today is a broad field, integrating comprehensive patient blood management strategies, very controlled blood donation practices, advanced immunohematology laboratory testing, rigorous screening for transfusion-transmitted infectious diseases (TTID), such as (HBV, HCV, HIV and Syphilis among others, also depending geographic region). Furthermore, careful observation of any adverse is important, such as non-hemolytic febrile reactions, allergic reactions, hemolysis, transfusion-associated graft versus host disease (TA-GvHD), transfusion-related acute lung injury (TRALI), and transfusion-associated circulatory overload (TACO), which can lead to fatal outcomes. [122]. This surveillance process, termed hemovigilance, is to ensure the safety of the blood transfused from collection to the follow-up of recipients [123]

WHO reports that 118.5 million blood donations take place annually in the world. In low-income countries, up to 54 % of blood transfusions are given to children under 5 years of age, whereas in high-income countries, the most frequently transfused patient group is over 60 years of age, accounting for up to 76% of all transfusions [124]. In Sweden, around 400 000 units of whole blood are donated each year. In fact, this number is decreasing as in many Western countries thanks to patient blood management (PBM) efforts and was ~372,000 in 2022 [125]. This medical product supports a broad spectrum of patients, from those having blood diseases and cancers to individuals undergoing surgical procedures, women facing obstetric emergencies and in massive bleeding following trauma. While in high-income nations, the elderly are the primary recipients of blood transfusions, in low-income countries, almost 54% of transfusions are received by children under the five years of age [124].

The common clinical practice is to separate donated blood into RBCs, platelets, and plasma components for targeted treatment to achieve so-called blood component therapy, though this approach varies globally. Recent trends highlight an emerging interest for reverting to transfusing whole blood in severe hemorrhages, challenging

the conventional preference for component therapy concept. Some researchers advocate for the advantages of using whole blood directly, suggesting potential benefits over separated blood components [126].

Every decision to transfuse is weighed carefully against the risk of possible adverse effects, which affect roughly 1% of recipients. The introduction of PBM strategies has significantly reduced transfusion rates, reflecting a shift towards more cautious and justified use for this life-saving intervention. This evolution towards stricter criteria and more critical evaluation before transfusion practice ensure patient safety and optimize blood use in medical treatment.

Antigen carriers on blood cells in transfusion medicine

An antigen (Ag) can be a part of a diverse array of molecular structures, including proteins, peptides (amino acid chains), polysaccharides (complex assemblies of sugar molecules), lipids or nucleic acids [3].

Antigens can be found on the surface of our own cells and are called self-antigens. These antigens are usually recognized as self by the immune system and thus are not attacked. Self-antigens are present on all tissues, but for the purpose of this study, they are mainly significant in the context of RBCs.

On the other hand, antigens that do not originate from one's own body are called non-self-antigens. These antigens are sometimes recognized by the immune system as foreign antigens and may therefore trigger an immune response, leading to activation of the innate and adaptive immune processes. For example, the transfusion of RBCs from another individual might stimulate the immune system to produce specific antibodies against the non-self RBCs antigens that the recipient lacks. These antibodies are called alloantibodies and often lead to opsonization and elimination of the foreign cells by complement attack or phagocytosis.

It is still not fully understood why some patients develop blood group antibodies while most do not, despite all the antigen differences their immune systems are challenged with. The reason why some antigen differences give rise to antibody formation whilst others do not remain a matter of debate. HLA restriction is one reason why not everybody lacking an antigen will be immunized against it. The danger model has been offered as another explanation. If the antigen is not presented in a context of a threat, the risk is less that the immune system is triggered to react against it. Danger signals may come in the shape of transiently expressed proteins during stress or injury and can provoke an antibody response that might either assist in suppress the triggering signals or lead to immunodeficiency, with Type I interferons highlighted as primary examples [127]. Alloimmunization occurs when patients develop harmful antibodies against donor blood antigens, presenting

significant risks in transfusions. Research suggests that factors associated with the donor, such as frequent donations or iron supplementation, may increase reticulocyte counts in donated blood, potentially triggering stronger immune responses in the recipient [128]. It has been suggested that increased levels of reticulocytes in donated RBC units may increase the risk of RBC alloimmunization in recipients. This is because they may contain components acting as immune triggers, leading to adverse reactions in patients. Despite the lifesaving role of transfusions, identifying RBC units with high immunogenic potential remains a critical, unmet need in clinical settings [129].

In transfusion medicine, the primary antigens of interest are the polymorphic molecules that are carried on the surface of RBCs, leukocytes, including neutophils, and platelets (PLTs). Antigen matching is important not only for blood transfusion but also plays a pivotal role in the success of transplantation. In the context of organ or tissue transplantation, compatibility of tissue antigens between the donor's and recipient is essential for better transplantation outcomes.

These molecules are traditionally characterized on leukocytes, neutrophils, PLTs and RBCs as human leukocyte antigens (HLA), human neutrophil antigens (HNA), human platelet antigens (HPA) and blood group antigens, respectively. Antibodies are usually stimulated following transfusion or in pregnancies.

The HLA system that represents the primary histocompatibility complex in humans, was initially discovered by Jean Dausset in 1958, through the detection of alloantibodies in the sera of patients receiving multiple transfusions [130, 131], leading to the identification of the first HLA antigens [132]. Dausset received the Nobel Prize in Medicine or Physiology in 1980 for his discovery [133]. There are two main classes of HLA molecules: class I (HLA I) and class II (HLA II). HLA I are expressed on the surface of almost all nucleated cells and shared by platelets and leukocytes including subsets such as lymphocytes, neutrophils and monocytes. They bind peptide antigens and display them for recognition by antigen specific $CD8^+$ T cells. HLA II are specifically expressed on the surface of certain immunocompetent cells including antigen-presenting cells (APCs), such as dendritic cells, macrophages and B cells. They bind peptide antigens and display them for recognition by antigen-specific $CD4^+$ T cells [3]. While RBCs are not considered to display HLA antigens (but having shed them during the later stages of erythropoiesis), some HLA antigens are reported to be weakly expressed on RBCs, such as HLA B7 (Bg^a), B17 (Bg^b) and A28 (Bg^c) [134, 135].

The HLA system's critical role in transplantation medicine was quickly acknowledged. This system features the highest degree of polymorphisms in the human genome. HLA molecules are crucial for the adaptive immune response to pathogens and can also trigger immune reaction against foreign antigens, leading to either Graft-versus-Host-Disease (GvHD) or graft rejection in the transplant setting. HLA antibodies can also cause TRALI.

The HNA system consists of glycoproteins that are carried on the surface of human neutrophils, one of the key cell types involved in the immune response. These glycoproteins are important in neutrophil activation, migration, and the modulation of the immune response. The recognition of these antigens by the immune system, leads to the production of allo- or auto-antibodies targeting these neutrophil antigens [136]. This immune response can result in various clinical complications, such as autoimmune neutropenia, neonatal alloimmune neutropenia, TRALI (especially due to anti-HNA-3a), failure of the hematopoietic stem cell transplants to engraft and the rejection of kidney transplants [136].

The HPA system is defined by polymorphisms carried on glycoproteins present on the platelet membrane. Individuals exposed to platelets from another person with different HPAs may produce alloantibodies. These antibodies can cause various clinical conditions such as fetal and neonatal alloimmune thrombocytopenia (FNAIT), post-transfusion purpura (PTP) and platelet transfusion refractoriness [137]. Currently, there are 35 HPA systems defined to be carried by seven different platelet glycoproteins [138], the most clinically important of which is HLA-1 with the two variants HLA-1a and HLA-1b. Anti-HLA-1a is the most common reason underlying FNAIT and only 1 in ~50 donors are HLA-1a negative among Europeans [139]. HPA antigens are defined by alloantibodies against platelet antigens, typically defined by a single nucleotide variant (SNV). If a whole platelet glycoprotein is missing in an individual, the antibodies made against this target are defined as isoantibodies and therefore do not currently fulfil the criteria to become an HPA.

Erythrocyte antigens – blood groups!

Blood group antigens are polymorphisms carried by surface glycoproteins and glycolipids on the extracellular surface of the RBC membrane, and identified by immune antibodies that develop in the sera of individuals who lack the specific antigen. Production of these antibodies can for instance be in response to transfusion, transplantation or to fetal bleeds during pregnancy and/or at delivery. These antibodies are usually IgG, but IgM develops in the early phase of an immune response which takes approximately ten days to switch to IgG [140]. Only IgG antibodies are capable of transplacental passage from mother to fetus [3]. In addition, naturally-occurring antibodies are made against antigens in our environment, e.g. glycan epitopes on gut bacteria [141]. These antibodies often remain as IgM throughout life and do not, or only partially, undergo class switching [142]. Interestingly, the characterization of a blood group antigen does not differentiate between allo- or isoantibodies. Both are considered antibodies against non-self and thereby good enough to define a blood group.

Blood group systems can be classified into two main types: carbohydrate-based blood group systems, in which the gene responsible encodes a glycosyltransferase. This enzyme attaches the sugar residue to a precursor substrate thereby creating a specific blood group antigen, which typically consists of at least sugar residues. Naturally-occurring antibodies are most common against carbohydrate antigens. On the other hand, most systems are protein-based blood group systems in which the gene directly encodes the protein that carries the antigen(s).

As of the the 17th of July 2023, there are 45 blood group systems containing 362 antigens recognized by the International Society of Blood Transfusion (ISBT) based on decision by its Working Party on Red Cell Immunogenetics and Blood Group Terminology (WP-RCIBGT). This WP has played an important role in creating a standardized nomenclature for blood group antigens, ensuring global consistency and clarity in the classification for the daily routine practice in the reference laboratory and at the bedside since its establishment in 1980. The WP-RCIBGT has established criteria for polymorphic antigens of proteins and glycans carried on RBC to be acknowledged as blood antigens, where each antigen must be defined by human antibodies in at least one person lacking the antigen in question, and also be shown to be inherited. Antigens belonging to the same blood group system are encoded by one gene or more than one closely linked genes with little or no homology between them. (These criteria are particularly relevant for paper I and III where the MAM and CD36 blood group systems were established).

At the time of writing, there are 50 genes that account for 45 blood group systems. Eight of these are carbohydrate-based blood group system and 37 protein-based blood group system (Table 2).

Additionally, there are 28 antigens known but not yet linked to a specific blood group system, and these are organized by the WP-RCIBGT into 1) collections, if more than one antigen is reported to be related in serological, biochemical or genetic terms but yet do not fulfill ISBT criteria to be assigned as a blood group system, or 2) one of two series when the antigen neither meets the system nor collection criteria. The series are divided into two categories based on the frequency of the antigen in the population: *700* and *901* when the antigen frequency is less than 1% or more than 90%, respectively.

Since Landsteiner's discovery of the ABO blood group antigens, the ABO system remains the most clinically important system among the eight carbohydrate blood group system reported to date. The *ABO* gene that encodes the glycosyltransferases synthesizing the A/B antigens was characterized 90 years later by Yamamoto et al. [143]. In this system, individuals have naturally-occurring antibodies to the ABO antigens that their RBCs lack due to immunization against ubiquitous antigens present in the environment. These antibodies usually arise in the first few months of life when the digestive system is colonized by the normal bacterial flora. They are usually IgM antibodies and react at both cold temperatures and 37°C. According to

Landsteiner's law, individuals produce these naturally-occurring antibodies to the ABO antigens their own cells lack. However, following hematopoietic stem cell transplantation (HSCT) with a non-identical ABO phenotype can lead to exceptions to this rule but is otherwise seldomly seen.

One of the most significant protein blood group systems is the Rh blood group system. In 1940, Landsteiner and Wiener identified the Rh system and named the antibodies "anti-rhesus" following their experiments of injecting rabbits or guinea pigs with RBCs from the rhesus monkey (Macaca mulatta) [144]. The Rh system is highly polymorphic consisting of 56 antigens. The complexity is due to two homologous genes, *RHD* and *RHCE*, which encode the RhD and RhCE proteins, respectively. Rh antibodies can lead to transfusion reactions and may cause immediate and delayed hemolytic transfusion reactions (HTR) as well as hemolytic disease of the fetus and newborn (HDFN). Rh antigens, especially anti-D, are notably immunogenic and can be triggered by the small number of RBCs present in platelet concentrates when transfused into an RhD-negative patient [145], although the risk is quite low [146]. In pregnancy, Rh immunization is prevented when anti-D of IgG type is administered at 28 to 34 weeks of gestation (antenatal Rh prophylaxis) and/or within 72 h following delivery (postnatal Rh prophylaxis). Currently, efforts at the global level have been initiated towards the prevention of RhD immunization in pregnancy. This work is led by the Worldwide Initiative for Rh Disease Eradication (WIRhE) since only around 50% of RhD– pregnant women globally who needs Rh prophylaxis will get it [147].

Blood group antigens are carried on the extracellular surface of molecules anchored in the RBC membrane. They are not only classified into different blood group systems but also based on structure or function. To elucidate the three-dimensional appearance of blood-group-active molecules, structural analysis by various methods has been employed once the molecular identities became known. In addition to traditional crystallography, computational modeling based on homologous structures has been applied and lately also single-particle cryo-electron microscopy, especially for multipass membrane proteins [148, 149]. Table 3 contains a list of all blood group systems to date, tentatively categorized according to their function [150].

Table 3. **Blood group system classified based on structure or known (and hypothesized) function**

Category	Blood group systems
Host defense, innate immunity (glycocalyx)	ABO, P1PK, LE, H, I, GLOB, FORS, SID
Receptors, adhesion molecules	LU, FY, XG, SC, LW, XK, IN, OK, JMH, CD36
Transporters and channels	RH, JK, DI, CO, RAPH, GIL, RHAG, JR, LAN, AUG, CTL2, PEL, MAM, ABCC1, ER
Enzymes	KEL, YT, DO, EMM
Glycoprotein structure or unknown functions	MNS, GE, VEL, KANNO
Complement regulation	CH/RG, CROM, KN, CD59

Table 2. Blood group systems recognized by the ISBT.

No	System name	System Symbol	Gene name(s)	Antigen(s)	Chromosomal location	CD number
001	ABO	ABO	*ABO*	4	9q34.2	
002	MNS	MNS	*GYPA, GYPB, GYPE*	49	4q31.21	CD235a, CD235b
003	P1PK	P1PK	*A4GALT*	3	22q13.2	CD77
004	Rh	RH	*RHD, RHC*	55	1p36.11	CD240
005	Lutheran	LU	*BCAM*	25	19q13.2	CD239
006	Kell	KEL	*KEL*	36	7q33	CD238
007	Lewis	LE	*FUT3*	6	19p13.3	
008	Duffy	FY	*ACKR1*	5	1q21—q22	CD234
009	Kidd	JK	*SLC14A1*	3	18q11-q12	
010	Diego	DI	*SLC4A1*	22	17q21.31	CD233
011	Yt	YT	*ACHE*	5	7q22	
012	Xg	XG	*XG, MIC2*	2	Xp22.32	CD99
013	Scianna	SC	*ERMAP*	7	1p34.2	
014	Dombrock	DO	*ART4*	10	12p13-p12	CD297
015	Colton	CO	*AQP1*	4	714p	
016	Landsteiner-Wlener	LW	*ICAM4*	3	19p13.2	CD242
017	Chido/Rogers	CH/RG	*C4A, C4B*	9	6p21.3	
018	H	H	*FUT1/FUT2*	1	19q13.33	CD173
019	Kx	XK	*XK*	1	Xp21.2	
020	Gerbich	GE	*GYPC*	11	2q14-q21	CD236
021	Cromer	CROM	*CD55*	20	1q32	CD55
022	Knops	KN	*CR1*	9	1q32.2	CD35
023	Indian	IN	*CD44*	6	11p13	CD44
024	Ok	OK	*BSG*	3	19p13.3	CD147
025	Raph	RAPH	*CD151*	1	11p15.5	CD151
026	John Milton Hagen	JMH	*SEMA7A*	6	15q22.3-q3	CD108
027	I	I	*GCNT2*	1	6p24.2	
028	Globoside	GLOB	*B3GALNT1*	2	3q25	
029	Gill	GIL	*AQP3*	1	9p13	
030	Rh-associated glycoprotein	RHAG	*RHAG*	3	6p12.3	CD241
031	FORS	FORS	*GBGT1*	1	9q34.13-q34.3	
032	JR	JR	*ABCG2*	1	4q22.1	CD338
033	LAN	LAN	*ABCB6*	1	2q36	
034	Vel	VEL	*SMIM1*	1	1p36.32	
035	CD59	CD59	*CD59*	1	11p13	CD59
036	Augustine	AUG	*SLC29A1*	4	6p21.1	
037	KANNO	KANNO	*PRNP*	1	20p13	
038	Sid	SID	*B4GALNT2*	1	17q21.32	
039	CTL2	CTL2	*SLC44A2*	4*	19p13.2	
040	PEL	PEL	*ABCC4*	1	13p32.1	

No	System name	System Symbol	Gene name(s)	Number of antigen	Chromosomal location	CD number
041	MAM**	MAM	*EMP3*	1	19p13.33	
042	EMM	EMM	*PIGG*	1	4p16.3	
043	ABCC1	ABCC1	*ABCC1*	1	16p13.11	
044	Er	Er	*PIEZO1*	5	16q24.3	
045	CD36**	CD36	*CD36*	1	7q21.11	CD36

Color code: Red text indicates systems based on carbohydrate antigens.

*Two additional antigens, Cs^a and Cs^b, were added to the ISBT 039 CTL2 system at the ISBT WP-RCIBGT meeting in November 2023 [151] but are not yet included in the official ISBT lists (April 4, 2024).

**Blood group systems were added as system ISBT 041 and ISBT 045 by the WP-RCIBGT based on Paper I and Paper III presented in this thesis [96, 152], respectively.

Blood groups and hematopoesis

Relatively recent studies have indicated that blood group molecules can be involved in the regulation of hematopoietic processes. Three examples will be given here: 1) The Atypical Chemokine Receptor 1 (ACKR1) protein, also known as Duffy Antigen Receptor for Chemokines (DARC) or simply Duffy blood group glycoprotein (FY-gp) on nucleated erythroid cells was elegantly shown to regulate granulopoiesis, i.e. the formation of neutrophil granulocytes [153]. The Fy(a−b−) blood group phenotype therefore results in benign ethnic neutropenia, and FY pheno- or genotyping to detect an altered GATA1-binding motif [154] has become a diagnostic help to investigate low neutrophil counts in children, particularly of African and Middle Eastern origin, since this is where this phenotype occurs most frequently [155]. 2) A second example relates to the SMIM1 protein carrying the Vel blood group antigen [156-158] . When this was discovered as a new human protein in 2013, its function was unknown, and it lacked homology with other human proteins but was evolutionarily conserved [156]. Interestingly, lower levels of SMIM1 on RBCs were associated with decreased mean corpuscular hemoglobin concentration (MCHC) in blood donors [158, 159] and a zebra fish knockout model indicated a possible erythropoiesis defect [158]. Thus, it was hypothesized that SMIM1 regulates formation of RBCs. 3) The third examples relate to ABO, where group B HSPCs were associated with accelerated erythropoiesis compared to group A [160]. These investigators also noted differences in the numbers of terminally differentiated RBCs, enucleation rates and hemoglobin amounts, and attributed their findings to increased levels of certain miRNAs in group B precursor cells. Thus, there are examples of blood group molecules appearing to regulate hematopoiesis. In this Ph.D. study, we have therefore investigated to what degree two relatively new blood group molecules have such regulatory properties.

A different principle by which blood groups can affect RBC formation involves alloimmunization. During the early stages of erythropoiesis, the emergence of specific blood group antigen-bearing proteins, notably Kell glycoprotein, may increase the risk of erythropoietic suppression if they are targeted by blood group antibodies. This becomes critically important during pregnancy, as antibodies formed by the mother against these fetal antigens can cross the placenta and subsequently attack fetal erythroid cells, leading to fetal anemia.

Unlike RhD incompatibility, antibodies to the KEL blood group system antigens may lead to early fetal anemia without the high bilirubin levels typically seen in RhD-mediated HDFN. The expression of Kell glycoprotein starts at the BFU-E stage during the culture of HSPCs toward erythroid cells, earlier than RhD [161, 162] suggesting that antibodies targeting e.g. the Kell antigen may inhibit the proliferation of Kell-positive erythroid progenitor cells, possibly through an apoptotic pathway. This results in a significant reduction in the number of mature erythrocytes and resulting in anemic disease of the fetus and newborn (ADFN). Similarly, several blood group antigens expressed during early erythropoiesis, such as those of the Ge, MNS, and JR blood group systems could lead to ADFN through different mechanisms reviewed Ohto et al. [163]. Interestingly, many of the cell surface markers used to characterize erythroid development during in vitro erythropoiesis, such as glycophorin A, CD44, Band 3, and CD36, are all classified as blood group systems. Notably, CD36 has been recognized as the most recently established blood group system, based on the research discussed in this thesis.

Furthermore, data mining of protein expression during erythropoiesis has revealed that various blood group proteins are expressed at distinct stages of culturing HSPCs [64], as illustrated in Figure 7. Collectively, these findings highlight the significance of this thesis towards understanding the function of the blood group-bearing glycoproteins, EMP3 and CD36, and uncover potential novel regulators crucial for understanding both healthy and disordered erythropoiesis.

Rare blood groups

A rare blood group is defined by the lack of a common (high-prevalence) antigen on the surface of RBCs. Individuals with such a phenotype can develop antibodies to these antigens following the initial exposure through pregnancy or transfusion [92]. Consequently, immunized individuals with rare blood groups should receive transfusion of matched, antigen-negative units of packed RBCs, which are often stored frozen in specialized blood banks. Unfortunately, the limited supplies in these banks often fall short of meeting the quantity demand, so such challenges in securing appropriate transfusion can crucially impact logistics of patient management, or even patient survival. Typically, when someone with a really rare blood group is

discovered, a family study is initiated to identify first-degree relatives with the same rare blood group who can donate. As discussed above Producing cultured RBCs from these identified donors may be a valuable approach in the future. Alternatively, stem cell source where the gene in question has been knocked out, could be used to produce the antigen-negative RBCs.

In vitro produced RBCs

Considerable efforts are being made to produce cultured RBCs (cRBC) for transfusion. It's reasonable to anticipate significant advantages from these cultured cRBC over donated blood. Notably, it has been demonstrated that cRBC exhibit a longer life span compared to the natural RBC populations. The enhanced life span of cRBCs can be attributed to their uniformly young age, in contrast to the varied age range found in the population of natural RBCs at donation time [164].

This attribute is very important within the scope of potential clinical use, suggesting a markedly improved efficiency of transfusion for this novel blood product. This could lead to fewer transfusions for transfusion-dependent and cancer patients, thereby reducing the overall risks associated with frequent transfusion, finding the correct donor and the costs associated with patient care if transfusion intervals can be increased [91].

As discussed earlier, there are 362 blood group antigens belonging to 45 systems and another 28 antigens not yet belonging to a system. Donor and transfusion recipients are primarily matched for ABO and RhD, although in some countries matching may routinely include K and even c (in women of child-bearing age, e.g. all females <50 of age). Almost 2-3% of all patients receiving blood transfusion develop antibodies against one or more blood group antigens [165], and as the number of transfusion increases, the percentage of patients developing these antibodies increases as well [166]. Among patient groups receiving regular transfusions, such as those with SCD, 20-40% develop alloimmunization to RBC antigens, leading in some cases to difficulties in finding suitable blood units for transfusion [167]. This is particularly troublesome if the donor population does not match the patient population, which is a common problem in Europe and USA for SCD patients, who are typically of African descent.

By choosing stem cell donors with specific red cell phenotypes, cRBC offer a potential solution for transfusing patients who have developed antibodies. It may even be possible to choose the patient’s own stem cells, or to reprogram fibroblasts or other cell types from a patient to become blood-forming [168, 169] cells.

The selection of stem cell donors based on desired RBC phenotypes may allow for the use of cRBC in the transfusion of alloimmunized patients. Research indicates that a single donor, chosen for the unique characteristics of their erythrocyte phenotype within the ten most immunogenic systems could possibly achieve compatibility with 95% of alloimmunized patients [89]. Furthermore, selecting a set

of few donors harboring these specific phenotypes could accommodate for 99% of alloimmunized patients [89]. Another innovative approach to enhance the availability of safe blood involve enzymatic conversion of blood group A, B, or AB to type as O, known as Enzyme Conversion to group O (ECO) [170-172]. We are approaching the much-anticipated idea of universal RBCs, a concept that has been pursued since the first blood group was identified in 1900. It is also possible that the ECO approach can be combined with antigen selection so that rare units of the "wrong ABO group" may be used for patients with rare non-ABO phenotypes. cRBC, their sources, advantages and limitations are reviewed in (Table 4) [173]. The *in vitro* generation of RBCs and PLTs is now possible. A promising proof of principle concept of the possibilities that cRBCs could provide were reported in a clinical trial in France [164] and currently, a clinical trial led by researchers in the UK is under evaluation. In addition to that, clinical trials for platelets generated from *in vitro* culture also showed promising results [174, 175] in the early phases of those trials. These innovative approaches could potentially support the need for rare blood [92]. In addition to that, these approaches could facilitate achieving the perfect blood transfusion scenario, which means the ability to produce blood entirely compatible with the recipient's eliminating immunization risks, safeguarding against infectious, health risks independent of donor source and preventing metabolic issues such as iron overload caused by hemolysis following transfusion. An overview of cRBCs from different sources is summarized in (Table 4). In addition to the limitations mentioned there, the major challenge right now is that it is incredibly costly and resource-consuming to produce the large number of RBCs required to transfuse a single patient so blood donors will probably be an invaluable resource for a long time ahead [173].

Table 4. Overview of cRBCs sources, advantages and limitations (based on [173]).

Abbreviation: Peripheral blood (PB), Umbilical cord blood (UCB), induced pluripotent stem cells (iPSCs), induced erythroid progenitors (iEPs)

	PB/UCB HSC	**iPSCs**	**iEPs**
Accessibility	• Highly accessible. • Well established international network of cord blood banks [176].	• Can be created from any type of differentiated adult cells	• Can be created from different source of HSCs resulting in unlimited source of erythroid progenitor [90].
Advantages	• Established use in treating blood diseases for over 3 decades. • Well established protocols for collection, preparation and preservation. • High proliferation and differentiation capacity with potential to produce hundreds of units from one sample [177]. • Genetically modified leading to fewer regulatory issues.	• Capable of unlimited *in vitro* proliferation and differentiation into any cell type [116]. • Can be sourced from selected voluntary donors. • Avoids ethical issues related to the use of embryonic stem cells [83].	• Potential for indefinite proliferation in the end differentiated state and differentiation into RBC on demand. • Share many advantages with iPSCs but with potentially higher proliferation and maturation capability.
Limitations	• Production is in batches and depends on donor.	• Genetically modified, they face stringent regulatory requirements • Enhancing proliferation remains a challenge.	• Genetically modified, subject to strict regulatory requirements.

Previous investigations of the elusive MAM blood group antigen

Background for Paper I and II

MAM is a high-prevalence blood group antigen, that belonged to the ISBT 901 series of unassigned high-prevalence antigens (901016), when the carrier molecule was unknown prior to the study presented in this thesis as Paper I.

The story of MAM started in 1992, when an antibody to an unknown high-prevalence antigen was detected in a 31-year old American woman of mixed Irish-Cherokee descent (M.A.M.) during her third pregnancy [178]. At week 31 of her pregnancy, sign of fetal cardiac distress led to the decision for a caesarean section delivery. The RBCs tested strongly positive on the DAT, yet the infant exhibited normal bilirubin levels and no evidence of clinical hemolytic disease, even though the mother's antibody exhibited a high titer at the time of delivery, rising to 256 from 4 at the time of detection. Instead, the baby turned out to suffer from severe thrombocytopenia and underwent a platelet transfusion. The thrombocytopenia was initially thought to be caused by anti-HPA-1a antibodies also found in the mother's plasma. The RBC antibody reacted strongly with all the RBCs tested and was predicted to be of clinical significance since the monocyte monolayer assay (MMA) was positive.

A second case of MAM immunization was reported in a pregnant woman of Arabic origin, resulting in severe HDFN and FNAIT. A decision of an emergency premature delivery was taken at week 24.5 after an unsuccessful intrauterine transfusion attempt [179]. Unfortunately, at six months of age, the infant died due to complications associated with the premature delivery, even though the hematocrit and bilirubin levels had been successfully normalized by the 13th week post-delivery. Subsequent investigations into the family's medical history revealed the presence of anti-MAM also in samples from the proposita's sister, who had one pregnancy in which the newborn was unaffected.

Since then, nine more cases of MAM– have been discovered, where anti-MAM has led to severe hemolytic disease of the fetus and newborn (HDFN) in some pregnancies, while having minimal or no impact in others. Typically, these cases remain undetected until complications arise during pregnancy, which complicates the provision of compatible blood and has resulted in fatalities [180].

Despite many efforts, the identity of the MAM blood group carrier molecule resisted identification almost 30 years until it was finally elucidated as a result of Paper I.

CD36: A brief overview of current knowledge and implications in transfusion medicine

Background for Paper III

CD36 was first discovered in 1973 by Kobylka and Carraway as a membrane protein in breast epithelial cells that could not be proteolyzed in milk fat globules [181]. By 1978, this molecule was recognized as the fourth major glycoprotein on the platelet surface (and therefore designated GPIV) [182], serving as a receptor for binding of thrombospondin-1 (TSP-1) in platelets [183]. CD36 is recognized by several names such as fatty acid translocase (FAT) [184], scavenger receptor class B, member 2 (SR-B2) [185] and plays a role in lipid metabolism.

CD36 exhibits a broad distribution across various tissues. Its expression is significantly noted in tissues characterized by high fatty acid metabolic activity such as adipose tissue and muscle [186], as well as many cancer cell lines [187]. Additionally, CD36 expression varies across the hierarchy of HSC maturation, with monocyte and megakaryocyte erythroid progenitor cells exhibiting the highest level of expression when compared to HSCs. Interestingly, CD36 expression remains elevated in platelets, whereas it is downregulated as erythroid progenitors differentiate into mature erythrocytes [187]. In platelets, it has been found to play an important role in activation [188].

Structure and genetics

CD36 is located on chromosome 7q21.11 and contains 12 coding exons, encoding a transmembrane glycoprotein found on the plasma membrane. The glycoprotein consists of a single peptide chain of 472 amino acid residues and has a molecular weight of 88 kDa [189], and is heavily glycosylated [190]. It consists of two transmembrane domains, nine confirmed N-linked glycosylation.

Disease association

Due to the role of CD36 in mediating the uptake of fatty acids, it has been extensively researched in various fields such as angiogenesis [191], atherosclerosis [192], metabolic disorders [193] and inflammation [194]. In solid cancer, it plays a major role in metastasis initiation [193, 195]. While in hematopoietic malignancies, its interaction with Apolipoprotein C-II (APOC2) drives acute myeloid leukemia (AML) progression via activation of ERK signaling *in vivo* [196]. In chronic myeloid leukemia (CML) CD36 expression defines the primitive cells that are less responsive to tyrosine kinase inhibitors treatment (Imatinib) [197].

The role of CD36 in malaria pathophysiology has been controversial, ranging from studies where CD36 appears to be a receptor for malaria parasites leading to severe

disease, to others where CD36 plays a crucial role in innate defense [198, 199]. No matter what, the geographic distribution of the CD36-deficient phenotype discussed below indicates that it may be protective against malaria.

CD36 deficiency

CD36 deficiency is divided into two types: Type I deficiency, which is characterized by the absence of CD36 expression across platelets and all other cell types [200, 201], and Type II deficiency, which is characterized by the absence of CD36 expression from platelets only [202]. While CD36 deficiency type I is rare in Europeans, it occurs with a prevalence of approximately 3% in Africans [203], 0.5%–1% among the Japanese [204, 205] and 0.5% in China [206], as recently summarized by Xu et al. [207]. Type I deficiency is of clinical significance, since it can be considered a null phenotype associated with the development of antibodies against CD36, also called anti-Nak^{a}. These antibodies have been implicated in various clinically important conditions, including platelet transfusion refractoriness [208-210], PTP [211] FNAIT [212], and in TRALI [213]. Testing hematopoietic progenitor cell donors for CD36 type I patients in need of stem cell transplantation has also been proposed due to the risk of incompatible transplantation [214].

A role for alpha-1-microglobulin in protection of hematopoietic cells

Background for Paper IV

Human RBCs typically circulate for approximately 120 days before they undergo senescence. At this stage, they are engulfed and broken down by tissue resident macrophages in the reticuloendothelial system found in the spleen, liver and bone marrow via a process called erythrophagocytosis [3]. Erythrophagocytosis plays an essential role in the natural disposal of senescent erythrocytes, utilizing their high antioxidant and iron-recycling capacities [215, 216]. The system is supported by haptoglobin (Hp) and hemopexin (Hpx), both scavenger proteins that bind to and neutralize cell-free hemoglobin and heme molecules, respectively.

Haptoglobin

Hp serves as a primary hemoglobin (Hb) clearance mechanism in humans, representing one of the most studied systems for Hb- and heme-detoxification [217, 218]. Hp binds to the extracellular Hb that is present in the bloodstream, forming a stable Hp-Hb complex. This complex is efficiently recognized and cleared from circulation through its interaction with the CD163 receptor on macrophages [219]. However, when the capacity of Hp is exceeded, the unbound oxyhemoglobin

undergoes auto-oxidation, leading to the production of reactive oxygen species (ROS) and the release of heme. The body counters these harmful effects through a network of antioxidative enzymes present in RBCs, including superoxide dismutase (SOD), glutathione peroxidase (Gpx), and catalase, which work together to neutralize ROS and repair oxidative damage [220].

Hemopexin

Free heme, which represents a significant toxicity risk, is tightly regulated in the blood through sequestration by albumin and more effectively by Hpx [221, 222]. Hpx has a high affinity for binding free heme, forming a complex that is readily cleared from the bloodstream by hepatocytes through an interaction with the CD91 receptor [219, 223]. This removal process is essential for preventing the pro-oxidant and pro-inflammatory effects of free heme. Additionally, within cells, heme oxygenase (HO) plays a pivotal role in metabolic breakdown of heme into biliverdin, free iron, and carbon monoxide (CO), further mitigating the potential for heme-induced toxicity. The process by which the Hpx-heme complex is eliminated through a receptor-dependent pathway bears notable resemblance to how macrophages clear Hp-Hb via CD163 [223].

Alpha-1-microglobulin (A1M)

A1M is found abundantly in plasma and various tissues. It can bind and degrade intracellular heme [224]. and has the capability to reduce methemoglobin (metHb). Its truncated form, t-A1M, participates in degradation of extracellular heme, and as such, acts as an additional protective mechanism [216, 220]. The interaction of A1M with heme not only prevents oxidative stress but also contributes to the broader defense against heme-induced cellular and tissue damage. This capability is supported by A1M's unique molecular properties, which facilitate its function as both radical scavenger, a reductase, and a heme-binding protein [220].

In vivo studies have shown that A1M is naturally present in RBCs and can be internalized when added exogenously [225]. Although the internalized A1M provided some degree of protection, the main protective effect against hemolysis is attributed to extracellular A1M [216]. This protective capability of A1M was observed across species as human recombinant A1M (rA1M) also safeguards murine RBCs against hemolysis *in vitro* [225]. These findings suggest that supplementing packed RBC products with A1M might be a beneficial strategy to stabilize RBCs and reduce hemolysis under various conditions.

Further insights were gained from experiments involving A1M knockout (KO) mice. The A1M-KO displayed a macrocytic anemia phenotype, indicating its potential role in the maturation of erythroid cells [225]. In line with this, the erythroid cell line K562 was found to express A1M, and we speculate that it may play a role in protecting against oxidative stress throughout erythropoiesis [226]. This suggests that A1M not only acts as a protector against hemolytic damage in

mature RBCs but may also be important in the formation and development of healthy RBCs.

Free heme plays an important role in the pathophysiology of Diamond Blackfan Anemia (DBA), an inherited anemia condition that is linked to mutation in ribosomal proteins disrupting ribosome synthesis leading to anemia and bone marrow failure. Recent studies showed that disrupting ribosome synthesis could affect globin mRNA translation, which leads to the accumulation of free heme, that could potentially contribute to the pathology of DBA [227-230]. These observations highlight the importance of the critical balance between globin synthesis and heme in erythropoiesis where A1M could play an important role in the regulation of RBC production.

Blood transfusion and protection against hemolysis

Blood transfusion is a life-saving treatment, and while most transfusions are successful and without complications, there is a notable risk of hemolysis, particularly in patients with antibodies against blood group antigens. This includes acute hemolytic events, and delayed hemolytic reactions that can appear days post-transfusion. Both acute and hyper-hemolytic episodes are known to cause renal damage [231].

A1M is of particular interest in this thesis due to its ubiquitous expression in plasma and various tissues [232], making its antioxidative stress and radical scavenging properties of great clinical interest in transfusion medicine, particularly for managing hemolytic anemias and improving outcomes of blood transfusion. Moreover, incorporating A1M into an RBC concentrate storage solution could possibly enhance the quality of stored blood by preventing and/or repairing oxidative damage, and could be particularly beneficial in blood units subjected to gamma irradiation, which risks increasing the degree of hemolysis in the unit.

Transfusion-dependent patients such as patients with SCD and thalassemia may develop antibodies against one or more RBC antigens as a result of multiple transfusions. Thus, chronic transfusion practices can lead to increased risk of hemolysis and require donor matching [220, 233]. SCD patients are also at risk for hyperhemolytic crises, which could benefit from protection against heme and ROS. Due to the protective role of A1M, particularly in protecting against renal damage during active RBC hemolysis, additional treatment with A1M could be beneficial in theory, but this notion requires more investigation.

Emerging research suggests that using whole blood transfusion for trauma and massive bleeding can be beneficial [126], however its benefits remain debatable. The use of group O blood to all patients carries a risk of hemolysis from anti-A and anti-B antibodies, highlighting the importance of selecting group O donors with low

anti-A and anti-B titers. In this setting, the use of A1M could be beneficial as a prophylactic measure [216, 220].

Several studies investigated the protective effect of A1M against hemolysis in RBCs, using A1M knockout mice and renal toxicity induced by ^{177}Lu-DOTATATE radiation therapy *in vivo* [216, 225]. *In vitro* studies with human RBCs showed that A1M reduced hemolysis under various conditions [225]. An *in vivo* model of radiation therapy showed that administration of A1M intravenously may protect the renal toxicity during peptide receptor radionuclide therapy [234].

Altogether, these studies indicate that A1M could play an important role in enhancing the safety and efficacy of blood transfusion across different medical scenarios. With this in mind, the study published in Paper IV set out to investigate the protective effects of A1M on the hematopoietic system following chemotherapy.

Aims of this work

Paper I: To elucidate the molecular and genetic background of the MAM-negative phenotype

Paper II: To investigate the role of EMP3 in erythropoiesis and identify candidate binding partners by proteomics and in silico approaches

Paper III: To establish CD36 as a new blood group system

Paper IV: To evaluate the effect of A1M in protecting the hematopoietic system from cytotoxic therapy

Summary of results

Paper I: Elucidating the molecular and genetic background of the MAM-negative phenotype

Aim

The aim of this study was to uncover the molecular and genetic bases underlying the MAM-negative phenotype and its consequences for erythropoiesis. A series of experimental procedures were performed by the international research team, including:

- A detailed serological characterization of MAM antigen.
- Next generation sequencing (NGS) to elucidate the potential carrier molecule for the MAM antigen.
- Comparison of MAM-positive vs. MAM-negative CD34+ cells during in vitro erythroid culture

Questions addressed

What is the expression of other high-prevalence RBC blood group antigens in MAM-negative individuals?

The serological characterization of MAM-negative individuals primarily aimed to evaluate the expression of RBC blood group antigens on MAM-negative RBCs. This study found that only antigens associated with the Indian blood group system, which are carried on the CD44 protein, were expressed weakly. Normal expression was observed for all other high-prevalence blood group antigens tested.

What is the molecular genetic basis of the MAM-negative phenotype?

Serological evidence had established a clear association between CD44 and MAM. However, an investigation into this relationship using Sanger sequencing for the erythroid-specific isoform of *CD44*, known as *CD44H* revealed no coding mutations in unrelated individuals lacking the MAM antigen. This finding suggested that CD44 is not responsible for the MAM-negative phenotype.

To uncover the underlying genetic basis of the MAM-negative phenotype, further genetic investigation was conducted using whole-exome sequencing (WES) analysis. While WES analysis confirmed the absence of any mutation in *CD44*, this analysis revealed that all MAM-negative individuals were homozygous for inactivating alterations in the gene encoding the Epithelial Membrane Protein 3 (*EMP3*) (Table 5).

Table 5. Genetic bases of the MAM-negative phenotype.

Allele name	Nucleotide change	Amino acid change	Exon	Accession number	rs number	reference
*MAM*01N.01*	c.123C>G	p.Tyr41Ter	3	NM121937	rs201392469	[152]
	c.373A>G	p.Ile125Val	5	NM121937	rs4893	[152]
*MAM*01N.02*	c.182-186_322+418del	p.Trp62_Ser108del	4	NM164486	NA	[152]
*MAM*01N.03*	c.323-231_492+338del	p.Val109_Ter164del	5	NM164487	NA	[152]
*MAM*01N.04*	c.1-3513_492+1379del	p.Met1_Ter164del	1 to 5	NM175569	NA	[152]
*MAM*01N.05*	c.1-3532_492+1361del	p.Met1_Ter164del	1 to 5	NM175570	NA	[152]
Not yet assigned	c.341 to IVS5+688	p.Gly114_Ter164del	5	NM164487	NA	[235]

To validate this finding, Sanger sequencing was specifically used to target *EMP3*. The results confirmed the presence of mutations in all MAM-negative samples analyzed, all of which were predicted to abolish the expression of EMP3. As expected, all nonsense mutations found in *EMP3* were rare, with c.123C>G (p.Tyr41Ter) being the most prevalent within the Genome Aggregation Database (Table 6). In addition to that, this mutation was the most common among MAM-negative individuals in this study. These investigations collectively, suggested that EMP3 could be the long-sought carrier protein of the MAM antigen, the lack of which caused the MAM-negative blood group phenotype.

Table 6. Population allele frequencies of the EMP3 missense variant, as reported in the Genome Aggregation Database (gnomAD v3.1.2, https://gnomad.broadinstitute.org/(ref).

Population	Allele frequency	
	rs201392469	**rs4893**
Middle Eastern	0.003165	0.04114
Ashkenazi Jewish	0.0008646	0.03573
Other	0.0004780	0.03113
Admixed American	0.00006551	0.02868
European (non-Finnish)	0.00005880	0.04117
African/African American	0.00004832	0.007611
European (Finnish)	0.000	0.04136
Amish	0.000	0.07143
South Asian	0.000	0.02342
East Asian	0.000	0.000

Is EMP3 essential for MAM expression?

Genetic analysis suggested that MAM expression is dependent on the presence of EMP3. To test this hypothesis, we modulated the expression of EMP3 in different cell lines. We performed shRNA knockdown and CRISPR/Cas9 gene editing independently in the human immortalized erythroid progenitors cell line BEL-A2 [90]. The undifferentiated state of BEL-A2 cells resembles erythroblasts, expressing EMP3 at a level similar to glycophorin A (GPA).

Knockdown studies using *EMP3*-specific shRNA resulted in the reduction of *EMP3* levels, correlating with a decrease in MAM expression. Two independent CRISPR/Cas9-directed *EMP3* knockouts targeting different exons in BEL-A2 cells resulted in abolished MAM expression.

Thus, MAM was not expressed without when the *EMP3* gene was manipulated. The next step was to perform the opposite experiment, i.e. to introduce the EMP3 gene and look for MAM appearance. For this over-expression study we used the Daudi cell line, which is a B lymphoblast cell line in which no detectable expression of MAM could be found. We used the full-length wildtype (WT) coding sequence of *EMP3,* but also generated a mutant EMP3 sequence based on the most common

mutation in MAM-negative individuals, c.123C>G (p.Tyr41Ter), as a negative control. Indeed, we found that overexpression of WT *EMP3* resulted in MAM expression while neither the empty vector control nor the mutant *EMP3* vector were able to induce MAM expression on the surface of Daudi cells.

Utilizing these three different experimental methodological approaches, our finding consistently corroborated that MAM antigen expression is dependent upon the presence of *EMP3*.

Is MAM a blood group system?

The MAM antigen belonged to the ISBT 901 series of unassigned high-prevalence antigens (901016). The identification of EMP3 as the carrier of the MAM antigen was sufficient evidence to elevate MAM to a new blood group system. The ISBT criteria to achieve this are shown in Table 7.

Is MAM expressed on platelets?

EMP3 expression has been observed across a broad spectrum of human tissues including hematopoietic cells [187, 236, 237].

Discrepancies in data concerning MAM expression on platelets, alongside observation of low platelet counts in neonates born to MAM-negative women with anti-MAM, led our investigation to evaluate MAM expression on platelets and whether the expression of MAM is affected by the activation status of platelets. Our findings demonstrate that MAM is present on platelets, and expression is not affected by the activation status of platelets.

Table 7. Criteria for the establishment of new blood group systems as applied to MAM.

ISBT Criteria	Yes/No	Evidence
The antigen must be defined by a human alloantibody.	Yes	Multiple examples known incl. antibodies made by pregnant women causing HDFN and fetal distress that led to hydrops in some cases [178, 179]
The antigen must be an inherited character.	Yes	MAM antigen is reprted to be inherited [179].
The gene encoding it must have been identified and sequenced.	Yes	All MAM-negative individuals had inactivating mutations in the gene encoding the epithelial membrane protein 3 (*EMP3*).
Its chromosomal location must be known.	Yes	*EMP3* is located on chromosome 19q13.3
The gene must be different from, and not a closely-linked homologue of all other genes encoding antigens of existing blood group systems.	Yes	*EMP3* lacks significant homology with other blood group molecules. The six other blood group genes on chromosome 19 are distant and lack homology.

A role for EMP3 in erythropoiesis: Higher output in culture!

To elucidate its role in erythropoiesis, an in vitro study was conducted. HSPCs from both MAM-negative individuals and MAM-positive controls were cultured under conditions promoting erythroid expansion and differentiation. This study revealed a significant increase in cell proliferation within the MAM-negative cultures, resulting in approximately 5- to 10-fold more erythroid output comparing to MAM-positive control cultures, suggesting that EMP3 may play a role as a brake of proliferation in normal erythropoiesis. Despite this observed increase, MAM-negative individuals displayed no signs of erythrocytosis or reticulocytosis, indicating that external factors or compensatory mechanisms might influence erythroid proliferation in vivo.

Paper II: Towards elucidating the role of EMP3 in erythropoiesis

Background

The absence of EMP3 on RBCs underlies the MAM-negative phenotype. EMP3 is a ubiquitous protein found at low levels in most tissues but with higher expression in hematopoietic cells [152, 187, 236, 237]. Low levels of CD44 are a signature of MAM− RBCs due its proposed physical interaction with EMP3, indicating that EMP3 stabilizes CD44 in the membrane [152]. Erythroid culture of CD34+ cells from peripheral blood from MAM−negative individuals showed an unexpected expansion of erythroid cells, suggesting that EMP3 acts as a brake on the erythropoietic pathway [152]. However, the function of EMP3 in erythroid cells is yet to be elucidated.

Aim

The aim of this study was to enhance our understanding of the function of EMP3 by investigating its molecular partners in erythroid cells, including but not limited to CD44. A series of experimental procedure were performed including:

- Global proteomics analysis study of MAM-negative and MAM-positive RBCs
- Flow cytometry and *in silico* investigation on how CD44 expression is correlated to EMP3 in individuals expressing MAM antigen and whether the expression of *EMP3* is affected upon *CD44* overexpression in addition to a selection of other blood group proteins.

- Co-immunoprecipitation of EMP3 from erythroid cells obtained from *in vitro* differentiated CD34+ HSPCs.
- *In silico* co-folding analysis of EMP3 with a comprehensive catalog of proteins identified throughout the stages of erythroid developments, including reticulocytes and RBCs, employing AlphaFold2 (AF2) protein co-folding prediction technology.

Questions addressed

What are the proteomic differences between RBC samples from MAM-negative and MAM-positive donors?

Our analysis showed that several proteins were significantly up- or downregulated in MAM-negative RBCs. STRING analysis for the up- and downregulated proteins identified showed pathways that are involved in RNA processing, translation and positive regulation of the MAPK cascade were among the downregulated. Proteins involved in homeostatic and cellular oxidant detoxification processes were upregulated.

How is CD44 correlated with EMP3 expression in MAM-positive individuals, and is EMP3 expression affected by CD44 overexpression?

We found that CD44 levels are directly correlated to MAM antigen expression on the RBCs of random healthy donors, where more than half of the variation in CD44 can be attributed to EMP3 expression level. We also evaluated MAM antigen expression on RBCs with the In(Lu) phenotype, which is characterized by downregulation of CD44 expression due to haploinsufficiency of the *KLF1* gene [59]. We found that EMP3 expression is not affected in In(Lu) RBCs, suggesting that the expression of EMP3 is independent of CD44. These results are in line with Paper I where we showed that EMP3 expression is not affected when CD44 is knocked out in an erythroid cell line [152]. However, another study in which CD44 was overexpressed in HEK293 cells resulted in upregulation of EMP3 (when we mined the data from this study), suggesting a level of co-dependency [238].

What are the binding partners of EMP3 in erythroid cells?

To identify proteins interacting with EMP3 in primary erythroid cells, co-immunoprecipitation (co-IP) experiments were conducted on erythroblasts obtained from cultured HSPCs. Due to the lack of good EMP3 antibodies observed in our previous study [152], we constructed EMP3 over-expression vectors coding for a product where a peptide tag (*FLAG*) was fused to either the N- or C-terminus of EMP3 to facilitate the pulldown using anti-FLAG-conjugated beads. This approach aimed to avoid any potential FLAG fusion-related artifacts affecting protein interaction. Utilizing different mass spectrometry methods for protein identification

and quantification, we found several potential candidate interacting proteins, some of which were found to be specifically interacting with the C-terminus while others specifically interacted with the N-terminus. CD44 was one of the proteins identified in this study which had been shown to interact with EMP3 in our previous study and served as a good positive control. In addition, we found that CD44 binding to EMP3 is preferred in the untagged C-terminus highlighting for the first time the potential site of interaction between these two proteins.

What is the erythroid interactome of EMP3?

To complement our co-immunoprecipitation results and narrow down our candidate target protein list, we performed *in silico* prediction of EMP3 potential protein interaction using AF2. We compiled a library of all the >6,000 proteins detected by different proteomics studies, capturing all the developmental stages of erythroid cells including reticulocytes and RBCs. Based on our co-IP results and AF2, we found that CD44 and alpha-enolase (ENOA) were the only two proteins overlapping in both co-IP and AF2. Having found that CD44 is among these two overlapped proteins confirmed the validity of our study approach. In addition to that, we were able to detail the structural interaction between EMP3 and CD44 and found that this interaction is facilitated through a specific salt bridge, providing a molecular mechanism for the interaction that could explain how EMP3 is stabilizing CD44 in the RBCs membrane. Yet other potential binding partners were also proposed but did not fulfill all criteria. Future studies will have to evaluate if these are real or false positive hits.

Paper III: Towards establishing CD36 as a new blood group system

Background

Polymorphic molecules expressed on blood cells are traditionally categorized as either blood groups, human platelet antigens (HPA) or human neutrophil antigens (HNA). Whilst some blood groups like Rh are mostly erythro-specific, others like ABO have wide tissue distribution and are termed histo-blood groups. Furthermore, some polymorphic systems are shared between blood cell lineages, e.g. the ABO and MAM blood groups are present on RBCs but also found on platelets. Similarly, Choline Transporter-Like Protein 2 (CTL2), both underlies a blood group system and carries the HNA-3a/3b antigens on neutrophils. However, CD36 is widely considered a platelet antigen (Nak^{a}) but is also highly expressed on erythroid progenitor cells: In fact, its expression in combination with CD34+ on HSPCs define the erythroid transition from BFU-Es to CFU-Es. In addition, clinical cases reported

that anti-CD36 can FNAIT in CD36-negative (type I) pregnant women. Despite this, CD36 was neither assigned as an HPA nor as a blood group system.

Aim

To evaluate a blood donor lacking a molecule normally expressed on both erythroid cells, platelets and selected leucocytes. Further, to investigate this observation and determine if CD36 fulfills the International Society of Blood Transfusion criteria for becoming a new blood group system. A series of experimental procedures were performed including:

- Surface markers monitored by flow cytometry on developing cells during erythroid culture of CD34+ HSPCs.
- Genetic and flow cytometric analyses on cultured erythroblast and peripheral blood cells.
- A literature study of available erythroid and platelets data, including mining of proteomics datasets, as well as search for clinical cases with anti-CD36 and symptoms involving the erythroid compartment.

Questions addressed

What does absence of CD36 expression lead to in erythroid culture?

We found that lack of CD36 antigen expression does not affect human erythroid cell differentiation *in vitro*, and the absence of CD36 did not have a detectable impact on upregulating or downregulating a limited but often used set of erythroid-specific cell surface markers during erythroblast maturation. CD36 expression on platelets showed complete lack of CD36, consistent with the *in vitro* erythroid culture results and a type I deficiency.

What is the genetic basis of the CD36 deficiency detected in the study?

Sequencing of CD36-cDNA identified homozygosity for c.1133G>T/p.Gly378Val in the CD36-negative donor. The minor allele frequency of rs146027667:T is 0.1% globally and results in abolished CD36 expression. The fact that CD36 is missing on both platelets and erythroblasts due to a germline variant previously implicated in CD36 deficiency, we concluded that the CD36 deficiency in this study is most likely type I.

Is CD36 expressed on reticulocytes and erythrocytes in peripheral blood?

Using a combination of erythroid- and platelet-specific cell surface markers in addition to dye for RNA content for flow cytometry analysis, we found that CD36 is highly expressed on platelets as expected. Moreover, we could show a small but

significant right shift compared to the isotype control, both on reticulocytes and RBCs, indicating that CD36 is indeed expressed on these cells, albeit at low levels. Interestingly, we identified and excluded from the analysis gate a small subset of cells double positive for the erythroid and platelet-specific markers and expressing CD36. Their size resembles that of normal RBCs but shares characteristics with both RBCs and platelets, indicating that they may represent platelets adhering to RBCs. These cell aggregates were present at low levels in all 20 normal donors tested.

Does CD36 fulfil the ISBT criteria to become a blood group system?

Based on experimental data from this study, as well as analysis of data from the literature, we asked the question if CD36 fulfills the formal ISBT requirements to form a novel blood group system. As shown in Table 8 we conclude that this is now the case.

Conclusion

We encountered a CD36-negative blood donor that prompted us to conduct a study including in vitro experiments, literature review and mining of publicly available proteomics data that resulted in acknowledgement of CD36 as a new blood group system. The WP-RCIBGT approved our proposal to make CD36 a new blood group system on 17 June 2023 at the ISBT congress in Gothenburg, substantiated not only by our experimental work but also by a detailed case report in which anti-CD36 was reported to cause a weak panagglutination in routine RBC antibody testing [239] .

This was later confirmed by another reference laboratory that had encountered blood samples from about 100 pregnant women of African origin, whose plasmas also reacted weakly positive in routine blood group antibody screening tests [240] All were shown to have anti-CD36 in their plasmas, that could be neutralized with recombinant CD36 protein.

Table 8. Criteria for the establishment of new blood group systems as applied to CD36.

ISBT Criteria[a]	Yes/No	Evidence
The antigen must be defined by a human alloantibody[b].	Yes	Multiple examples known incl. antibodies made by pregnant women and causing fetal anemia [178, 179].
The antigen must be an inherited character.	Yes	CD36 is well known to be inherited [179].
The gene encoding it must have been identified and sequenced.	Yes	Multiple variants are known to cause the $CD36_{null}$ phenotype.
Its chromosomal location must be known.	Yes	*CD36* is located on chromosome 7q21.11
The gene must be different from, and not a closely-linked homologue of all other genes encoding antigens of existing blood group systems.	Yes	CD36 lacks significant homology with other blood group molecules. The three other blood group genes on chromosome 7 are distant and lack homology.

Paper IV: Elucidating the potential advantages of A1M in protecting the hematopoietic system from radio-therapy

Background

^{177}Lutetium-[DOTA°,Tyr^3]octreotate (^{177}Lu-DOTATATE) peptide receptor radionuclide therapy (PRRT) is used clinically to treat metastasized or unresectable neuroendocrine tumors (NETs). Although ^{177}Lu-DOTATATE is mostly well tolerated in patients, bone marrow suppression and long-term renal toxicity are still side effects that should be considered. Amino acids are often used to minimize renal radiotoxicity, however, they are associated with nausea and vomiting in patients. α_1-microglobulin (A1M) is an antioxidant with heme- and radical-scavenging abilities. A recombinant form (rA1M) has previously been shown to be renoprotective in preclinical models, including in PRRT-induced kidney damage.

Aim

To evaluate the potential effect of rA1M on hematopoietic and renal protection as well as circulatory biomarkers and in a mouse ^{177}Lu-DOTATATE model in terms of the administration route and dosing regimen, and as a combined therapy with an intravenous amino acid solution (Vamin).

Results

- Intravenous (i.v.) administration of rA1M reduced albuminuria levels and circulatory levels of the oxidative stress-related protein fibroblast growth factor-21 (FGF-21).
- Dual injections of rA1M (i.e., at 0 and 24 h post-^{177}Lu-DOTATATE administration) preserved bone marrow cellularity and peripheral blood reticulocytes.
- Administration of Vamin, alone or in combination with rA1M, did not show any protection of bone marrow cellularity or peripheral reticulocytes.

Conclusion

This study suggests that rA1M, administered i.v. for two consecutive days in conjunction with ^{177}Lu-DOTATATE, may reduce hematopoietic and kidney toxicity during PRRT with ^{177}Lu-DOTATATE. However, it needs to be considered that these data were obtained in experimental models and further work needs to be done before this can be considered for clinical use.

Discussion and future perspectives

Paper I: The identification of the epithelial membrane protein 3 (EMP3) as the carrier of the MAM antigen established MAM as a new blood group system and resolved a case reported 3 decades ago, as well as a series of subsequent, similar cases. We demonstrated that EMP3, a molecule thought to act as a tumor suppressor in various cancers, is essential for MAM expression. This study bridges blood group antigens with oncology, providing a novel perspective on how these fields intersect. The observation that EMP3 disruption not only abolishes MAM expression but also leads to enhanced erythroid proliferation and higher reticulocyte yields in *in vitro* cultures of HSPCs suggests a potential role of EMP3 in regulating RBC development and specifically, in regulating the proliferation of early erythroid cells. Furthermore, the identification of the underlying genetic basis in MAM-negative individuals and the detailed characterization of the MAM-negative phenotype contributes significantly to the field of transfusion medicine and provides tools to screen for MAM-negative donors genetically. The possibility know EMP3 down to create MAM-negative cRBCs in the future is now a solution within reach.

Exploring how EMP3 regulates RBC production and its interaction with CD44 within the erythroblast membrane is essential. Such insights could result in innovative treatments for conditions affecting RBC production. Secondly, re-evaluating EMP3 as a therapeutic target in cancer, given its functions as both a tumor suppressor and a regulator for cell proliferation is tempting. Lastly, investigations into the EMP3-CD44 relationship in different cell types might reveal novel targets with a potential for therapy and diagnosis in a range of diseases. Finally, further evaluation if genetic manipulation of EMP3 in HSPCs could potentially enhance the output of in vitro cRBCs and PLTs for transfusion.

Paper II: Both global and targeted proteomics techniques were used to map out the protein interaction landscape of EMP3 within erythroid cells. The analysis not only reconfirmed the significant downregulation of CD44 in the RBC proteome of MAM-negative samples in line with prior findings in Paper I but also revealed an additional 32 proteins with significant expression changes in MAM-negative RBCs, indicating the potential role of EMP3 in erythroid biology beyond its interaction with CD44. Integrating the results of co-immunoprecipitation proteomics of EMP3 from *in vitro* cultured erythroblasts with *in silico* AlphaFold2 protein-complex prediction for EMP3 against the erythroid proteome narrowed down a long list of potential partners to CD44 and ENOA, determining these as significant interactors.

While CD44 had been previously reported, the novel association with ENOA opens avenues for exploring the interplay between metabolic pathways and transcriptional regulation in erythropoiesis, which could explain the mechanism by which EMP3 influences erythroid differentiation and maturation.

Future investigations should aim to expand upon these findings by utilizing advanced proteomics techniques, such as single-cell proteomics, to overcome the limitation posed by small sample size and heterogeneity of analyzed cells. We have generated EMP3 knock-out cell lines using CRISPR/Cas9 technology for validating the functional roles of EMP3 and its binding partners in a more controlled setting. In addition to that, the potential formation of EMP3 homodimers, as suggested by AlphaFold2, presents an intriguing hypothesis deserving further experimental exploration. Both this homodimer interaction and the specific salt bridge predicted to stabilize the EMP3-CD44 heterodimer interaction could be experimentally tested by mutagenesis of implicated amino acids.

This study sets the stage for an in-depth understanding of the potentially significant role of EMP3 in erythropoiesis. Further exploration of EMP3's interaction network could reveal novel therapeutic targets for erythroid disorders, offering insights into the manipulation of erythroid progenitor cells for clinical applications.

Paper III: During our research work on erythroid cultures in the first project, we encountered a donor whose erythroid progenitor cells lacked CD36 expression, a cell surface marker critical for defining the transition from BFU-Es to CFU-Es in vitro. This observation provided us with a unique opportunity to address a question regarding the function of CD36: Will lack of CD36 change the normal process of erythropoiesis? CD36-negative individuals appear to be healthy and can serve as blood donors. Therefore, a reasonable hypothesis is that CD36 is dispensable for human hematopoiesis, especially since there is a mouse model suggesting there is only limited impact on normal hematopoiesis when the *CD36* gene is knocked out [241]. Even if our study was not designed to investigate this question, we did not find any signs of altered in vitro erythropoiesis, based on the percentages of various cell stages during the whole culture. The other question asked in our study is both simple yet controversial in the field of transfusion medicine. Can CD36 be considered a blood group antigen?

Initially, we assessed whether CD36 meets the criteria for establishing a blood group system as outlined by the ISBT, WP-RCIBGT. The ISBT criteria imply, but do not clearly mandate, that a blood group antigen be present on RBCs, probably because this has been taken for granted in the past. This criterion becomes particularly problematic in the case of CD36, which is highly expressed in early erythroid cell stages but has been challenging to detect on mature RBCs and reticulocytes in peripheral blood. Addressing this issue, we found evidence of CD36 expression on gated RBCs free from a platelet-specific marker, thereby resolving the controversy surrounding its presence on RBCs. We also confirmed our findings by finding CD36

peptides in datasets from purified reticulocytes and mature RBCs, while at the same time being unable to find peptides from any of the seven HPA-carrying glycoproteins. Having presented these, data, ISBT approved our proposal based on the evidence provided in our study.

The CD36 blood group system includes a high-prevalence antigen, CD36.1. This was previously dubbed Nak^{a}, initially identified as a platelet antigen with over 97% prevalence among Japanese individuals. Family studies have indicated that the Nak^{a} antigen is inherited as an autosomal co-dominant trait. Nak^{a} was first detected approximately four decades ago in a 36-year-old woman (S. Nak), later found to be carried on CD36 (Glycoprotein IV). The patient manifested transfusion-related alloimmunization after receiving multiple units of random donor platelets during her remission from acute myeloid leukemia. She had never been pregnant but otherwise anti-CD36 appears to quite a common finding during and after pregnancy. While it is not possible to designate CD36 as ISBT 036 due to numbering constraints (but instead ISBT 045), the clinical relevance of CD36 in platelet immunology suggests it could be named an HPA system (if so currently HPA-36 since there are currently 35 recognized systems).

Our analysis of CD36 expression on RBCs from peripheral blood revealed a subset of cells double-positive for GPA, an erythroid-specific marker, and CD61, a platelet-specific marker. These cells, resembling normal RBCs in size and sharing characteristics of both RBCs and platelets, possibly represents platelets adhering to RBCs, a phenomenon previously documented. In healthy individuals, the frequency of these Platelet-RBC complexes (PLT-RBCs) up to 1%, as confirmed by our findings and others [242, 243]. However, the prevalence of circulating PLT-RBCs in patients post-splenectomy significantly increases around 60 days post-operation. Very recently, a study proposed that platelets mark RBCs for phagocytosis, a phenomenon they termed prophagocytosis which could be observed both in mice and humans [244]. These findings are intriguing in the context of the RBC lifespan and that it is still under debate how senescent RBCs are cleared from circulation after 120 days. It remains to investigate what this PLT-RBC interaction means and how important it is.

Paper IV: We showed the protective effect of A1M on the kidneys during PRRT radiotherapy, and pointed to its potential as supportive therapy.

Future directions include the following:

- To investigate administration methods and dosing regiments to optimize the protective benefits of A1M.
- To expand research to assess when it would be most beneficial to enroll test subjects in in vivo trials to reduce the hematopoietic and kidney toxicity observed in PRRT patients and potentially also cancer patients who undergo

other cytotoxic treatment that affect the hematopoietic system and cause cancer-related anemias.

- To explore a combination of A1M with amino acid solutions and other potentially protective compounds to develop comprehensive strategies against PRRT-induced toxicities.

In the longer perspective, it would be of interest to plan for clinical trials to validate A1M efficacy in the in-patient setting, and to integrate A1M into other solid tumor treatment protocols to test its potential in other cytotoxic challenges in cancer therapies.

General remarks and endnote

Clarification of the molecular and genetic basis of blood groups has provided practical solutions for the field of transfusion medicine. We have also come to realize that many blood group phenotypes constitute human knockouts of molecules of great interest. These knockouts are the result of evolution, either in the form of random variants or sometimes likely as a response to evolutionary pressure. By carefully recognizing, characterizing and molecularly defining differences in human blood groups, the transfusion medicine community has not only provided the knowledge to enable DNA-based blood group typing and synthesis of recombinant blood group substances for practical use in reference laboratories. We have also provided researchers with primary human knockout cell models, through which we can create new knowledge about important erythroid (and other) molecules. In addition, blood donors and others with these interesting phenotypes may be interested to act as consenting volunteers in future research studies to dissect how a certain knockout phenotype affects not only mouse models but real humans. Very recently, a study reporting how hundreds of rare Vel-negative individuals compare to Vel-positive controls when it comes to clinical chemistry data, body weight and energy expenditure [245]. This kind of research requires well-coordinated study cohorts and access to biobank data, in addition to the blood group phenotype or genotype. It is hereby predicted that these kinds of studies involving knockout humans, databases and biobanks will be useful to investigate the function of blood groups in the future.

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