# A Feasibility Study Examining the Potential for a Predictive Test for Vaso-Occlusive Crises in Sickle Cell Disease Patients.

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DClinSci 2020

# A Feasibility Study Examining the Potential for a Predictive Test for Vaso-Occlusive Crises in Sickle Cell Disease Patients.

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A thesis submitted in partial fulfilment of the requirements of the Manchester Metropolitan University for the award of Doctorate in Clinical Science Manchester Metropolitan University.

Department of Life Sciences Manchester Metropolitan University, in collaboration with NHS Blood and Transplant and University College Hospital, London.

2020

**Abstract**

**Background**

There are 15,000 adults and children with Sickle Cell Disease (SCD) in England. Patients with homozygous (HbSS) SCD are at risk of painful Vaso-Occlusive Crises (VOCs), and haemolysis, caused by deoxygenated, sickle shaped erythrocytes occluding the microcirculation. VOCs may be exacerbated by the release of procoagulant Red Cell Derived Particles (RCDP), including autophagic vesicles (AV) into the bloodstream. RCDP release occurs during erythrocyte maturation and as part of sickling and unsickling of the erythrocyte upon repeated deoxygenation and reoxygenation.

**Aims**

The primary aim is to determine if AV numbers are elevated in SCD patients who are in SCD crisis when compared with steady state and healthy controls. Secondary aims include; whether levels of microvesicles correlate with VOC; whether levels of microvesicles correlate with length of admission and whether levels of microvesicles correlate with numbers of circulating reticulocytes in SCD patients admitted in VOC.

**Methods**

Samples from 11 SCD patients, admitted to University College London Hospital (UCLH) in VOC were analysed using confocal microscopy and flow cytometry techniques during admission, to quantify RCDP and AV on the RBC and in their plasma, respectively. Results were compared to healthy control samples and patients in a steady state of SCD.

**Results**

In confocal microscopy studies, statistically significant ($p<0.0001$) numbers of PS-positive RCDP, and GPA-pos RCDP were identified which is concordant with previous studies. In flow cytometry studies, a heterogeneous population of RCDP was present in the plasma of both healthy controls and SCD patients in steady state and in VOC. RCDP of an inside out orientation, most likely AVs, were present in the plasma of SCD patients in both steady state and VOC but were not significantly increased in either cohort. This is a novel finding. No population of MV was found to be a statistically significant predictor of VOC, which was limited by the experimental methodology.

**Conclusion**

The study of MV in SCD provides research opportunities into the prevention of SCD symptoms. Future studies should utilise imaging flow cytometry, to examine potentiators of the symptoms of SCD. They must be used to build a complete picture of the complex interactions in SCD patients; addressing the current slow progress in treatment and monitoring.

**Dedication**

This thesis is dedicated to my wife Kelly and to my two children Oliver and Henry. Without their never-ending love and support, none of my achievements would be made possible, nor be quite as worthwhile in the end. I love you all xxx

**Acknowledgments**

This doctoral thesis completes an epic 17-year journey for me personally, from trainee Biomedical Scientist to Consultant Clinical Scientist; something I never envisaged would happen at the start of my career. Many people have helped me along the way, far too many to thank here, but I would like to thank the following, in particular, for their continued support and advice throughout my Doctoral programme. Dr Edwin Massey, Dr Tom Latham, Dr Mark Williams, Ruth Evans, Dr Shubha Allard, Dr Nay Win, and all my colleagues in the NHSBT Red Cell Immunohaematology (RCI) laboratories.

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Special thanks go to Dr Elisa Allen, Principal Statistician, and Senior Statistician, Mark Jones Statistics and Clinical Studies, NHS Blood and Transplant for their help and advice with the statistical analysis of this thesis data.

Most importantly, I would like to thank the patients involved in this study for consenting to have samples taken during their admission for what is an extremely painful and difficult to manage hospitalisation episode. Without this, research cannot begin to answer questions about disease pathophysiology, diagnosis and treatment, leading to improvements in care for others.

Finally, I would like to thank my wife Kelly and my sons Oliver and Henry for their love and support, and my extended family and friends for patiently putting up with me, and for always asking “how’s the training going?” over the past 5 years. I’m going to have to find something else to talk about now!

**Declaration**

No portion of this work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institution of learning.

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**List of abbreviations**

| | |
|---|---|
| ACS | Acute Chest Syndrome |
| AE1 | Anion Exchanger 1 |
| APPG | All Party Parliamentary Group |
| ARDS | Acute Respiratory Distress Syndrome |
| ATP | Adenosine triphosphate |
| AV | Autophagic vesicles |
| BITS | Bristol Institute of Transfusion Science |
| BSH | British Society for Haematology |
| CPD | Continuing Professional Development |
| CR1 | Complement Receptor 1 |
| EQA | External Quality Assessment |
| FACS | Fluorescence Activated Cell Sorting |
| FBC | Full Blood Count |
| FC | Flow Cytometry |
| FITC | Fluorescein isothiocyanate |
| FS | Forward Scatter |
| GPA | Glycophorin A |
| GvHD | Graft Versus Host Disease |
| Hb | Haemoglobin |
| Hct | Haematocrit |
| HbF | Foetal haemoglobin |
| HbSC | Heterozygous Haemoglobin S / Haemoglobin C disease |
| HbSS | Homozygous Sickle Cell Haemoglobin |
| HLA | Human Leukocyte Antigen |
| HPHF | Hereditary Persistence of Haemoglobin F |
| HS | Hereditary Spherocytosis |
| HSCT | Haemopoietic Stem Cell Transplant |
| HTA | Health Technology Assessment |
| IBGRL | International Blood Group Reference Laboratory |
| ISTH | International Society of Thrombosis and Haemostasis |
| mAb | Monoclonal Antibody |
| MAHA | Microangiopathic Haemolytic Anaemia |

| | |
|---|---|
| MP | Microparticle |
| MSD | Matched Sibling Donor |
| MUD | Matched Unrelated Donor |
| mMUD | Mismatched Unrelated Donor |
| MV | Microvesicle |
| MVB | Multivesicular Bodies |
| NAD | Nicotinamide Adenine Dinucleotide |
| NEQAS | National External Quality Assessment Scheme |
| NHR | National Haemoglobinopathy Registry |
| NHSBT | National Health Service Blood & Transplant |
| NICE | National Institute for Health and Care Excellence |
| NIHR | National Institute of Health Research |
| NO | Nitric Oxide |
| PDPU | Protein Development and Production Unit |
| PE | Phosphatidylethanolamine |
| PHE | Public Health England |
| PNH | Paroxysmal Nocturnal Haemoglobinuria |
| PPP | Platelet Poor Plasma |
| PS | Phosphatidylserine |
| QA | Quality Assurance |
| RBC | Red Blood Cell(s) |
| RCDP | Red Cell Derived Particles |
| RCT | Randomised Controlled Trial |
| RCDV | Red Cell Derived Vesicles |
| RR | Ribonucleotide Reductase |
| SCA | Sickle Cell Anaemia |
| SCD | Sickle Cell Disease |
| SE | Succinimidyl Esters |
| SIRP-α | Signal Regulatory Protein Alpha |
| SS | Side Scatter |
| STOP | Stroke Prevention Trial in Sickle cell disease |
| TCD | Transcranial Doppler |
| TFP | Tetrafluorophenyl |
| TO | Thiazole Orange |

| | |
|---|---|
| TSP1 | Thrombospondin 1 |
| TTP | Thrombotic Thrombocytopenic Purpura |
| UCH | University College Hospital |
| UKAS | United Kingdom Accreditation Service |
| VOC | Vaso-Occlusive Crises |
| vWF | von Willebrand’s Factor |
| WHO | World Health Organisation |
| 2,3-DPG | 2,3-diphosphoglycerate |

# CHAPTER 1. INTRODUCTION

## 1.1 Historical and Genetic Background

Sickle cell disease (SCD) was first described by Dr James Herrick in 1910 in a West Indian patient named Walter Clement Noel. Following this discovery, further cases of SCD were subsequently reported (Washburn, 1911; Cook & Myer, 1915). The molecular association of the disease was first described by Pauling & Itano *et al*. in 1949; and in 1956, Ingram described the Glutamic acid (Glu) to Valine (Val) point mutation at codon 6 in the β-globin gene ($\beta^S$). This single point mutation is responsible for formation of the sickle haemoglobin tetramer ($\alpha_2\beta_2{}^s$). Inheritance of SCD follows an autosomal recessive pattern. Inheritance of two $\beta^S$ genes (HbSS) leads to symptomatic homozygous SCD, while heterozygous inheritance of HbA/HbS results in sickle cell trait. Sickle cell trait typically does not manifest any symptoms (Ware *et al.* 2017) and affords limited protection to heterozygous carriers from endemic *Plasmodium falciparum* malaria infection at a population level. (Nagal, 1990; Killian *et al.* 2015).

SCD can also be inherited in a compound heterozygous form with other haemoglobinopathies which are prevalent within distinct populations, such as haemoglobin C (HbC) with HbS (HbSC), HbS with β-thalassaemia (HbS/$\beta^0$-thalassaemia or HbS/β+-thalassaemia), and HbS with other beta-globin variants such as HbSD or $HbSO_{Arab}$, all of which express sufficient HbS to cause intracellular sickling. The genotypic variants of SCD and their clinical severity are shown in Table 1.1. The different mutations responsible for the haemoglobinopathies are shown in Table 1.2.

**Table 1.1 Genotypic variants of SCD with genotype percentage and their associated severity** (adapted from Rees, 2013).

| ***Types of SCD and genotype percentage*** | | |
|---|---|---|
| **Very mild SCD** | **Mild SCD** | **Severe SCD** |
| HbSE (1-2%) | HbSC (25-30%) | HbSS (Sickle cell anaemia) (50-60%) |
| HbS/Hereditary Persistence of foetal haemoglobin (1-2%) | HbS / $\beta^+$-thalassaemia (5-10%) | HbS / β-thalassaemia (1-3%) |
| | HbS / Lepore (1-2%) | HbS / $O^{Arab}$ (1-2%) |
| | | HbS / $D^{Punjab}$ (1-2%) |

**Table 1.2. Genetic mutations associated with haemoglobinopathies**
(adapted from Bain, 2008).

| *Types of mutation responsible for haemoglobinopathies* | | |
|---|---|---|
| **Types of mutation** | **Consequence** | **Examples** |
| **Point mutations**<br>Within coding sequence. | **Same sense or neutral mutation**<br>Mutant codon codes for the same amino acid as normal codon – no consequences. | Many mutations are this type. One third of point mutations result in no amino acid change. |
| | **Missense mutations**<br>Mutant codon codes for a different amino acid to the normal codon. | HbS; HbC; HbE disease. |
| | **Nonsense mutations**<br>Mutant codon does not code for an amino acid but functions as a premature stop codon. | Haemoglobin McKees Rocks. Premature stop codon leading to α-thalassaemia. |
| | **New-sense mutations**<br>Conversion of a stop codon to a coding sequence. | Haemoglobin Constant Spring. |
| | **Gene conversion +/- point mutation** | Haemoglobin F-Port Royal |
| Within a non-coding sequence | **New splice site leading to structurally abnormal DNA** | Some β- thalassaemias |
| | **Mutation of an enhancer** | Some β- thalassaemias |
| | **Reduced mRNA synthesis** | Some β- thalassaemias |
| **Deletion or duplication of one or more genes** | **Loss of expression or function of gene** | Most α-thalassaemias, some β-thalassaemias. |
| | **Loss of function of one gene but enhancement of another gene** | Hereditary persistence of fetal haemoglobin. |
| **Abnormal cross-over during meiosis leading to gene fusion.**<br>Simple crossover | **Loss of one α gene but normal α-chain is encoded** | $\alpha^{3,7}$-thalassaemia. |
| | **Reduced rate of synthesis of structurally abnormal globin chain** | Haemoglobin Lepore.<br>Haemoglobin Parchman. |
| Double crossover<br>Gene fusion | **Synthesis of a variant haemoglobin in addition to increased production of haemoglobin F** | Haemoglobin Kenya. |

The genetic association of SCD was first described by Pauling & Itano *et al*. in 1949. It was subsequently isolated to a defect in the β-globin gene in 1956 by Ingram. There are two globin genes, *HBA* located on chromosome 16, which codes for α-globin, and *HBB*, located on chromosome 11, which codes for β-globin. Normal adult haemoglobin consists of two alpha (α) subunits and two beta (β) subunits (Hagar & Vichinsky, 2008). Defects in the *HBB* gene give rise to the various haemoglobinopathies. Mutations causing quantitative defects in globin chain synthesis result in thalassaemia, whereas qualitative defects result in haemoglobin variants (Rees, 2013) such as SCD. (see figure 1.1)

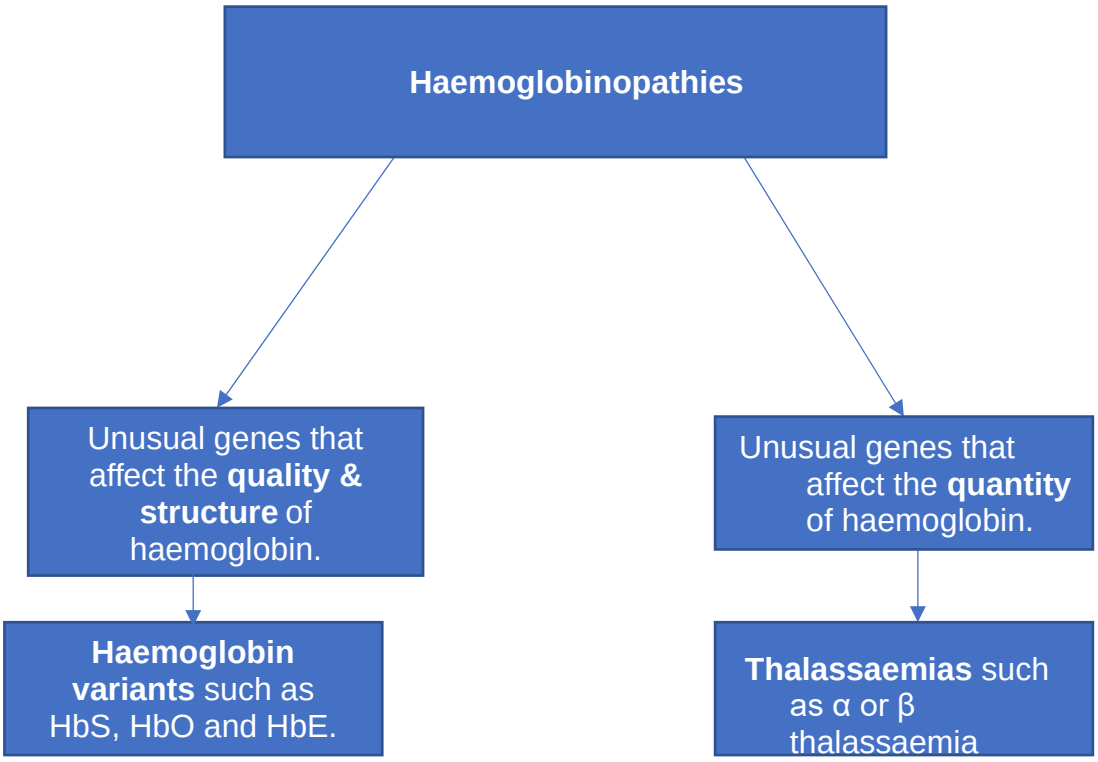



**Figure 1.1. Categorisation of the Haemoglobinopathies.**

Haemoglobinopathies can be subdivided into two categories; where mutations cause qualitative variants in expression of the haemoglobin molecule, leading to SCD among other structurally abnormal haemoglobinopathies, Alternatively, quantitative variants of haemoglobin expression are referred to as the thalassaemia's. (Adapted from PHE, 2013).

SCD is an autosomal recessive disease caused by a point mutation in the β-globin gene (*HBB*), found on chromosome 11p15.5. The point mutation, at codon 6 causes a substitution of Glutamic acid (Glu) to Valine (Val) and results in the formation of the sickle haemoglobin tetramer ($\alpha_2\beta_2^s$), if inherited from both parents in a homozygous form. Homozygous (HbSS) SCD is now the commonest inherited single gene disorder in the UK (Streetly & Clark *et al.* 2008) affecting 50-60% of SCD sufferers and it is clinically the most severe. The heterozygous form, with inheritance of one normal *HBB* allele and one abnormal HbS *HBB* allele is termed sickle cell trait.

Other forms of sickle cell disease include compound heterozygous conditions such as HbSC disease, where inheritance of a haemoglobin C (HbC) allele along with a HbS allele results in the compound expression of both HbS and HbC. HbC inheritance is governed by a point mutation in codon 6 of the β-globin gene (*HBB*), at the same position as that of the HbS mutation, but on the alternative *HBB* allele, but in HbC disease the point mutation is a substitution of Glutamic acid (Glu) to Lysine (Lys). HbSC heterozygotes have clinical symptoms similar to those with homozygous HbSS disease, and is characterized by erythrocyte dehydration, intracellular sickling and increased blood viscosity (Pecker *et al.* 2017). Although considered a milder sickle cell disease (SCD) variant, HbSC is associated with potentially severe morbidities and therefore requires surveillance and intervention (Pecker *et al.* 2017; Nagel *et al.* 2003). Other compound heterozygote haemoglobinopathies include HbS / β-thalassaemia, which results in severe clinical symptoms akin to those in homozygous HbSS disease.

A diagnosis of SCD is established by identification of significant quantities of HbS with or without an additional abnormal β-globin chain variant identified by a haemoglobin assay in screening tests, as described previously, or by identification of a bi-allelic compound *HBB* pathogenic variant where at least one allele is the variant *HBB* gene (e.g., heterozygous *HBB*.Glu6Val; Glu6Lys in HbSC disease) on molecular genetic testing. (Bender, 2017; Steinberg & Sebastiani, 2012).

### 1.2 Worldwide distribution and mortality

Homozygous SCD prevalence rates have been reported to be as high as 3% of births in Sub-Saharan Africa, whilst heterozygous SCD (trait) has been detected in up to 45% of populations within this region. SCD is also found in considerable numbers in those of Indian, Arabic and Greek decent. (Figure 1.2). Mortality rates in Sub-Saharan Africa are reported to be in the range of 50-90%, however significant studies in this area are scarce due to a severe lack of funding and associated healthcare resources in this region (Grosse & Odame *et al.*, 2011). One population study estimates that by 2050 the number of affected infants worldwide could increase by 30%, especially within Africa. (Piel *et al.* 2013).

Globally, SCD is more common in areas where malaria is endemic (Figure 1.3) as it provides limited protection against infection with some forms of malaria. The protective effect of sickle cell trait (HbAS) over those affected by sickle cell disease (HbSS) was first described by A. Allison in 1954. Allison showed that not only was the HbS gene frequently detected in areas with high malarial transmission, but also that HbAS (sickle trait) individuals seemed to have less symptomatic episodes of severe malarial infection. Subsequent studies (Garlick, J.P. 1960 & Thompson, G.R. 1962) and epidemiological data have shown that sickle trait individuals do get malaria, but their blood contains lower numbers of parasitized red cells.

The 2015 Global Burden of Disease report (GBD, 2015) states SCD as “causing more than 100,000 deaths, a 6% p.a. increase in prevalence since 2005”.
At present, it is estimated there are more than 15,000 adults and children with SCD in Britain, with most patients living in or near in the larger cities such as London, Birmingham and Manchester (Figure 1.4). Every year about 350 babies are born in the UK with SCD (Sickle Cell Society, 2017). As the population of the United Kingdom becomes more diverse it is becoming increasingly difficult to manage these patients. They require blood transfusion support from a largely Caucasian donor pool that have differences in expressed antigens on the red blood cell (RBC) surface, particularly those in the Rh, Kell, MNSs, and Duffy blood group systems. This leads to a higher rate of alloimmunisation and alloantibody

production, making subsequent transfusions more challenging and increasing the risk of acute and delayed haemolytic transfusion reactions, and episodes of hyperhaemolysis (Win, N. 2009).

Despite NHS Blood & Transplant (NHSBT) marketing campaigns (NHSBT, 2014), recruitment of Black, Asian and Minority Ethnic (BAME) donors remains a challenge and they are under-represented in the UK donor population, where less than 5% of blood donors who gave blood in the last year were from black, Asian and minority ethnic communities. (NHSBT, 2019).

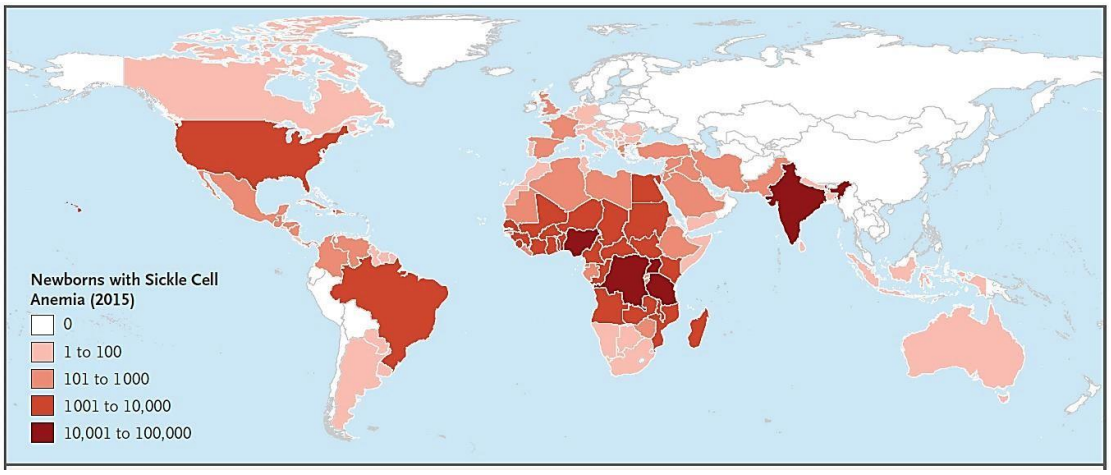



**Figure 1.2 Number of Newborns with Sickle Cell Disease by Country (2015).**
Data are based on estimates from Piel *et al*. 2017.

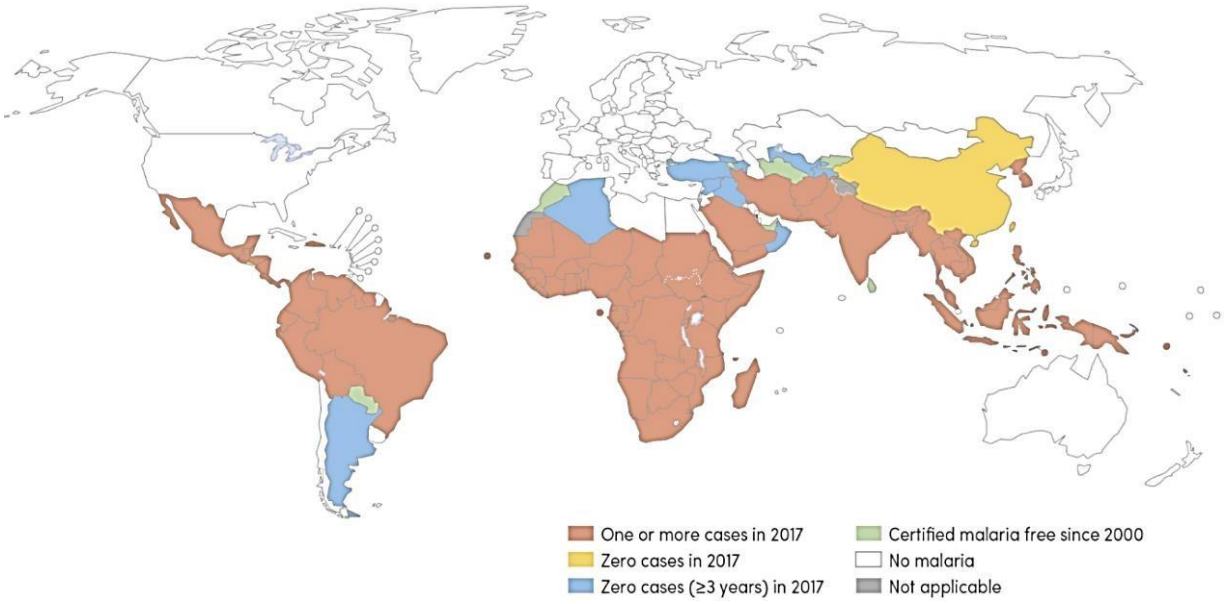



**Figure 1.3 Countries with reported indigenous cases of malaria in 2000 and their status by 2017.** Countries with 0 cases over the last 3 years are considered to be malaria free (World Health Organization, 2018).

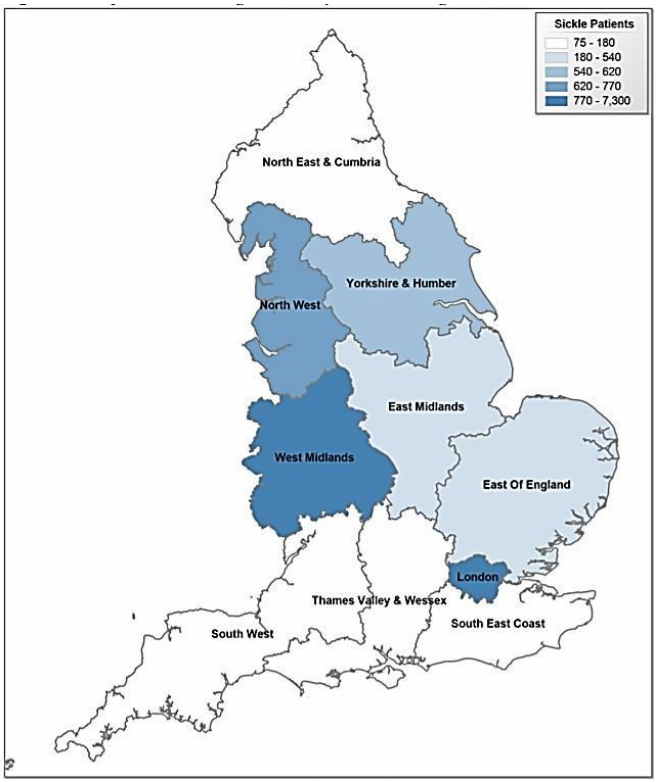



**Figure 1.4. Map of Sickle Cell Disease registrations by commissioning hub 2016/17.** (Figure from NHR annual report 2016/17).

## 1.3 Pathophysiology

In SCD, the erythrocytes undergo rapid but reversible shape change upon deoxygenation and reoxygenation. Repeated deoxygenation and reoxygenation causes haemoglobin S to align, forming long parallel rods of HbS polymers, resulting in the cell becoming sickle-shaped (figure 1.5). The HbS polymers dissolve upon reoxygenation, restoring the original biconcave disc shape. Eventually, these HbS polymers form into a helical fibre structure with 14 HbS molecules in each section which reduces, and eventually prevents the erythrocyte membrane from being flexible and deformable (Serjeant & Serjeant, 1992). The role of the HbS single point mutation from glutamic acid to valine in the formation of long HbS polymers is that, once formed, the helical fibres enable hydrophobic contacts to form between the valine of one HbS molecule and alanine, phenylalanine and leucine from an adjacent HbS molecule, reducing deformability through the aggregation of the helical fibres (Vekilov, 2007). The decreased erythrocyte deformability leads to erythrocyte membrane damage, vaso-occlusion and subsequent episodes of acute pain crises.

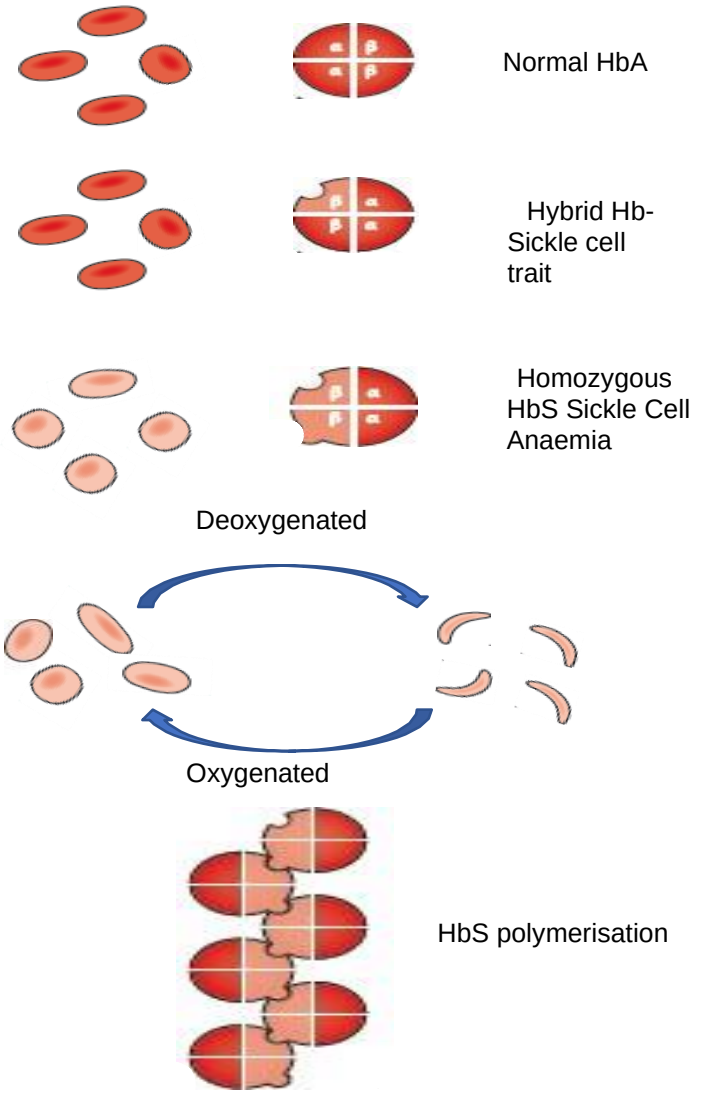



**Figure 1.5. Induction of Sickling by polymerisation of deoxyhaemoglobin S.**
Normal haemoglobin A (HbA) is formed by two α-globin subunits and two β-globin subunits, which are encoded by *HBA* and *HBB* respectively. The sickle Hb (HbS) *HBB* allele occurs when an adenine-to-thymine substitution results in the replacement of glutamic acid with valine at position 6 in the mature β-globin chain. SCD occurs when both *HBB* alleles are mutated and at least one of them is the βS allele. Sickle haemoglobin (HbS) polymerises upon repeated oxygenation and deoxygenation. HbS polymers stiffen the erythrocyte membrane, leading to reduced deformability. Individuals with one βS allele have the sickle cell trait (HbAS) but not SCD; individuals with sickle cell SCD, the most common SCD genotype, have two βS alleles (βS/βS). (Adapted from Kato *et al.* 2018)**.**

Eventually the HbS erythrocyte becomes irreversibly damaged, leading to increased cation permeability and dehydration (Hannemann *et al.* 2015; Kato *et al.* 2018). Normally, RBC cation homeostasis is maintained by an ATP-driven Na+/K+-pump in addition to passive permeability through various additional transport pathways (figure 1.6). High cation permeability causes RBC to lose intracellular solutes, become dehydrated and shrink, which increases intracellular concentrations of HbS (Lew and Brookchin, 2005) and results in the characteristic sickling seen (figure 1.7). Polymers of HbS affect structural membrane proteins including the band 3 anion transport protein, Anion Exchanger 1 (AE1) (Westerman *et al.* 2008). These changes cause membrane microvesiculation, the release of erythrocyte microparticles and exposure of prothrombotic phosphatidylserine (PS) on the erythrocyte surface. (De Jong *et al.* 2001; Yasin *et al.* 2003; Piccin *et al.* 2007).

SCD erythrocytes have a reduced lifespan in the circulation (10 - 20 days) when compared to erythrocytes in a healthy individual (90 – 120 days) (Piel *et al.* 2017). Reduced erythrocyte lifespan results in increased levels of haemolysis and release of haemolytic by-products such as haem, which aggravates pro-inflammatory mediators, and scavenges the vasodilator, Nitric Oxide (NO) causing vasoconstriction, activation of endothelial cells and platelets (Kato *et al.* 2018). Premature RBC degradation and destruction leads to increased erythropoiesis and reticulocytosis as the bone marrow is stimulated to produce more RBC due to the underlying anaemia, which in turn leads to an increased number of vesicles, including Autophagic Vesicles (AV’s) released as part of reticulocyte maturation in the peripheral circulation. (Platt, 2000; Sharma *et al.* 2007; Griffiths *et al.* 2012; Mankelow *et al.* 2015).

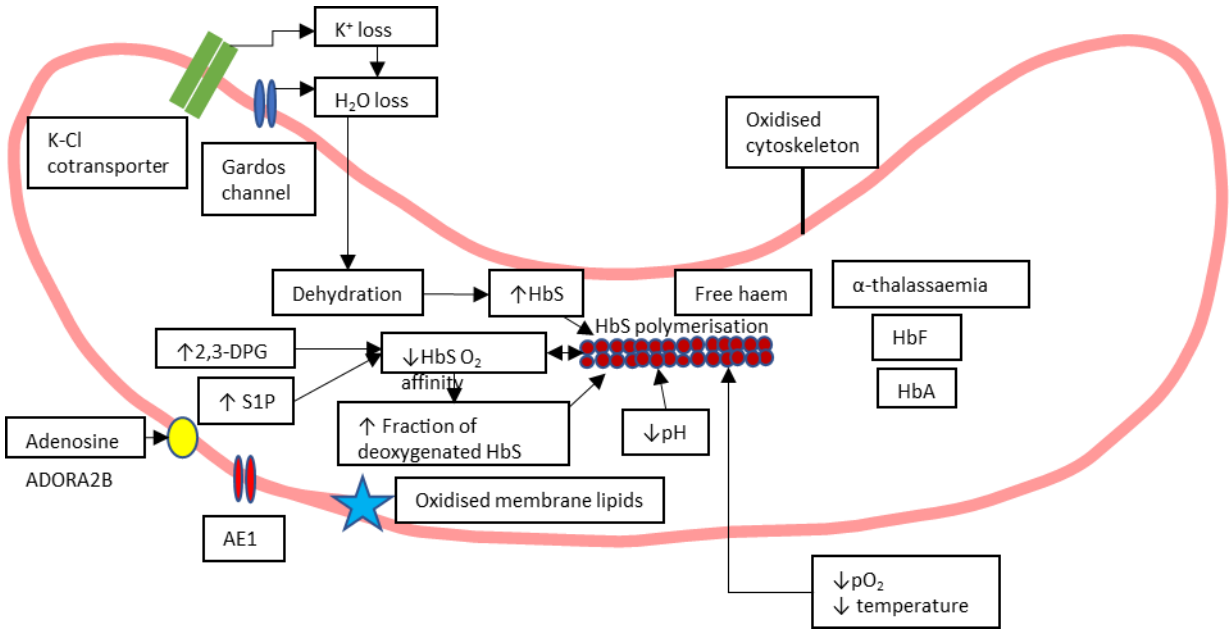



**Figure 1.6. HbS polymerisation and erythrocyte deformation**. Long polymers of sickle haemoglobin (HbS) eventually align into parallel rods within the erythrocyte. The polymerization of HbS depends on a number of contributing factors, including concentration of HbS, partial pressure of oxygen (pO2), temperature, pH, 2,3-diphosphoglycerate (2,3-DPG) concentration and the presence of different Hb molecules.
Cation homeostasis is abnormal in sickle erythrocytes, leading to the dehydration of cells. Potassium loss occurs via a calcium-activated potassium channel, protein 4 (also known as the Gardos channel) and K–Cl cotransporter. (Adapted from Kato et al. 2018).

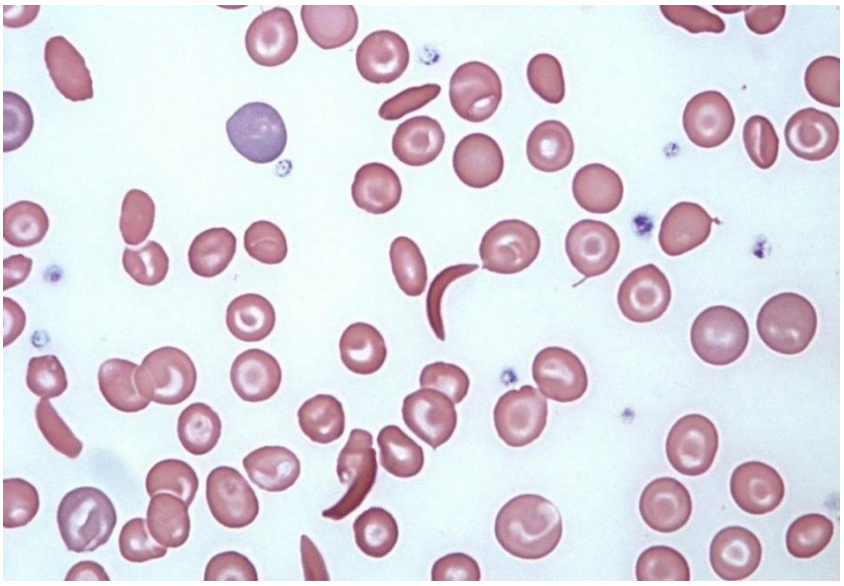


**Figure 1.7. Photomicrograph of a blood film. Sickle cell disease (homozygosity for haemoglobin S)** Shows sickled cells, boat-shaped cells, and target cells. (Bain, 2012).

Although the primary pathophysiology of SCD is reduced deformability leading to aggregation, vaso-occlusion and episodes of acute pain (termed crises), combined with a haemolytic anaemia; the complex interactions between blood cells, plasma factors and the vascular endothelium lead to many of the symptoms associated with the disease (figure 1.8). These include vascular–endothelial dysfunction, functional nitric oxide deficiency, inflammation, oxidative stress and reperfusion injury, hypercoagulability, increased erythrocyte adhesion, increased neutrophil adhesiveness, and platelet activation (Piel *et al*. 2017)

Existing research into the causative agents of vaso-occlusive crises has focussed upon many of the different physiological and non-physiological aspects of the condition, from abnormalities in structure of erythrocytes (Kaul *et al.* 1996), adhesiveness of erythrocytes (Kaul *et al* 2006; Joneckis *et al.* 1993; Brittain *et al.* 2004; Manodori *et al.* 2000) the pro-inflammatory state caused by elevated haemolysis (Hoppe, 2014; Owusu-Ansah *et al.* 2016), pro-thrombotic markers (Westerman *et al.* 1999; Ataga & Orringer, 2003), elevated reticulocytosis (Joneckis *et al.* 1993), to microparticle / microvesicle release by platelets, monocytes, endothelial cells and erythrocytes and their precursors (Hebbel & Key, 2016; Kasar *et al.* 2013).

Additional modifiers of disease severity include inherited genotype, as discussed, with less severe clinical manifestations observed in compound heterozygotes who inherit another globin gene mutation such as Hereditary Persistence of Haemoglobin F (HPHF) or α- thalassaemia, in addition to HbS (Thein, 2017).

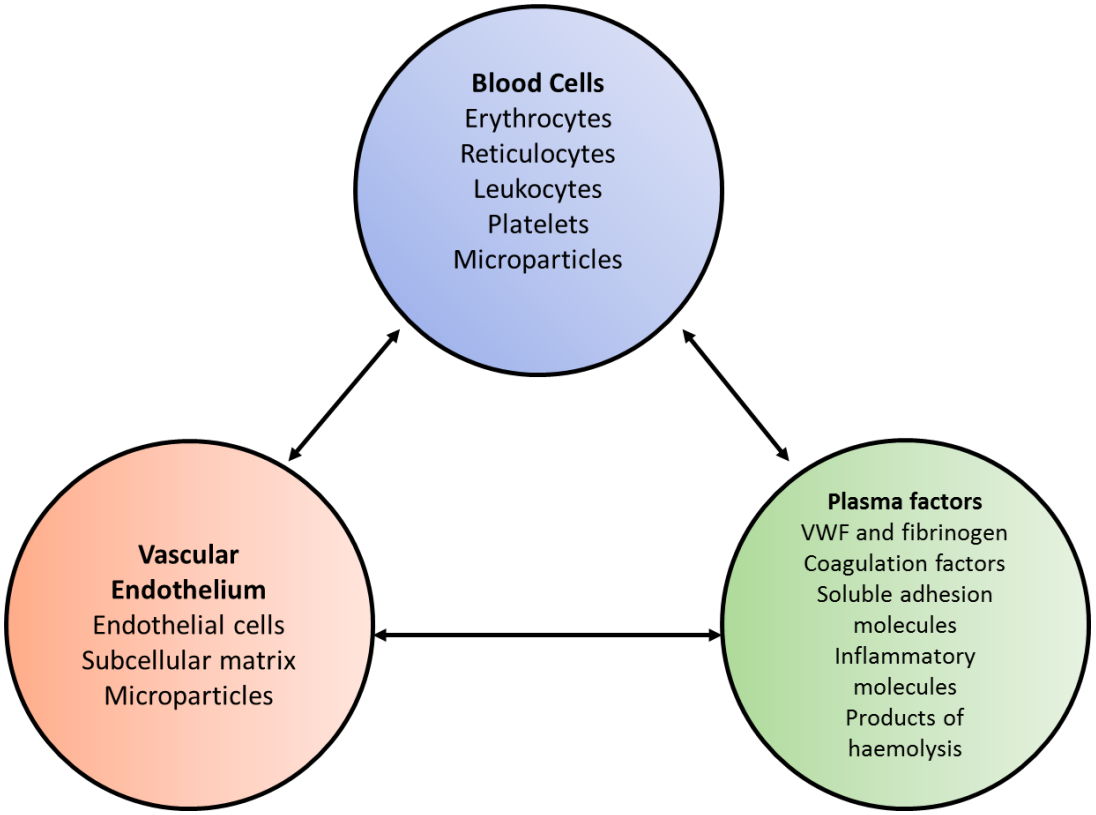



**Figure 1.8 Complex interactions in SCD which lead to vaso-occlusion and tissue damage**. These include interactions between Blood cells, Plasma factors and Vascular endothelium which contribute towards a cumulative effect of VOC and tissue damage due to deoxygenation. The absence of a predictive test for VOC means that clinicians are unable to predict when a patient experiences VOC and cannot proactively manage the condition. Microparticle associated factors, both in the vascular endothelium and from the blood cells, are associated with this triad of VOC interaction. (Adapted from Ware & De Montalembert, *et al.* 2017)

Non-physiological modifiers attributable to disease severity include wind speed, temperature, altitude, air quality and poverty (Jones *et al.* 2005; Tewari *et al.* 2015). An association has been made between higher wind speed and low humidity causing rapid changes in skin temperature due to direct cooling and rapid evaporation of sweat (Jones *et al.* 2005). This has been linked to vaso-occlusion and pain in physiological studies and clinical observations (Mohan *et al.* 1998;

Resar & Oski, 1991). The subsequent complex interaction between these physiological and non-physiological processes plays a role in the disease pathophysiology, and requires a multimodal, multidisciplinary therapeutic approach, or emerging gene therapy to provide either amelioration of symptoms, or a cure in the best-case scenario.

### 1.4 Clinical symptoms

A wide range of clinical symptoms are experienced in SCD patients, making them extremely complex to manage (table 1.3). SCD can affect the cardiothoracic, nervous, reticuloendothelial, musculoskeletal, urogenital and gastrointestinal systems (figure 1.9). Many of the symptoms result from vaso-occlusion and impairment of oxygen supply to tissues and organs, due to rigid sickle cells occluding the microcirculation, as previously discussed. In addition, there are also associated changes in vascular endothelial cells due to excess levels of plasma free haem, released upon intravascular haemolysis and extravascular removal by the reticuloendothelial system. (Ware & De Montalembert *et al.* 2017)

**Table 1.3. Acute clinical complications of Sickle Cell Anaemia, including common manifestations, presumed pathophysiology and treatment options.**

(Adapted from Ware & De Montalembert *et al*. 2017)

| | **Manifestation** | **Pathophysiology** | **Treatment** |
|---|---|---|---|
| Painful event | Dactylitis; pain in the sternum/ribs; pain in the long bones; priapism | Vaso-occlusion; hypoxia; ischaemia-reperfusion | Hydration; analgesia |
| Infection | Bacteraemia / sepsis; meningitis; osteomyelitis; pneumonia; malaria | Splenic dysfunction; inflammation; necrotic bone | Antibiotics; surgery |
| Anaemia | Splenic sequestration; transient aplastic crisis; transfusion reaction; papillary necrosis | Erythrocyte sickling; infection; sequestration; hyperhaemolysis | Transfusion |
| Organ damage | Stroke; acute chest syndrome; splenic infarction; papillary necrosis | Ischemia; infarction; haemorrhage | Hydration; transfusion |

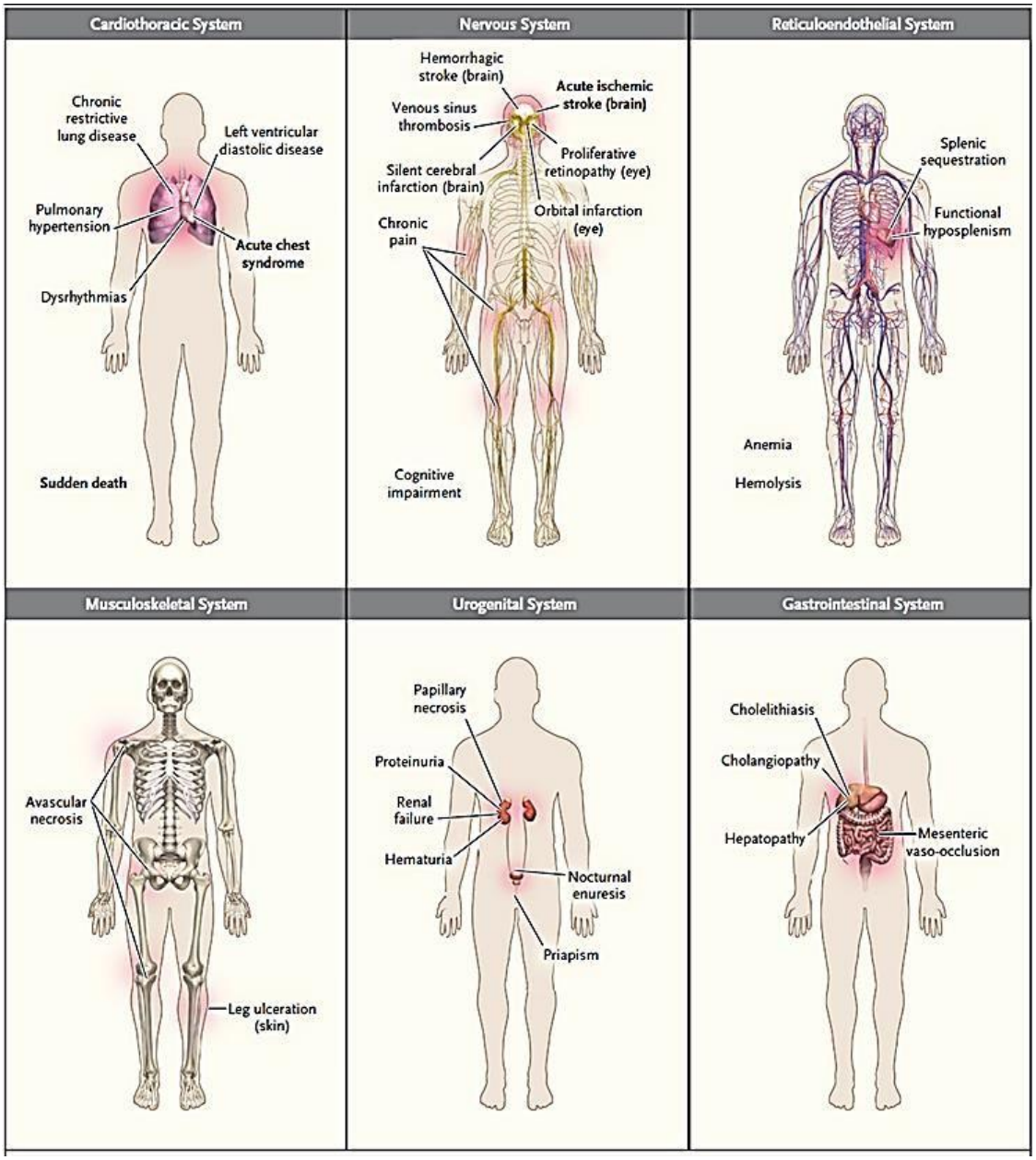



**Figure 1.9. Common complications from Sickle Cell Anaemia**.
Acute complications are shown in **bold** (adapted from Piel *et al.* 2017).

Clinical symptoms can vary in severity and frequency between individuals with the same inherited HbSS phenotype, which can make the condition extremely difficult to manage and treat, as prediction of clinical severity is often not possible, with a previous clinical history (if available) often an important predictor of likely ongoing clinical support.

Data on reported serious events from the national haemoglobinopathy registry (figure 1.10) indicates a wide range of clinical symptoms, experienced across

different age groups. The most common single clinical symptom encountered is Acute Chest Syndrome (ACS).

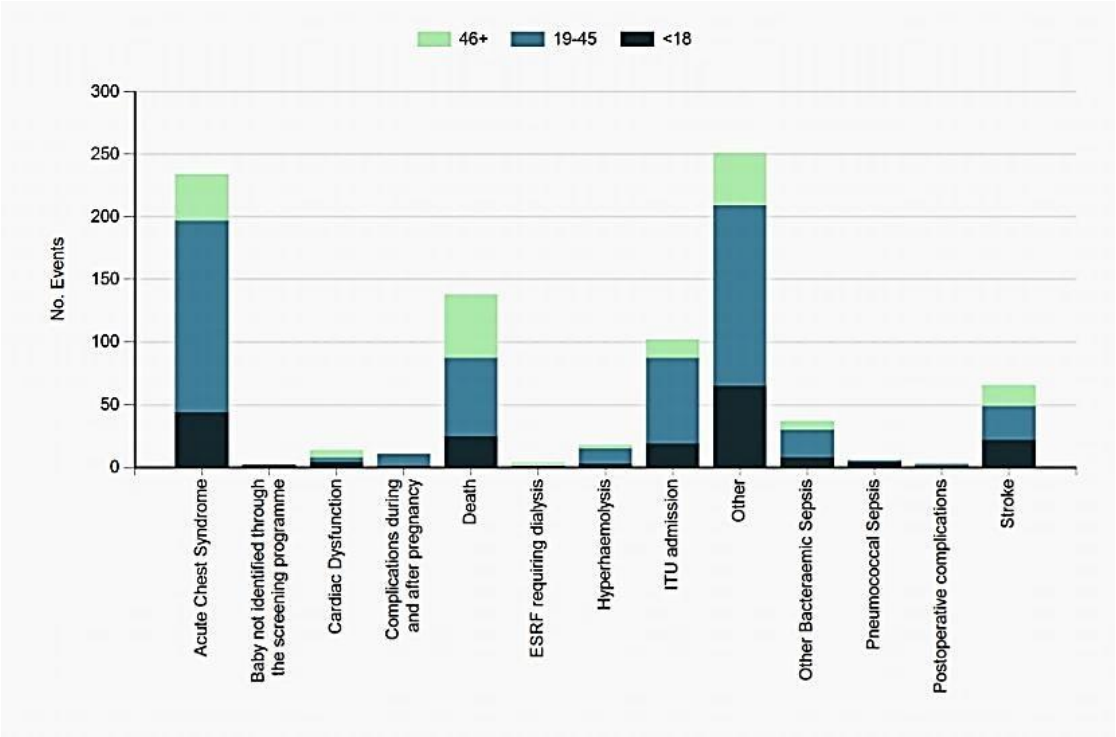



**Figure 1.10. Total reported serious events - Sickle Cell Disease** (All Years by age group - Figure from National Haemoglobinopathy Registry annual report 2016/17.

ACS is an acute illness characterized by fever and/or respiratory symptoms, accompanied by a pulmonary infiltrate on chest X-ray (Charache *et al.* 1979; Ballas *et al.* 2010).

ACS is a leading cause of morbidity and mortality in SCD patients (Howard *et al.* 2015) and is the third most common cause of death reported in adults with the disease (Lucas *et al.* 2008). ACS in adults tends to be a severe illness marked by hypoxia, which further exacerbates sickling of RBC, leads to a higher requirement for transfusion and higher mortality. The process of ACS is described as acute lung injury that can progress to acute respiratory distress syndrome (ARDS) progressing, albeit infrequently, to acute multi-organ failure. Half of patients present initially with a painful vaso-occlusive crisis and then develop this complication whilst in hospital. ACS will often develop 24–72 h after the onset of severe pain and is commonly seen following pulmonary infection, fat embolism

and/or pulmonary infarction. Transfusions, either a simple top up, or exchange (manual or automated) are recommended, based upon the clinical severity of the ACS (Howard *et al.* 2015; Davis *et al.* 2017b). ACS can also develop post-operatively, if patients are not given pre-operative blood transfusion before surgery, such as abdominal surgery. The Transfusion Alternatives Preoperatively in Sickle Cell Disease (TAPS) study (Howard *et al.* 2013) found that preoperative transfusion was associated with decreased perioperative complications in patients with sickle-cell disease who underwent low-risk to medium-risk surgery.

An additional significant clinical complication includes increased risk of stroke, particularly in childhood where prior to the implementation of routine trans-cranial doppler (TCD) screening, childhood stroke occurred in 5-10% of cases (Ohene-Frempong *et al*. 1998; Balkaran *et al.* 1992; Mazzucco *et al.* 2017). The consequences of stroke include permanent motor, cognitive, and psychological damage. TCD screening measures the maximum time-averaged mean velocity (TAMV) of blood flow in the intracranial arteries, enabling the identification and treatment of children, with abnormal velocities (≥200 cm/s) who are at highest risk of developing stroke (Belisário *et al.* 2018; Adams *et al.* 1992).

Patients are also particularly susceptible to bacterial infections, due to damage to the spleen in infancy. Repeated splenic vaso-occlusion causes fibrosis and progressive atrophy of the spleen (autosplenectomy) and are generally asplenic by 5 years of age (Brousse *et al.* 2014). This leaves patients at risk of life-threatening pneumonia, sepsis and meningitis. Patients are managed with ongoing penicillin prophylaxis and immunization from an early age with routine vaccinations in accordance with the child immunization programme. Additional vaccinations are required, and include Pneumococcal polysaccharide vaccine, Hepatitis B, seasonal *Haemophilus influenzae* and meningitis if they are travelling to areas of high risk for meningitis (NICE, 2016).

## 1.5 Treatment

Treatment for SCD is limited when compared to other conditions of a similar prevalence, despite increasing knowledge of the pathophysiology of the disease. Therapies are multimodal, and require a multidisciplinary approach, with the aim to improve survival, reduce acute and chronic complications, and improve overall quality of life. Patients with SCD are advised to take proactive measures to prevent symptoms occurring or the worsening of existing symptoms. These measures include drinking plenty of fluids to avoid dehydration, wearing appropriate clothing to keep warm and avoiding sudden drops in temperature, such as swimming in cold water (NHS, 2016). Whether these measures are wholly effective in preventing the development of recurrence of symptoms is debatable. Research suggests that self-management strategies do not always transfer from one disease to another (Greenhalgh, 2009), and that the onus of prevention should not rest with the patient, and should engage the patient, family members and professionals alike, to ensure understanding of the individuals physical, psychological and socioeconomical factors, which can all contribute to the effective management of the condition.

### 1.5.1 Access to care

In the NHS there is a recognised inequality in access to consistent, good quality treatment and care for sufferers of SCD and Thalassaemia (All Party Parliamentary Group on Sickle Cell and Thalassaemia 2009). As the most common genetic condition in England, SCD is more prevalent than cystic fibrosis, yet awareness of the health, social and educational impact its symptoms and treatment have upon sufferers is not widely known. The publication of standards for the clinical care of adults with sickle cell disease in the UK (Olujohungbe *et al*. 2008; 2018) provides a suggested framework with a wide range of recommendations for treating adults with the condition, with the aim of reducing levels of morbidity and mortality and improving the experience of haemoglobinopathy patients by reducing inequities in treatment, inequality of care and providing timely access to high quality expert care. Implementation of healthcare interventions such the establishment of a national haemoglobinopathy

screening programme, national haemoglobinopathy registry, haemoglobinopathy coordinating centre networks and routine transcranial doppler screening to reduce the risk of childhood and adult stroke have improved the identification and treatment of patients dramatically, but there is still a lot of progress to be made.

### 1.5.2 Transcranial Doppler Scanning

The use of transcranial Doppler (TCD) ultrasonography identifies children with SCD who are at high risk for stroke, which is a serious complication of SCD. The risk is particularly increased during childhood due to smaller lumens in the microcirculation, with an estimated incidence of 11% of patients with homozygous SCD having a stroke by the age of 20 years (Ohene-Frempong *et al.*1998). The publication of the Stroke Prevention Trial in Sickle cell disease (STOP) trial (Adams *et al*. 1998; Lee *et al.* 2006), led to the publication, of standards for TCD screening in children in the UK (UK Forum for haemoglobin disorders, 2016). The standards recommend annual monitoring of arterial blood velocities by TCD to determine the relative risk of stroke in patients from the age of 2 years until at least the age of 16 years. With low (velocities <170cm/s) borderline (170-199cm/s) and high (≥200cm/s) risk categories defined, with subsequent recommended follow up actions, including chronic transfusion regimens in those at risk.

In response to the identification of a paucity of data on European screening practices or on adherence to the STOP TCD screening protocol (Mazzucco *et al*. 2017). A European multi-centre study was undertaken to promote the standardisation and delivery of effective screening. It found that gaining competency in stroke screening using transcranial Doppler scanning (TCD) in SCD is easier for professionals with an ultrasound imaging background. It identified the need for a quality assurance (QA) system and suggested that further work was in progress to develop an achievable and reproducible QA program (Inusa *et al.* 2020).

### 1.5.3 Treatment of pain

Episodes of pain experienced by SCD patients are managed with the aims of improving quality of life and helping patients understand the different types of pain that they are experiencing, whether it be acute or chronic “every day” pain. Management includes medical and psychological intervention, with personalised care the optimal approach. (NICE, 2012) Medication can include strong opioid painkillers, which have a number of side effects, including addiction. One survey of hospital staff showed that over half of emergency department doctors and nearly a quarter of haematologists believed that one in five of SCD patients were ‘addicted’ to analgesics (Shapiro *et al.* 1997; Elander *et al.* 2004). This may lead to an unintended consequence of the patient being treated as an addict, with effective pain relief denied inappropriately by less experienced clinicians. As pain can be hard to manage in SCD, it is important that patients and staff use all available resources including psychological coping mechanisms such as cognitive behavioural therapy in addition to conventional pharmacological treatment (Smith *et al.* 2008).

### 1.5.4 Prevention of infection

The spleen is the first organ to be injured in sickle cell disease (Brousse *et al.* 2014), with patients rapidly becoming functionally asplenic due to sequestration and infarction (figure 1.11). This leads to increased susceptibility to infection, due to the spleen’s role in immune defence. The spleen eliminates blood-borne bacteria through direct recognition and phagocytosis by red pulp macrophages, and the generation and maintenance of IgM memory B cells which produce natural IgM antibodies, allowing an efficient T-cell dependent immune response which produces high affinity antibodies. (William and Corazza, 2007) Splenic injury reduces the trapping of blood-borne bacteria, which can impair the generation or maintenance of IgM memory B cells, in addition to increasing the risk of life- threatening infections with encapsulated bacteria such as *Streptococcus pneumoniae, Nesseiria meningitidis* and *Salmonella* – all of which can cause life- threatening sepsis. Following the Prophylactic Penicillin Study

(PROPS) Group study (Gaston *et al*. 1986), children with SCD now receive ongoing penicillin prophylaxis and immunization with routine vaccinations in accordance with the child immunization programme. Additional vaccinations include Pneumococcal polysaccharide vaccine, Hepatitis B, seasonal *Haemophilus influenzae* and meningitis if they are travelling to areas of high risk for meningitis (NICE, 2016).

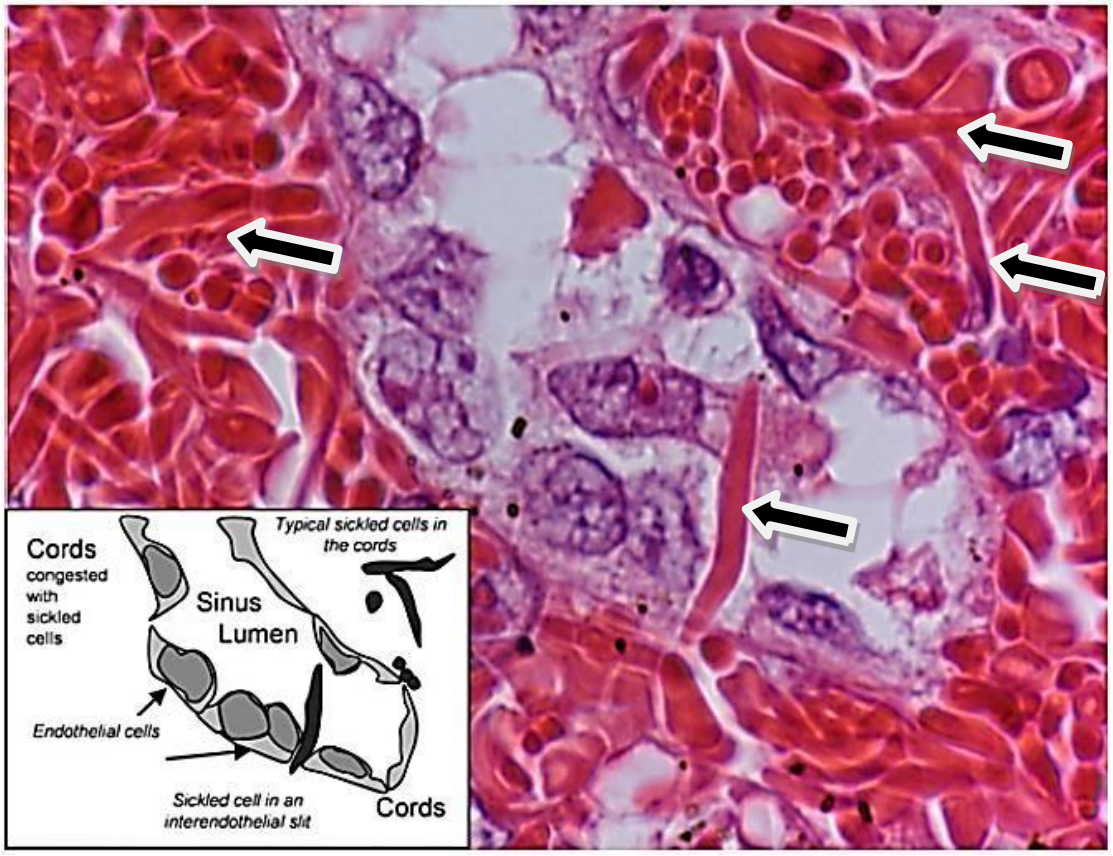



**Figure 1.11. Haematin-eosin-saffron stained spleen sample following splenectomy for acute splenic sequestration in a 5-year-old girl with sickle cell anaemia**, showing sickled red cells (arrows) retained upstream of the sinus inter endothelial slits. Original magnification 91000. (Adapted from Brousse *et al.* 2014).

### 1.5.5 Treatment of anaemia

The resulting anaemia and associated symptoms experienced by SCD patients are treated by RBC transfusion, normally initiated by monitoring of HbS% levels or as a result of symptoms experienced when a patient is admitted in acute chest crisis (ACS), a complication which cannot be predicted. Indications for RBC transfusion in SCD are either acute or elective, with elective subdivided into one-off transfusions prior to surgery (Howard *et al.* 2013) or ongoing transfusion as part of a longer-term programme. SCD patients often continue to live a normal quality of life at considerably lower Hb levels than the acceptable normal range for healthy individuals (130-170g/l males / 120-150g/l females Dacie *et al.* 1975), and as a consequence should be managed according to symptoms experienced, rather than an arbitrarily defined transfusion trigger. Historically, some indications for transfusion remain questionable, with little evidence behind the efficacy of the treatment, although the publication of recent BSH guidelines should standardise treatment and improve outcomes (Davis *et al.* 2017a, 2017b). Transfusion should be initiated on a case-by-case basis, after analysis of the associated risks and benefits, especially for unproven or controversial indications such as hyperhaemolysis syndrome (Win, 2009) and severe sepsis. SCD patients should therefore receive ABO, Rh (CcDEe) and K compatible, HbS negative units (<7days old for exchange / <10 days old for top up) as routine. In addition, blood should also be antigen negative for clinically significant antibodies that are currently or have previously been detected. (Davis *et al.* 2017a).

Emergency transfusion for acute anaemia, defined as a rapid fall in haemoglobin ≥20 g/l below the steady state value, or if the Hb <50g/l, is usually indicated for conditions such as aplastic crises, splenic or hepatic sequestration, haemodynamic compromise or when there is concern about multiple organ failure (Davis *et al*. 2017). Transfusion in SCD is performed by two transfusion approaches, simple transfusion (sometimes referred to as top up), which is the transfusion of one unit of blood at a time to correct a mild anaemia, or exchange transfusion, a whole-body volume exchange of patient RBC with donor RBC, undertaken either manually, by removing a volume of whole blood before replacing with an equal volume of RBC, or via automation using a Spectra Optia apheresis

machine as part of an automated exchange procedure The choice of transfusion method, i.e. simple (top up) or exchange, should be based on clinical judgement of individual cases, taking into account the indication for transfusion, the need to avoid hyperviscosity and minimise alloimmunisation, maintenance of iron balance, venous access issues and available resources (Davies *et al.* 2017).
Automated exchange transfusion is usually indicated for conditions such as aplastic crises, splenic or hepatic sequestration, haemodynamic compromise or when there is concern about multiple organ failure (Schwartz *et al*. 2016) and should be available in the UK at all specialist centres, with manual RBC exchange training available in addition. The target of exchange being to reduce the HbS% to <30 and increase the haematocrit (Hct) to >30% to alleviate ACS or stroke (Padmanabhan *et al.* 2019). Risks of regular transfusion include iron overload, and consideration must be given to the need for iron chelation. While rare, iron cardiomyopathy is detectable in about 2.5% of chronically transfused SCD patients (Meloni *et al*. 2014). Automated exchange transfusion is preferable to manual exchange transfusion, as there is anecdotal evidence that it reduces alloimmunisation in addition to the greater benefit of lowering the need for iron chelation (Porter & Huehns, 1987; Kim *et al*, 1994; Michot *et al.* 2015; Dedekan *et al*. 2018).

However regular exchange transfusion and top up transfusions still result in alloimmunisation in many patients. and can increase the risk of alloimmunisation to erythrocyte antigens to 20%-50%, through exposure to multiple blood donors with different red cell phenotypes due to the ethnic background of the donor population (Yazdanbakhsh *et al*. 2012). The most common alloantibodies developed in SCD patients are to those in the Rh blood group system (Rosse *et al.* 1990, Chou *et al.* 2013) as the number of RHD and RHCE gene variants are greater within patients of black African or black Afro Caribbean ancestry and therefore immunisation to donor units which may also carry variant RH genes is more common. Recent studies have shown RH gene variants in 29% of RHD and 53% of RHCE alleles in black African and Afro Caribbean patient and donor cohorts (Chou *et al*. 2018), making alloimmunisation effectively unavoidable, unless genotype matched blood is provided, but this is currently unsustainable due to the lack of ethnically matched donors (Chou *et al*. 2018). A full RBC genotype,

which is able to identify the most common Rh variants found in populations where SCD is more prevalent should also be performed. This can make compatibility testing and blood provision easier should a multi-transfused patient make multiple alloantibodies. However, many of these tests are currently performed in batches due to the cost of the reagents and therefore are subject to long turn-around times which render them ineffective in an urgent scenario where genotype matched blood is required imminently.

### 1.5.6 Hydroxycarbamide (Hydroxyurea)

Pharmacological treatment with hydroxycarbamide (also known as hydroxyurea), is the only drug that has been shown to modify the symptoms of Sickle Cell Anaemia. Hydroxyurea was first licenced for use by the United States Food and Drug Administration (FDA) in 1967 (McGann & Ware, 2015), and is currently the only medication licensed in the UK for the prevention of recurrent painful crisis in patients with SCD (Qureshi *et al.* 2018). No alternative pharmacological therapy has been introduced since that time. A large collaborative placebo controlled, randomised Multicentre Study of Hydroxyurea (MSH study) was undertaken by Charache *et al.*, in 1995. It comprised 299 adult patients, and found lower annual rates of pain crises, longer time between episodes of crisis, in addition to a lower overall incidence of ACS, a reduced need for blood transfusion and increased total Hb and haemoglobin F levels. The study was so beneficial that it was terminated early and all patient cohorts were given hydroxycarbamide. The Baby HUG study, performed in children, found that hydroxyurea was associated with statistically significantly lower rates of initial and recurrent episodes of pain, dactylitis, acute chest syndrome, and hospitalization. (Thornburg *et al.* 2012)

The exact mechanism of action of hydroxycarbamide is unknown, and responses to therapy are variable. The most important effect appears to be blocking of the ribonucleotide reductase system resulting in inhibition of DNA synthesis (eMC, 2018). This inhibition raises the amount of foetal haemoglobin in red blood cells (Maier-Redelsperger *et al*. 1998; Kinney *et al*. 1999; Zimmerman *et al.* 2004) and preserves erythrocyte shape through reducing the mutated, polymerised form of β-

globin found in HbSS, in addition to reducing intracellular adhesion through integrin downregulation and increasing vasodilation by increasing Nitric Oxide (NO) levels, thereby reducing sickling. The dose is usually 15mg/kg/day (adults) or 20mg/kg/day (children) with a therapeutic target of an increase on HbF, in addition to maintaining WBC and RBC indices. (Charache *et al*.,1995). Current evidence shows that hydroxycarbamide is effective at decreasing the frequency of acute painful episodes and raising fetal haemoglobin levels in the blood of people with SCD (Qureshi *et al.* 2018; Sheehan *et al.* 2014). Hydroxycarbamide is also effective at preventing strokes in patients who are at an increased risk of stroke (Kratovil *et al.* 2006). Side effects include bone marrow suppression, with leucopenia as the first and most commonly occurring sign, in addition to painful leg ulcers which are usually difficult to treat and require cessation of therapy. Other risks include teratogenicity and reduced spermatogenesis, which means some SCD patients, for example pregnant women, are not able to take it, exposing them to a higher level of risk. As a consequence, strict adherence to the hydroxycarbamide regimen remains an ongoing challenge (Candrilli *et al.* 2011; Badawy *et al*. 2017).

### 1.5.7 L-Glutamine

More recently, phase III trials of the amino acid L-Glutamine have shown promising results (Niihara *et al.* 2018). L-Glutamine plays a physiological role in the production of Nicotinamide Adenine Dinucleotide (NAD). NAD is a co-factor involved in redox reactions in the erythrocyte, enabling oxidation and ATP production as part of the Embden-Meyerhof pathway (Brown, 1996). The uptake of L-glutamine by HbS erythrocytes is greater than that of normal red cells as they seek to increase the total intracellular NAD level due to increased oxidative stress and cellular damage (Niihara *et al.* 1997). By reducing oxidative stress through administration of L-Glutamine, the red cells of SCD patients treated for 4 weeks showed reduced endothelial adherence when compared to an untreated SCD control (Niihara *et al.* 2005 & 2018). Updated guidance now states that L-glutamine can be considered in patients aged 5 years and older who are intolerant to hydroxycarbamide or who continue to have painful events while on hydroxycarbamide. (BMJ best practice, 2020).

### 1.5.8 Haemopoietic Stem Cell Transplants

Currently, curative hematopoietic stem-cell transplantation (HSCT) is only available to a limited cohort of patients, with the NHS funding HSCT only in those up to 19 years of age with severe SCD and a suitable sibling donor. Even then, its use is restricted by the high cost, toxicity, and limited availability of suitable donors (Hsieh & Fitzhugh *et al.* 2011). Indications for HSCT in adults have historically been limited, due to the increased risk of mortality from aggressive conditioning regimens. Most published studies on adults with SCD report positive outcomes of HSCT, but they are studies involving small numbers of patients, who have been transplanted with different types of conditioning regimens and most frequently from Human Leukocyte Antigen (HLA) identical siblings. HLA matched sibling donor (MSD) transplants offer the best outcomes for SCD, but less than 14% of patients with SCD have a sibling that is HLA matched (Shenoy, 2013; Gluckman *et al.* 2017). Other sources of HSCT include matched unrelated donors (MUD) or mismatched unrelated donor (mMUD), an umbilical cord blood (UCB) product or a haplo-identical (half-matched) donor from the family. Each of these transplant approaches are associated with complications innate to the graft source and transplant approach used and include Graft Versus Host Disease (GVHD) and delayed immune reconstitution, each of which can result in unacceptable outcomes. However, emerging therapies such as gene therapy may offer a solution to these obstacles.

### 1.5.9 Gene therapy

Gene therapy treatment has shown early promise and is in the very early stages of clinical trials. In a recent study by Ribeil *et al.* (2017), the researchers treated a patient with lentiviral vector–mediated addition of an anti-sickling β-globin gene into autologous hematopoietic stem cells. This showed encouraging results, with 15-month post-transplant outcomes suggesting engraftment of transduced stem cells that were capable of long-term repopulation. The patient experienced no recurrence of sickle crises and had correction of the biological hallmarks of the disease. Further clinical trials are underway, evaluating the efficacy and safety of LentiGlobin drug product in Beta-Thalassemia Major and SCD, and looking at the long-term follow -up of subjects with haemoglobinopathies treated with *ex-vivo* gene therapy (NCT02151526 & NCT02633943 respectively). The cost is currently prohibitive for routine treatment, with estimated costs in the region of £380,000-£540,000. Full calculations of a cost benefit ratio, particularly for using the therapy in young patients, are yet to be done, and the benefits of gene therapy may outweigh the long-term costs of treatment and care. In addition, the consequences of genome editing, including the effects of the semi- random nature of lentivirus integration leading to off-target mutations, and the sustainability of any therapeutic effects all await additional long-term study (Steinberg, 2020).

### 1.5.10 Inhibitors of Haemoglobin S polymerisation

One emerging therapy, Voxelotor, is designed to specifically target the sickle haemoglobin (HbS) molecule. Voxelotor is an HbS polymerisation inhibitor that reversibly binds to haemoglobin to stabilise the molecule in its oxygenated state, therefore inhibiting HbS polymerisation and subsequent sickling of the erythrocyte (Vichinsky *et al.* 2019). A phase III double-blind trial of Voxelotor (HOPE trial) found that it significantly increased haemoglobin levels and reduced markers of haemolysis. Concerns remain regarding the increase in haemoglobin causing an increased risk of vaso-occlusive events. Additionally, the increase in blood flow to the brain of young patients on Voxelotor may result in an increased chance of white matter injury, where current treatment with hydroxyurea has shown some neuroprotection against white matter ischaemia. (Fields *et al.* 2019).

### 1.5.11 Inhibitors of cellular and vascular interaction

The complex interaction between physiological processes and abnormal expression of cellular adhesion molecules in SCD has been previously described (Zennadi & De Castro *et al.* 2008; Koehl & Nivoit *et al.* 2017; Cartron & Elion 2008; Canalli & Proença *et al.* 2011; Conran & Costa, 2009; Manwani & Frenette, 2013). An increased propensity for interaction between cellular surface molecules and activation of vascular endothelium through inflammatory processes are thought to be the underlying cause for VOC, in addition to anaemia-induced reticulocytosis and inefficient maturation to erythrocytes in SCD. A number of adhesion molecules are present on reticulocytes but are lost or reduced on maturation to erythrocytes, and this is a common theme throughout the research literature. Cell surface proteins thought to be responsible for HbS erythrocyte / vascular endothelium interaction and adhesion include Lu/BCAM (B Cell Adhesion Molecule), P-selectin, E-selectin, CD44 and ICAM-4 (Inter-Cellular Adhesion Molecule) among others.

The number of cellular and vascular endothelial interactions however are complex, (figure 1.12) and the exact cause of a hypercoagulable state and of the increased tendency of thrombosis and VOC in SCD has not been fully elucidated, suggesting a number of biological interactions are responsible for the vaso-occlusive pathophysiology. Ongoing research aims to identify and affect the interaction(s) of most significance in the pathophysiology of VOC.

Novel, anti-adhesive therapies are therefore developed with the aims of either blocking or reducing these interactions between HbS erythrocytes and the vascular endothelium. Of particular interest are the selectins, adhesion molecules which are present on RBCs and on the vascular endothelial surfaces. Increased expression of selectins (P-selectin and E-selectin) are associated with severity of SCD and are known to contribute to vaso-occlusion (Johnson and Telen, 2008). Novel therapies include panselectin inhibitors, such as Rivipansel (RESET trial; NCT02187003), which did not meet its primary or secondary endpoints and was unfortunately not effective (Clinicaltrials.gov, 2020) or specific selectin inhibitors, such as Crizanlizumab, which was trialled in the SUSTAIN study (Ataga *et al*.

2017).

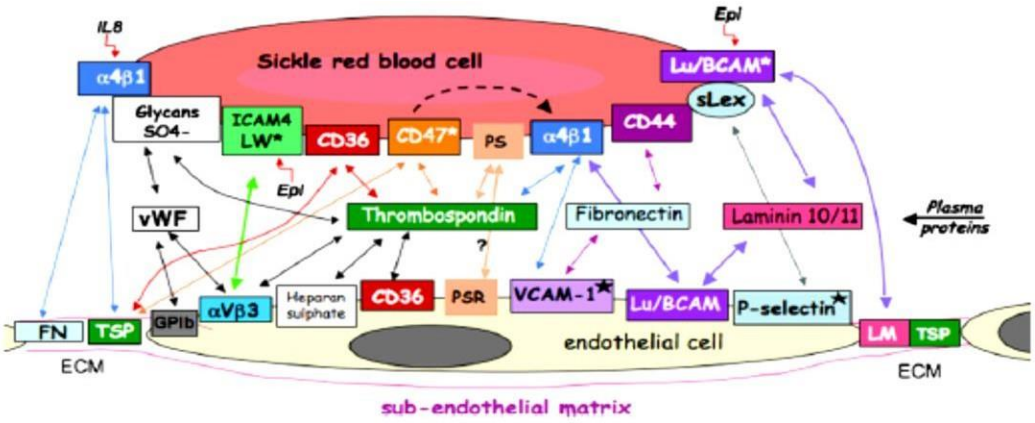



**Figure 1.12 Interactions between Sickle Red Blood Cells and the sub-endothelial surface.** (Adapted from Cartron & Elion, 2008)

In the SUSTAIN study, Crizanlizumab, a monoclonal antibody directed toward P-selectin, was found to block cell to cell interaction (figure 1.13). It was therefore proposed that it may reduce the risk or incidence of sickle cell pain crises. P-selectin is found in endothelial cells and platelets and is transported to the cell membrane on activation of the cell during processes such as inflammation. Studies in P-selectin-deficient transgenic mice with sickle cell disease have shown that they have defective leucocyte recruitment to the vessel wall and are protected from vaso-occlusion (Turhan *et al.* 2002). It is thought that increased expression of P-selectin in endothelial cells and platelets contributes to the pathogenesis of SCD. In the SUSTAIN study, high-dose Crizanlizumab therapy significantly reduced the frequency of sickle cell-related pain crises when compared with the placebo cohort. When compared with patients who were on hydroxyurea, where the annual rate of crisis was 32.1%, sickle cell-related crisis events were 50% lower with high-dose Crizanlizumab compared to those patients not taking any hydroxyurea therapy (Ataga *et al.* 2016; Ataga *et al.* 2017).
However, there was no reduction in haemolysis, and the Crizanlizumab did not prevent all episodes of vasoocclusive crises, indicating that P-selectin is not solely responsible for episodes of vasoocclusive crises and haemolysis, and that these physiological processes may involve the interaction of other cell surface proteins and microparticles.

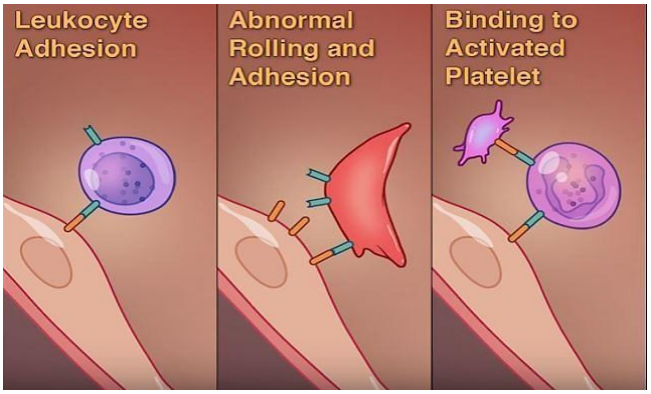



**Figure 1.13 P-Selectin (in orange) interactions in vascular endothelial adhesion.** (Adapted from Ataga & Kutlar *et al*. 2016).

## 1.6 Phosphatidylserine (PS) and thrombosis

One of the key cell surface proteins known to be contributory to VOC and a potentiator of thrombosis in SCD is Phosphatidylserine (PS) (Setty *et al.* 2002; Tait & Gibson,1994; Kuypers *et al*. 1996). PS is located on the inner leaflet of the membrane bilayer and it plays a role in thrombosis via enhanced interactions with receptors on endothelial cells and platelets, thereby contributing to micro-vascular occlusion (Lentz, 2003). A deficiency in PS, known as Scott syndrome, is associated with increased bleeding (Zwaal *et al.* 2004). In Scott syndrome, the mechanism for translocating PS to the platelet membrane is defective, resulting in impaired thrombin generation (Toti *et al.* 1996).

There are two main mechanisms by which PS is transported in the erythrocyte. Both are aided by the actions of two translocase enzymes, Flippase and Scramblase. Flippase-mediated transport of PS is an ATP-driven process, which maintains PS expression in the inner membrane leaflet, whilst the calcium ion ($Ca^{2+}$) dependent scramblase moves PS quickly in a bidirectional process which causes disruption of phospholipid asymmetry (Williamson *et al.* 1992; Weiss *et al.* 2010). In normal RBCs, PS is largely confined to inner leaflet, through the dominant action of the flippase whilst the scramblase remains quiescent.

In SCD, repeated deoxygenation and reoxygenation of the erythrocyte results in damage and dehydration, which causes $Ca^{2+}$ influx which results in abnormally increased exposure of PS on the outer RBC membrane leaflet through inhibition of

ATP-driven flippase and activation of scramblase (figure 1.14) (Wagner *et al.* 1985; Lubin *et al.* 1981; Dekkers *et al.* 1998; Yasin *et al.* 2003). Additionally, PS exposure and generation of procoagulant erythrocyte microvesicles can be caused by alternative mechanisms such as protein kinase C (PKC) activation (De Jong *et al.* 2002).

The increased PS exposure on SCD erythrocytes contributes to a shortened erythrocyte life span due to increased complement recognition and cell destruction via eryptosis (Wang *et al.* 1993; Lim *et al.* 2013; Bogdanova and Lutz, 2013). PS is well documented as present in greater numbers in circulating RBCs in splenectomised SCD patients (Kuypers *et al*. 1996; Tait & Gibson,1994; Setty *et al.* 2002), and subpopulations of PS positive erythrocytes have been implicated as the cause of stroke in SCD patients and they are thought to be one of the major contributors to VOC (Styles *et al.* 1997).

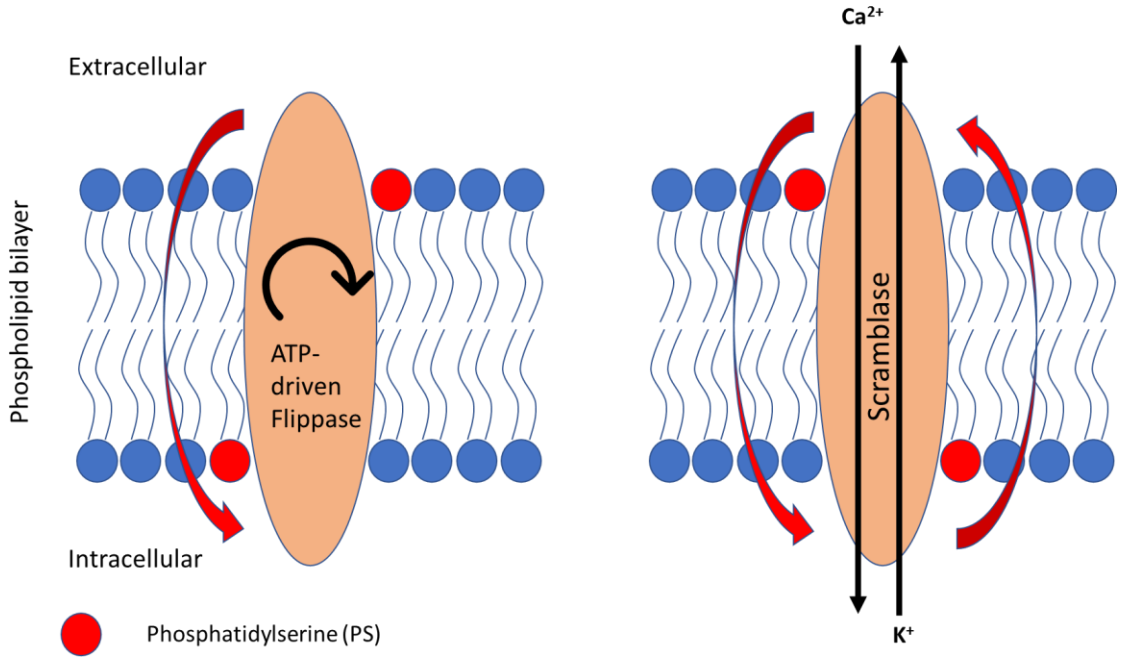



**Figure 1.14 Phosphatidylserine transport in the SCD erythrocyte**

Actions of the ATP-dependent Flippase enzyme and subsequent inhibition of ATP by $Ca^{2+}$ influx, causing translocation of PS to the external erythrocyte surface by scramblase. This externalisation of PS results in haemolysis by eryptosis and is also considered to be a contributory factor to VOC in SCD.

An early study by Wood *et al.* in 1996 examined the association of PS in patients with SCD. They measured exposed levels of PS on the outer surface of the erythrocyte membrane based on addition of fluorescein-labelled Annexin V to whole-blood specimens and subsequent flow cytometric (FC) analysis. The physiological role of annexins within the body is not completely understood. Annexins are mainly cytosolic but can also be found as extracellular proteins. They are involved in a wide range of intra- and extracellular biological processes, most of them directly related with the ability to bind to phospholipid bilayers: membrane trafficking, membrane-cytoskeleton anchorage, ion channel activity and regulation, as well as anti-inflammatory and anticoagulant activities. (Lizarbe *et al.* 2013). Annexin is able to bind in the presence of Calcium ions ($Ca^{2+}$) to phosphatidylserine to form a membrane-bound two-dimensional crystal lattice. (van Genderen *et al.* 2008). The PS affinity is such that labelled annexin molecules are used in both the lab and clinically for PS identification. *In vivo,* annexin's anticoagulant role is dependent on its ability to block the adhesion of clotting factors V and VIII to PS displayed on endothelial surfaces (Thiagarajan *et al.* 1991; Ahmad *et al.* 2000). It is the binding capacity of labelled annexins and their anti-inflammatory and anticoagulant activities that have made analysis of PS and annexin targeted therapy a focus of study among researchers of SCD.

Wood *et al.* (1996) looked at 17 SCD patients and 2 β-Thalassaemia patients and found that when compared to normal controls, levels of PS were elevated in 96% of patients studied. The percentage of annexin-positive cells always decreased after transfusion and levels of PS were higher in those patients who had recently been in SCD crisis. The observed decrease could not have arisen through the dilutional effect of transfusion alone and it was suggested that it may be due in part to suppression of erythropoiesis and to faster clearance of abnormal erythrocytes. Wood *et al.* (1996) concluded that because of the thrombogenic nature of exposed PS, and the dependence of coagulation reactions on the percentage of PS in the membrane, small numbers of HbS erythrocytes could have a substantial thrombogenic effect, especially if they are concentrated at a site of vascular occlusion.

In experiments to characterise the subpopulations of PS-exposed sickle RBCs, De Jong *et al.* (2001) described populations of PS-exposed cells that are present in both high and the very low-density fractions of sickle RBCs, in the very young, transferrin receptor–exposing reticulocytes and also in more mature RBCs. These findings correlate with PS exposure at the reticulocyte cell maturation stage, as described by Mankelow *et al.* (2015) and the effect of cell membrane damage on older, mature erythrocytes due to calcium ion influx caused by repeated sickling *in vivo*, leading to induction of membrane phospholipid scrambling, as described (Williamson *et al.* 1992; Weiss *et al.* 2010).

One later paper by Kennedy (2015) hypothesises the use of annexin V to terminate ongoing sickle cell crises. Kennedy describes how approximately 1/3 of sickle erythrocytes are prematurely senescent, causing them to display PS molecules on their surface and that the adhesive and procoagulant character of PS is responsible for a haemolysis and VOC in SCD. Kennedy proposed that annexin V dosing may aid biological levels of annexin V in the blocking of endothelial PS receptor, thrombospondin, therefore reducing the tendency towards coagulation. However, examples given by Kennedy *et al.* were in animal models only, and currently in-human trials have not been performed to date.

A more recent, in-human study by Whelihan *et al*. (2016) showed RBC PS levels were significantly elevated in SCD patients when compared with controls, and that there was a correlation between increased RBC PS exposure when compared to reticulocyte count. The authors state that this could be due to localisation of phosphatidylserine to a few discrete areas of the membrane in normal reticulocytes, as well as on red cells from SCD patients. The localised patches of PS shown in Whelihan *et al.* (2016) are very similar to that shown as the site of AV extraction in SCD and splenectomised individuals as described by Mankelow *et al.* (2015). Whelihan *et al.* proposed that the large population of PS+ RBCs and reticulocytes with a significantly shortened half-life accelerates protein S clearance from the circulation and results in the low levels of protein S and subsequent pro-thrombotic state seen in SCD, a finding previously described in a study by Lane *et al.* (1994).

Other studies in this area which examine cell surface adhesion in the study design have demonstrated that PS is a major determinant of RBC–endothelial adhesion when compared to other cell surface adhesion molecules, such as erythrocyte adhesion receptor, CD36. (Setty *et al.* 2002). Further investigations into the mechanisms by which PS positive erythrocytes interact with the vessel wall need to be performed, and the full implications of erythrocyte PS exposure in relation to the vascular pathology of SCD require further research. (Setty et al. 2002). Better predictors of the severity of SCD could lead to more precise treatment and management (Piel et al 2016). Correlation between levels of circulating PS positive microvesicles and sickle cell crises could be a possible method of predicting severity and frequency of crises. When combined with apheresis methodologies that can remove the causative cells or cellular microparticles of interest, this could offer a novel treatment for patients with SCD who are at increased risk of VOC.

### 1.7 RBC Microparticles and Autophagic vesicles - Microparticles, Microvesicles, Autophagic Vesicles, Exosomes, Ectosomes & Apoptotic Blebs.

There are varying accounts in the literature as to what constitutes a microparticle (MP), and the nomenclature surrounding the topic is confusing. The stated size range of MPs varies from 100–3000nm and does not exclude other small particulates. At the top end of the scale, particulate matter could include apoptotic blebs from end-stage cells (500– 3000nm), and at the bottom end are exosomes (70–120nm), immune complexes, lipoprotein particles and viral particles. In the literature, the term ‘extracellular vesicles’ can include exosomes, ectosomes and apoptotic blebs, despite their distinct generative origins and detection methodologies (Pol *et al*, 2016). Red cell microparticles are generally smaller in size than those described for other cell types above. They are approximately 150nm in diameter and are accompanied by a smaller type of vesicle of approximately 60nm, termed nanovesicles (Allan *et al.* 1980; Piccin *et al*. 2007). All cells release particulate matter as they mature or degrade as part of cell development or apoptosis. When referring to microparticle release in blood, microparticles are released by RBCs, platelets, white cells and vascular

endothelial cells, and all may contribute to the complex processes involved in SCD crises. The focus of this review will be on RBC-derived Microparticles (RCDP), Autophagic Vesicles (AVs), and their potential effect on SCD crises.

### 1.8 Red Cell Derived Microparticles and red cell storage lesion

Red blood cell microparticle (RCDP) formation occurs throughout normal erythrocyte maturation, and the phenomena *in vivo* was first described by Dumaswala and Greenwalt in 1984. During early maturation, RBCs lose approximately 20% of their volume, increasing intraerythrocytic haemoglobin (Hb) concentration by approximately 14% (Said and Doctor, 2017) through the release of RCDP into the circulation. Once mature, and under normal circumstances, while in the circulation, RBCs accumulate damage as they move throughout the vasculature (Crosby and Benjamin, 1957). In the final stage of the erythrocyte lifecycle, after approximately 120 days, senescent RBCs are removed from the circulation. This gradual RBC maturation and final senescence is associated with a decrease in deformability, with the eventual removal of the aged RBC from the circulation by splenic macrophages through the process of erythrophagocytosis (Kaestner and Bogdanova, 2014).

The effects of RBC vesiculation include loss of haemoglobin and RBC surface area, and as a result the RBC becomes smaller and denser (Allan et al, 1982; Piomelli and Seaman, 1993; Willekens *et al.* 2003). Vesiculation and the mechanism of selective removal of both the released RCDP and the mature, senescent RBC by splenic macrophages, located in the red pulp of the spleen, explains the efficient removal of damaged content while leaving the RBC intact but reduced in size. However, the exact molecular mechanism by which macrophages in the spleen facilitate specific RCDP removal is still unknown.

The deterioration of the RBC as it ages is a gradual process, and there is no accumulation of signalling molecules which leads to the removal of senescent RBCs. In fact, these RBC surface signalling molecules appear as a rapid and non-linear cascade of events at the terminal stage of the aging process shortly before RBC removal (Franco *et al.* 2009; Mebius and Kraal, 2013). To initiate this,

changes in the conformation of the following membrane-bound proteins, Anion Exchanger-1 (AE-1), PS, CD47 and Complement Receptor 1 (CR1) lead to the appearance of a senescent cell-specific antigen phenotype which is recognized by specific, naturally occurring autologous immunoglobulin G (IgG) antibody. This marks senescent erythrocytes for removal by macrophages, such as Kupffer cells in the liver and spleen (Kay *et al.* 2005). See figure 1.15.

AE-1 (also referred to as Band 3) is a transmembrane protein that is ubiquitously found throughout the RBC membrane. It has been found to be a major target of IgG antibodies and is implicated in macrophage-mediated clearance of senescent and damaged RBC. It is thought that oxidative damage to haemoglobin occurs during the RBCs lifecycle. This results in the formation of hemichromes which bind to AE-1, and in time lead to AE-1 clustering which results in the IgG antibody-mediated clearance of the RBC (Pantaleo *et al.* 2008; Arashiki *et al.* 2013).

As previously described, the loss of phospholipid asymmetry and exposure of PS on the RBC surface in apoptotic cells may be a general trigger for RBC removal. Phosphatidylserine (PS) exposed on the RBC membrane can directly bind Stabilin-2, Tim-1, Tim-4, or CD300 on the macrophages and is presumed to give a pro-phagocytic signal. Throughout their lifecycle, red blood cells spontaneously shed PS-positive MVs. It is estimated that each RBC generates approximately 230 vesicles during its lifespan (Bosch *et al*. 1994). It is estimated that if there was a total erythrocyte mass of 25 x $10^{12}$ in a healthy male, this would produce 580 x $10^{6}$ vesicles per second. These would have to be removed at the same time, assuming homeostasis. In their study, the total amount of erythrocyte-derived vesicles measured in the blood was 850 x $10^{6}$, indicating that it takes only 0.7secs to clear half of all the vesicles from the circulation (Willekins *et al.* 2008). Vesicles have been observed in erythrocytes of all ages, and have been shown to accumulate after splenectomy (Reinhart and Chien, 1988)

CD47 is a signal regulatory protein alpha (SIRP-α) acts as a strong “don’t eat me” signal for phagocytosis and functions as a marker of “self” on RBC. Reduced expression of CD47 may contribute to the uptake of these RBC by macrophages. Some studies suggest that there is evidence that points in this direction, with

murine models showing a >20% reduction in CD47 expression in older RBC (Fossati-Jimack *et al.* 2002; Burger *et al.* 2012).

Complement Receptor 1 (CR1) on human RBC binds opsonised complement particles bearing C3b/C4b in the circulation. A recent study has shown that signal transduction downstream of CR1 following binding of complement resulted in alterations in RBC membrane deformability and CR1 clustering on the surface of the RBC. This enhanced binding of more complement and more importantly, it leads to ATP secretion, which has a direct stimulatory effect on particle uptake and removal by phagocytes (Melhorn *et al.* 2013)

A study by Willekens *et al.* (2008) concluded that vesiculation provides a mechanism for the removal of erythrocyte membrane patches associated with removal molecules, thereby delaying the premature elimination of otherwise healthy erythrocytes. Research continues into which stages of erythrocyte production and maturation the most vesiculation occurs, with some studies concluding that vesiculation peaks in the latter third of erythrocyte life cycle and some studies finding increased vesicle release during the later stages of maturation of the reticulocyte into the erythrocyte within the circulation (Willekens *et al.* 2008).

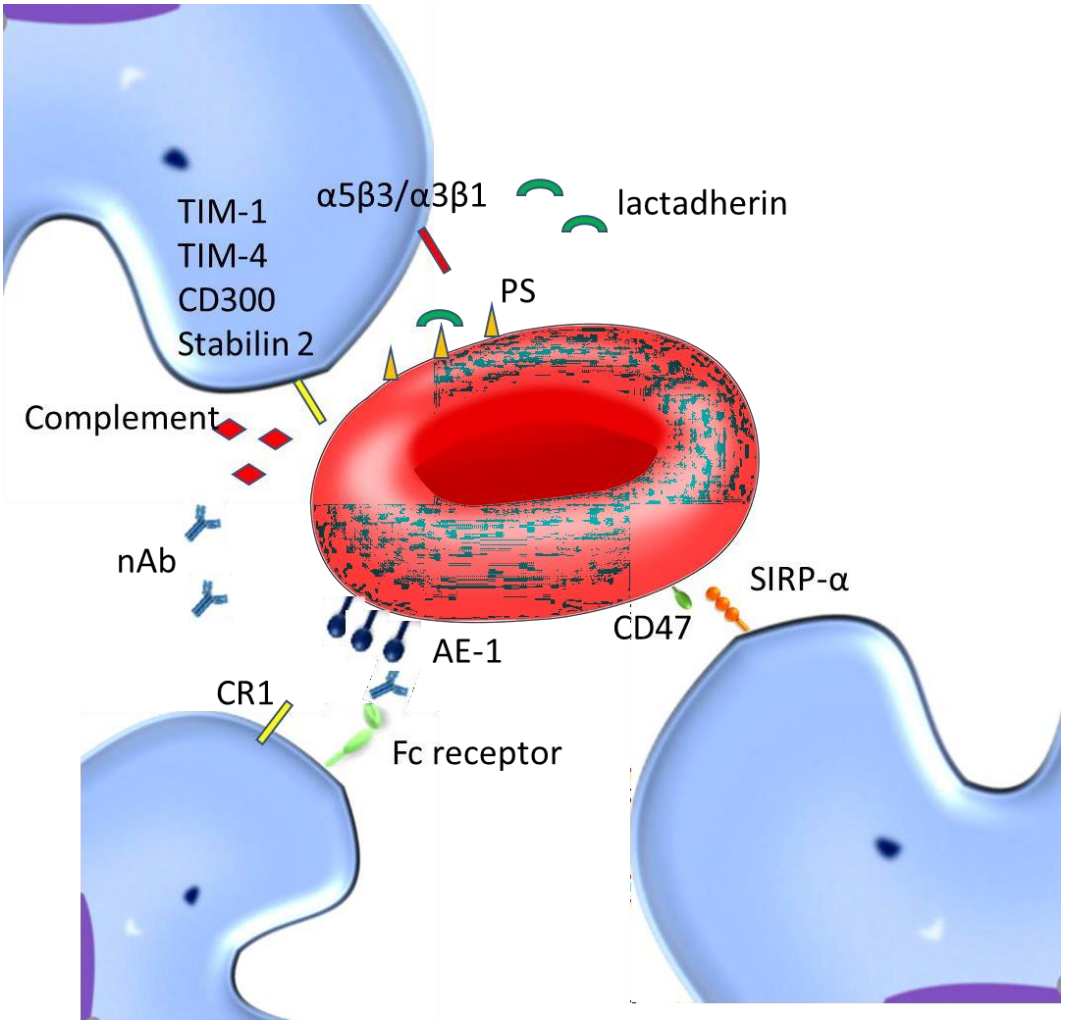



**Figure 1.15 Signals involved in interaction between macrophages and red blood cells (RBC) regulating clearance**. RBCs and macrophages interact with each other through ligand–ligand interactions. Phosphatidylserine (PS) exposed on the RBC membrane binds to macrophage receptors Stabilin-2, Tim-1, Tim-4, or CD300 on the macrophages to stimulate phagocytosis. AE-1 clusters form and promote opsonization with naturally occurring IgG antibodies (nAb) and complement on the RBC. This enables binding to the macrophage *via* Fc receptors and CR-1 and facilitates phagocytosis. As a counterbalance, CD47–SIRPα interactions inhibit phagocytosis of RBCs by the macrophage (adapted from Klei *et al.* 2017).

RBC microparticle formation is increased during storage of red cells for transfusion in a process termed storage lesion (Kim-Shapiro *et al.* 2011). Storage lesion can be further defined as a variety of changes identified within the RBC and storage media during RBC preservation that are correlated with reduced tissue oxygenation and the potential concern of transfusion-associated adverse effects (Tzounakas *et al.* 2017; Steiner *et al.* 2015; Lacroix *et al.* 2011).The effects of storage lesion include the loss of Adenosine triphosphate (ATP), 2,3-diphosphoglycerate acid (2,3-DPG), Hb and cell viability, in addition to

biomechanical changes to the erythrocyte, for example, the modification of the RBC shape. Decreased ATP deregulates cation homeostasis and disorders membrane symmetry through inhibition of flippase activity and activation of scramblase, exposing the phospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) which, as described earlier are normally in the inner membrane bilayer. This combination of accumulated damage and expression of leads to microparticle formation (Yoshida *et al.* 2019).

Over time, stored RBCs become more rigid, which limits their ability to pass through capillaries. This reduces tissue oxygenation efficiency (Hovav *et al.* 1999; Blasi *et al.* 2012). The normal discoid RBC changes shape, and becomes a spherical cell, and in doing so, passes through intermediate echinocyte and sphero-echinocyte shapes. (Delobel *et al.* 2016). During this shape change, RBCs release increasing amounts of Microvesicles (MVs) (figure 1.16). Numerous studies have examined red cell storage lesion, and there have been a number of large Randomised Controlled Trials (RCT) in various clinical settings which have compared fresh (6-12 days on average) to standard-issue or moderately aged RBC units (~3 weeks on average) which found no differences in mortality or morbidity, indicating that there is no inferiority in transfusing RBCs using standard practice (oldest units available) when compared to transfusing fresher RBCs (freshest available) (Lacroix *et al.* 2011; Garraud, 2017; Chai-Adisaksopha *et al.* 2017).

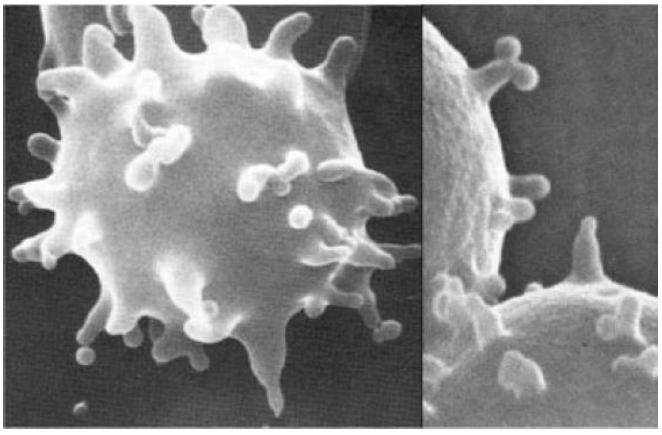


**Figure 1.16 Echinocytosis and Microvesiculation in Erythrocytes**

Left image: Stored blood first undergoes echinocytosis. It then begins to fragment by budding and microvesiculation from the surface of the RBC. (Right image) Buds and microspherules seen with a scanning electron microscope. (From Greenwalt, 2006)

## 1.9 Microparticles and Disease

The ability for various cell types to form microparticles is integral to physiological coagulation pathways. Aminophospholipids, such as PS and PE on the surface of platelet and endothelial cell microparticles provide binding sites for factors IXa, VIII, Va and IIa in addition to tissue factor (del Conde *et al.* 2005). Microparticles have long been considered as cell fragments or debris without any biological function. Although their true biological function is still unknown, there are more and more indirect evidence that MPs are involved in a broad spectrum of biological activities. (Rubin *et al.* 2008) An increase in MP formation is observed under conditions of stress and injury and so MPs have been investigated as potential disease biomarkers. MPs have been the focus of research due to their potential involvement in inflammatory and autoimmune diseases, as well as in cardiovascular disorders and diabetes (Leroyer *et al.* 2008a, 2008b). For example, circulating monocyte, platelet and endothelial cell-derived microparticles are found in septic patients (Raeven *et al.* 2018) with one study concluding that the presence of microparticles may predict a more favourable outcome in severe sepsis (Soriano *et al.* 2005). The extent to which they contribute to the disease process in other conditions is not fully known, although numerous papers describe their contribution to disease pathophysiology.

Thrombotic thrombocytopenic purpura (TTP) is a microangiopathic haemolytic anaemia (MAHA) characterised by failure to cleave very large multimers of von Willebrand's Factor (vWf) in the circulation. TTP causes extensive clots (microscopic thrombi) to form in small blood vessels throughout the body and if not treated quickly can be fatal. Normally, these large multimers of vWF are cleaved by the enzyme ADAMTS13, however in TTP ADAMTS13 stops working because of autoimmune anti-ADAMTS13 production (Tsai & Lian, 1998). Increased levels of platelet microparticles circulate during the acute and chronic phases of TTP and express the proteolytic enzyme calpain on their surface. Expression of calpain is associated with the early generation of microparticles which contain functional adhesive receptors with procoagulant activity on their surface. The activation of calpain leads to degradation of actin filaments and breaking of bonds between the cytoskeleton filaments and the phospholipids. This weakening of protein fibrils in

the cytoskeleton initiates the budding and shedding of MVs (Dekkers *et al.*1998; Kalra *et al.* 2016). These MVs function to disseminate procoagulant activity and stabilize the formation of platelet clots, contributing to the formation of thrombi in the microcirculation in TTP (Kelton *et al.* 1992).

Microparticles are increased in the circulation of patients with paroxysmal nocturnal haemoglobinuria (PNH), and are derived from platelets, monocytes and endothelial cells (Hugel *et al*.1999). PNH is an acquired disorder of the blood characterised by intravascular haemolysis and thrombophilia due to the absence of glycosylphosphatidylinositol-anchored proteins (CD55 and CD59) on the membrane surface of blood cells (Rosse, 1997). These microparticle subpopulations have been shown to carry prothrombotic activity, offering an explanation for the increased incidence of thrombosis in PNH. In addition, microparticles caused by complement activation (C5b-9) have high expression of binding sites for factors Va and VIIIa, further contributing to the prothrombotic state.

RCDPs have been more specifically associated with disease states including hereditary spherocytosis (HS) and SCD (Piccin *et al*. 2007; van Beers *et al.* 2009). Rubin *et al.* (2013) describe how RCDPs increased thrombin generation, even in the absence of tissue factor, suggesting that tissue factor may be expressed on the RCDP surface, contributing to their pro-coagulant effect (del Conde *et al.* 2006).

### 1.10 Red Cell Derived Microparticles and SCD

As discussed, most SCD patients do not have a functional spleen as a result of splenic infarction and autosplenectomy at an early age (Brousse *et al.* 2014). The consequences of this are multifactorial, and lead to aggravation of the patient's symptoms. Studies have shown that circulating RCDP are 8-10 times higher in SCD patients than in normal controls (Mahfoudhi *et al.* 2012) as the SCD patient is unable to remove RCDPs from the circulation via splenic macrophage activation. This results in elevated numbers of pro-coagulant PS positive RCDP and RBCs in the circulation (van Beers *et al.*2009) increasing the tendency to clot and the associated risk of VOC and stroke.

Generation of RCDP is also enhanced by inflammatory conditions, such as those experienced in SCD. When engulfed by circulating phagocytic cells, RCDP have been found to significantly enhance the production and secretion of pro-inflammatory cytokines IL-6, TNF-α, and IL-1β from macrophages, markers of systemic inflammation and promoters of endothelial cell adhesion (Pathare *et al.* 2003; Awojoodu *et al.* 2014). Up to one-third of cell-free heme in SCD may be carried by circulating RCDP. Such heme-laden RCDP transfer haem directly to vascular endothelium, causing oxidative stress and endothelial apoptosis. Such linkage of haemolysis to endothelial injury has been proposed as a trigger for SCD vaso-occlusive crises. (Camus *et al.* 2015). Heme-laden microparticles react with Nitric Oxide (NO) about 1000 times faster than with intact erythrocytes. In *in vivo* studies by Donadee *et al.* (2011) they showed that haemoglobin, even at concentrations below 10μmol/L (in heme), produces potent vasoconstriction.

However in the published literature, there is some disagreement about the origin of the causative MVs in SCD, with some attributing RBC-derived MVs as greater in number (Shet *et al*. 2003; van Beers *et al.* 2009; Mahfoudi *et al.* 2012; Camus *et al*. 2012), and some studies finding endothelial, monocyte or platelet-derived MVs as the larger subpopulation (Nebor *et al.* 2014).
Shet *et al*. (2003) found that RCDP always constitute the majority of blood MPs in patients with SCD. They found that RBC and monocyte-derived MPs were significantly increased in both steady state and crisis compared with healthy

control subjects, with RCDP the largest population detected. The monocyte-derived MPs were triple-labelled with Cy5-labeled annexin V, a cell type–specific PE-labelled MoAb against either CD14 (macrophage-specific lipopolysaccharide ligand) or CD144 (endothelial-bound calcium-dependent cell–cell adhesion ligand) and a FITC-labelled MoAb against Tissue Factor (TF). TF is a transmembrane receptor for Factor VII/VIIa (FVII/VIIa). It is constitutively expressed by cells surrounding blood vessels. Breakage of the endothelial barrier leads to exposure of extravascular TF and rapid activation of the clotting cascade (Mackman, 2009). Shet *et al.* (2003) found that patients in crisis had significantly higher total TF positive MPs compared with those in steady state or healthy subjects, and subjects in steady state had significantly higher numbers than healthy subjects. They concluded that markers of coagulation were elevated in patients with sickle cell disease versus control subjects and correlated with total MPs and TF-positive MPs, and that their findings support the concept that SCD is an inflammatory state with monocyte activation and abnormal TF activity. They did not, however, consider the PS content and procoagulant activity of the RCDP which were the most abundant type of MV detected in both steady state and crisis SCD patients in the study.

A study by van Beers *et al.* (2009) found that the majority of microparticles originated from both platelets and erythrocytes. There was no significant difference in MP number between SCD patients in crisis and those in steady state. They observed that numbers of all types of microparticles were lower in healthy controls than in patients during baseline conditions. This difference, was, however, clearer for erythrocyte-derived microparticles. This is concordant with the findings of other studies (Setty *et al.* 2002; Shet *et al.* 2003). Importantly, they identified a distinctive population of microparticles exposing CD71, the transferrin receptor, but lacking glycophorin A. This correlated to the percentage of reticulocytes found in the SCD patients studied. Van Beers *et al.* postulated that the CD71+ microparticles were selectively shed from reticulocytes during erythrocyte maturation. An observation that had been reported previously in animal studies (Chitambar *et al*. 1991). They authors state that the large difference in the number of circulating CD71+ microparticles between patients and controls reflects the enormous difference in hematopoietic rate between the two groups of subjects.

This finding regarding large numbers of RBCMV shed by reticulocytes was echoed in the findings of other studies (Johnstone *et al.* 1986; Hillery *et al.* 1996 and Mankelow *et al.* 2015)

Nebor *et al.* (2014) looked at 3 cohorts of Jamaican SCD patients. A severe group (n=12), a non-severe group (n=17) and a negative control group (n=20). They found that the plasma concentration of MPs shed by platelets was greater in number in both the severe and non-severe groups, when compared to RBC-derived MPs. They concluded that PS-positive MPs from platelets was a biomarker of vaso-occlusive phenotype-related severity. However, due to the small size of the study, they concluded that larger studies focusing on the relationship between plasma concentrations of these sub-cellular elements and the other SCD clinical manifestation should be undertaken.

A study by van Tits *et al*. in 2009 examined annexin V, an intracellular protein present in endothelial cells and platelets and is released upon tissue injury. Annexin V has high affinity for PS and has been shown to inhibit several PS-mediated pathophysiological processes (Kennedy, 2015). They could show that HbSS sickle cell patients have elevated plasma concentrations of annexin V compared to healthy controls. Increments were seen in both HbSS and HbSC patients at presentation with a painful crisis. However, they found no significant differences in the annexin A5/MP–PS ratios at presentation with a painful crisis as compared to the steady state. This would, in theory be expected to increase if annexin calcium-dependent cell–cell adhesion levels were associated with increased PS levels in crisis.

A more recent study in 2012 by Mahfoudhi *et al.* used a triple Annexin V/CD41/GPA staining method enabling them to detect and quantify both platelets-derived and erythrocyte-derived MPs in the same sample. These findings agreed with the previous findings of other studies in that erythrocyte-derived MPs were increased in their cohort of SCD patients. Patient MPs were monitored before and after exchange transfusion therapy. They observed a selective reduction in the number of erythrocytes-derived MPs after treatment, while the number of platelets-derived MPs and total MPs remained unchanged.

Camus *et al.* (2012) looked at vasoocclusion in kidneys using murine models. They induced erythrocyte MPs using Thrombospondin-1 (TSP1). They found that TSP1 triggered rapid erythrocyte conversion into spicule-covered echinocytes, followed by MP shedding. The shed MPs were then administered back into the mice, where they triggered immediate renal vasoocclusion. The effects were inhibited by saturating MP phosphatidylserine with annexin-V. The increased number of MVs released by the unstable sickle erythrocytes as they undergo sickling and unsickling upon deoxygenation and reoxygenation respectively, levels of circulating MVs in sickle cell patients are increased when compared to normal healthy controls.

Gerotziafas *et al.* (2012) Examined changes in thrombin generation (TG) to see if there was a link with the concentration(s) of erythrocyte- or platelet-derived microparticles (Ed-MPs and Pd-MPs) in a cohort of patients with steady-state SCD. They compared steady-state SCD patients, of whom some (19) were receiving treatment with hydroxyurea, with healthy age- and sex-matched controls. They found that the steady-state SCD patients who were not taking hydroxyurea had a significantly accelerated propagation phase of TG which correlated with the dual labelled Ed-MP $PS^+$ subpopulation. This led them to postulate that the acceleration of the propagation phase of TG is driven by Ed-MP/$PS^+$. They suggested that the presence of these MP may contribute to blood coagulation in patients with steady-state SCD.

There remains a lack of clarity with regard to the RBC sub-population most responsible for RCDMV production. Some suggest that senescent RBCs are responsible for the majority of RCDMV production *in vivo*, while others have shown that during storage younger RBC populations produce the majority of RMPs. In all previous studies examining RCDMV, the authors utilise antibodies to extracellular epitopes, predominantly on GPA, which won't detect inside out AV. In addition, those that dual stain with anti-GPA and annexin V will detect a PS positive vesicle that isn't attributed as derived from a red cell as it will be GPA negative AVs very little is known about the very early stages of erythrocyte development, few previous studies have examined vesicle release during this

stage of erythropoiesis, and no previous studies have accounted for Autophagic Vesicle (AV) numbers, especially for SCD in VOC.

### 1.11 Autophagic vesicles and reticulocyte maturation in the circulation

Vesicle Formation during reticulocyte maturation was first described by Johnstone *et al.* in 1996**.** During the process of erythropoiesis, the premature enucleated erythrocyte (reticulocyte) must undergo a series of physiological processes to transform into a mature erythrocyte. The term reticulocytes normally refers to erythroid cells that have undergone enucleation and are not yet fully biconcave, whereas in reality, reticulocytes are a heterogeneous population of erythroid lineage cells at all stages in between enucleated reticulocytes and erythrocytes.

Heilmeyer and Westhauser first described diversity within the reticulocyte population in the early 1930's (Heilmeyer and Westhauser, 1932). With further work performed by Mel *et al.* (1977) subdividing reticulocytes into class 1 (R1) and class 2 (R2) reticulocytes based upon a two-stage living cell cytological classification in animal studies on rats. Since then, a number of methodologies have been used to differentiate reticulocyte maturation based upon staining with the RNA stain, Thiazole Orange (TO), but these methods have been found to be unreliable in the presence of parasitaemia or erythrocyte inclusions found in certain haematological malignancies (Choi and Pai, 2001; Wollman *et al*. 2014).

The transferrin receptor (TfR or CD71) has now become a standard marker for reticulocyte maturation. Levels of TfR decrease throughout reticulocyte maturation. Once haemoglobin synthesis is complete, reticulocyte Tfr1 is rapidly downregulated to prevent continued iron importation, and iron-mediated toxicity (Ney, 2011). Studies have found that when dual stained with CD71/TO, it is now possible to differentiate four stages of reticulocyte maturation from early reticulocytes (R1 stage) to late reticulocytes (R4 stage) (Malleret *et al.* 2013; Ovchynnikova *et al.* 2017).

To adopt a bi-concave erythrocyte shape, membrane stability and deformability, reticulocytes undergo a maturation process within the circulation. Membrane-cytoskeleton rearrangement is an important step allowing the transition from an

unstructured reticulocyte to a morphologically biconcave and functional erythrocyte. Reticulocytes are released from the bone marrow into the circulation, and at the point of release contain nuclear material and cytosolic organelles which must be exuded from the cell by through enucleation, endosomal, exosomal pathways and autophagy. The reticulocyte loses up to 20% of its cell surface area and volume during this enucleation step, as well as the nuclear material and cytosolic organelles which are not required in the mature erythrocyte (figure 1.17). Reticulocyte maturation is accompanied not only by a loss of cytosolic organelles, but also by an intense membrane remodelling. It takes 1–2 days in the circulation for the reticulocyte to obtain bi-concaveness and mature into fully functional erythrocyte. Unfortunately, not much is known about how this process of volume loss and membrane reorganisation occur during differentiation.

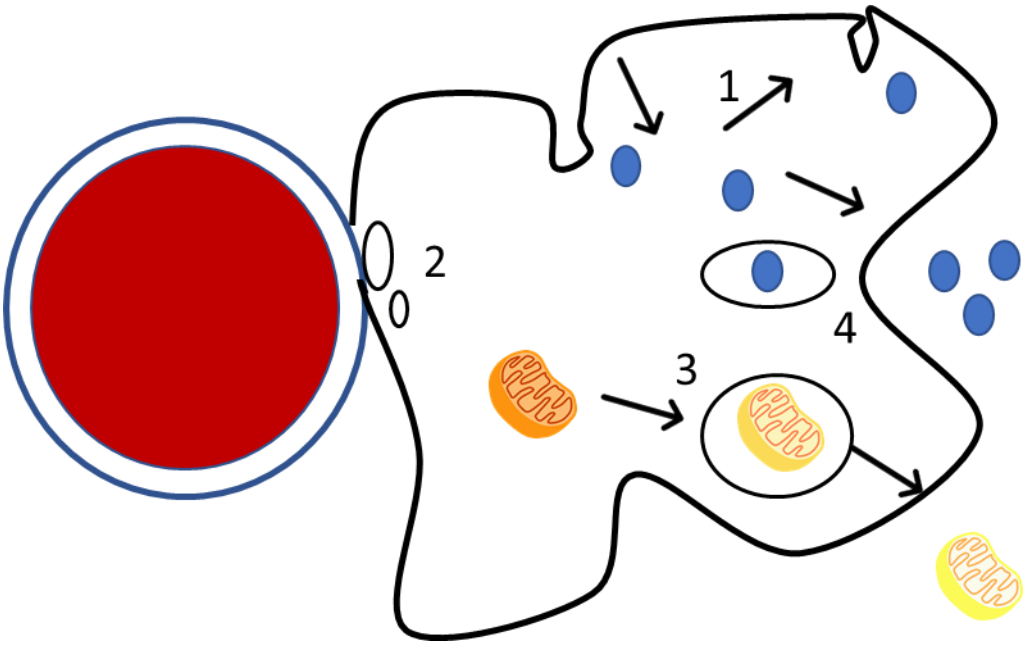



**Figure 1.17 Normal reticulocyte maturation**.

There are four main steps in reticulocyte maturation. 1) Iron bound to transferrin is internalized via endocytosis into an endosomal compartment. After acidification of the endosomal compartment and release of transferrin-bound iron, transferrin is recycled to the cell surface. 2) Vesicles congregate at the nuclear cytoplasmic junction, creating a new membrane. The sides are pinched inwards by the combined action of vesicle trafficking and microfilaments. 3) Vesicles are recruited and surround cellular organelles in an autophagic process. Organelles are subsequently degraded and eliminated by exocytosis. 4) Membrane is lost, and transferrin receptor and other membrane proteins are downregulated through the exosomal pathway (adapted from Ney, 2011).

The role of splenic and hepatic macrophages in the development of reticulocytes has been
extensively studied (Klei et al. 2017), however the influence of the shear stress forces, and capillary interactions involved in reticulocyte development remains unclear. It is known that reticulocytes and erythrocytes of SCD patients share many ligands. Levels of surface expressed adhesion proteins facilitate adhesion with the vascular endothelium in both reticulocytes and erythrocytes in SCD leading to VOC in the latter instance. Proteins such as α4β1 integrin, which is widely expressed on reticulocytes interacts with VCAM-1, and CD47 which binds to thrombospondin, and Lu/BCAM which interacts with laminin, a major constituent of the vascular basement membrane (Rasmussen and Karsdal, 2016). These interactions with the endothelium serve no known biological purpose, whether shear stress forces combined with enhanced endothelial adhesion result in removal via physical forces of membrane bound constituents or MVs is yet to be seen.

The dogma regarding MV formation during reticulocyte maturation has followed the theory that loss of plasma membrane and organelles was facilitated by 2 separate mechanisms. The first mechanism, involving plasma membrane loss via an endosome-exosome pathway, describes a process where small plasma membrane vesicles are endocytosed and incorporated into multivesicular endosomal bodies which then fuse with the plasma membrane, releasing unwanted material as exosomes. This is mediated by endosomal sorting complexes required for transport (ESCRT) machinery (Saksena *et al.* 2007). The ESCRT machinery comprises four complexes (ESCRT 0, I, II, III) that are involved in MV formation. It is generally believed that ESCRT complexes recognize membrane proteins that need to be sorted into MV to be excluded from the maturing reticulocyte as a discrete MV (Odorizzi *et al.*1998; Hislop and von Zaslow, 2011).

The second mechanism is an autophagic pathway, where unwanted cellular material is enclosed in a double membrane to form an autophagosome. First described by Kent *et al.* (1966) autophagy, or “self-digestion”, is an intracellular process for recycling cellular components including cytoplasm, protein aggregates

and organelles such as mitochondria (Ney, 2011). These are delivered to lysosomes and expelled from the cell (Johnstone *et al.*1987; Ney, 2011). As described, these two processes are considered to be distinct from each other, with each serving a separate biological purpose. A further study by Fader *et al.* (2008) suggested that there may be overlap at the cellular and molecular levels. Fader *et al.* used K562 cells from an erythroleukemic cell line that generates multivesicular bodies to demonstrate that autophagosomes fuse with endosomes, forming so called amphisomes, a pre-lysosomal hybrid organelle. Fader *et al.* described how amphisome formation is driven by intracellular $Ca^{2+}$ influx, indicating that fusion of multivesicular bodies (MVBs) with the autophagosome compartment is a calcium-dependent event.

At the cellular level, both pathways are able to terminate with exocytosis of vesicles of intracellular components, suggesting a common mechanism. However, the mechanisms and pathways involved in the formation and fusion of the vesicles with the membrane and their subsequent secretion are still not yet fully understood. Earlier work on erythropoiesis and reticulocyte maturation had been performed using animal-derived bone marrow cells and reticulocytes from animals forced (by stimulation with erythropoietic agents or phlebotomy) to undergo stress erythropoiesis. (Mel *et al.* 1977; Chasis *et al.* 1989).

Recently an *ex vivo* culture system has been developed that allows for the relatively easy study of human erythropoiesis and reticulocyte maturation. Using this culture system, Griffiths *et al.* (2012) were able to replicate human erythropoiesis using peripheral blood CD34+ stem cells which had been isolated from the mononuclear cell fraction discarded from blood donations during routine processing. Their work provided more detail regarding the interaction of the endosome-exosome and autophagic pathways. Using their *ex vivo* culture system, they also concluded that reticulocyte maturation occurs in two stages.

The first stage occurring in the bone marrow, when plasma membrane components not required in the mature erythrocyte such as CD71, CD98, and α4β1 are eliminated via the endosomal-exosome pathway. The second stage occurs in the circulation when large plasma membrane vesicles internalize, then

fuse with autophagosomes, and the contents are mechanically expelled, during passage through the spleen. They described how this process may also result in the loss of plasma membrane by “blebbing” of large GPA positive vesicles, termed autophagic vesicles (AV) from the reticulocyte membrane (see figure 1.18). It is this continuous process that defines the reticulocyte’s final maturation step to an erythrocyte, which then gives the erythrocyte stability and deformability characteristics that sustain it through multiple passages through the peripheral circulation.

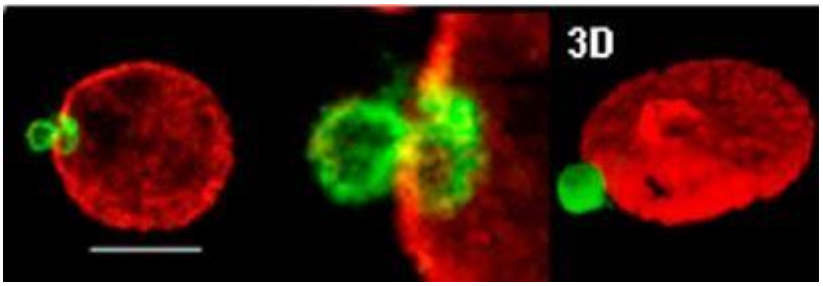



**Figure 1.18 Release of PS-exposed, inside-out autophagic vesicles is a normal mechanism of reticulocyte maturation**. Reticulocytes produced by the method as described by Griffiths *et al.* 2012. Cultured reticulocytes were treated with the proteolytic enzyme trypsin and then fixed and stained with an anti–GPA antibody (red). Following permeabilization, they were then stained with an anti–trypsin-sensitive GPA antibody (green). Inside-out GPA positive Autophagic Vesicle (AV) release shown (green). Image from Mankelow *et al*. 2015. Scale bars 7μm.

Mankelow *et al.* (2015) then utilised the findings by Griffiths *et al.* (2012) to study autophagic vesicles and their secretion from R2 stage reticulocytes in more detail. The R2 reticulocytes are less motile, more mechanically stable and comprise ~2% of circulating RBCs. Mankelow *et al.* used two monoclonal antibodies (mAb) to Glycophorin A (GPA), R10 and BRIC 256, to label two different epitopes on the glycoprotein extracellular domain of R2 stage reticulocytes. The R10 epitope was sensitive to trypsin but the BRIC256 was not. The mAbs when incorporated into immunofluorescence and confocal microscopy experiments confirmed that the endocytosed plasma membrane, which fuses with the autophagosome is subsequently expelled from the reticulocyte in an intact state and that there was interaction between the membrane removal and autophagic pathways as previously described, resulting in the release of a GPA positive AV (figure 1.19). The group looked at the orientation of these extruded vesicles, by staining cultured reticulocytes for the intracellular epitopes PS and with mAb to intracellular

epitopes BRIC163 (GPA) and BRIC155 (AE1). Using these intracellular-specific epitope markers, they were able to confirm that the large GPA-positive AVs described by Griffiths *et al.* (2012) were inside-out at the point of expulsion from the maturing R2 stage reticulocyte. There found no evidence to suggest that the vesicle membrane fuses with the plasma membrane, which would be expected if residual organelle material were expelled by exocytosis prior to blebbing, as previously described.

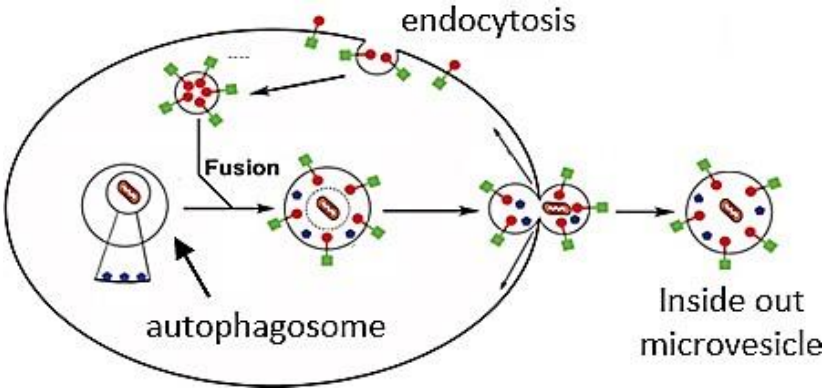



**Figure 1.19. Membrane Remodelling and Autophagy in late stage (R2) Reticulocytes**.
A GPA positive endosome is formed which then fuses with an autophagosome to create an autophagosome / GPA positive endosome hybrid. The contents of this are then expelled from the reticulocyte as a discrete PS-decorated RCDP, termed an autophagic vesicle. Note the inside-out conformation of the autophagic vesicles. (adapted from Mankelow *et al.* 2016).

The observation that these AV were inside-out when expelled from the reticulocyte led the researchers to postulate whether the expression of intracellular proteins, which would now be expressed extracellularly could be contributory to the adverse effects observed in some haematological disease states, with SCD a particular area of focus. As these AV are inside-out they express PS, a procoagulant phospholipid that, due to the action of a phospholipid translocase, is normally located on the intramembrane side of the plasma membrane (Mankelow *et al* 2015). If the AVs have an inside-out orientation, they would not be detected using existing monoclonal antibodies used in previous studies which have examined extracellular red cell epitopes on MVs and erythrocytes. There remains a possibility that this particular subset of RBC-derived PS-decorated AV exists in plasma from SCD patients that has previously been undetected.

The association between PS and prothrombotic states has been studied extensively (Setty *et al.* 2002; Tait & Gibson,1994; Kuypers *et al*. 1996). Levels of PS are known to be up to 8 times higher in SCD patients (Kuypers *et al*. 1996, Wood & Gibson *et al.*1996). Most SCD patients have hyposplenism before 12 months of age (Brousse *et al.* 2014) and therefore have no functioning spleen, where the elimination of vesicles occurs. It had previously been presumed that PS expression was uniform over the cell surface, but the studies by Mankelow *et al*. (2015) showed, through confocal microscopy, that PS was isolated in discrete regions on cultured reticulocytes, corresponding to AVs. To confirm the PS-positive cells observed by microscopy correlated with those detected by flow cytometry, PS-positive SCD cells were sorted using a cell sorter and subsequently imaged. All cells positively sorted for PS showed fluorescence exclusively in large vesicles. Negatively sorted cells did not show any PS fluorescence. The study showed increased levels of PS-decorated AV in steady state SCD patients, when compared with healthy controls and highlighted that previous flow cytometric studies, which label extracellular PS on erythrocytes, may have detected erythrocytes carrying an AV and assumed as they were positive for PS that it decorated the whole cell rather than just a small patch. It is likely in these studies that the PS positive red cell population detected was in fact two sub populations, reticulocytes and young erythrocytes with an attached AV and older senescent red cells with PS expressed across the entire plasma membrane. Mankelow *et al.* (2015) hypothesized that in the absence of a functional spleen (as is the case in SCD) that the AV produced as part of reticulocyte maturation would eventually be released into circulation, this, however, has never been tested (Discher & Ney, 2015).

### 1.12 Quantification of Microvesicles

Flow cytometry (FC) is the standard method to detect, quantify and characterize MVs in physiological and in pathological conditions. However, current literature is based on limited sampling, because MV have been defined by flow cytometry characteristics that were technically achievable at the time. RBC-derived vesicles are generally smaller in size than those of other cell types. They are approximately 0.15μm in diameter and are accompanied by a smaller type of vesicle,

approximately 0.06µm in size, termed a microparticle (Allen *et al*. 1980). This presents a problem when trying to enumerate vesicles using flow cytometric techniques, as some flow cytometers are not able to distinguish particles of such a small size. Flow cytometers are primarily designed to characterise intact cells rather than MPs. Until very recently, this meant particles that ranged from 500–1000 nm in size and expressed PS, as detected by annexin V binding (Freyssinet & Toti, 2010; Lacroix et al, 2010) were classed as MVs. This specific criteria meant there was the potential to miss the greater proportion of extracellular vesicles that are smaller and/or PS negative (Hebel & Key, 2016).

Other techniques including atomic force microscopy, dynamic light scattering, confocal microscopy and nanoparticle tracking analysis can be used for smaller particle enumeration (van der Pol *et al.* 2016). However, they lack the ability to differentiate the phenotypic origin of MVs. Using flow cytometry, two main approaches have been employed in analysing RBC-derived MVs.

- Selection of microvesicles based on a size, followed by analysis of the fluorescent signals of events limited to this preselected size.
- Initial selection based on the fluorescent signals then limiting the analysis by scatter gating to these signals

However, several variables must be considered when performing MV quantitation and classification by flow cytometry. Pre-analytical variables in sample preparation can lead to falsely elevated numbers of MVs. Centrifugation of the sample before freezing is a crucial step, to avoid residual RBCs and platelets falsely elevating MV counts. Lacroix *et al.* (2013) recommended dual centrifugation of blood samples at 2500 g for 15 min at room temperature to ensure that the plasma is truly cell free before freezing prior to analysis.

Analytical variables associated with FC analysis are generally related to issues with particle size. These issues are generally encountered with older FC technologies and are corrected in modern FC by utilisation of latex bead size calibrators and careful set up of the FC software to ensure the correct populations are gated and subsequently captured.

The choice of reagent used in FC can affect study outcomes. In many studies, Annexin V has been used to label PS-Positive MVs. Whether this is specific enough to differentiate between platelet or RBC-derived MVs is debatable. The

IBGRL PDPU department in Bristol also produce a large number of antibodies to intracellular epitopes on the cytoplasmic portions of RBC antigens. These include BRIC163 (GPA), BRIC155 (AE-1), BRIC132 (AE-1), BGRL100 (glycophorin C) and BRAC67 (glucose transporter-1). They all have been used to detect AVs on RBCs (Mankelow *et al.* 2015) in SCD patients in both flow cytometric tests and confocal microscopic analysis.

Additionally, newer technologies for analysing small cellular particles and debris are being employed including imaging flow cytometry. Imaging flow cytometry is a benchtop, multispectral, imaging flow cytometer which is able to acquire up to 12 channels of cellular imagery. By collecting large numbers of digital images per sample and providing a numerical representation of image-based features, imaging flow cytometers are able to combines the per cell information content provided by standard microscopy with the statistical significance afforded by large sample sizes common to standard flow cytometry (Litwin and Marder, 2011). They are, in effect a combination between an advanced flow cytometer and a confocal microscope. However, they are currently not used in clinical diagnostic settings, but are becoming increasingly utilised in research.

Taking the described pre-analytical variables into account, using effective control measures including sample preparation methodologies to minimise microvesicle formation *in vitro*, cell particle size gating bead technology and specific mAbs that target intracellular reticulocyte epitopes such as those described by Mankelow *et al.* (2015) on a more modern flow cytometer platform, would it be feasible to enumerate the numbers of PS-decorated AVs, attached to erythrocytes and/or free in circulation to see if these were significantly raised in SCD patients who were admitted in VOC? Additionally, from the results of this testing, would it then be feasible to develop a prognostic test to predict when SCD patients are likely to experience VOC and to administer an interventional therapeutic measure such as apheresis, chemotherapy or transfusion? This feasibility study aims to seek answers to such questions.

# Chapter 2: Aims and Objectives

## 2.1 Hypothesis

The overarching aim of this study is to explore the feasibility of a prognostic test, which could be used to predict the likelihood of VOC in SCD patients, to aid earlier intervention and improve the clinical management of this condition.

The null hypothesis is that there are **no significant differences** in numbers of AVs between SCD patients in VOC and steady state / healthy controls.

The alternative hypothesis, therefore, is that there **are significant differences** in numbers of AVs between SCD patients in VOC and steady state /healthy controls.

## 2.2 Primary aim

To determine if AV numbers are elevated in SCD patients who are in SCD crisis when compared with steady state and healthy controls.

## 2.3 Secondary aims

To determine whether levels of microvesicles correlate with VOC?
To determine whether levels of microvesicles correlate with length of admission?
To determine whether levels of microvesicles correlate with numbers of circulating reticulocytes in SCD patients admitted in VOC?

## 2.4 Objectives

This feasibility study is performed in collaboration with the Microvesicles and coagulation in sickle cell disease (MACS) study at University College London Hospital (UCLH). This is an observational case control study involving SCD patients from the UCLH. All samples from SCD patients and healthy controls will be obtained through collaboration with Dr Sara Trompeter at UCLH, Consultant

Haematologist and Paediatric Haematologist Joint Red Cell Unit who is also a Consultant Haematologist at NHSBT.

In order to achieve the above aims the following objectives were addressed:

- To identify the RBC-derived AV content of three distinct populations using flow cytometry and confocal microscopy and monoclonal antibodies, specific for intracellular AV proteins.
- To determine if microvesicles, in particular RBC-derived autophagic vesicles (AV), are elevated in SCD in patients admitted in crises.
- To establish whether there is a correlation between AV number and disease severity or likelihood of VOC.
- To establish whether these then decrease once treatment has commenced.
- To deduce whether the aims indicate the feasibility of the development of a prognostic test for VOC in SCD.

# Chapter 3: Materials and Methods

## 3.1 Ethics

Ethical approval for this study was granted by the Health Research Authority, NRES committee London – Harrow, including the use of Human blood samples, kindly provided by Dr Sara Trompeter, Consultant Haematologist and Paediatric Haematologist, Joint Red Cell Unit, University College London NHS Foundation Trust. (REC Reference: 10/H0715/61; IRAS Project ID: 59193)

## 3.2 Participant Recruitment

Participants were recruited to the study at UCLH. Participant number was determined by projected number of admissions and likelihood of participant consent over the duration of the study period. Participants were identified and recruited to the study by a team of National Institute of Health Research (NIHR) funded research nurses, working in the emergency department, using the following set of inclusion and exclusion criteria.

Patient inclusion criteria:

- Adult SCD patient presently stable.
- Patient able to give informed consent
- Compound heterozygote or homozygote for a sickling disorder
- Having a blood test in any case

Patient exclusion criteria:

- Unable to meet all inclusion criteria

Control inclusion criteria:

- Adult able to give informed consent
- Must know their sickle cell status or be highly unlikely to be HbSS through ethnicity.

Control exclusion criteria:

- Unable to meet all inclusion criteria

### 3.3 Patients

A total of 11 (homozygous HbSS) SCD patients in VOC were recruited to the study. None of the patients were on hydroxyurea therapy. In crisis was defined as presenting in the Emergency Department at UCLH in pain. They were identified by the investigators from the inpatient setting as meeting the inclusion criteria. The study was explained to them verbally in addition to the provision of a patient information sheet (see appendix 2). Patient participants were consented using a written patient consent form (see appendix 5). Patient participants were consented to have samples drawn at regular intervals throughout the course of their admission, at the following intervals:

- 0 hours (day of admission after VOC stabilised)
- 24 hours (day 1)
- 48 hours (day 2)
- 72 hours (day 3)
- 120 hours (day 5)

Samples were taken only during the length of a patient's hospitalisation and so could be prematurely stopped by discharge. There was no payment for participation in the study.

### 3.4 Controls

Six SCD (homozygous HbSS) steady state controls were recruited to the study. None of the steady state controls were on hydroxyurea therapy. Steady state was defined as attending the outpatient haemoglobinopathy clinic at UCLH for a routine red cell exchange transfusion and not having had a transfusion in the previous 4 weeks. They were identified by the investigators from the outpatient setting as meeting the inclusion criteria. The study was explained to them verbally in addition to the provision of a patient information sheet (see appendix 2). Patient participants were consented using a written patient consent form (see appendix 5).

Samples were taken only during the length of a patient's hospital visit for monitoring / treatment and so one sample only was obtained for steady control SCD patients. There was no payment for participation in the study.

Four healthy control participants were recruited to the study. They were identified by the investigators as meeting the inclusion criteria. The study was explained to them verbally in addition to the provision of a healthy control information sheet (see appendix 3). Participants were consented using a written healthy control consent form (see appendix 4).
Healthy control participants were consented to have samples drawn at regular intervals throughout the course of the study to duplicate those taken from the SCD crisis patients. Samples were collected at the following intervals:

- 0 hours (day of admission after VOC stabilised)
- 24 hours (day 1)
- 48 hours (day 2)
- 72 hours (day 3)
- ≥120 hours (day 5)

There was no payment for participation in the study.

Serial samples were not obtained for SCD steady state controls, as they were day case patients, and some SCD VOC patients were not admitted for long enough in order to obtain serial samples. Longitudinal data throughout the study reflects those study participants where serial samples were obtained only.

### 3.5 Sample Preparation and Transport

On arrival at the Emergency Department (ED) at UCLH, and once stabilised and consented, a 5ml whole blood sample was taken from the participant, into a sodium citrate vacutainer® (Becton Dickinson, Oxford). The whole blood was then centrifuged (Sorvall Legend T, Thermo Fisher, UK) at 2000 x g for 10 minutes at room temperature with no brake.

The plasma was separated from the cells (buffy coat and red cells) in a sterile laminar flow hood, using sterile tips and sterile tubes, leaving a small layer of plasma behind to ensure no cells were transferred into the plasma. Tubes were labelled with anonymised participant numbers and the date and time of sampling. Tubes containing the separated plasma and cells were stored at 4°C until collection, to minimise in vitro vesicle release. All samples were processed and stored within 2 hours of collection. Samples were collected by courier and transported to NHSBT, Filton, Bristol for analysis by flow cytometry and confocal microscopy. Upon arrival in Bristol, the platelet poor plasma was centrifuged (Sorvall Legend RT, Thermo Fisher, UK) at 2500 x g for 15 minutes at room temperature with no brake, to ensure full removal of cells and that only vesicles were present before analysis. Plasma samples were separated into 150µl aliquots in a sterile laminar flow hood, using sterile tips and were frozen at -80°C in polypropylene cryo-tubes (2 ml cryo-tubes (Sarstedt Ltd. Leicester, UK) until flow cytometric analysis. Sterile Alsever’s solution (Inverclyde Biologicals, Strathclyde,

UK) was added to the packed RBC in a sterile laminar flow hood, using sterile tips, and was thoroughly mixed to ensure adequate suspension of cells.
Steady state control samples were drawn from SCD patients attending routine haemoglobinopathy clinic appointments at UCLH. Single samples, at 0h only, were obtained from this cohort due to the period of their admittance as day case patients. Healthy controls samples were taken from healthy volunteers at NHSBT Filton, samples were taken at the different time points; 0, 24, 48 72 and ≥120 hrs to mimic that of the crisis samples. All control samples were processed identically to those from patients in SCD crisis.

### 3.6 Confocal Microscopy

#### 3.6.1 Live cell confocal microscopy

Live cell imaging was used to determine the number of erythrocytes with a PS positive vesicle attached in SCD patients undergoing episodes of sickle cell crises, steady state SCD patients and healthy volunteer participants. PS was detected using Annexin-V fluorescein isothiocyanate (FITC) conjugated mAb (Annexin V-FLUOS Staining Kit (Roche)) as directed by the manufacturer.

##### 3.6.1.1 Materials

Human blood sample, RBC only.
Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Indianapolis, USA)
4% w/v BSA / Phosphate Buffered Saline

- Albumin Bovine Fraction V (Parks Scientific, Northampton, UK)
- Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich, Gillingham, UK)

8-well u-chamber slide (Ibidi, Martinsried, Germany)

##### 3.6.1.2 Method

A 100µl volume of packed RBC added to 500µl of 4% w/v BSA / Phosphate Buffered Saline and left to incubate for 20 mins at room temperature. The cells were then washed once in BSA / Phosphate Buffered Saline and resuspended in 400µl incubation buffer from the Annexin-V-FLUOS staining kit (Roche Diagnostics, Indianapolis, USA). Resuspended cells (50µl) were aliquoted into an 8-well u-chamber slide (Ibidi, Martinsried, Germany) for analysis (see figure 3.1).

##### 3.6.1.3 Live Cell Imaging

Morphological analysis of slides was undertaken. Samples were imaged at 19°C with the use of 40x oil-immersion lenses on a Leica DMI6000 B inverted microscope with phase contrast connected to a Leica TCS-SP8 confocal laser

scanning microscope (Leica) using an Argon laser set at 5% to read fluorescence at 488nm. Images were obtained using Leica LAS software (LAS AF 1.6.3 build 1163) (Nuβloch, Germany) confocal microscope, with installed accompanying LAS software. 5 random stacks were taken. AV number and total RBC number were counted at 3x magnification to give a total number of AVs present, as described in Mankelow *et al.* 2015.


**Figure 3.1. Confocal Microscopy - Live Cell Slide** – Healthy control 3, 48h and 168h samples suspended in 150µl Annexin-V-FLUOS incubation buffer.

### 3.6.2 Fixed Cell Confocal Microscopy

Fixed cell imaging was used to determine the AV number within the erythrocytes in participants undergoing episodes of sickle cell crises, steady state controls and healthy volunteer participants. R10, a mouse monoclonal antibody to a trypsin sensitive epitope of extracellular Glycophorin A (GPA) was used to stain fixed, permeabilised erythrocytes, in particular GPA-decorated intracellular AV. These were then labelled with a goat anti-mouse antibody conjugated with fluorescent dye (488).

#### 3.6.2.1 Materials

Human blood sample, RBC only.
Phosphate Buffered Serological Saline (PBS) pH7.0 (Source Bioscience, Nottingham, UK).
Bovine Trypsin 2.5mg/ml (Sigma Aldrich, Gillingham, UK).
Poly-L-Lysine solution 0.1% w/v in $H_2O$ (Sigma Aldrich, Gillingham, UK).
Hank's Balanced Salt Solution (Sigma Aldrich, Gillingham, UK).
Superfrost Microscope slides (VWR International, Leighton Buzzard, UK).
Coverslips (VWR International, Leighton Buzzard, UK).
Vectashield™ mounting medium for fluorescence (Vector Laboratories, Peterborough, UK).
Monoclonal antibodies:

- R10 intracellular anti-GPA (IBGRL PDPU, Bristol, UK)
- Goat anti-Mouse 488 (IBGRL PDPU, Bristol, UK)

Rinse Buffer:

- PBS pH7.0 (Source Bioscience, Nottingham, UK)
- Albumin Bovine Fraction V (Parks Scientific, UK)
- D - Glucose (BDH Laboratory supplies, Poole, UK)

1% w/v formaldehyde / 0.01% w/v in PBS:

- PBS pH7.0 (Source Bioscience, Nottingham, UK)
- 36% w/v Formaldehyde (TAAB Laboratories, Reading, UK)
- 25% w/v Gluteraldehyde (Sigma Aldrich, Gillingham, UK)

Glycine Rinse Buffer:

- Rinse buffer (as above)
- 1M Glycine, pH8.0 (Sigma Aldrich, Gillingham, UK)

0.1 % w/v Triton-X in Rinse Buffer:

- Rinse buffer (as above)
- Triton-X 0.1% w/v (Sigma Aldrich, Gillingham, UK)

#### 3.6.2.2 Method

40µl of whole blood was washed 4 times with 1ml PBS and centrifuged at 4000rpm for 1min. 10µl of packed RBC were added to 40µl of Bovine Trypsin 2.5mg/ml solution and incubated at 37ºC for 30 minutes. After trypsin treatment, the packed RBC were washed a further 4 times, as before. The packed, washed RBC were resuspended in rinse buffer at a ratio of 1:400. (2 µl packed cells and 798µl of rinse buffer).

An appropriate number of glass coverslips were covered with Poly-L-Lysine solution and left to incubate at room temperature for 30 minutes in preparation for the seeding of the trypsin-treated erythrocytes. Excess Poly-L-Lysine solution was aspirated off and the coverslips were washed twice by flooding the coverslip with a solution of Hank's Balanced Saline.

300µl of trypsin-treated erythrocytes were seeded onto the coverslips and left at room temperature for 60 minutes to allow attachment to occur. Excess rinse buffer was aspirated, and cells were fixed with 1% w/v formaldehyde / 0.01% w/v in PBS at room temperature for 20 minutes. The coverslips with the fixed erythrocytes were then washed once in rinse buffer, once in glycine rinse buffer, to reduce autofluorescence when viewed on the confocal microscope, and once again with rinse buffer before the permeabilization step.

Fixed, washed cells were permeabilised with 0.1% w/v Triton-X in Rinse Buffer at room temperature for 5 minutes and were then washed twice in rinse buffer before labelling.

Fixed, permeabilised cells, seeded onto coverslips were labelled with 80µl of a 1:100 dilution of the primary R10 mAb at room temperature for 60 minutes. They were then washed twice in rinse buffer before being labelled with a secondary 4% Alexa Fluor 488-conjugated goat anti-mouse mAb and left to incubate at room temperature for 60 minutes. After incubation the coverslips were washed twice in rinse buffer and mounted onto microscope slides. One set of coverslips was retained as a negative control and had been labelled with 4% Alexa Fluor 488-conjugated goat anti-mouse mAb only.

The coverslips were mounted onto microscope slides using Vectashield™ mounting medium and were sealed with clear lacquer to prevent the coverslips from moving during confocal microscopy (see Figure 3.2).

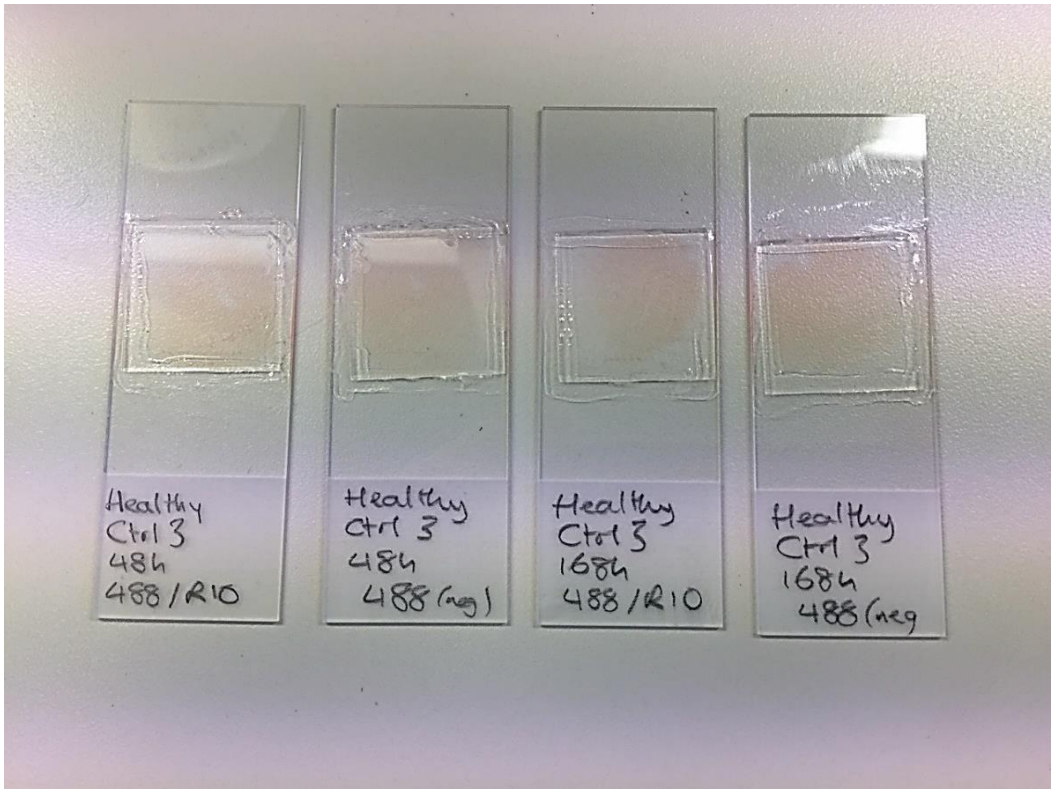



**Figure 3.2 Confocal Microscopy – Fixed Cell Slides** – Healthy control 3, 48h and 168h samples dual stained with primary R10 and secondary Alexa Fluor 488 goat anti-mouse mAb and negative controls labelled with 4% Alexa Fluor 488-conjugated goat anti-mouse mAb only.

### 3.6.2.3 Fixed Cell Imaging

Morphological analysis of slides was undertaken. Samples were imaged at 19°C with the use of 40x oil-immersion lenses on a Leica DMI6000 B inverted microscope with phase contrast connected to a Leica TCS-SP8 confocal laser Scanning microscope (Leica) using an Argon laser set at 5% to read fluorescence at 488nm. Images were obtained using Leica LAS software (LAS AF 1.6.3 build 1163) (Nuβloch, Germany) confocal microscope, with installed accompanying LAS software. 5 random stacks were taken. AV number and total RBC number were counted at 3x magnification to give a total number of AVs present.

## 3.7 Flow Cytometry

### 3.7.1 Monoclonal Antibody Labelling

#### 3.7.1.1 Materials

BRIC163, 1mg/ml (IBGRL PDPU Bristol, UK).
BRIC256, 1mg/ml (IBGRL PDPU Bristol, UK).
Molecular probes kit - Alexa Fluor 647 (Red) / Alexa Fluor 488 (Green) (Life technologies, Carlsbad, Ca)
Nanodrop 2000 (Thermo Fisher, UK)

#### 3.7.1.2 Method

Two murine monoclonal antibodies (BRIC163 and BRIC256) were supplied for the study.
BRIC163 is a mAb to intracellular glycophorin A (GPA) epitopes found in the cytoplasm (Okubo *et al.* 1988). BRIC256 is a mAb to an extracellular trypsin-resistant epitope on GPA (Reid *et al.* 1997). Monoclonal antibodies were supplied as unconjugated supernatants of concentration 1mg/ml and required labelling and titration / dilution to a functional concentration before use.
BRIC163 and BRIC256 mAbs were conjugated to fluorescent dyes Alexa Fluor 647 and Alexa Fluor 488, respectively, using a molecular probes kit. (Life technologies, Carlsbad, Ca). A full methodology can be found at https://www.thermofisher.com/uk/en/home/life-science/antibodies/secondary-antibodies/fluorescent-secondary-antibodies/alexa-fluor-secondary-antibodies.html
Briefly, a 1M solution of sodium bicarbonate was prepared by adding 1ml of deionised water ($dH_2O$) to a vial of sodium bicarbonate (kit component B). The sodium bicarbonate solution (pH8-9) was thoroughly mixed by pipette before use. 100µl of the sodium bicarbonate solution was added to 900µl of each mAb to be labelled. 100µl of this solution was transferred to the vial of reactive dye and then gently mixed and left to incubate at room temperature for 1hr, with gentle agitation every 10-15 minutes.
Purification was undertaken to remove unconjugated dye from the dye-conjugated antibody. Purification columns were set up as in figure 3.3.

1.5 ml of purification resin (kit component C) was added to the column. The column was centrifuged (Sorvall Legend RT, Thermo Fisher, UK) at 1100 x g for 3 minutes to enable the column buffer to permeate throughout the column.
100µl of labelled mAb was added, dropwise onto the centre of the spin column, and was allowed to absorb into the resin bed.

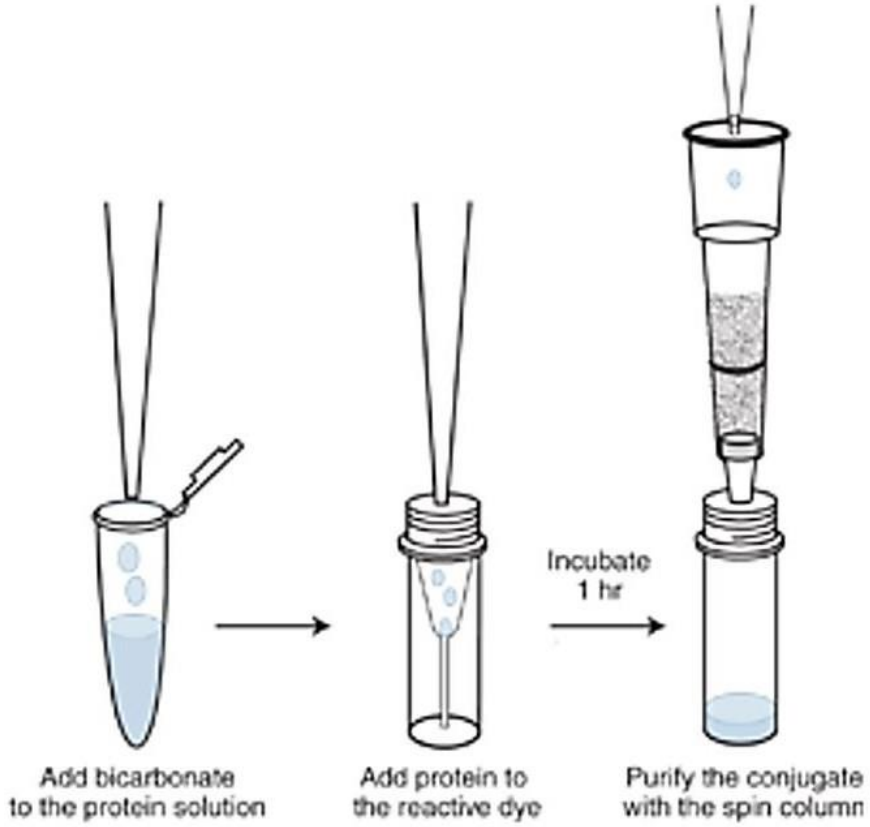



**Figure 3.3 Labelling and purification of monoclonal antibodies BRIC163-488 and BRIC256-647.** (Source: Life Technologies, 2017: online)

The spin column was placed into an empty collection tube and centrifuged (Sorvall Legend RT, Thermo Fisher, UK) at 1100 x g for 5 minutes.
After centrifugation, the column contained 100µl of the labelled mAb, pH 7.2, with 2mM sodium azide. The spin column was then discarded, and the procedure repeated for the other mAb.

Following purification, the concentration of the labelled mAbs was determined using the NanoDrop® 2000 spectrophotometer (Thermo Fisher, UK). A 0.5µl volume of labelled, purified sample was pipetted directly onto the optical measurement surface. Absorbance was measured at A280 and the concentration

of purified labelled/conjugated protein was determined by the NanoDrop® 2000 operating software (version 1.6).

### 3.7.2 Titration of Monoclonal Antibodies

Conjugated antibodies BRIC163-488 and BRIC256-647 were titrated to determine a working dilution for the assay. Starting points for serial dilutions were determined from previous studies performed using the mAbs. (Mankelow *et al.* 2015).

#### 3.7.2.1 Materials

Human Blood sample – Platelet Poor Plasma. (PPP)
BRIC163-488, 1.6µg/ml (IBGRL PDPU, Bristol, UK).
BRIC256-647, 0.4µg/ml (IBGRL PDPU, Bristol, UK).
Sterile Filtered Binding Buffer (Roche, Welwyn Garden City, UK)
0.2-µm filter (Pall, Ann Arbor, MI)

#### 3.7.2.2 Method

All buffers were sterile and filtered using a 0.2-µm filter. Binding buffer (Roche, UK) was used mAbs BRIC163 and BRIC256 were diluted in serial dilutions to the following concentrations (table 3.1):

| BRIC163-488 | BRIC256-647 |
|---|---|
| 1.6µg/ml | 0.4 µg/ml |
| 0.8 µg/ml | 0.2 µg/ml |
| 0.4 µg/ml | 0.1 µg/ml |
| 0.2 µg/ml | 0.05 µg/ml |
| 0.1 µg/ml | 0.025 µg/ml |

**Table 3.1 Titrated dilutions of BRIC163 and BRIC256** mABs that were used in flow cytometric testing to determine a functional dilution for the analysis of AV's in plasma.

A 20µl volume of Human PPP was added to 80µl of each concentration of diluted antibody. A ‘blank’ control, consisting of 20µl of Human plasma and 80µl of binding buffer was included.
Samples were incubated on ice, in the dark for 30 minutes before the addition of 300µl of binding buffer (Roche, Welwyn Garden City UK). Samples were run at low flow speed on a Navios flow cytometer (Beckman Coulter, Wycombe, UK) with Kaluza Version 1.2. 50,000 events were measured before analysis was undertaken.

### 3.7.3 Flow Cytometer Size Gating

Flow cytometric size gating was undertaken using Megamix™ beads, a blend of monodisperse fluorescent beads of four diameters (0.1 µm, 0.3 µm, 0.5 µm and 0.9 µm). Gating was set to capture RBC-Derived microparticles, which have an average size of 0.15µm (Piccin *et al.* 2007).

#### 3.7.3.1 Materials

Megamix™ beads (BioCytex, Marseilles, France)

#### 3.7.3.2 Method

A full methodology can be found at
https://www.biocytex.com/fileadmin/user_upload/80-Biocytex/kits/EN_package%23insert%23Megamix_7801_20161001.pdf
Briefly, following agitation, 500µL of Megamix™ reagent was pipetted into a flow cytometry tube and analysed at low flow speed on a Navios flow cytometer (Beckman Coulter, Wycombe, UK) with Kaluza Version 1.2. Events were measured for 1 minute before analysis was undertaken. Regions of interest were gated according to size, with a of range of 0.5µm-0.9µm.

### 3.7.4 Flow Cytometric Analysis

#### 3.7.4.1 Materials

Human Blood sample – Platelet Poor Plasma.
BRIC163-488, 0.8µg/ml (IBGRL PDPU, Bristol, UK).
BRIC256-647, 0.05µg/ml (IBGRL PDPU, Bristol, UK).
Annexin-V FITC, Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Indianapolis, USA).
Flow Buffer pH7.4:

- PBS (Source Bioscience, Nottingham, UK)
- 1% BSA (Parks Scientific, UK)
- 0.05% sodium azide (Sigma Aldrich, Gillingham, UK)

Binding Buffer pH7.4 (Roche Diagnostics, Indianapolis, USA):

- 50mM HEPES
- 700mM NaCl
- 12.5nM $CaCl_2$

AccuCount® Counting beads [$1x10^6$ particles/ml = 49655 particles / 50µl] (Spherotech, Chicago, IL, USA)
0.2-µm filter (Pall, Ann Arbor, MI, USA).

#### 3.7.4.2 Method

Two murine monoclonal antibodies (BRIC163-488 and BRIC256-647) and one commercially supplied recombinant marker (Annexin-V FITC) were used in the study. BRIC163 is a mAb to intracellular glycophorin A (GPA) epitopes found in the cytoplasm (Okubo *et al.* 1988). BRIC256 is an extracellular trypsin-resistant epitope on GPA (Reid *et al.* 1997). Annexin V is a $Ca^{2+}$-dependent phospholipid-binding protein with high affinity for phosphatidylserine. Recombinant Annexin-V is produced in *E.Coli* (strain $NB_{42}$) and is purified by standard purification protocols and conjugated with a FITC marker.
Working dilutions of fluorescein-conjugated BRIC163-488, BRIC256-647 and Annexin-V FITC mAbs were prepared in concentrations which had either been

found to be optimal from previous experiments, or from the recommended concentration from the supplied instructions for use (Roche Diagnostics, 2018). All MAbs were centrifuged at 13,000 rpm prior to use. All buffers were sterile and filtered using a 0.2-µm filter.

A 20µl volume of Human platelet poor plasma was added to 80µl of each concentration of diluted MAb. A negative control, consisting of 20µl of Human plasma and 80µl of binding buffer was included in addition to three blank controls, consisting of 20µl of each mAb and 80µl of binding buffer (see table 3.2).

**Table 3.2 Flow cytometry test layout**. Tubes include negative control, Single specificity, dual stain and blank controls for each single stain.

| Tube number | Sample | Antibody |
|---|---|---|
| **1** | Plasma | Buffer (Negative Control) |
| **2** | Plasma | BRIC256-647 |
| **3** | Plasma | BRIC163-488 |
| **4** | Plasma | Annexin-V FITC |
| **5** | Plasma | BRIC256-647 / BRIC163-488 (Dual Stain) |
| **6** | Plasma | BRIC256-647 / Annexin V FITC (Dual Stain) |
| **7** | Buffer | BRIC256-647 (Blank Control) |
| **8** | Buffer | BRIC163-488 (Blank Control) |
| **9** | Buffer | Annexin-V FITC (Blank Control) |

Samples were incubated on ice, in the dark for 30 minutes. 20µl of well vortexed AccuCount® counting beads were added to tubes 2-7. 300µl of binding buffer was added to each tube immediately before testing.

Samples were tested at low flow speed on a Navios flow cytometer (Beckman Coulter, Wycombe, UK) with Kaluza Version 1.2. Forward Scatter (FS) and Side Scatter (SS) were set in a log scale, detecting MPs of interest based upon size and granularity respectively. Threshold parameters were set using the FS parameter. MP gating was accomplished by preliminary standardization experiments using fluorescent microbeads of different sizes as described earlier. Events in the MP gate were further assessed for labelling with BRIC163-488; BRIC256-647 and Annexin V-FITC to distinguish true events from electronic noise

and thereby increase the specificity of MP detection as described by Shet *et al.* 2003. Flow cytometry protocols were stopped once either 50,000 events were measured or after 300 seconds before final analysis was undertaken.
For MP quantification, a known quantity of 7.2µm AccuCount® counting beads were added to tubes 2 to 7. The diameter of these beads allowed discrimination from the MP population on light scatter and could be counted using a separate gate. Counting bead quantities / ml were supplied by the manufacturer (Spherotech, Chicago, IL, USA) and the following formula (as described in Nielsen *et al.* 2014) was applied to calculate MP quantity $\mu l^{-1}$, where N is the number of positive events recorded.

$$\mathbf{(N \times Beads_{added}) / (Beads_{counted} \times test\ volume)}$$

The number of beads per µl was provided by the manufacturer and the test volume was 400µL.

### 3.8 Statistical analysis

Statistical analysis of the results was performed by Microsoft Excel using unpaired t-test to determine the statistical significance between the means of all groups tested with +/- SD included, where appropriate. The values were taken as a significant difference when $p \leq 0.05$. A Binomial Generalised Linear Model (GLM) with logit link was used to describe the likelihood of occurrence of VOC in sickle cell patients when compared to the measured variables in this study. All statistical analyses were performed using SAS Enterprise Guide v7.1 software (SAS Institute Inc., Cary, NC, USA). Assistance in the statistical analysis was provided by the NHS Blood and Transplant statistics department.

# Chapter 4: Results

## 4.1 Confocal Microscopy

### 4.1.1 Live Cell Confocal Microscopy

Previous studies have shown that localised PS expression is increased in SCD patients in steady state when compared to healthy controls (Wood *et al*. 1996; Kuypers *et al*. 1998; Setty *et al.* 2001). This localised PS expression has been demonstrated on mature R2 human reticulocytes which have not been removed by splenic macrophages due to functional asplenia in SCD patients. (Mankelow *et al*. 2015).

Analysis of erythrocyte-bound PS in our three cohorts at 0h using annexin-V-FITC and live cell confocal microscopy showed accordance with these previous studies. The live cell imaging of PS positive RBCs in adult healthy control, steady state control and SCD crisis patient RBC samples stained with Annexin-V-FITC PS marker showed significant differences between the percentage of PS-positive vesicle number in the studied cohorts. A 60-fold, statistically significant (t-test, $p<0.0001$) difference in (live cell) vesicle percentage between the healthy controls (mean 0.09%; SD +/- 0.12%) and the SCD crisis cohort (mean 5.47%; SD +/- 3.45%) was observed. A 30-fold statistically significant (t-test $p<0.0001$) increase in PS-positive vesicles was observed between healthy controls (mean 0.09%; SD +/- 0.12%), and steady state controls (mean 2.77%; SD +/- 1.55%) and a 2-fold statistically significant (t-test, $p<0.05$) increase was observed between steady state controls (mean 2.77%; SD +/- 1.55%) and the SCD crisis cohort (mean 5.47%; SD +/- 3.45%). (see figure 4.1).

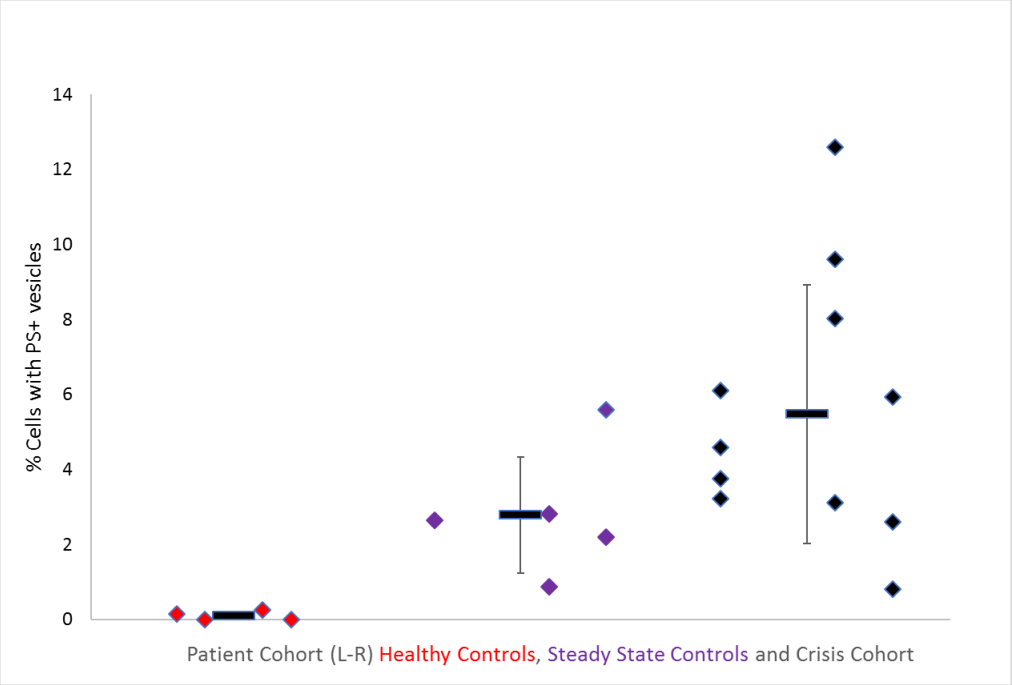



**Figure 4.1 Quantification of Phosphatidylserine (PS) expression by live cell confocal microscopy**. Localised PS positive vesicles were stained with Annexin-V fluorescein isothiocyanate (FITC) conjugated mAb following incubation with 4% w/v BSA / Phosphate Buffered Saline. % of PS positive vesicles was quantified. Random stacks, n=5 were counted and a mean average % of PS positive vesicles calculated. Solid bars indicate mean average of each cohort with error bars showing +/- 2SD  A 60-fold increase in PS positive vesicles was observed between healthy controls and the SCD crisis cohort.

Over the period of admission, PS positive vesicles were quantified in SCD crisis patients where serial samples were received (Crisis patients 1, 2, 3, 4, 5, 8 and 9), and compared to serial samples in the healthy control cohort (Healthy controls 1-4). Significantly higher levels of PS positive vesicles were observed in the SCD crisis cohort than the healthy control cohort, where serial samples were tested. An overall decreasing trend in PS positive vesicle number was observed throughout the course of admission in the SCD crisis cohort in all patients studied. (See figure 4.2).

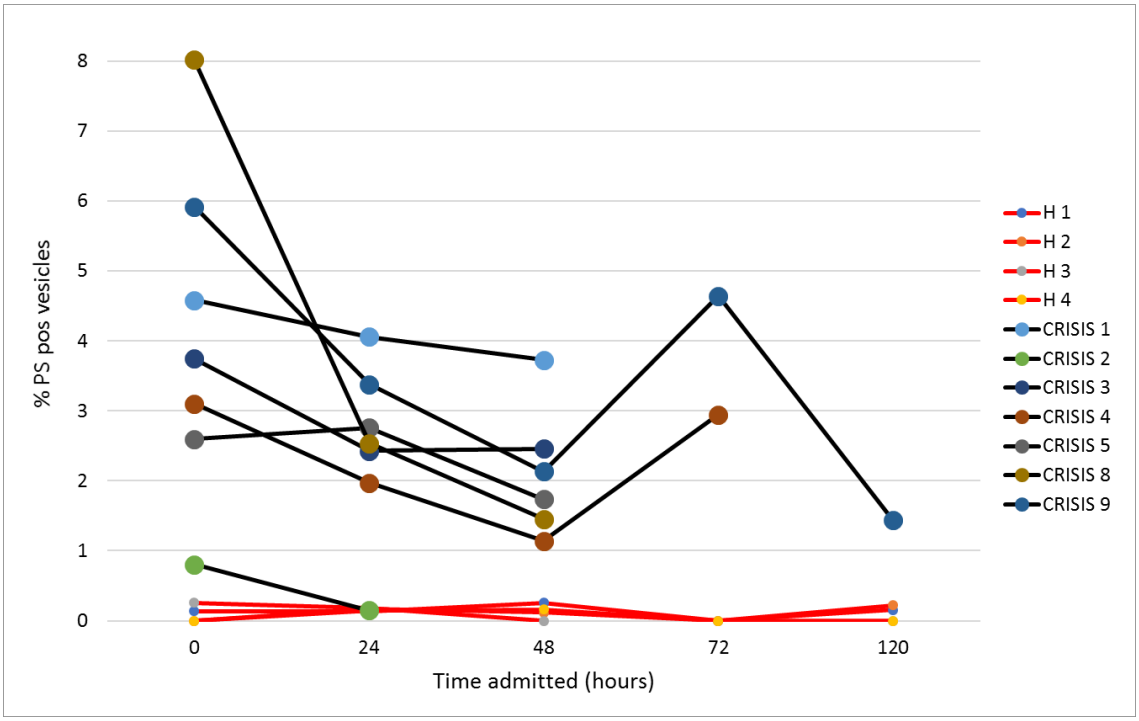



**Figure 4.2 Serial quantification of Phosphatidylserine (PS) expression by live cell confocal microscopy**. Localised PS positive vesicles were stained with Annexin-V fluorescein isothiocyanate (FITC) conjugated mAb following incubation with 4% w/v BSA / Phosphate Buffered Saline. Random stacks, n=5 were counted and a mean average % of PS positive vesicles for each timepoint calculated. % of PS positive vesicles was quantified over serial time points for SCD crisis patients 1, 2, 3, 4, 5, 8 and 9. These were compared to healthy controls 1-4. PS positive vesicle number was up to 7-fold higher in SCD patients with serial samples upon admission than those in healthy control cohorts (range 0.7%-7.75%) Overall, a decreasing trend was observed in PS positive vesicle number during the course of admission. SCD crisis patients 4 and 9 showed a rise in PS positive vesicle number near the end of their admission period, which correlated with the extended time of their admission.

It is of interest to note that two of the SCD crisis patients (crisis patient 4 and crisis patient 9) were admitted for >72h before they were discharged. In both patients, the PS positive vesicle at admission was similar to that seen in patients with a shorter period of admission. It was observed that PS positive vesicle number rose slightly before they were discharged from hospital following the episode of VOC. It may be that this rise in PS positive vesicle number may be associated with VOC severity, and hence length of admission, due to the contribution of PS to thrombosis in SCD patients. These findings are in line with previous studies that have described the externalization of PS on sickle erythrocytes and their potential to cause thrombosis. (Wood *et al*. 1996; Kuypers *et al*. 1998; Setty *et al.* 2001)

Images from the live cell confocal microscopy experiments clearly showed PS positive stained vesicles (figures 4.3, 4.4 and figure 4.5), measuring approximately 0.8µm in diameter. This is in accordance with published findings which report RBC-derived vesicle size to be in the range of 0.1-1.0µm in diameter (Rubin *et al.* 2008; Nielsen *et al.* 2017). The AV's observed by live cell imaging in this study are the same size/shape as previously observed in this laboratory by Mankelow *et al.* 2015.

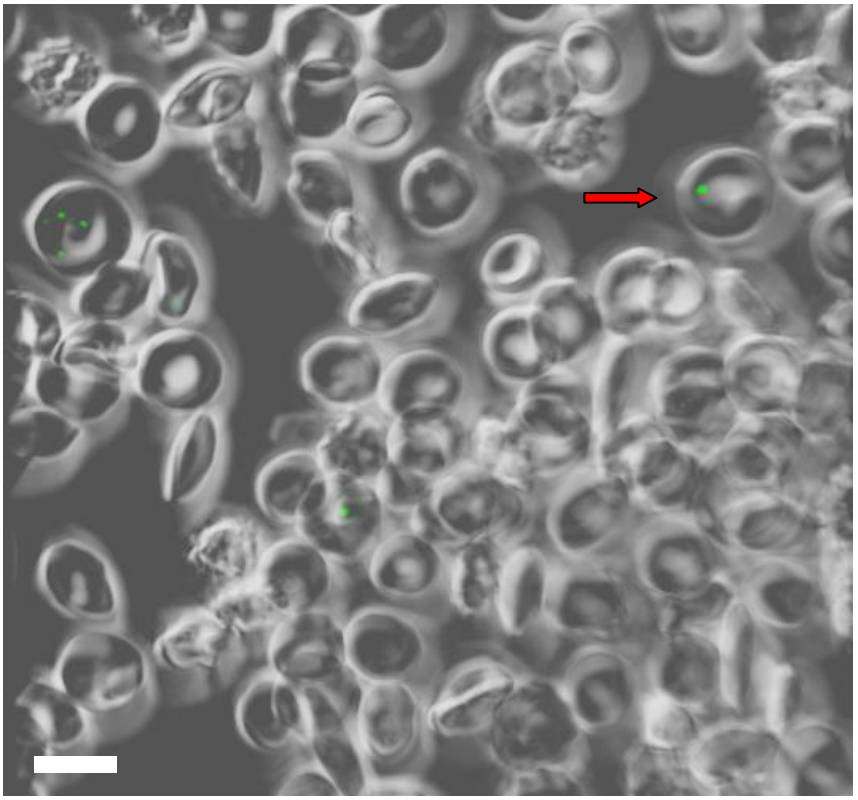


**Figure 4.3 SCD Crisis Patient 3D image - Live cell confocal microscopy.** Sample taken from SCD crisis patient at 0h time interval and stained using Annexin-V fluorescein isothiocyanate (FITC) conjugated mAb. The presence of green FITC-conjugated Annexin-V Phosphatidyl Serine / Autophagic Vesicles can be seen (red arrow). Scale bars 7µm.

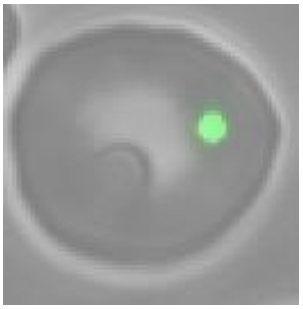


**Figure 4.4 SCD Crisis Patient - Live cell confocal microscopy**. Sample taken from SCD patient at 0h time interval and stained using Annexin-V fluorescein isothiocyanate (FITC) conjugated mAb. The presence of a FITC-conjugated Annexin-V Phosphatidyl Serine / Autophagic Vesicle can be seen (stained green).

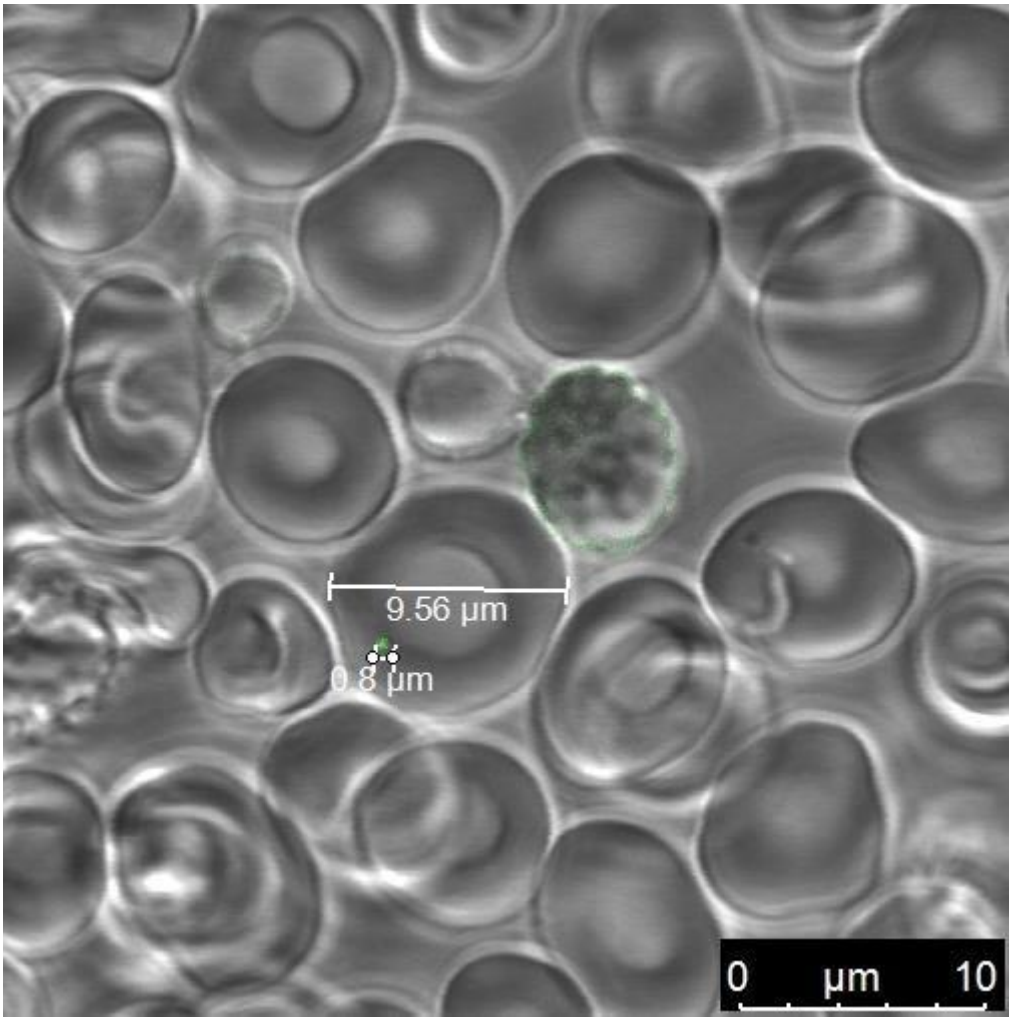



**Figure 4.5 SCD Crisis Patient - Live cell confocal microscopy**. Sample taken from SCD patient at 0h time interval and stained using Annexin-V fluorescein isothiocyanate (FITC) conjugated mAb Live cell confocal microscopy. Measurements performed using a RBC measuring 9.56µm in diameter shown, in addition to 0.8µm diameter, FITC-conjugated Annexin-V Phosphatidyl Serine / Autophagic Vesicle (green).

Live cell confocal images taken from serial SCD crisis samples and healthy control samples clearly showed the difference in PS positive vesicle number. Significantly more ($p<0.0001$) PS positive vesicles could be seen in the SCD crisis cohort both at 0h and throughout the period of admission when compared to the serial samples from the healthy controls. Steady state samples (taken at 0h only) showed a visually noticeable, smaller number of PS positive vesicles when compared to the SCD crisis cohort, and more PS positive vesicles when compared to the healthy control cohort. (See figures 4.6, 4.7 and 4.8).

**Live cell confocal microscopy**

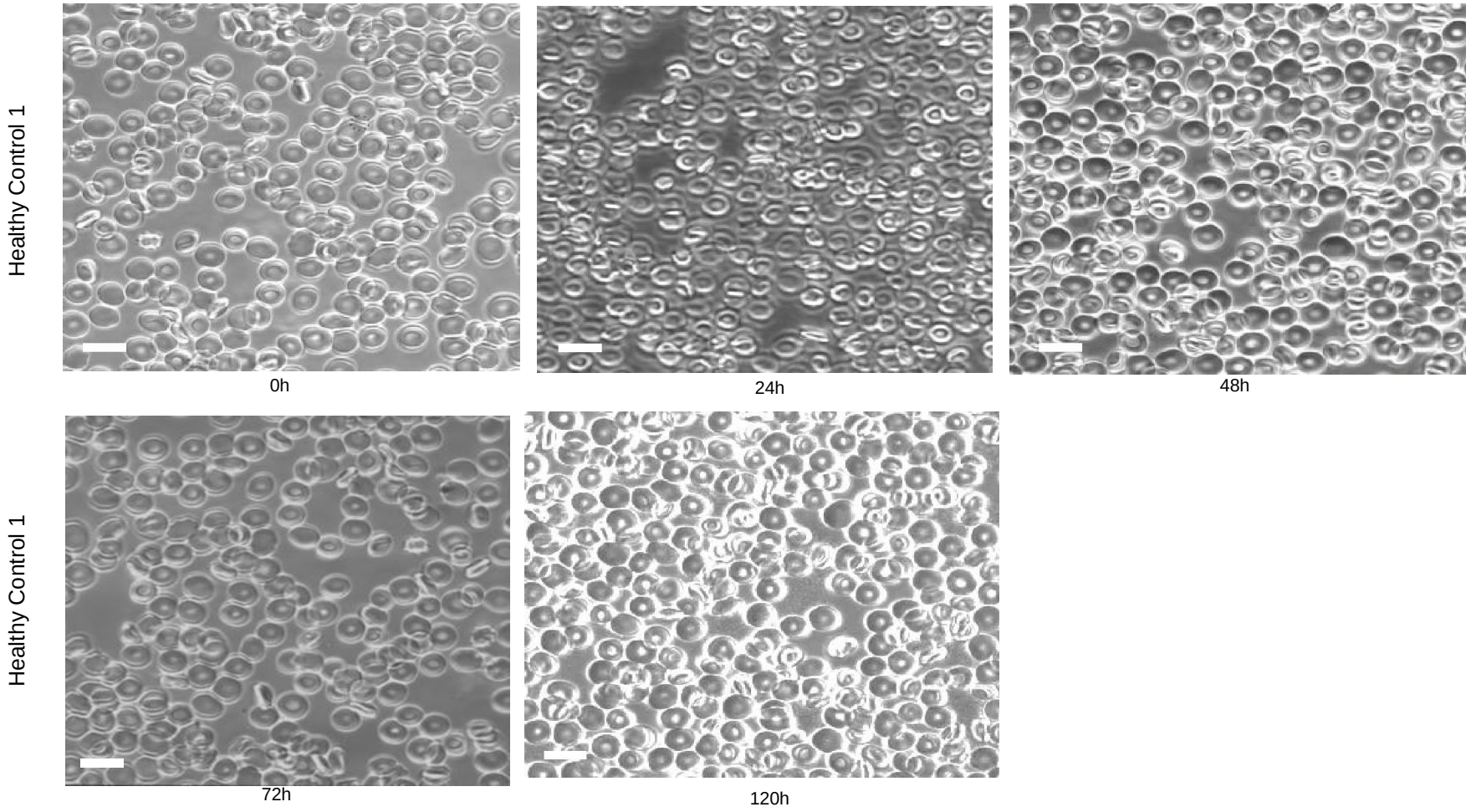



**Figure 4.6 Healthy control 1 - Live cell confocal microscopy**. Serial images from samples taken at 0h**,** 24h**,** 48h**,** 72h and 120h intervals. Note the normal red cell morphology and the absence of FITC-conjugated Annexin V Phosphatidyl Serine / Autophagic Vesicle marker (green). Scale bars 7µm.

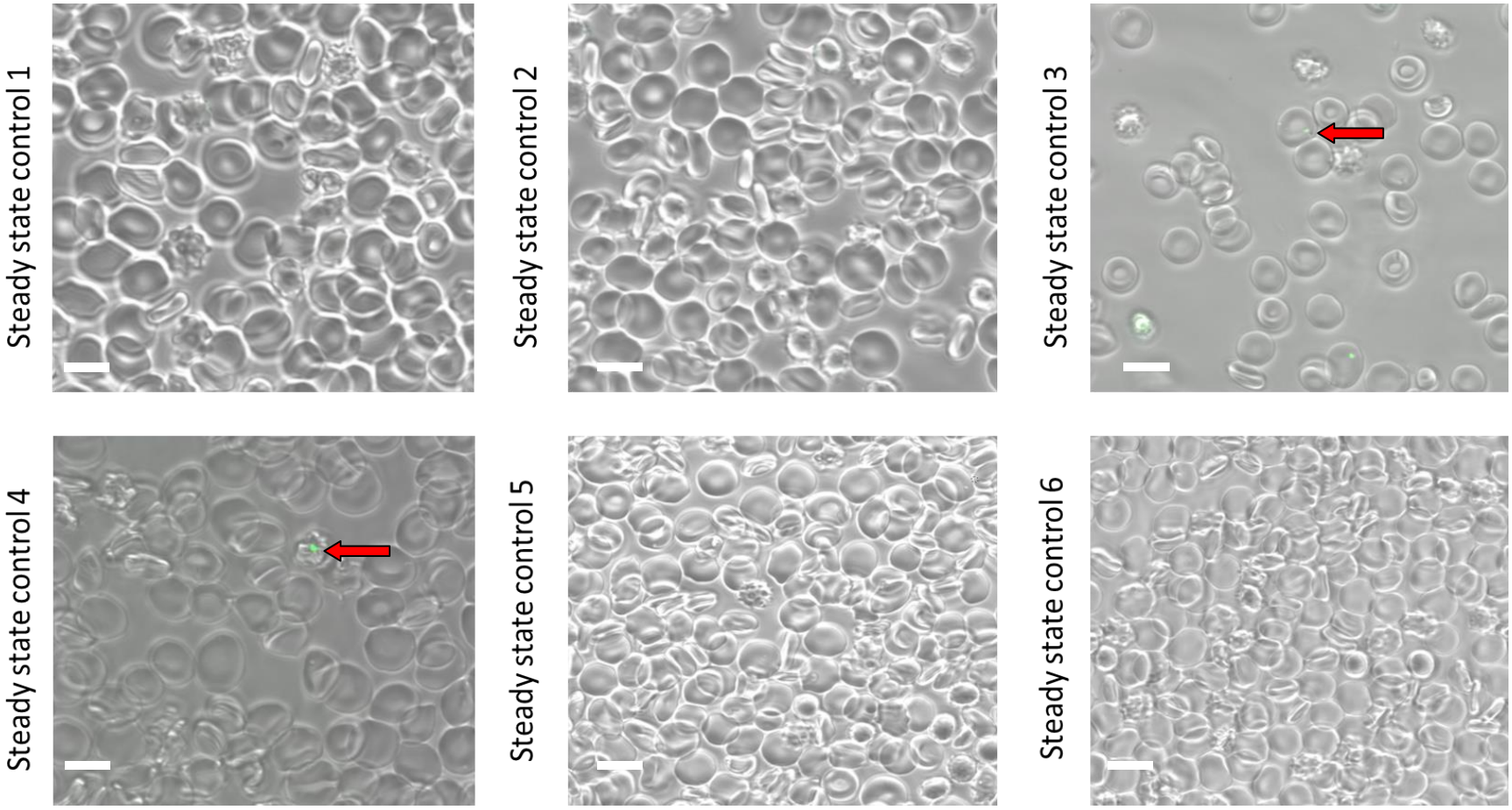



**Figure 4.7 Steady State SCD control – Live cell confocal microscopy.** Images from samples taken at 0h. Note the presence of FITC-conjugated Annexin V Phosphatidyl Serine / Autophagic Vesicle marker (red arrows). Scale bars 7µm.

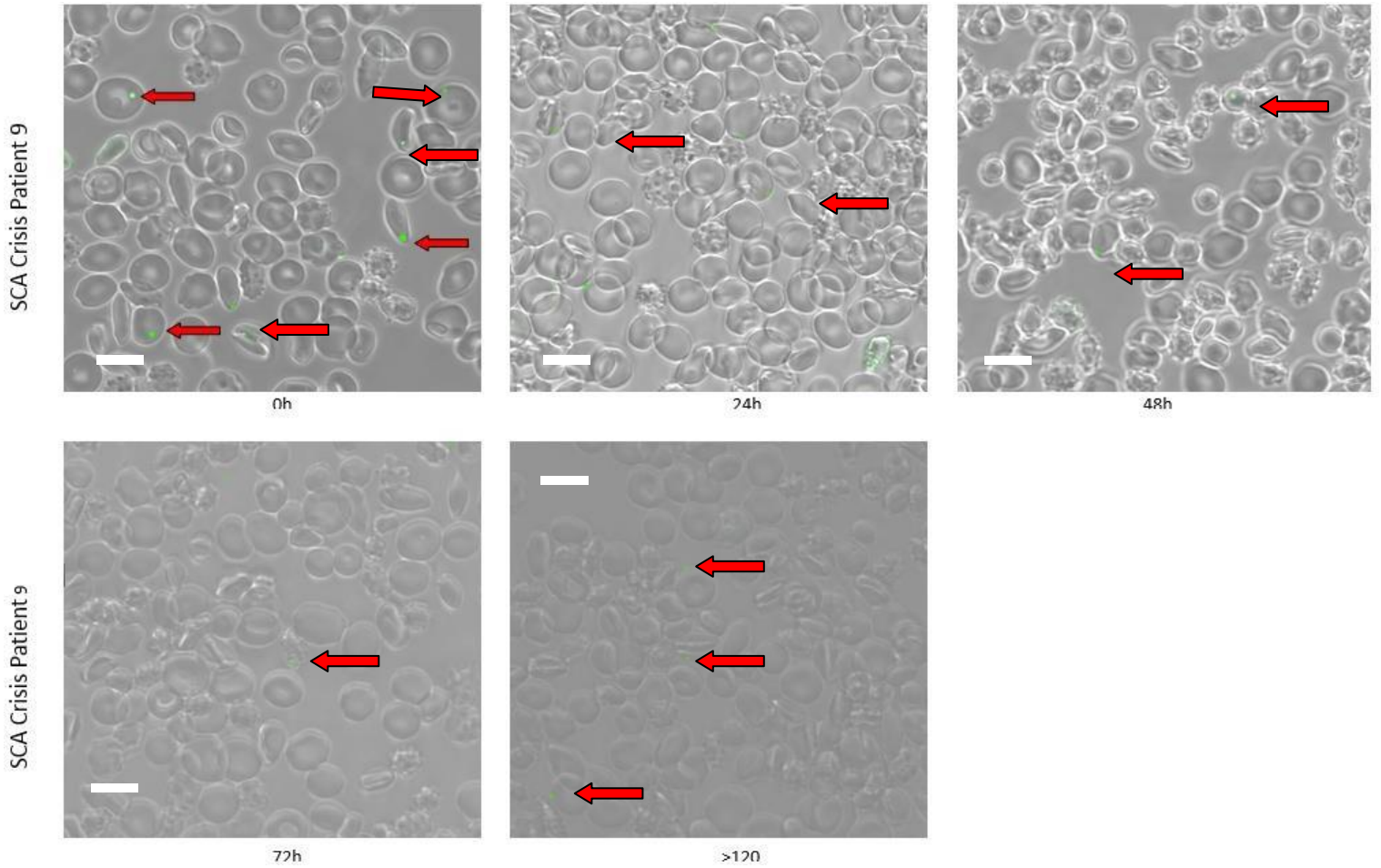



**Figure 4.8 SCD Crisis Patient - Live cell confocal microscopy.** Serial images from samples taken at 0h**,** 24h**,** 48h**,** 72h and >120h intervals. Note the presence of green FITC-conjugated Annexin V Phosphatidyl Serine / Autophagic Vesicle marker (red arrows) at 0h showing a decreasing trend throughout the admission period. Scale bars 7µm.

Where reticulocyte count was measured in the SCD patient cohort, a loose correlation between PS positive vesicle number (%) and reticulocyte count (%) was seen (see figures 4.9 and 4.10). In the SCD crisis patients with serial samples available, PS-positive vesicle percentage decreased with length of admission, as did reticulocytopenia (reticulocyte NR: 0.5-2.5% (50-100 x $10^9$/l), Dacie and Lewis, 2001) but not in absolute amounts. The reticulocyte count only decreased by approximately 0.5%, but the percentage of PS positive vesicles decreased by 5%.

This discrepancy could suggest that red cells with an AV get caught up in the occluded vessel to a greater degree than those without an AV present, which would result in circulating reticulocytes captured in RETIC counts on automated haematology analysers being AV negative. However, the limited amount of data available made a strong association between reticulocyte count and PS positive vesicle percentage impossible, but PS positive vesicles would be expected in increased numbers in patients who have increased reticulocyte counts due to an underlying haemolytic anaemia.

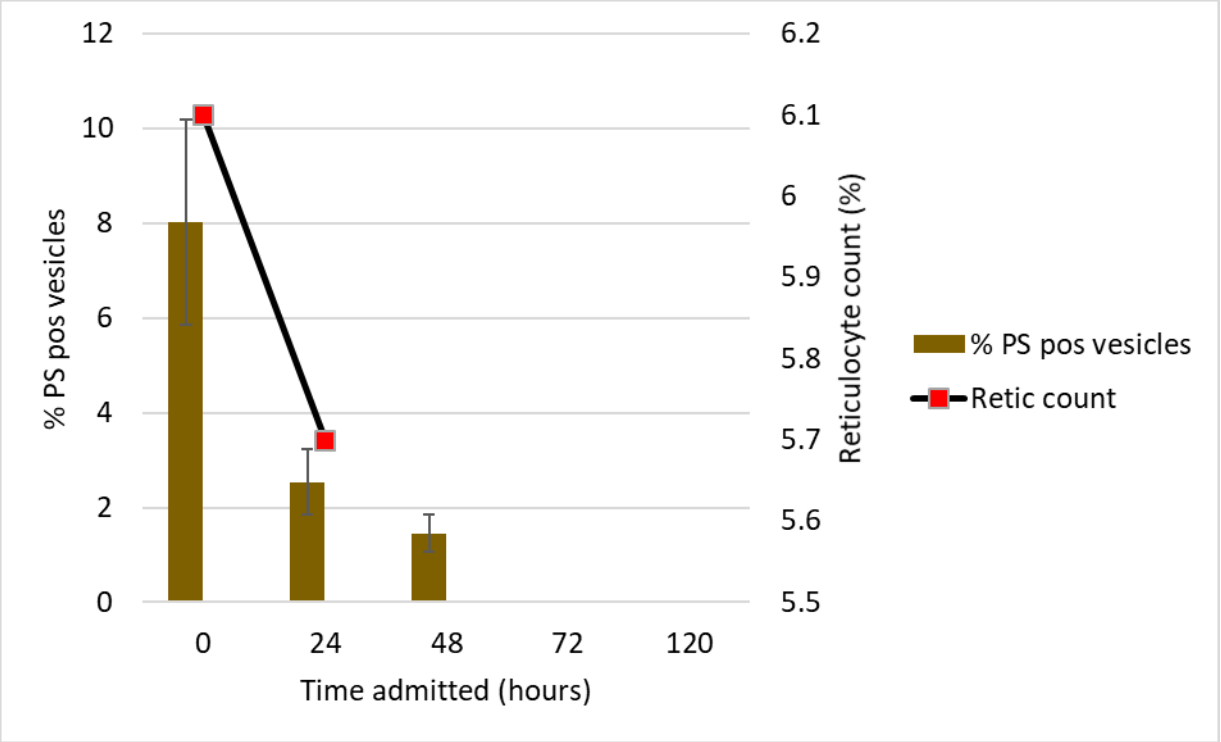



**Figure 4.9 SCD crisis Patient 8 PS positive vesicle (%) and Reticulocyte count (%).** Measurement of PS positive vesicles (%) in serial samples received over the course of admission by live cell confocal microscopy compared with reticulcyte count (%), measured using the RETIC function on the FBC analyser at UCLH. Random stacks, n=5 were counted and a mean average % of PS positive vesicles for each timepoint calculated is shown with error bars showing +/- 2SD. A decrease in PS positive vesicle number and a corresponding decrease in reticulocyte count was observed during the course of admission.

In SCD crisis patient 9, where serial samples had been received during the course of admission, an increasing trend in reticulocyte count towards the end of admission correlated with an increasing PS positive vesicle number. (See figure 4.10). The inverse relationship between PS positive cells and number of reticulocytes seen at 0h correlates with the prothrombotic action of PS during a VOC potentiated by a free circulating haem following haemolysis, and the following reticulocytosis to correct the anaemia (Manwani *et al.*2013). The number of these PS positive vesicles would make them likely candidates for involvement in the pathology of SCD due to their involvement in prothrombotic biological pathways (van Tits et al. 2009; Noubouossie et al. 2016). Therefore, an increasing number of PS-decorated vesicles would be anticipated in a patient who has a longer admission due to the severity and subsequent treatment of VOC.

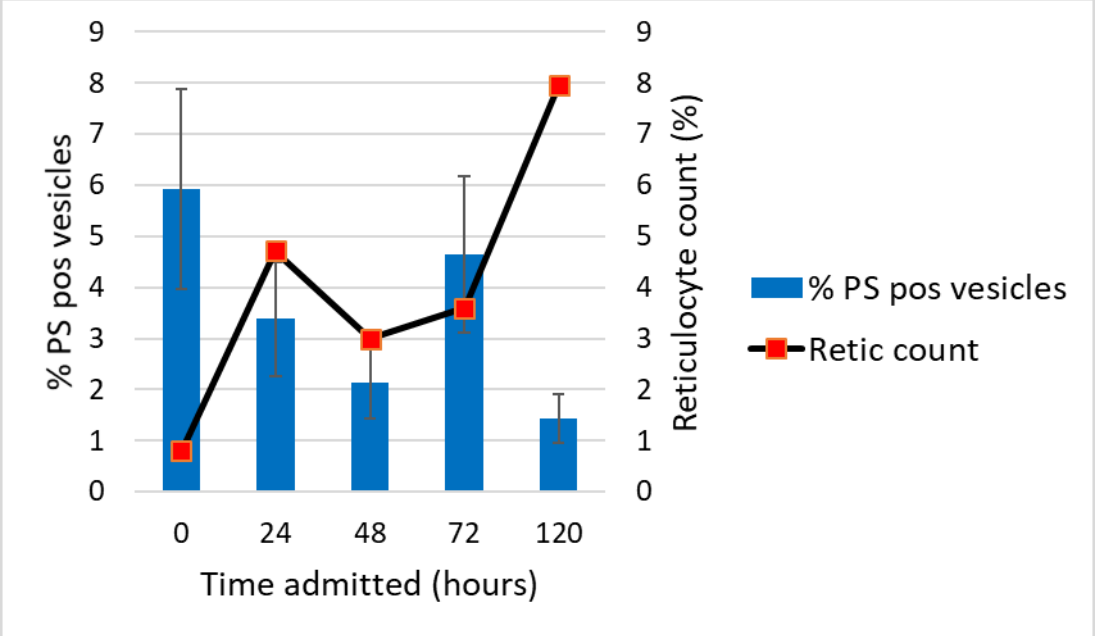



**Figure 4.10 SCD crisis Patient 9; PS positive vesicle (%) and Reticulocyte count (%).** Measurement of PS positive vesicles (%) in serial samples received over the course of admission by live cell confocal microscopy compared with reticulcyte count (%), measured using the RETIC function on the FBC analyser at UCLH. Random stacks, n=5 were counted and a mean average % of PS positive vesicles for each timepoint calculated is shown with error bars showing +/- 2SD. PS positive vesicle number and reticulocyte count was variable throughout admission, but showed a rising trend at 72h, correlating with PS positive vesicle percentage.

### 4.1.2 Fixed Cell Confocal Microscopy

Fixed cell confocal microscopy analysis of an extracellular trypsin-sensitive GPA epitope using a mAb to trypsin-sensitive GPA (R10) showed significantly increased levels of trypsin-sensitive GPA-decorated vesicles when compared to a healthy control (see figure 4.11). This difference is in accordance with previous work (Mankelow et al. 2015). However, a greater difference in trypsin-sensitive GPA-decorated vesicles, when compared to healthy controls was observed in this study than had previously been reported.

A significant 23-fold increase in R10-positive vesicles was observed between healthy controls (mean 0.54%; SD +/- 0.52), steady state controls (mean 12.4%; SD +/- 12.3%) and SCD crisis cohort (mean 13%; SD +/- 13.3%). These differences were statistically significant between the healthy controls and crisis patients ($p<0.0001$), with the mean vesicle percentage being significantly larger in the latter than in the former group. There was no significant difference in R10-positive vesicle expression between the steady state and SCD crisis cohorts.

Although the findings show no significant difference between steady state and SCD crisis patients, this may be explicable due to a variation in the repeatability of the manual experimental procedure. It was noted that during the course of the permeabilization stage of the experiment, cell lysis occurred with some of the patients studied, leading to a lower overall cell count, and a subsequent variation in R10-positive percentage value. This variation in quality of fixation and staining, and of the subsequent result is reflected in the wide standard deviation observed in this part of the study.

The poor quality of staining in fixed cell confocal microscopy experiments may also be due to the damage to the plasma membrane and/or cytoskeleton in SCD, due to repeated sickling and unsickling during de-oxygenation and re-oxygenation. The SCD RBC are therefore more fragile, and more susceptible to damage as part of the experimental protocol. The confocal microscopy

method used was initially established using healthy erythrocytes in healthy controls and may need further optimising for future use on more fragile cells such as those in SCD.

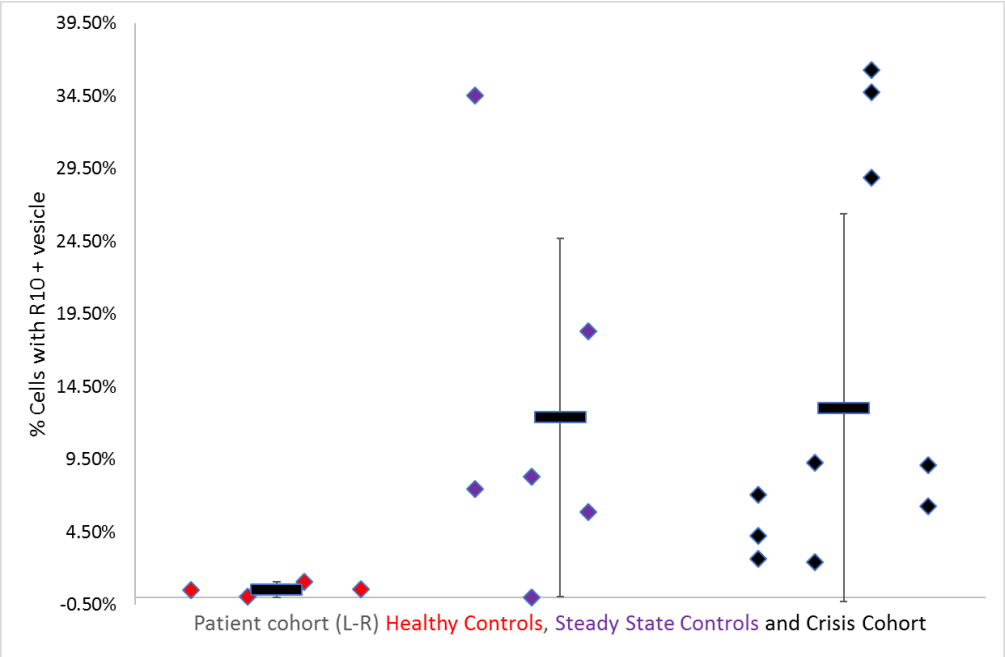



**Figure 4.11 Quantification of Trypsin-sensitive GPA expression by fixed cell confocal microscopy** internal R10 positive vesicles were stained with R10 conjugated mAb following incubation with 4% w/v BSA / Phosphate Buffered Saline and the % of R10 positive vesicles were quantified. Random stacks, n=5 were counted and a mean average % of R10 positive vesicles calculated and a mean average % of R10 positive vesicles for each timepoint calculated is shown with error bars showing +/- 2SD. An average 13-fold increase in R10 positive vesicles was observed between healthy controls and the steady state control and SCD crisis cohort.

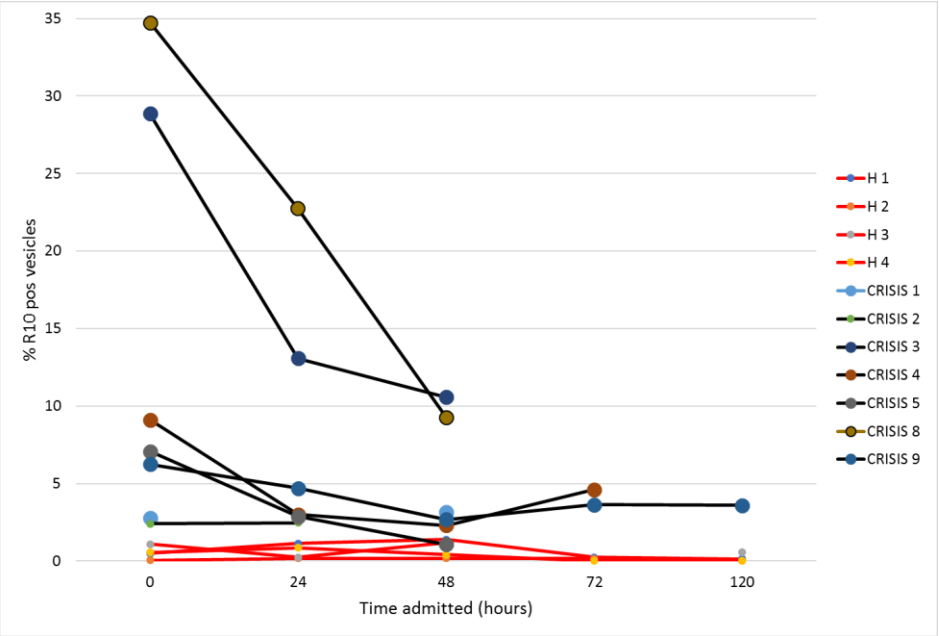



**Figure 4.12 Quantification of Trypsin-sensitive GPA expression by fixed cell confocal microscopy** SCD red cells were trypsin treated then fixed, permeabilized, and stained with trypsin-sensitive GPA mAb R10 to detect intracellular GPA. Random stacks, n=5 were counted and a mean average % of R10 positive vesicles calculated. An average 13-fold increase in R10 positive vesicle number at the point of admission, in addition to a decreasing trend of R10 positive vesicle number was observed when comparing SCD crisis patients with healthy controls.

Overall the percentage of R10-positive vesicles fell over time during the period of admission but did not fall to levels seen in healthy controls (see figure 4.12). This may be due to the increased reticulocytosis in these patients compared to healthy individuals, even in a steady state where the effects of an underlying anaemia are manifested in an increased reticulocyte count as the body seeks to replace erythrocytes lost to the haemolytic process (See Figure 4.13).

In two SCD patients, (SCD patients 3 and 8) high levels of R10-positve vesicles were observed throughout the course of their admission. Higher levels of R10 positive vesicles did not have any correlation to length of stay, with both patients discharged at 48h following admission. It was noted that patients with lower levels of R10 positive vesicles were hospitalised for longer periods throughout the course of the study.

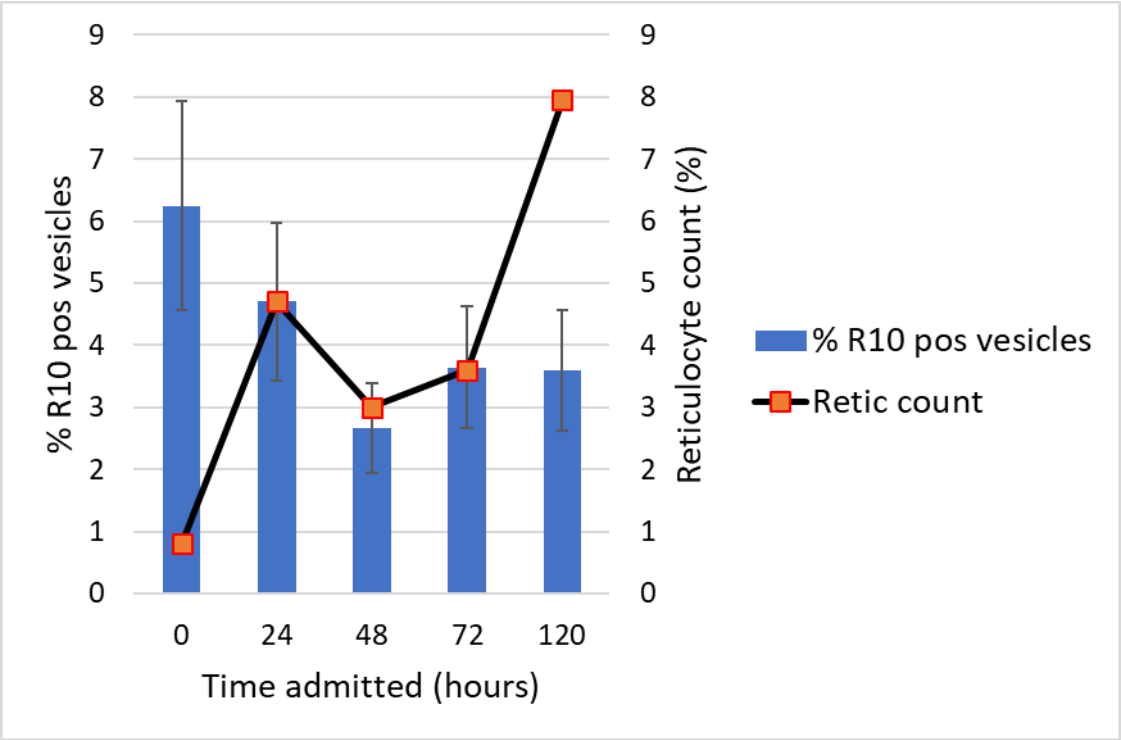



**Figure 4.13 SCD crisis Patient 9 R10 positive vesicle (%) and Reticulocyte count (%).** Measurement of R10 positive vesicles (%) in serial samples received over the course of admission by fixed cell confocal microscopy compared with reticulcyte count (%), measured using the RETIC function on the FBC analyser at UCLH. Random stacks, n=5 were counted and a mean average % of R10 positive vesicles for each timepoint calculated is shown with error bars showing +/- 2SD. R10 positive vesicle number and reticulocyte count was variable throughout admission, but showed a rising trend at 72h.

Images of fixed cell R10 positive staining are also provided (see figures 4.14, 4.15, and 4.16). Increased numbers of R10 positive vesicles in steady state and SCA crisis patients can be seen when compared to healthy controls.

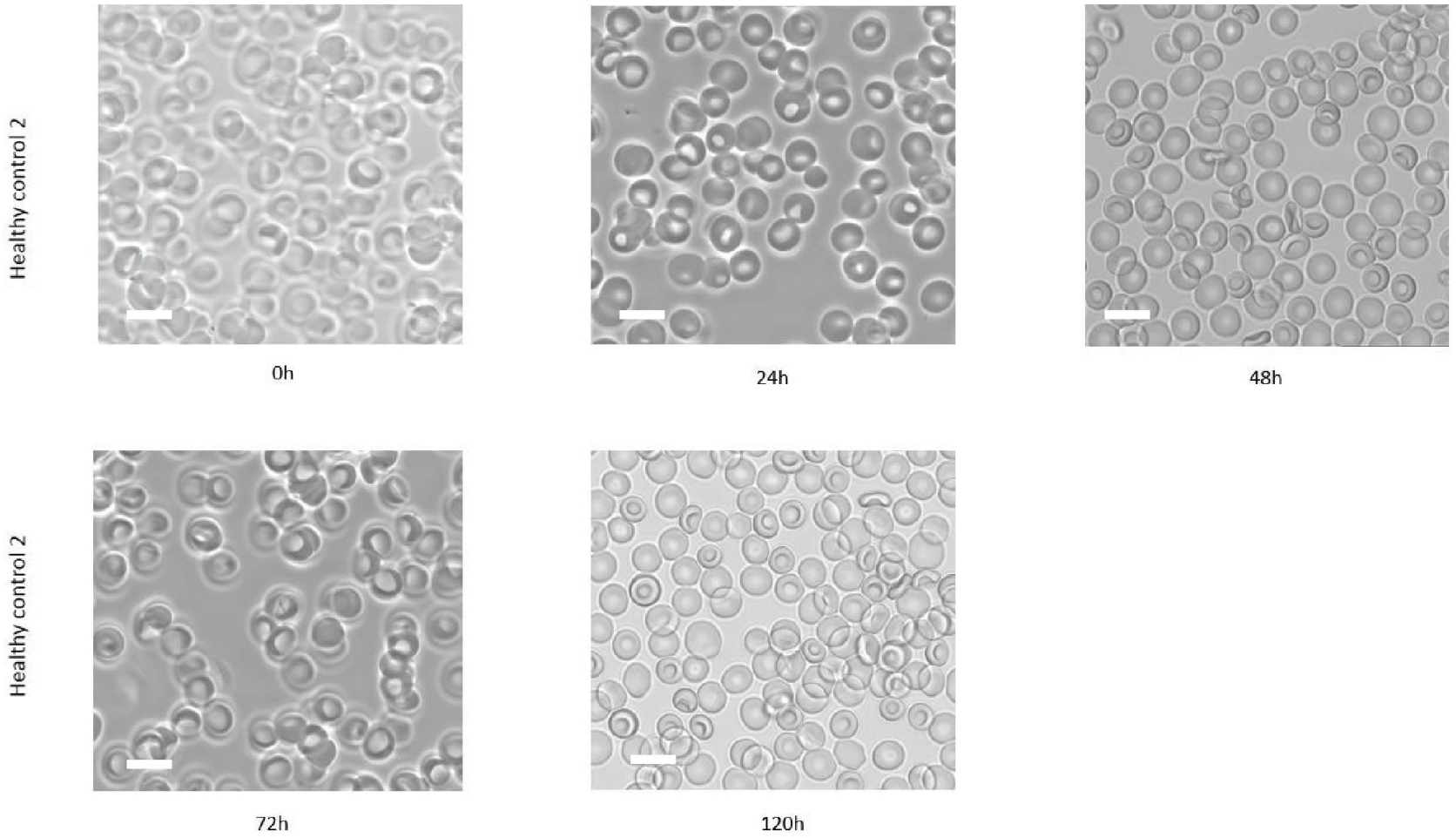



**Figure 4.14 Healthy control 2 - Fixed cell confocal microscopy** Serial images from samples taken at 0h**,** 24h**,** 48h**,** 72h and 120h intervals. Note the normal red cell morphology and the absence of anti-trypsin sensitive GPA Vesicle mAb marker, R10. Scale bars 7µm

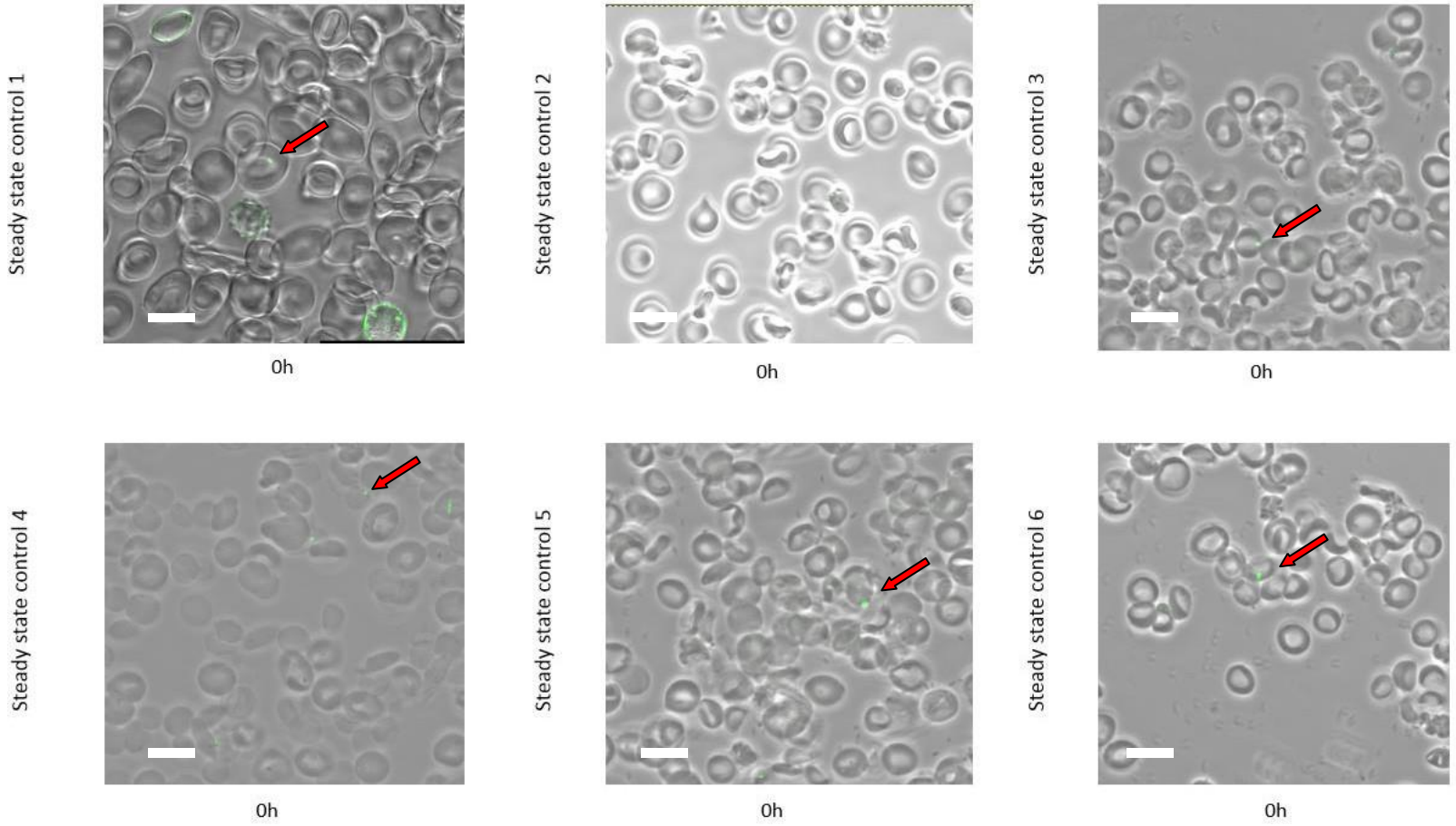



**Figure 4.15 Steady state control - Fixed cell confocal microscopy** Images from samples taken at 0h. Note the presence of anti-trypsin sensitive GPA Vesicle mAb marker, R10 (red arrows). Scale bars 7µm

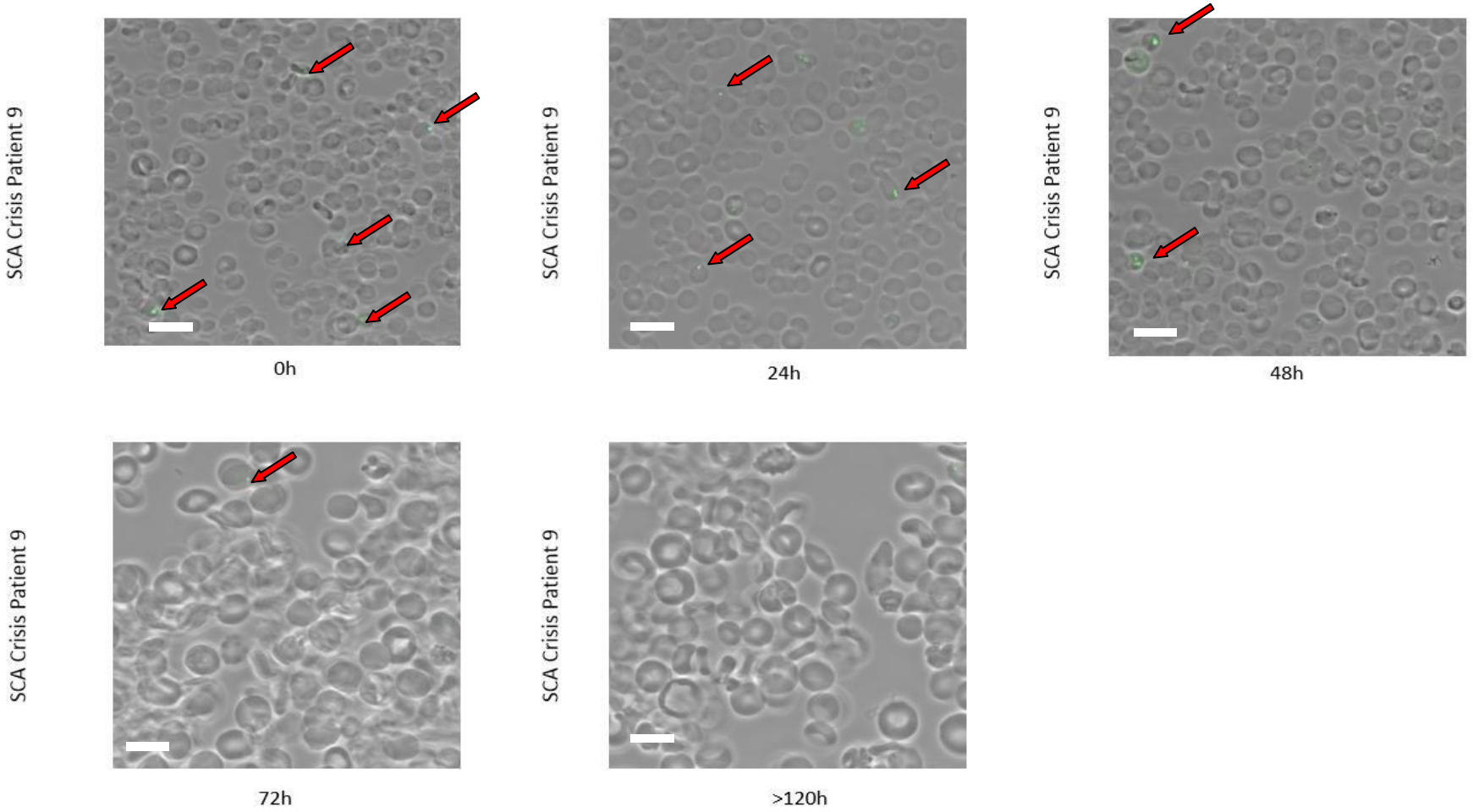
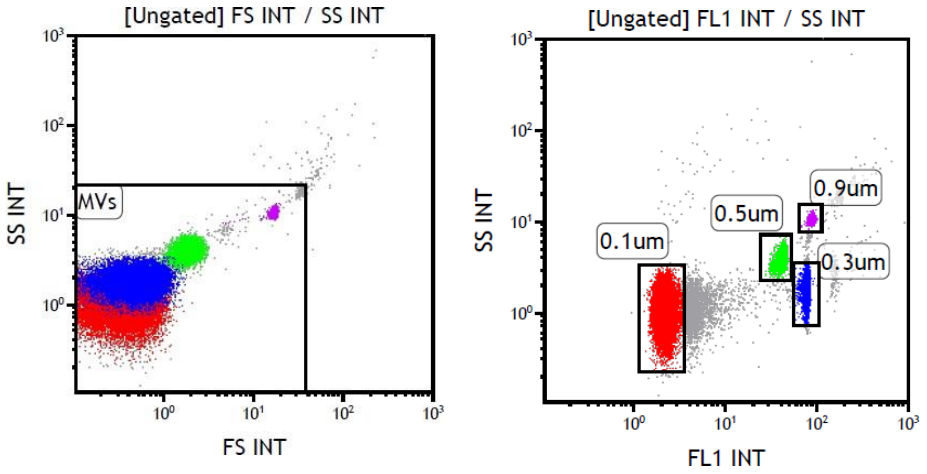



**Figure 4.16 SCD Crisis Patient - Fixed cell confocal microscopy**. Serial images from samples taken at 0h**,** 24h**,** 48h**,** 72h and >120h intervals. Note the presence of anti-trypsin sensitive GPA Vesicle mAb marker, R10 (red arrows) at 0h showing a decreasing trend throughout the period of admission. Scale bars 7µm

## 4.2 Flow cytometry

### 4.2.1 Size gating

a) b)




c)

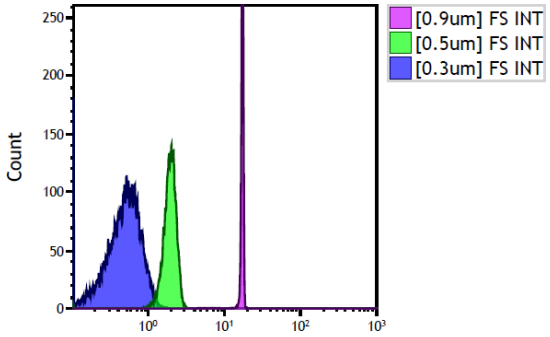



**Figure 4.17 Identification of RBC microparticles (MPs) based upon a fluorescent threshold and utilizing the mixture of absolute counting and calibration beads to define the light scatter parameters**. (a) Side Scatter (SSC) and forward Scatter (FSC) parameters of RBC MPs (b). Side Scatter (SSC) and forward Scatter (FSC) parameters of RBC MPs in relation to the 0.1, 0.3, 0.5 and 0.9µm beads mixture being analysed (c). Representative histogram of the mixture of absolute counting standard microbead and calibration beads (0.3, 0.5 and 0.9µm)

Results of flow cytometric size gating undertaken using Megamix™ beads, as described in methods, showed discrete populations of reagent microparticle beads (see figure 4.17). Some studies define MPs as events by flow cytometry that are 2µm or less, but this may include platelet-derived MPs.

Therefore, we defined MPs as vesicles that are 1µm or less in size following a prior ultracentrifugation step. Gating was set to capture these RBC-Derived microparticles, which have an average size range of 0.15µm- 0.9 µm (Piccin *et al.* 2007; Xiong *et al.* 2013). Positive events were defined as those that were detected in the gate in (a) figure 4.17 which subsequently exceeded threshold fluorescence values defined in the methods.

### 4.2.2 Antibody Titration

The two mAbs used in this study, BRIC256 and BRIC163 were used because they stain for right-side out Red Cell Derived Microparticles (RCDP) and inside-out RCDP respectively. Following fluorescent labelling with fluorescent dyes Alexa Fluor 647 and Alexa Fluor 488 respectively, and subsequent purification they had the following concentrations (mg/ml) when assayed at A280 on the NanoDrop® 2000 spectrophotometer (Thermo Fisher, UK).

- **BRIC256-647 0.53mg/ml**
- **BRIC163-488 0.91mg/ml**

From these starting concentrations, working dilutions were determined by serial dilution and titration of the mAbs. Working concentrations were chosen from gated scatterplots (figures 4.18, 4.19, 4.20 and 4.21), which showed clear separation of positive and negative events in the respective FL6 and FL1 channels. Following titration, the following working concentrations were chosen:

- **BRIC256-647 0.05µg/ml**
- **BRIC163-488 0.8µg/ml**

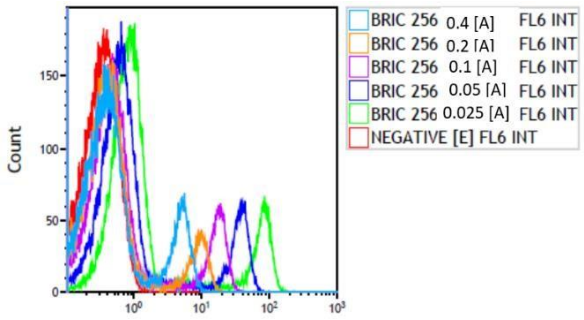



**Figure 4.18 Overlaid histogram plot labelled BRIC256-647** Flow cytometric titration analysis of labelled GPA positive vesicles from 0.4µg/ml to 0.025 µg/ml dilutions colour labelled in the legend above and negative (flow buffer) control shown in red.

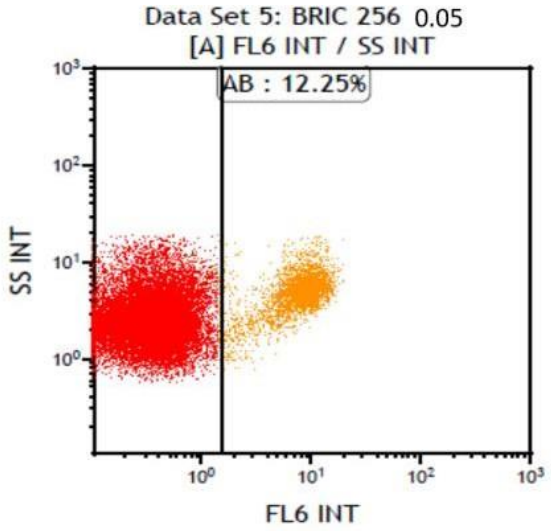



**Figure 4.19 Gated Scatterplot for labelled BRIC256-647** Flow cytometric titration analysis at the chosen dilution of 0.05 µg/ml, showing separation of BRIC256-647 (extracellular GPA) postive (orange) and BRIC256-647 negative (red) populations in the FL6 channel on the Navios flow cytometer

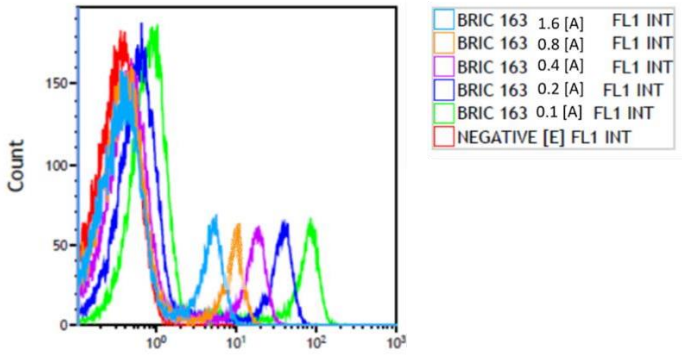



**Figure 4.20 Overlaid histogram plot labelled BRIC163-488** Flow cytometric titration analysis of labelled GPA positive vesicles from 1.6µg/ml to 0.1 µg/ml dilutions colour labelled in the legend above and negative (flow buffer) control shown in red.and negative (flow buffer) control – Red.

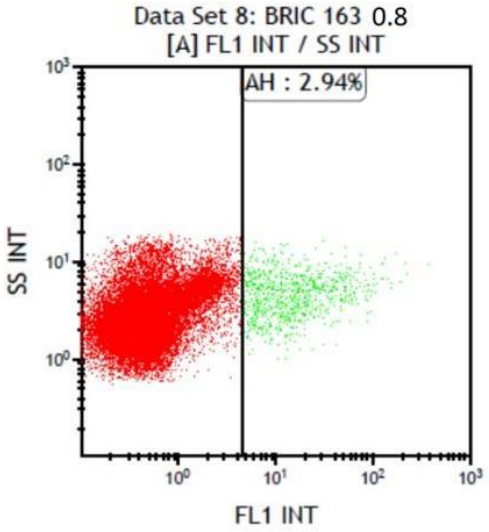



**Figure 4.21 Gated Scatterplot for labelled BRIC163-488** Flow cytometric titration analysis at the chosen dilution of 0.8 µg/ml, showing separation of BRIC163-488 (Intracellular GPA) postive (green) and BRIC163-488 negative (red) populations on the Navios flow cytometer.

### 4.2.3 Flow Cytometric Analysis

To mitigate against the amount of *in vitro* vesicle release the following measures were taken.

All samples were collected in Sodium citrate vacutainer® tubes as described by Xiong *et al.* (2012). The whole blood was then centrifuged (Sorvall Legend T, Thermo Fisher, UK) at 2000 x g for 10 minutes at room temperature with no brake. This ensured separation of any cells from the plasma. Samples were processed under sterile conditions, to prevent microbial growth which may affect any vesicles present and can also show up as false positives in flow cytometric analysis. Within 2 hours of collection all samples were processed, and the tubes containing the separated plasma and cells were stored at 4°C until collection. Upon arrival in Bristol, the platelet poor plasma was centrifuged (Sorvall Legend RT, Thermo Fisher, UK) at 2500 x g for 15 minutes at room temperature with no brake, to remove any remaining cells and large extracellular debris, and to ensure only small vesicles or microparticles were present before analysis.

In order to detect all Red Cell Derived Microparticles (RCDP) in SCD plasma we stained with BRIC256, an antibody recognising an extracellular epitope on GPA, and BRIC163, an antibody recognising an intracellular epitope on GPA, so we could distinguish between right-side out vesicles (BRIC256 positive) and inside-out AVs (BRIC163 positive) respectively. To detect PS positive vesicles, we stained with FITC-conjugated Annexin V. Dual stain tubes were also included to establish whether the different populations of RCDMV observed were extracellular GPA / PS positive, or extracellular GPA / intracellular GPA positive which would therefore mean that they were likely to be unsealed membrane fragments from lysed red cells rather than discrete RCDP.
Serial samples for 0h, 24h, 48, 72h and ≥120h were received for all healthy controls in the study. For steady state SCD patients, only 0h samples were received, as these were obtained at routine day haemoglobinopathy day unit appointments. Serial samples were received for SCD crisis patients 1, 2, 3 , 4, 5, 8 and 9. This is reflected in the graphs displaying monitoring of RCDP over time which omit the data for steady state controls and those SCD crisis patients for whom serial samples were not obtained (SCD crisis patients 1, 6, 7, 10 and 11).

### 4.2.4 BRIC256-647 - Right-side out RCDP

At 0h, when comparing the SCD crisis patients (mean: 2210 events/µl; SD: +/- 1852 events/µl) with healthy controls (median: 572 events/µl; SD:+/- 234 events/µl), there was a 4-fold increase in right-side out RCDP. There was a larger 6-fold increase in steady state SCD patients (median: 3612 events/µl; SD: +/- 2695 events/µl) when compared to healthy controls (see figure 4.22). The differences in right-side out RCDP between healthy controls and SCD crisis patients was not statistically significant (p=0.10), neither was the difference between healthy controls or steady state SCD patients (p=0.11).

There were outliers in the data, most notably steady state controls 3 and 4, and this may have skewed the data. These were excluded from the average

calculation by use of a median average. However even when this was taken into account, the average RCDP number for each cohort do not alter. Figure 4.22 appears to show a slight trend towards increased BRIC256-647 extracellular GPA-positive vesicles in SCD crisis patients which may have been more pronounced given a larger dataset. The fact that more data may have informed a more accurate outcome is one of the recognised limitations of the study.

Comparison of the levels of extracellular GPA-positive RCDMV over the course of admission did not show any signficant difference between healthy controls and SCD patients in crisis, with levels of BRIC256-647 extracellular GPA-positive RCDMV generally stable throughout the period monitored (see figure 4.23). SCD crisis patient 1 showed an increase in extracellular GPA-pos RCDMV over the course of their admission before they were discharged at 24h. SCD crisis patient 5 also showed an increase, which then fell at 48h before discharge.

The elevated levels of MVs in patients 1 and 5 are consistently high in flow cytometry studies throughout the study, but these findings of increased levels of MV in their plasma were not associated with an increased length of admission in either of these patients.

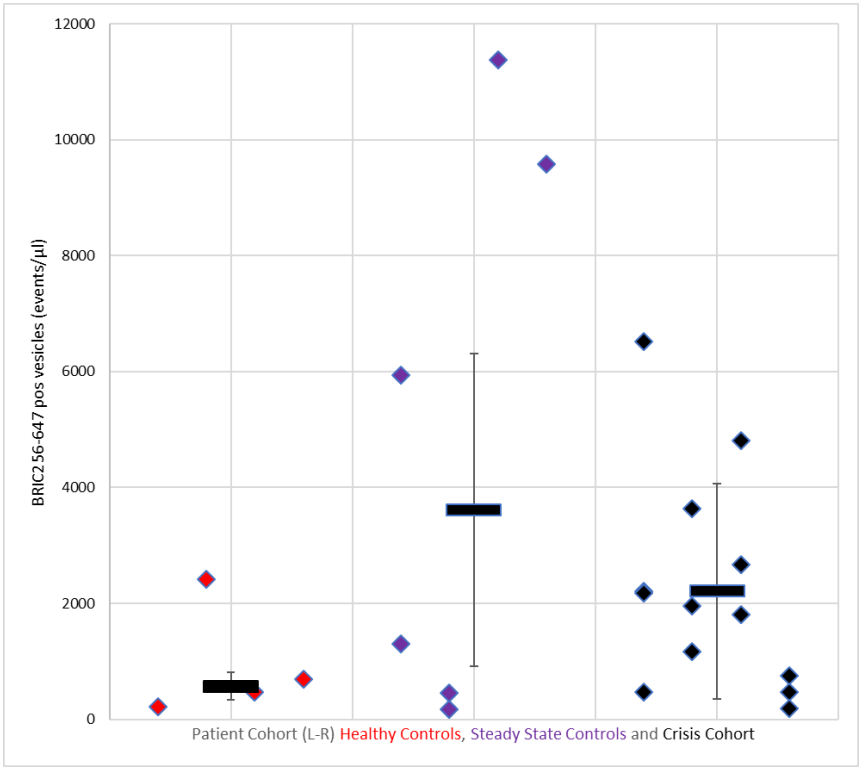



**Figure 4.22 Flow cytometric analysis of BRIC256-647 labelled RCDMV at 0h in 3 studied cohorts**; Healthy controls, Steady State SCD patients and Crisis Cohort SCD patients. There was a 4-fold increase in extracellular-GPA pos RCDMV between healthy controls and SCD crisis patients. There was a larger 6-fold increase between steady state SCD patients and healthy controls. The differences between the cohort means were not statistically significant.

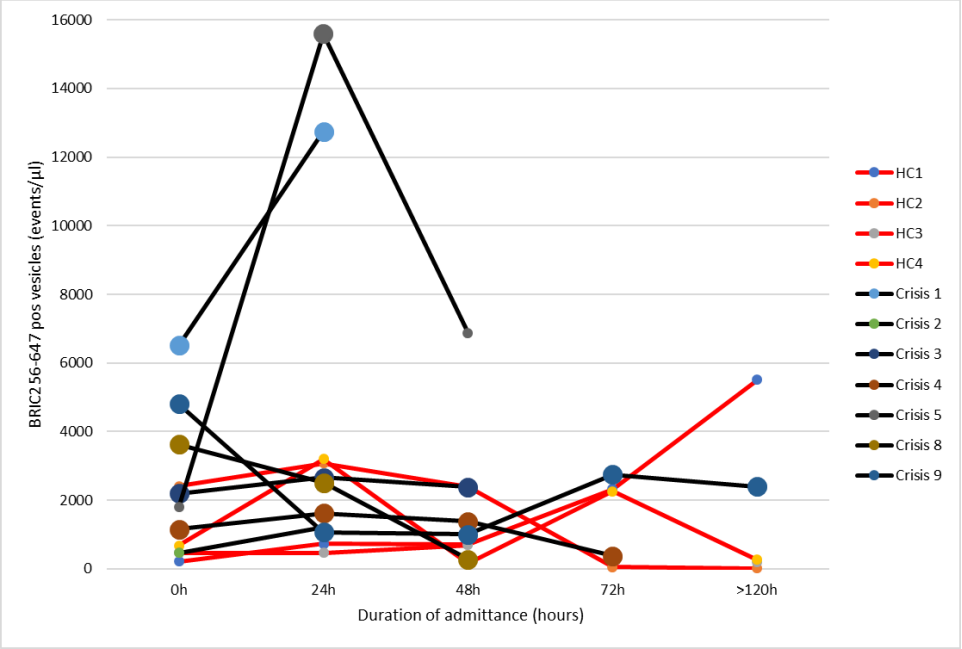



**Figure 4.23 flow cytometric analysis of BRIC256-647 labelled vesicles (events/µl) over time admitted (h).** Studied cohorts where serial samples obtained only; Healthy controls (red line, small markers) and Crisis Cohort SCD patients (black line, large markers)

### 4.2.5 BRIC163-488 – Inside-out Autophagic Vesicles

When quantifying AVs using BRIC163-488 at 0h, there were approximately equal numbers of AVs observed in the healthy control cohort (median: 1367 events/µl; SD +/- 1013 events/µl) compared to the SCD crisis patients (Mean: 1068 events/µl; SD +/-1307 events/µl). The steady state SCD patients had on average, twice as many AVs in their plasma (median: 2654 events/µl; SD +/- events/µl as the SCD crisis cohort (median: 1068 events/µl; SD +/- 1307 events/µl) and healthy controls (median: 1367 events/µl; SD +/- 1013 events/µl) (see figure 4.24). The differences between the averages of the cohorts studied were not statistically significant when comparing healthy controls to SCD patients in crisis (p=0.42) or healthy controls to steady state SCD patients (p=0.37) or finally, steady state SCD patients to SCD crisis patients (p=0.34).

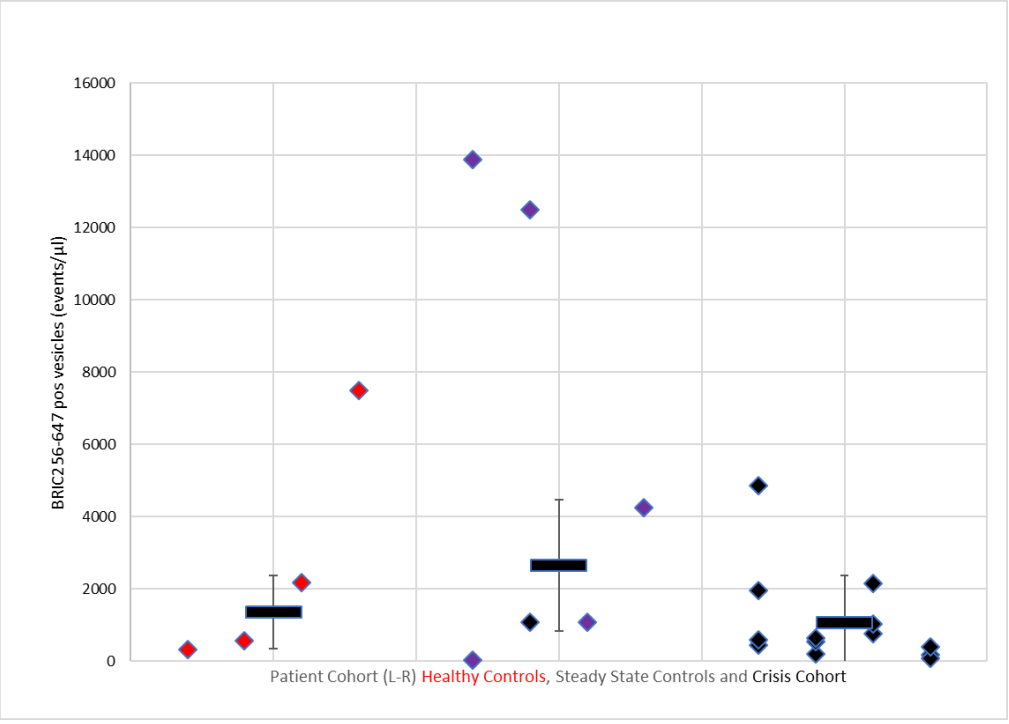



**Figure 4.24 Flow cytometric anlysis of BRIC163-488 labelled vesicles at at 0h in 3 studied cohorts**; Healthy controls, Steady State SCD patients and Crisis Cohort SCD patients. There was a no real observed increase in intracellular GPA-pos RCDMV between healthy controls and SCD crisis patients. The steady state SCD patients had on average, twice as many AVs in their plasma as the SCD crisis cohort and healthy control, although the differences between the cohort averages were not statistically significant.

Over the period of admission, AV levels declined for SCD crisis patients 1 and 5, but AV number remained stable for the other SCD crisis patients studied, except for patient 2, whose AV numer rose before discharge (see figure 4.25). For healthy control 4, AV number was higher at admission, but fell after 24h. However the numbers of AVs for healthy control 4 were higher throughout the study period than any of the SCD crisis patients. These results suggest that AV number is not a significant contributory factor to the pathophysiology of VOC.

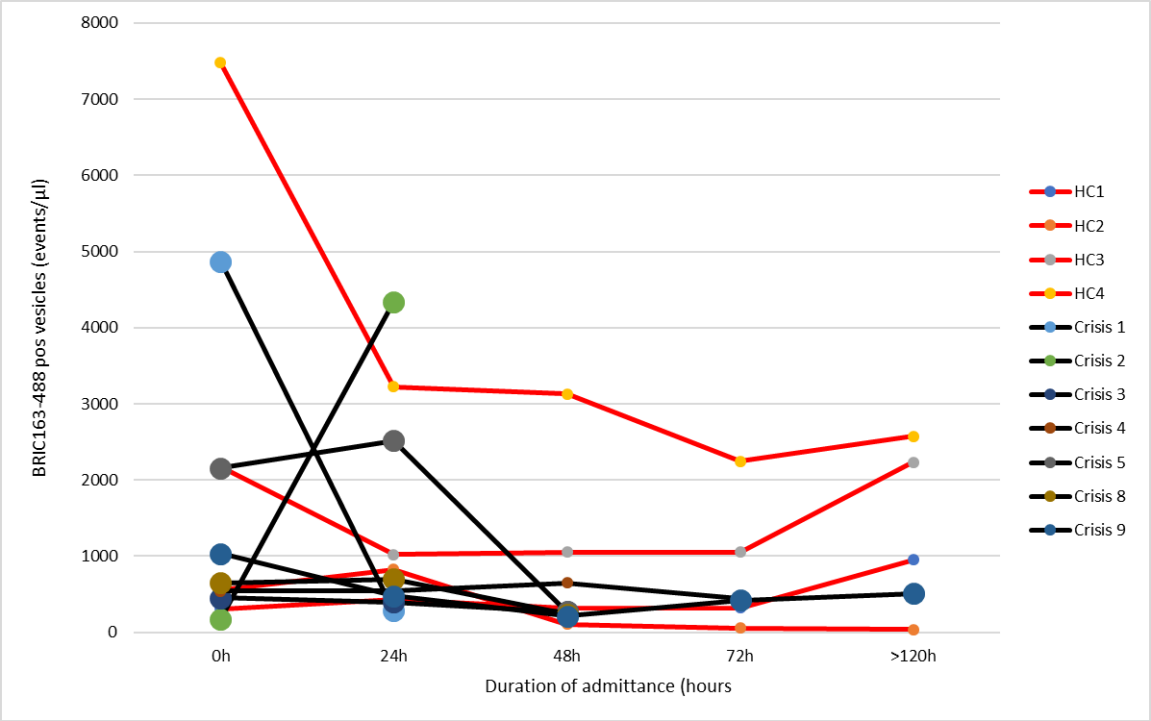



**Figure 4.25 BRIC163-488 labelled vesicles (events/µl) over time admitted (h).** Studied cohorts where serial samples obtained only. Healthy controls (red line, small markers) and Crisis Cohort SCD patients (black line, large markers)

### 4.2.6 Annexin V FITC labelling – PS positive MVs

Annexin V FITC staining for PS positive MV showed that these were the most numerous population out of all subpopulations of MVs studied (see figure 4.26 and 4.32). These results reflect the observations in live cell confocal microscopy analysis and are in line with other studies (de Jong *et al.* 2001; Westerman *et al.* 2008; van Tits *et al.* 2009). PS positive vesicle number was approximately equal between SCD crisis patients (mean:13630 events/µl; SD +/- 7741 events/µl) and SCD steady state controls (mean:13334 events/µl; SD +/- 9562 events/µl). PS positive MV number in SCD crisis patients was 30% higher than healthy controls (median: 9939 events/µl; SD +/- 7025 events/µl). however there were no statistically significant differences between the 3 cohorts with the differences in p values not <0.05, where SCD crisis patients Vs healthy controls (p=0.97).

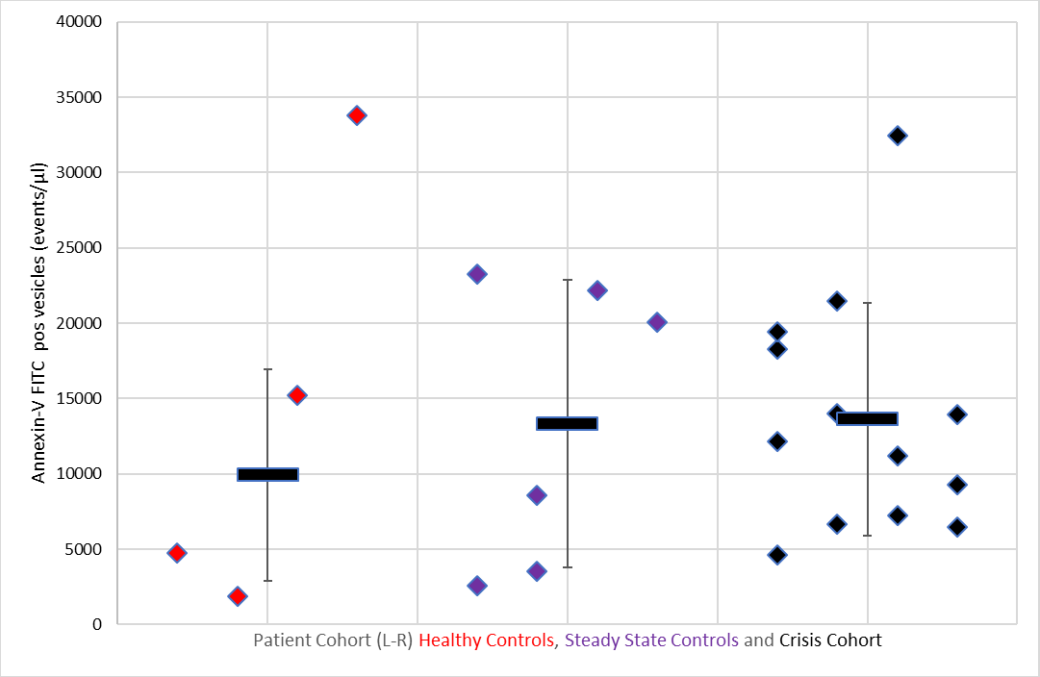



**Figure 4.26 Anexin-V FITC labelled vesicles at 0h in 3 studied cohorts**; Healthy controls, Steady State SCD patients and Crisis Cohort SCD patients. There was a 50% increase in PS pos RCDMV between healthy controls and SCD crisis patients, but this was not significant. No significant difference was observed between SCD crisis patients and SCD steady state patients.

Throughout the course of the study, PS positive MVs did not remain stable in either the healthy controls or the SCD crisis patients studied (see figure 4.27). There was no observable difference in the levels of PS pos MVs between healthy controls and SCD crisis patients studied, and no decreases in PS positive MV number were noted over the time period observed. Variation in PS expression may be accounted for by the ongoing inflammatory state present in SCD. Alternatively, limitations in the methodology particularly in the use of annexin V, rather than an alternative PS-specific label, such as lactahedrin, may have led to the variation seen, and are further explored in the discussion.

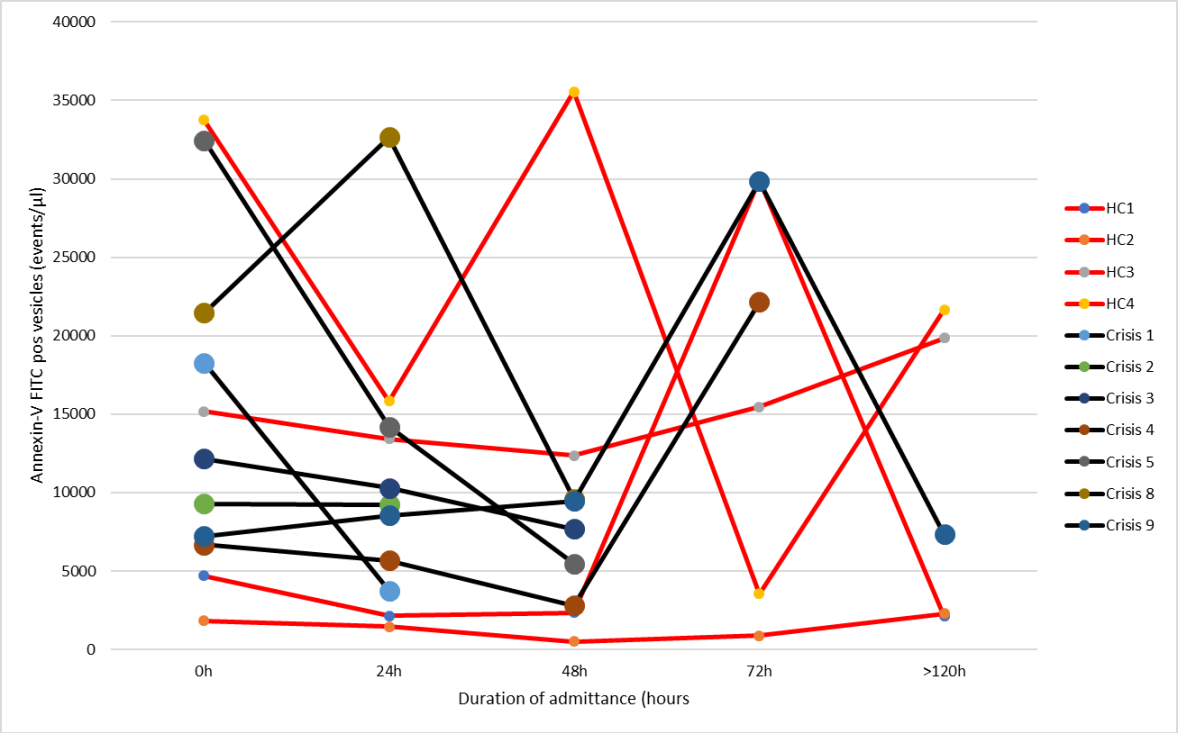



**Figure 4.27 Annexin-V FITC labelled vesicles (events/µl) over time admitted (h).** Studied cohorts where serial samples obtained only. Healthy controls (red line, small markers) and Crisis Cohort SCD patients (black line, large markers)

### 4.2.7 Dual-labelled BRIC256-647 / BRIC163-488 RCDP – Unsealed RBC Membrane Fragments

There was no significant difference observed between in cohorts dual-labelled with BRIC256-647 / BRIC163-488 RCDP (see figure 4.28). These MVs, which most likely represent unsealed membrane fragments from lysed red cells were the smallest subpopulation of MVs detected, although it is known that they vary greatly in size and therefore would not all be captured by our size gating protocol. There was an approximate two-fold increase in dual-labelled events detected in SCD steady state patients (mean:1893 events/µl; SD +/- 1800 events/µl) compared to SCD crisis patients (mean:765 events/µl; SD +/- 790 events/µl), and healthy controls (median: 923 events/µl; SD +/- 341 events/µl). However, no statistically significant differences were observed between any cohort. The increase observed in steady state controls was not significantly higher when compared to healthy cohorts or SCD crisis patients ($p=0.31$ and $p=0.69$ respectively). In addition, SCD crisis patients did not differ significantly from healthy controls ($p=0.50$) or SCD steady state patients ($p=0.69$).

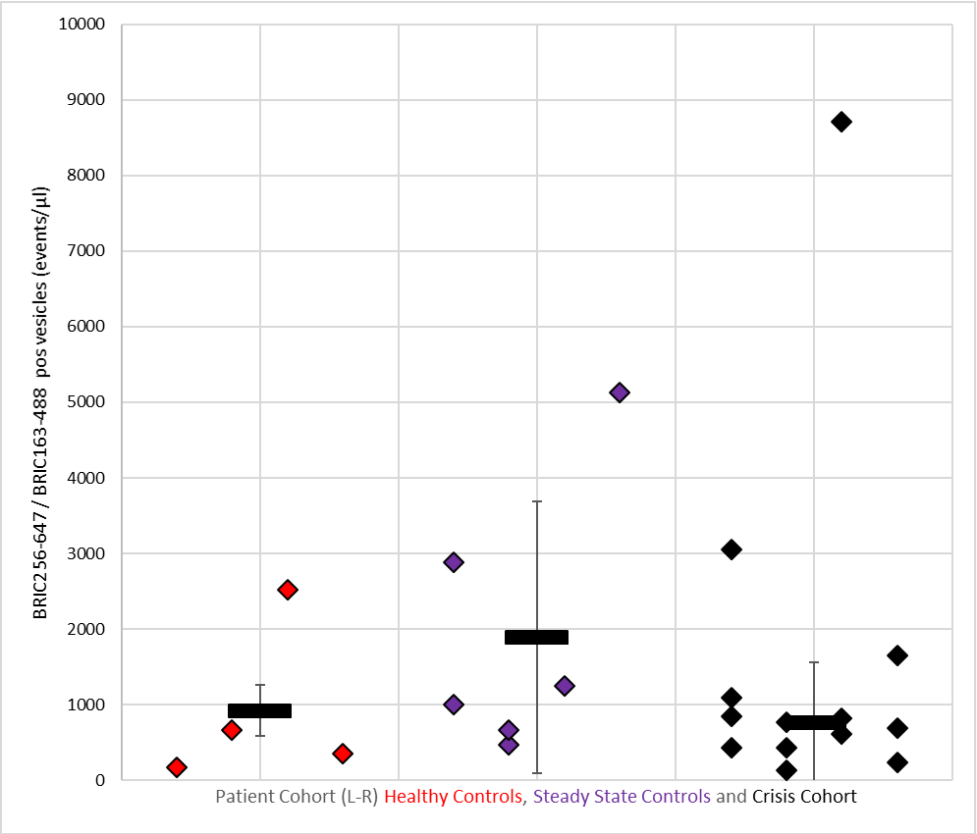



**Figure 4.28 BRIC256-647 / BRIC163-488 dual-labelled vesicles** at 0h in 3 studied cohorts; Healthy controls, Steady State SCD patients and Crisis Cohort SCD patients. There was a 2-fold increase in dual labelled extracellular-GPA pos RCDP between SCD steady state patients compared to SCD crisis patients and healthy controls. However, the differences between the cohort averages were not statistically significant.

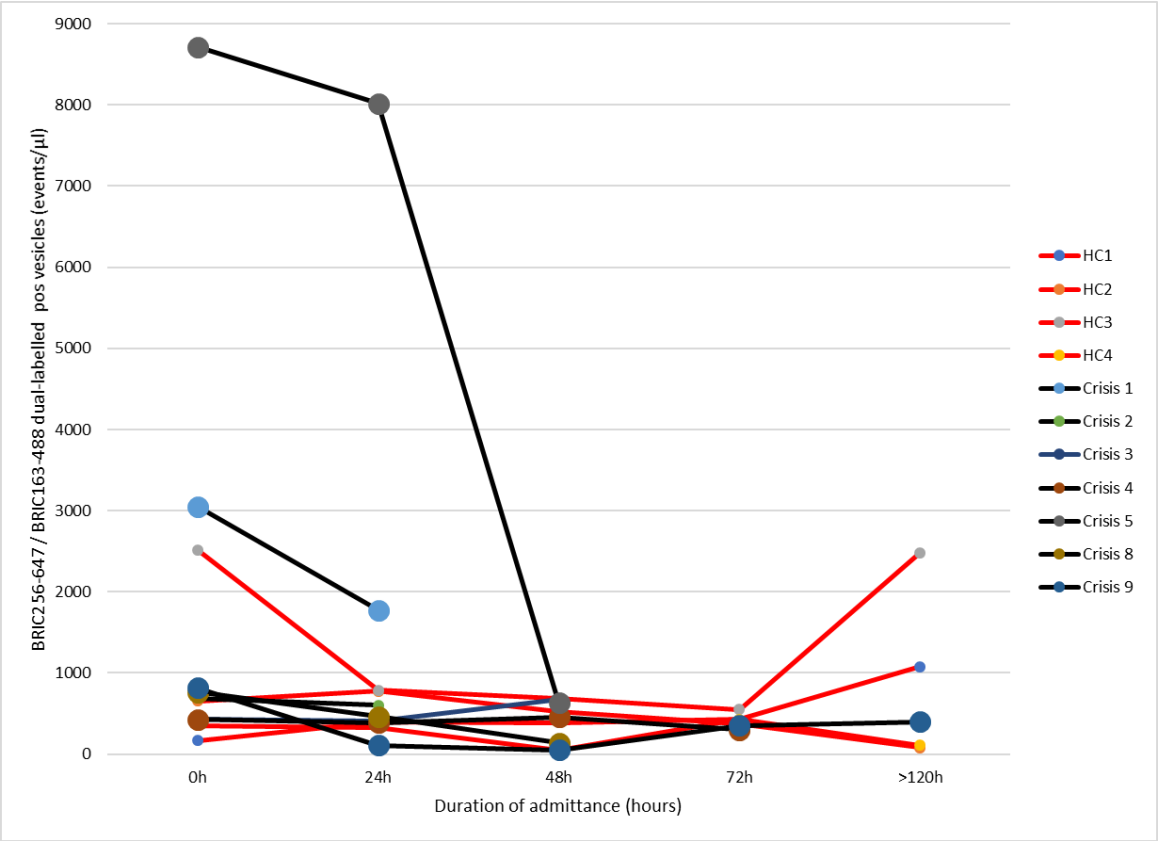



**Figure 4.29 BRIC256-647 / BRIC163-488 dual-labelled vesicles (events/µl) over time admitted (h).** Studied cohorts where serial samples obtained only; Healthy controls and Crisis Cohort SCD patients

During the course of admission, crisis patients 1 and 5, and healthy control 3 had higher levels of dual-labelled membrane fragments in their plasma which decreased at, or shortly after 24 hours following treatment (see figure 4.29). The levels of fragments in the plasma did not have effect on the period of admission, and therefore the severity of crisis as other patients (patient 9) were admitted for longer. However, this could have been due to other co-morbidities or contributing social factors, rather than the severity of the VOC event. The majority of patients and healthy controls had stable levels of dual-labelled red cell fragments in their plasma throughout the study period, suggesting that these do not contribute to VOC.

### 4.2.8 Dual-labelled BRIC256-647 / Annexin V FITC – PS-positive RCDP

Dual-labelled extracellular GPA-pos RCDP, which express procoagulant PS were 6-fold higher on average in SCD crisis patients (mean:5402 events/µl; SD +/- 3612 events/µl) when compared to healthy controls (mean:811 events/µl; SD +/- 650 events/µl), and 50% higher than steady state SCD patients (mean:3520 events/µl; SD +/- 2837 events/µl) (see figure 4.30). These findings were not significant however, with no statistically significant differences observed between the averages of the SCD crisis patients and healthy controls (p=0.19), or SCD crisis patients and SCD steady state patients (p=0.42)

The presence of increased RCDP which express PS in SCD patients, whether in steady state or in VOC is not surprising. These findings are in concordance with the exisiting literature and are in line with the results of the confocal microscsopy testing on the three cohorts studied.

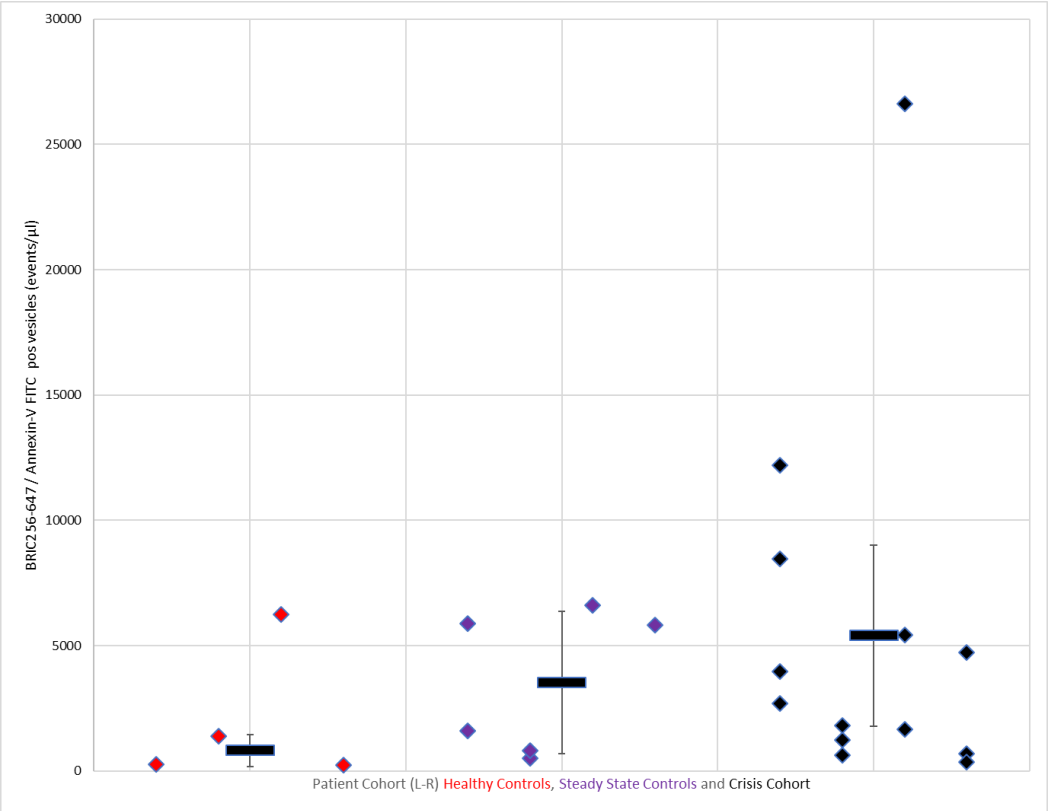



**Figure 4.30 BRIC256-647 / Annexin-V FITC dual-labelled vesicles at 0h in 3 studied cohorts**; Healthy controls, Steady State SCD patients and Crisis Cohort SCD patients. Dual-labelled extracellular GPA-pos RCDP, which express procoagulant PS were 6-fold higher on average in SCD crisis patients when compared to healthy controls, and 50% higher than steady state SCD patients .However, the differences between the cohort averages were not statistically significant.

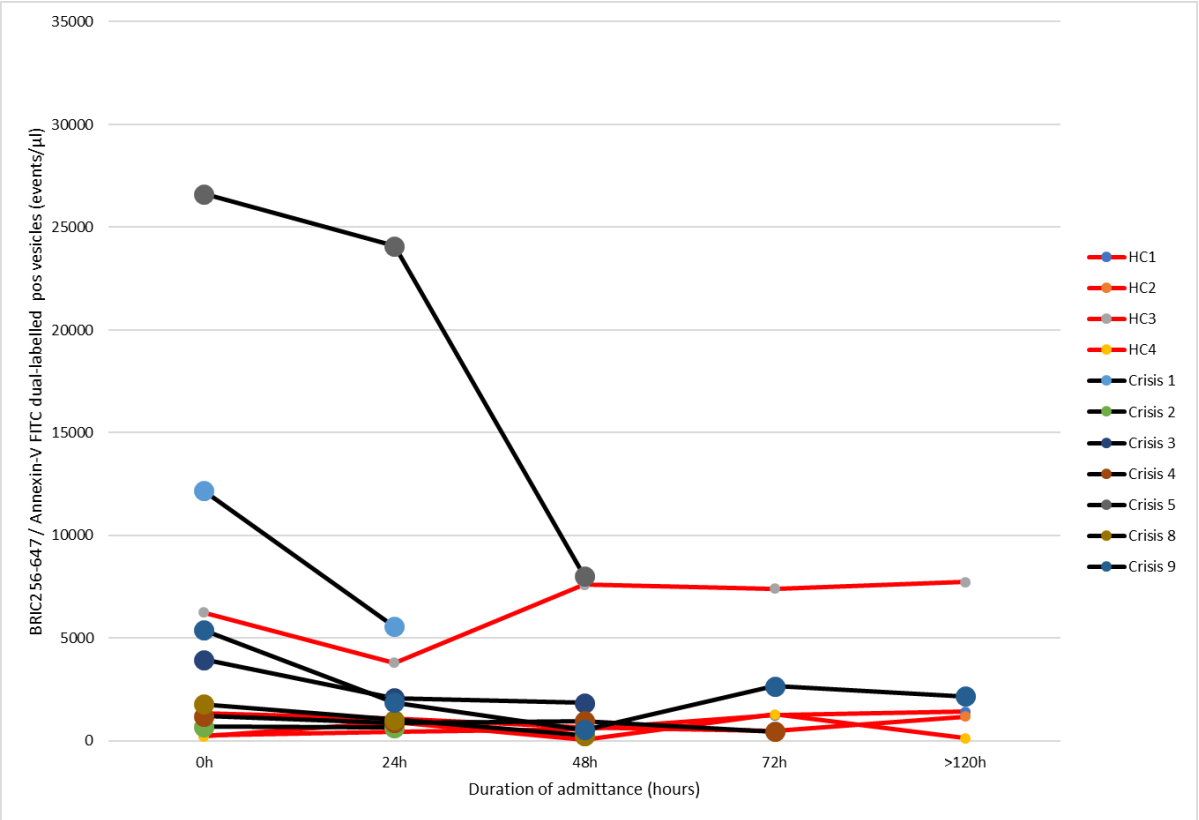



**Figure 4.31 BRIC256-647 / Annexin-V FITC dual-labelled vesicles (events/µl) over time admitted (h)**. Studied cohorts where serial samples obtained only; Healthy controls and Crisis Cohort SCD patients

Over the course of the study, levels of dual-labelled extracellular GPA-pos / PS pos RCDP, remained stable for the majority of the SCD crisis patients and healthy controls tested, which suggests following treatment no increase or decrease in PS positive RCDP occurs (see figure 4.31). For the two patients with the highest number of PS pos RCDP, patients 1 and 5 fell over the course of admission and treatment, however PS pos RCDP did not have any correlation with length of stay as they were discharged at 24h and 48h respectively, when compared to patient to 9 who had much lower PS positive RCDP present and a longer period of admission. Healthy control 3 had higher PS pos RCDP levels than most of the SCD crisis patients tested, but with no manifestation of thrombosis, nor any declared significant past medical history.

### 4.3 Microvesicle subpopulation comparison

Of all of the subpopulations of MV detected, Annexin V PS positive MV were the most numerous in the plasma of the cohorts studied. With these microparticles being higher in number in both the SCD crisis patients (mean:13630 events/µl; SD +/- 7741 events/µl) and steady state patients (mean:13334 events/µl; SD +/- 9562 events/µl) than the healthy control cohort (median: 9939 events/µl; SD +/- 7025 events/µl). When comparing the numbers of these PS-pos MV with ones which were RBC in origin (BRIC256-647 / Annexin V dual-labelled), RCDMV which were extracellular GPA-positive as well as PS positive were the second most numerous particle detected, but were not as numerous as the PS positive, single stained MVs, suggesting a subpopulation of MVs that are not RBC-derived which were not excluded from analysis by pre-analytical processing and setup protocols established to reduce containation by other MVs.

The majority of the subpopulations of MVs studied were elevated in SCD crisis and SCD steady state patients when compared to healthy controls, with the exception of Intracellular GPA (BRIC163) stained AVs, where AV number was higher in the healthy control and SCD steady state cohorts than the SCD crisis cohort. However, this difference in increase MV number was not statistically significant in any of the cohorts studied.

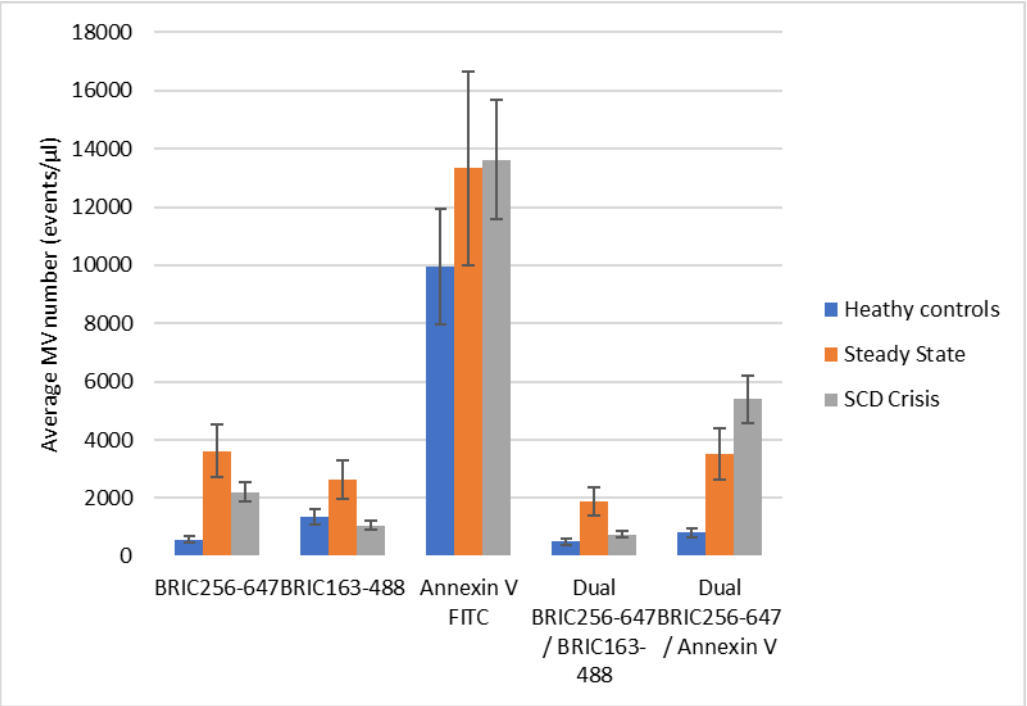



**Figure 4.32. Average microvesicle number categorised by flow cytometric labelling.** Average (Mean average +/- 2 SD) MV numbers in each MV subpopulation were measured using single markers to both extracellular (BRIC256-647) and Intracellular (BRIC163-488) RBC-derived GPA, and Phosphatidylserine (Annexin V FITC), in addition to dual staining to detect RBC-derived fragments and RBC-derived right-side out and inside-out PS positive fragments. PS positive MV were the largest subpopulation detected. MVs were increased in all SCD crisis and steady state patients when compared with healthy controls with the exception of BRIC163- 488 labelled AV. Mean averages (and SE) of each patient cohort shown.

### 4.4 Is there a significant threshold for MV number to predict VOC?

To determine if there was any correlation for any of the MV subpopulations in plasma to predict VOC, statistical analysis, using univariate analyses and a Binomial Generalised Linear Model (GLM) was performed, examining the following 7 variables;

(i) Antibody BRIC256
(ii) Antibody BRIC163
(iii) Antibody Annexin V
(iv) Antibody BRIC256/163
(v) Antibody BRIC256/Annexin V
(vi) Sickle Cell Haemoglobin (HbS) percentage
(vii) Reticulocyte count.

The results from fitting a Binomial Generalised Linear Model with logit link for a patient experiencing VOCs at the time of admission are shown below, in Table 4.1. The only variable (at the 5% significance level) found to have a statistically significant explanatory value associated with VOCs in these sickle cell patients was the HbS percentage, with increased percentages being associated with greater likelihood of patients experiencing VOCs. The evidence however is weak, as the 95% confidence interval (CI) for the odds ratio includes 1.00, which denotes no change in the likelihood of patient's crisis for increasing values of HbS. The results of the analysis showed no statistically significant correlation between any microvesicle subpopulation number studied and VOC. These results are limited by the small number of observations included in the analysis.

**Table 4.1. Factors affecting whether Sickle Cells patients experience Vaso-Occlusive Crises.**

| Factor | Number | Odds ratio | 95% CI | P value |
|---|---|---|---|---|
| Sickle Cell Haemoglobin (HbS) percentage | 13 | 1.16 | 0.862 - 1.557 | 0.0003 |
| *Other factors considered:*<br>Number of vesicles in the plasma of patient using antibody BRIC256 (p=0.76)<br>Number of vesicles in the plasma of patient using antibody BRIC163 (p=0.88)<br>Number of vesicles in the plasma of patient using antibody Annexin V (p=0.42)<br>Number of vesicles in the plasma of patient using antibody BRIC256-163 (p=0.86)<br>Number of vesicles in the plasma of patient using antibody BRIC256 Annexin V (p=0.35)<br>Reticulocyte count (p=0.24) | | | | |

# Chapter 5: Discussion

The pathological symptoms of SCD are caused by the repeated sickling and unsickling of the HbS erythrocyte leading to polymerisation of HbS, a reduction in membrane deformability, subsequent membrane damage and membrane loss via membrane blebbing, producing microvesicles, which express surface exposed PS (Westerman *et al.* 2008; van Tits *et al.* 2009; van Beers *et al*. 2009; Gerotziafas *et al.* 2012). This premature destruction of the HbS erythrocyte also results in an increase in the production of their progenitor, the reticulocyte (Bernard *et* al. 2006). Previous work by Griffiths *et al.* (2012) describes a third mechanism of microvesicle formation during the later stages of reticulocyte maturation, which results in the production of a subset of microvesicles termed the Autophagic Vesicle (AV). Later, studies by Mankelow *et al.* (2015) showed that AV's had an inside-out orientation, resulting in discrete areas of surface-exposed PS and exposure of the cytoplasmic domains of red cell plasma membrane proteins. They further demonstrated that the numbers of red cells with AVs are elevated in steady-state SCD patients and patients who had been splenectomised. They postulated that ordinarily these AVs would be removed by splenic macrophages in the reticuloendothelial system, but in the absence of a spleen they would eventually be released directly into plasma where, due to the expression of PS on the AV surface they could contribute to the prothrombotic state that causes VOC. They suggested that by using apheresis it may be possible to remove PS-positive vesicles from circulating red cells *ex vivo*, and that this may provide a useful additional therapy for patients with SCD, potentially reducing the incidence of VOC. However, the numbers of AV in SCD patients in VOC was not known, and therefore the need and potential efficacy of this proposed intervention was unknown.

In this feasibility study we describe the analysis of MV in samples obtained from three cohorts; healthy individuals, SCD patients in a steady state and SCD patients in VOC. We use a combination of confocal microscopy and flow cytometric analysis to quantify MV numbers on RBC and in plasma

respectively. MV subpopulations quantified include right-side out RCDPs, inside-out AVs, PS-positive MVs, RBC-derived membrane fragments and PS-pos RCDP. Subpopulations in these three cohorts were examined to see if there are any statistically significant differences between healthy individuals, steady state SCD patients and those SCD patients in VOC.

**5.1 Confocal Microscopy**

Using both live cell and fixed cell confocal microscopy experiments to examine the RBC, PS-positive vesicles and GPA-positive vesicles were labelled with monoclonal antibodies (Annexin V-FITC and R10-488), and subsequently quantified. In both live cell and fixed cell confocal microscopy studies, statistically significantly higher numbers of PS-positive and GPA-positive vesicles were seen when comparing healthy controls to steady state SCD patients and SCD patients in VOC.

In live cell confocal microscopy experiments, which looked at PS-positive vesicles, the differences in PS-positive vesicle percentage were statistically significant when comparing healthy controls and crisis patients ($p<0.0001$) with the mean vesicle percentage being significantly larger in the latter than in the former group.

In fixed cell confocal microscopy experiments, which looked at extracellular trypsin sensitive Glycophorin A (GPA) positive vesicles, using the mAb R10, statistically significant differences were observed between the healthy controls and crisis patients ($p<0.0001$), with the mean vesicle percentage being significantly larger in the latter than in the former group. There was however no significant difference in R10-positive vesicle expression between the steady state and SCD crisis cohorts. There were differences in amounts of PS and R10 labelling in both live cell and fixed cell confocal microscopy experiments, respectively. If these represent PS decorated AVs then this indicates that externalisation of PS had already taken place, but there were further AVs to become externalised, and therefore express PS, potentially

increasing the amount of procoagulant phospholipid at the RBC membrane surface.

During the period of admission, both PS-positive and GPA-pos vesicle number on RBC declined in the SCD crisis patients when compared with the healthy control cohort. This may be due to the patients receiving either exchange or top-up transfusion during the course of their admission, reducing the MV number by reducing the circulating volume of HbS RBC by either replacement or dilution by way of addition of healthy donor-derived RBC.

### 5.2 Flow Cytometry

Flow cytometric analysis of the plasma showed that there were different subpopulations of RCDP as well as a large number of PS-pos microparticles/microvesicles. This suggests that the situation is not as straightforward as there just being a regular population of “red cell derived vesicles” present. Of all subpopulations of MV detected, PS-positive MV were the most numerous in the plasma of all the cohorts studied. This is in line with the confocal microscopy studies which showed that statistically significant numbers of PS-positive MV were seen in SCD patients in VOC and in steady state SCD patients when compared to healthy controls.

However, unlike the confocal microscopy studies, levels of PS-pos MVs in the plasma were not elevated enough to achieve statistical significance and did not decrease during the period of the study but were variable from day to day. Day-to-day variation in MP in the blood of SCD patients will occur due to fluctuations in inflammatory intensity (van der Pol *et al.* 2016). It is unknown whether these detected PS-pos MV were all reticulocyte or erythrocyte-derived, or whether they were particles derived from either platelets or endothelial cells (van Beers *et al. .*2009; del Conde *et al.* 2005) but it is likely that from their larger number when compared to dual-labelled RBC-derived PS-positive MV, they are of a combination of cellular origin.

This would indicate that the although the sampling and processing mitigation measures taken to reduce cellular contamination appear effective, MV and MP contamination cannot be removed or reduced by the processing measures taken in this study. The finding that PS-positive MV were raised in all cohorts but were higher in both steady state and VOC SCD patient cohorts when compared to healthy controls, is in line with other published studies (Wood *et al.* 2006; Kennedy, 2015; Whelihan *et al*. 2016) and is indicative of the role that PS plays in VOC and thrombogenic states (Setty *et al.* 2016).

When looking specifically at RCDPs, the largest subpopulation detected were the dual-labelled RCDP, which were PS-positive (BRIC256-647 / annexin V). Levels of these remained relatively stable throughout the course of the study in most patients and controls observed. Two of the VOC SCD patients (patients 1 ad 5) had increased numbers of BRIC256-647 / annexin-V dual-labelled vesicles at admission, but increased levels of these dual-labelled vesicles were not associated with increased length of admission, and in one healthy control, levels of these dual-labelled MV were higher than five of the VOC SCD patients but were not associated with any clinical symptoms or associated disease state. The presence of this subpopulation as the largest RCDMV population correlates with previous studies and the effects of RBC-exposed PS in the pathophysiology of SCD (Wood *et al.* 2006; Kennedy, 2015; Whelihan *et al*. 2016)

There was a difference in the overall number of single-labelled (annexin V labelled) PS-positive MV, described above and dual-labelled RCDP, which were PS-positive (BRIC256-647 / annexin V). This would also support the suggestion that there were large numbers of PS-positive MVs or small particulate matter that was not RBC-derived and may be microparticles from platelets or endothelial cells (van Beers *et al.* 2009; del Conde *et al.* 2005). Additionally, it may also be that the binding of BRIC256-647 / Annexin V mAbs was not effective due to steric hindrance. This occurs when the size of the antibody molecule (150,000 Daltons for IgG) interferes with the reaction of the antibody molecules with the surface antigenic determinant, particularly if the

antigenic determinants are closely spaced (Kent *et al*. 1978). If this is the case the two mAbs would not be able to bind to their respective sites due to the small size of the MV and the binding sites upon it. While annexin V has been the most commonly used label for the detection of externalized PS, the failure of the low scatter small particles to bind annexin V in addition to BRIC256-648 may also be due to the curvature of these sub-cellular particles rather than an absence of PS. A study by Shi *et al.* (2004). suggested an alternative PS marker, lactadherin. Lactadherin may be a more specific and sensitive marker in studies such as these by virtue of its ability to bind to MP having smaller radii of curvature.

Of the remaining RCDP detected by flow cytometry, small populations of right-side out (BRIC256-647 positive) RCDP and inside-out (BRIC163-488 positive) AV respectively were detected in the plasma. Of these two subpopulations, right side out MV were the most numerous, reflecting MV produced as part of HbS-positive erythrocytes undergoing cellular blebbing during repeated cycles of cellular sickling and recovery (Allen *et al.* 1982). A smaller subpopulation of inside-out MV, most likely AV, which are produced in the latter stages of reticulocyte maturation (Griffiths *et al.* 2012; Mankelow *et al.* 2015) were detected in the plasma. In both of these subpopulations of right-side out and inside-out RCDP, the RCDP were more numerous in steady state SCD patients than patients in VOC.

The increased presence of RCDP, in particular AV in steady state SCD patients when compared to SCD in VOC is a novel finding, however the differences in MV number were not significant enough to achieve statistical significance and the small numbers of patients in each of the cohorts may have skewed the results. They did not decrease over time in either healthy controls or SCD patients, with numbers remaining relatively constant in most participants studied, which would indicate that they are being produced constantly and are not affected by treatment during the course of admission.

Quantification of dual stained extracellular / intracellular GPA-pos RCDP, (BRIC256-647 / BRIC163-488 labelled), which most likely represent unsealed RBC membrane fragments showed these were the smallest subpopulation of MV detected. Flow cytometric gating was set to capture small, lower scatter events, measuring <1µm, and more specifically RCDP, which have an average size of 0.15µm (Piccin *et al.* 2007). As some RBC membrane fragments will have been greater than this size it is likely that they were excluded from the endpoint analysis and are therefore not a significant subpopulation in this study. Despite their size range, these MVs would have expressed PS and could still be a significant factor in VOC.

Finally, when looking at whether MV quantification enabled prediction of VOC, no statistical significance was found in any of the MV subpopulations studied, with the only significant predictor of VOC being HbS%, which is in agreement with current guidelines regarding the monitoring and management of SCD patients (Davis *et al.* 2017b).

### 5.3 Limitations

Limitations associated with the study included the small size of the cohorts, which affected the analysis of results where outliers were present in the flow cytometry experiments where single specificity mAb labelling was used. If more participants had been recruited to the study, it would have increased the confidence in the findings and significance of the statistical analysis. Factors affecting recruitment included a limited cohort at a single study site, consenting and total time allocated for the study.

We encountered difficulty in serial monitoring of patients during admittance for VOC, as some patients were admitted for very short (<24h) time periods before discharge. This meant that with some patients only 1 sample was obtained for analysis from admission to discharge, which then made serial monitoring impossible. Day-to-day variation in MV in the blood of SCD patients will occur due to fluctuations in inflammatory intensity, even within

steady state patients. By not having this information for the steady state cohort, as they were day case patients, and some of the SCD VOC cohort patients due to their recovery and discharge made longitudinal analysis and trending of results problematic.

No association was found between length of admission and MV number, with some patients discharged at earlier points even when their vesicle number throughout the study was higher. The length of admission however may not be solely attributable to severity of crisis, as additional factors such as social and personal issues may have affected the ability to discharge the patient at an earlier stage.

Fixed cell confocal microscopy experiments showed larger variation in results due to difficulties with fixation and staining. The impact of loss of cells during these experimental procedures, which have previously been optimised for healthy RBC populations, may have adversely affected the results, but they were still different enough to achieve statistical significance. In addition, expertise of the author with new techniques performed in the study may have introduced potential bias in the protocol.

Challenges associated with the flow cytometric experimental protocol included agglutination of the samples if left for longer than 30 minutes before analysis. In the original protocol patient's plasma was mixed with the respective mAb and then left to incubate on ice in the dark for 30 minutes. Next, 300µl of binding buffer was added to each tube and they were loaded onto the flow cytometer for analysis. The latter tubes in the flow cytometer run failed to acquire sufficient events to enable interpretation, and upon examination, agglutination was observed in the tubes which had been waiting for analysis. As the tubes contained citrated plasma and the annexin V binding buffer reagent contained $Ca^{2+}$ in order to facilitate binding of Annexin V to PS it was thought that this could potentiate agglutination in the sample if left for prolonged amounts of time before analysis.

To overcome this agglutination, tests were run as single tube assays, with annexin V binding buffer added to the test immediately before analysis on the flow cytometer. This prevented agglutination and enabled the analysis of the sample and acquisition by the flow cytometer (see figure 5.1). As mentioned earlier, annexin V has been the most commonly used label for the detection of externalized PS. The milk-derived protein, lactadherin may have been a more appropriate marker to use in this study, due to its increased specificity and sensitivity over annexin V, due to its ability to bind to MP having smaller radii of curvature. In addition, as its binding does not require re-calcification of citrated plasma samples which can lead to agglutination and an invalid test result, it may therefore be a more suitable reagent for PS pos MV analysis in future studies.

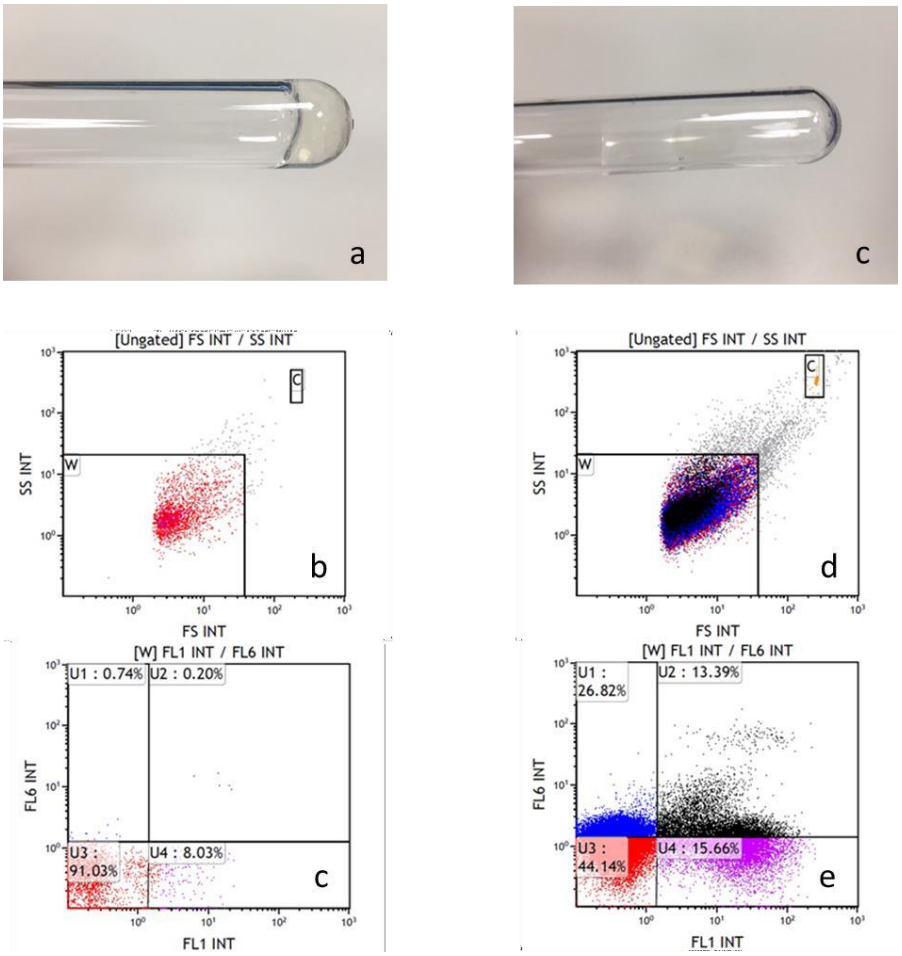



**Figure 5.1. Agglutination observed in flow cytometry testing.** Agglutination observed in multiple tube tests **(a)** caused low event number in gated FS/SS and FL1/FL6 scatter graphs **(b)** and **(c)**. No agglutination in single tube tests **(c)** or in the subsequent gated FS/SS and FL1/FL6 scatter graphs **(d)** and **(e)**. Caused by activation of clotting factors in patient serum due to the addition of $Ca^{2+}$ contained in the Annexin V flow buffer.

There remains an ongoing challenge to develop a method to accurately categorise and enumerate MV in plasma. In this study, despite attempts to identify and subsequently quantify small (<0.15µm) RCDP in plasma by including protocols to mitigate against contamination with microparticles and particulate matter from other cellular sources, it appears, from the levels of PS-positive microparticles, that this has not been wholly successful. There remains no consensus for flow-cytometry analysis of MPs (Hebbel and Key, 2016), which has led to the creation of numerous protocols which then make the most accurate strategy unclear. Flow cytometric analysis has historically been the most commonly used methodology for MP detection (Shet *et al.* 2003; van Beers *et al.* 2009; Piccin *et al.* 2015), but its ability to accurately detect and categorise the smallest particles is limited, which has led to varying reports of RCDP percentage, ranging from 3%-75% of total MV detected (Shet *et al.* 2003; van Beers *et al.* 2009; Piccin *et al.* 2015). The use of flow cytometry sizing beads may not be appropriate for calibration of modern flow cytometers to detect and define MV and MP of such small size. They have a different refractive index to RBC-derived vesicles and may therefore not be a true representative calibrating material. However, it should also be noted that all bead-based calibration strategies have been criticized for the fact that other standards with refractive indices closer to MP may be preferable for size calibration (van der Pol et al, 2014).

Studies have described a limitation of flow cytometric analysis in its inability to detect ‘swarms’ of MVs (Harrison and Gardiner, 2012). As MV’s are small, it is highly likely that they will be in increased concentrations in the analyte as it passes through the flow cell. Consequently, multiple swarming MVs may be detected at once by the laser beam of the flow cytometer. These swarming MVs will have a greater forward scatter and may be excluded from analysis if incorrectly size gated as a single large particle or cell. Dilution of the sample should decrease MV concentration and mitigate against MV swarming, decreasing the probability that multiple MVs passing through the laser beam will generate a false signal which is subsequently excluded from analysis.

Until standardisation of collection, processing and analysis is achieved, then accurate enumeration and classification of MVs by flow cytometry is an ongoing challenge. Attempts at standardisation have been made in workshops run by the International Society of Thrombosis and Haemostasis (ISTH) with a new initiative to standardize EV measurements
started in 2014. (van der Pol *et al.* 2014). Reference materials and samples were circulated to 33 laboratories worldwide for analysis by a specified technique. From this exercise, dedicated software has been developed which can automatically correct for differences in optical configurations of the flow cytometers used, and to correct for differences in light scattering between reference materials and EVs (van der Pol *et al.* 2014).

Presently, the study of MV in SCD provides further opportunities for future research into the prevention of the pathophysiological symptoms of the disease. However, the complexity of symptoms experienced and subtle differences in the haemoglobinopathy genotypes may mean that monitoring approaches may be too varied and complex to be achievable by all but a very few specialised centres, which may inhibit their feasibility and application. Until the development of an effective technology and standardised process that allows an optimised approach to this monitoring is available, then the current multi-faceted approach to management of these patients with existing and emerging new drugs, such as selectin inhibitors or L-Glutamine is the best and only option for these patients. Whilst gene therapy offers promise, it is too far on the horizon for many patients in the western world and an unachievable dream for the vast majority of patients in the developing world.

### 5.4 Further work

A potential technological solution may come in the form of imaging flow cytometry. A recent study by Smith *et al.* (2020) examined the plasma of SCD patients in both steady state and crisis compared with healthy controls, to ascertain the role Red Cell Derived Particles (RCDP) in the pathology of SCD and whether their removal by apheresis would be of therapeutic benefit to patients, by reducing their risk of VOC.

The authors used imaging flow cytometry to quantify RCDP and AV in SCD plasma. The use of imaging flow cytometry allowed the authors to identify RCDP which varied in size, ranging from large macrovesicles (MaV) which are similar in size to erythrocyte ghosts, to microvesicles. The heterogeneity in size of RCDP was unsurprising given the destructive cellular haemolytic process and associated inflammation associated with SCD, particularly in those patients in crisis.

Similar to this study, of the RCDP detected, the majority bound antibodies to extracellular epitopes on glycophorin A (GPA) and were right-side out in their orientation. A second, minor population of inside-out AVs was detected with antibodies to epitopes on the intracellular domain of GPA. This was confirmed by electron micrograph experiments using transmission electron microscopy and immunolabelling techniques. The large, right-side out GPA pos MaV exposed PS at the membrane surface. The subsequent removal of these right side-out MaV from SCD patient plasma samples using anti-GPA coated magnetic beads resulted in a seven-fold increase in the plasma clotting time.

These results suggest that removing RCDP from plasma of SCD patients would be of therapeutic value by minimising the pathological effects of hypercoagulation. Potentially leading to a new therapeutic approach to managing VOC, avoiding the need for an intensive transfusion regime, with the associated risks of haemolysis, alloantibody formation and iron overload.

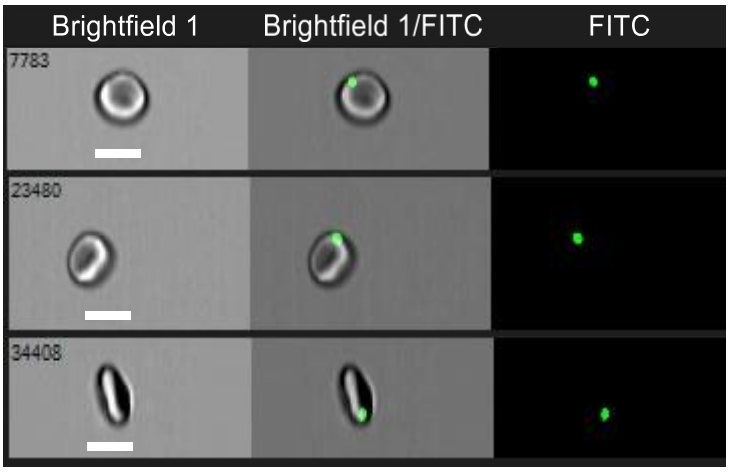



**Figure 5.2 Cellular fluorescence "spot" analysis using Imaging flow cytometry to detect emerging AV.** Annexin V FITC stained erythrocytes can be examined using an ImageStream®X Mark II imaging flow cytometer (Luminex, USA) and the data generated analysed using the "Spot Analysis" functionality of IDEAS 6.2 software (Luminex, USA) whereby fluorescent positive events (cells) can be sorted into negative, positive, and positive but only in a small region of the cell surface (a spot). This type of analysis is much faster and less subjective than live cell confocal imagining and because you can rapidly calculate the percentage of AV positive cells from 100,000 cells (c.f. manually counting approximately 2000 cells by confocal), you would expect it to be more accurate. Preliminary data using 3 healthy samples gave a mean % of AV positive cells of 0.07%. Two steady state SCD samples have been analysed by both live cell confocal imaging and ImageStream spot analysis, one gave a figure of 2.3% of AV positive cells by confocal and 2.7% by ImageStream, the other 1.0% by confocal and 1.2% by ImageStream (personal Communication T Mankelow). Scale bars 7μm.

# Chapter 6: Conclusion

The results show that a heterogeneous population of MV were present in the plasma of both healthy controls and SCD patients in steady state and in VOC. In confocal microscopy studies, statistically significantly elevated numbers of PS-positive MV, and GPA-pos MV were identified which is concordant with previous studies (Mankelow *et al.* 2015). In flow cytometry studies, RCDP of an inside out orientation, most likely AVs, are indeed present in the plasma of SCD patients in both steady state and VOC but were not significantly increased in either cohort when compared to healthy controls. Overall, no population of MV was found to be a statistically significant predictor of VOC in this study, which was limited by the small cohorts examined and limitations in the existing experimental methodology used. Future studies in this area should utilise newer, more accurate methodologies, such as the ImageStream imaging flow cytometer, to differentiate MVs from RBCs/ platelets and other cellular debris, and to examine their clinical significance as potentiators of the pathophysiological symptoms of SCD. As the population of the United Kingdom changes and the prevalence of SCD increases, the need for an effective prognostic test for SCD becomes more pressing. Current treatments are largely defensive in their nature, with bone marrow transplantation the only curative option available for very few patients. While gene therapy offers future promise of a cure, long-term outcomes are unknown, and the cost is currently prohibitive to most healthcare systems. As new reagents and technologies become available, they must be used to build a complete picture of the complex interactions in SCD patients and address the current relatively slow progress made in the treatment and monitoring of patients with SCD.

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# Chapter 8: Appendices

## Appendix 1

C2 Proforma - Submitted to Manchester Metropolitan University and the Royal College of Pathologists

# DClinSci C2 Module Proforma

Completed forms should be emailed to admin@mahse.co.uk

| Trainee Details | |
|---|---|
| **Name:** | Tom Bullock |
| **Student ID:** | 9841490 |
| **University:** | Manchester Metropolitan University |
| **HSST Specialism:** | Transfusion Science |
| **Place of work:** | NHSBT |
| **Date proforma submitted:** | 11/01/2017 |

| Research Project Details | |
|---|---|
| **Research dissertation working title:** | A Feasibility Study Examining the Potential for a Predictive Test for Vaso-Occlusive Crises in Sickle Cell Disease Patients. |
| **Name of proposed workplace supervisor:** | Dr Tosti Mankelow |
| **Contact email of proposed workplace supervisor:** | Tosti.mankelow@nhsbt.nhs.uk |

**Description of proposed research (500 words maximum)**
***Please include:***

***(a) Aims of the research***

Patients with homozygous (HbSS) SCD are at risk of painful Vaso-Occlusive crises (VOCs), and haemolysis, caused by deoxygenated, sickle shaped erythrocytes (red blood cells) occluding the microcirculation. Recent studies have indicated that VOCs may also be associated with the release of inside-out autophagic erythrocyte membrane vesicles into the bloodstream. The release of inside-out autophagic erythrocyte membrane vesicles is essential to erythrocyte maturation.

Ordinarily these vesicles would be removed from the circulation through the process of sequestration by splenic macrophages. However sickle cell patients have a non-functioning spleen, or have been splenectomised. This results in increased numbers (8-10 fold) of circulating vesicles in this group of patients. Unpublished work has shown that these inside-out vesicles express phosphatidylserine (PS), which acts as a substrate for the prothrombin/thrombin complex, potentially increasing the propensity of thrombosis and VOC.

***(b) Principal research question(s)***

To ascertain if there is a correlation between numbers of circulating autophagic erythrocyte membrane vesicles and the severity and likelihood of VOCs.

***(c) Proposed methods***

Monoclonal antibodies (BRIC256 and BRIC163) have been developed at the Bristol Institute of Transfusion Science (BITS). These monoclonal antibody clones are specific for the PS structures on the outside of the vesicles. There is potential for these monoclonal antibodies to be used in flow cytometry experiments to quantify the numbers of autophagic vesicles in the patient's plasma. Confocal microscopy will also be used to quantify the numbers of vesicles located on the erythrocytes of the same patients.

***(d) Potential impact of research***

Quantification of autophagic vesicles may form the basis of a clinical diagnostic testing regimen, in order to predict, and therefore prevent VOC's in this cohort of patients

***(e) A summary of patient and public involvement in the research***

Patients attending UCL will be consented to allow for an additional sample to be taken in order to perform the analysis. The sample will be taken when they are admitted in VOC, in addition to existing diagnostic sampling. It is envisaged that the innovation could lead to novel therapeutic interventions in the management of this patient cohort.

| Research Governance | | |
|---|---|---|
| | **Yes:** | **No:** |
| **(a) Does your proposal involve animal experimentation?** If yes, do you and/or your proposed supervisor hold a valid and current animal licence? (please give details) **Click here to enter text.** | ☐ | x |
| **(b) Does your proposal involve human participants?** | x | ☐ |
| **(c) Does your proposal involve samples covered under the Human Tissue Act (HTA)?** | x | ☐ |
| **If you answered yes to either (b) or (c) above;**<br>• Is ethical approval required?<br>• If required, has ethical approval been obtained? (please give details)<br>Ethical approval obtained by Dr Sara Trompeter – Consultant Haematologist – UCL. | x<br>x | ☐<br>☐ |

| Research Costings | | |
|---|---|---|
| | **Yes:** | **No:** |
| **(a) Has the project been costed?** | ☐ | x |
| **(b) Are funds in place to cover the costs?** If funds are not in place, outline the approach to securing these costs: **Click here to enter text.** | x | ☐ |

## For Office Use Only:

| Approval of C2 Research Project by University | |
|---|---|
| **HSST Lead:** | Select HSST Lead from list. |
| **Notes:** | Click here to enter text. |
| **Signed:** | |
| **Date approved:** | Click here to enter text. |

| Approval of C2 Research Project by Royal College of Pathologists (Life Sciences only) | |
|---|---|
| **Name:** | Click here to enter text. |

| | |
|---|---|
| **Notes:** | Click here to enter text. |
| **Signed:** | |
| **Date approved:** | Click here to enter text. |

## Appendix 2

Patient Information Sheets

**GENERIC PATIENT INFORMATION SHEET V3 12.2.13**
**PROTOCOL V5 12.2.13**
**UCL PROJECT ID: 10/0270**
**NHS REC REFERENCE: 10/H0715/61**
**TITLE OF PROJECT: MACS STUDY: MICROVESICLES AND COAGULATION IN SICKLE CELL ANAEMIA**

**PARTICIPANT INFORMATION:**

**PARTICIPANT STUDY NUMBER:**

Chief Investigator: Prof. John Porter 020 7380 9638

**PART 1:**

**Why am I being asked to be in this Research Study?**

We are inviting you or your child to take part in a research study.
Research is a way of attempting to gain new knowledge. A person who participates in research study is called a "subject". Before you decide we would like you to understand why the research is being done and what it would involve for you. **One of our team will go through the information sheet with you and answer any questions you have.** We would suggest this should take at least 30 minutes. Talk to others about the study if you wish.
Part 1 tells you about the purpose of the study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study. Ask us if there is anything that is not clear.

**What is the purpose of this study?**

The purpose of the research study is to understand the role of microvesicles in Sickle Cell Anaemia. Microvesicles are broken bits of cells that circulate in the blood. Research done so far, shows that these microvesicles are sticky. This may be important in Sickle cell diseasewhere we know that many of the problems are caused by cells becoming sticky and blocking the blood vessels. We will measure the levels of the microvesicles and related aspects of blood stickiness in the blood and see if that helps identify people who have more problems with their Sickle Cell Anaemia. We hope in the future these measures may be used to predict who may get complications of their disease or to guide doctors when to start or stop treatments for the complications. We will publish the results of the study and present it at meetings. We will provide the study centres with a leaflet explaining the outcome of the study that they can distribute to participants.

**Why have I (my child) been invited?**

You (or your child) are being asked to take part in this study because you (or your child) have Sickle Cell Anaemia. This study will simply involve a blood test and does not involve any treatment changes. It is expected that a total of approximately 300 patients will participate in this study from UCLH, The Whittington Hospital and Guys and St Thomas' Hospital.

This research information sheet explains why this study is being done, what is involved in participating in the research study, the possible risks and benefits of the research study, and your rights as a research subject. The decision to participate or not is yours. Your participation in this research study is voluntary and you can stop being in this study at any time. Leaving the research study will not affect your medical care in any way. If you decide to participate, please sign and date at the end of the consent form. We will give you a copy so that you can refer to it while you are involved in this research study.

**Do I have to take part?**

It is up to you to decide to join the study. We will describe the study and go through the information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

**What is involved in the research study?**

The study doctors and nurses will make sure that you (or your child) meet the study requirements to take part in the study. After your study doctor determines that you (or your child) are eligible to participate in the study then you (or your child) will have an additional amount of blood taken at the time of a routine blood test. This will be in the form of an additional blood bottle that in small children takes about a teaspoon of blood and in a larger child or adult, five teaspoons of blood. If you are having an apheresis procedure, we will take the left over blood that is usually diSCDrded also. You (or your child) will not need an extra needle for the study. The samples will be taken to the laboratory and prepared for later analysis at University College London, University College Hospital London or the National Blood Service. We will also be comparing the results of this test with your other blood results and measurements taken in clinic.

**Will there be any cost or compensation for my participation?**

You (or your child) will not be paid for participating in this trial. Participation in the study will not involve any additional cost to you.

**What are the possible disadvantages and risks of taking part?**

There are no drugs involve in this study. The possible risks related to the study are listed below.

Risks associated with blood tests: Risks include a possible bruise or infection at the needle site. Suitable precautions will be taken to minimize risk. Children will be offered local anaesthetic cream (EMLA / Ametop) or cold-spray to numb the skin prior to the blood test. The blood is being taken when other blood tests are being taken as a matter of normal care in any case. No extra needle will be needed. There will be one further sample taken at that time for the study (one teaspoon in a small child, five teaspoons in a larger child or adult). If you are having an apheresis procedure, we will take the left over blood that is usually diSCDrded also.

**What are the possible benefits of taking part?**

Taking part in this research study may or may not provide direct benefit to you (or your child). We hope the information learned from this research study will help the research doctors learn more about your (or your child's) future treatment and that of other patients with Sickle Cell Anaemia.

**Will my participation in the study be stopped?**

Your (or your child's) participation in the study may be stopped by your (or your child's) doctor, even without your consent, for medical reasons. The sponsor of this study, UCL (University College London) can end the study at any time, for any reason.

**What if there is a problem?**

The risks of participation in an observational study are minimal. However, in the event of an injury resulting directly from your (or your child's) participation in this research study, medical treatment will be provided. Provision of such medical care does not imply any negligence or other wrongdoing on the part of Hospital or any of the physicians or other personnel involved in the study. The NHS indemnity scheme will apply and the sponsor will provide insurance for negligent harm.

**Will my taking part in the study be kept confidential?**

Yes. We will follow ethical and legal practice and information about you will be handled in confidence. The details are included in Part 2.

This completes part 1

**Part 2**

**What if relevant new information becomes available?**

Sometimes we get new information about the investigation being studies. If this happens and it may affect your care, we will let you know.

**What are my rights as a study participant?**

You (or your child) are entitled to certain rights. These rights include, but are not limited to, your (or your child's) right to have information about the purpose and nature of the research, the procedures and any treatment, any discomfort or other risks, any benefits to you (or your child) or to others and available medical care if complications occur. You (or your child) have the right to receive answers to all of your questions, as best as your doctors can explain them to you (or your child).

If you decide that you want to withdraw/destroy any of your (or your child's) blood samples during the study, please notify the Chief Investigator or study staff members at your facility in writing (their contact information is on the first page of this form). Since the blood samples stored in the repository (storage facility) and the clinical database will have all your identifying information removed at the end of the study, you (your child) will not be able to withdraw/destroy your (or your child's) samples once the database has been sealed and all identifiers and means of linking your samples back to you have been removed at the end of the study. Blood tests completed before you request that the sample be withdrawn/destroyed will be kept by the study.

**What if I have more questions? What if there is a problem?**

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your question. You can contact the Principle or Chief Investigators on the numbers above.
For Complaints please contact the Patient advisory Liaison Service (PALS) on …….. or the complaints department at this site on …….

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for legal action for compensation against UCL or your hospital trust, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

**Will my study records be kept confidential?**

General Principles: All our procedure for handling, processing, storage and destruction of their data adhere to the Caldecott principles, the Data Protection Act (1998) and the NHS Data Protection Charter. If you join the study, some parts of your medical records and data collected for the study will be looked at by authorised persons form the sponsor (UCL). They may also be looked at by authorized people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty. Data collected during this study may be sent to associated researchers to countries where the law's don't protect your privacy to the same extent as the law in the UK but all reasonable steps will be taken to protect your privacy.

Medical Records: Medical information collected during this study will become part of your (or your child's) hospital record only if it is determined to be pertinent to the care you (or your child) receive at University College London / The Whittington / Guy's and St Thomas' Hospitals. Medical records are considered permanent records; therefore, materials cannot be deleted from the record. Medical records are available to health care professionals at University College London / The Whittington / Guy's and St Thomas' Hospitals and may be reviewed by Hospital staff in their course of carrying out their responsibilities; however, they are required to maintain confidentiality in accordance with applicable laws and Hospital policies.

Research Records: Information collected during the study that does not become part of your (or your child's) medical record will be stored in separate research files maintained by the investigator and other research staff. The records will be stored on an encrypted memory stick and backed up on the encrypted component of a designated computer hard-drive. Any transfer of information will be encrypted, also. All data will be managed in accordance with the Data Protection Act. The data will be pseudoencrypted. That is, it will have most personal data removed, but the study team will be able to trace back whose sample was whose, if the need arose. Records are kept for 20 years at the first instance. This is standard procedure.

**Storage and use of study specimens**

Blood will be collected at the hospital and then tested at University College London (UCL). Some of the sample will be tested within the week, others at a later date. The sample will be stored at University College London (UCL). Samples will be kept for 5 years at the first instance. The samples will be used for the purpose of the study. If you have chosen to "gift" your sample, then that will give us permission to use any left-over sample for a future study (please see consent form).

**Involvement of the General Practitioner / Family Doctor (G.P.)**

We will inform your G.P. that you have been involved in a research study. We will ask for your permission for this on the consent form.

**What will happen to the samples I give?**

Your sample will be taken to your NHS laboratory where it will be prepared and stored for later transfer to UCL for analysis. Those working the departments have access to the samples. Your (or your child's) blood sample will not be labeled with your (or your child's name) or other direct personal information. On the consent form you will be able to tell us whether we can use any sample left over at the end of the planned testing for other research or whether it must be destroyed. This is known as "gifting" your sample for future research.

**What will happen to the results of the research study?**

We hope to publish the research in peer reviewed journals and at national and international meetings. We will present the findings to the UK forum for Haemoglobinopathies, to the Sickle Cell Society and to the teams in the centres taking part in the study.

**Who is organising/funding the research?**

The cost is being covered by the NHS and the Chief Investigator. UCL is the research Sponsor.

**Who has reviewed this study?**

All research in the NHS is looked at by an independent group of people called a Research Ethics Committee, to protect your interest. This study has been reviewed and given favourable opinion by Central Research Ethics Committee 4.

## Appendix 3

Patient Consent form

**PATIENT CONSENT FORM V3 12.2.13**
**PROTOCOL V5 12.2.13**
**UCL PROJECT ID: 10/0270**
**NHS REC REFERENCE: 10/H0715/61**
**TITLE OF PROJECT: MACS STUDY: MICROVESICLES AND COAGULATION IN SICKLE CELL ANAEMIA**
**CENTRE NUMBER:**
**PARTICIPANT INFORMATION:**

**PARTICIPANT STUDY NUMBER:**

1. I confirm that I have read and understand the information sheet dated 12.2.13 (Version 3), for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I confirm that I have had sufficient time to consider whether or not I want to be included in the study. ☐

3. I understand that my participation is voluntary and that I am free to withdraw my consent at any time, without giving any reason, without my medical care or legal rights being affected. ☐

4. I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by responsible individuals from regulatory authorities, from the NHS trust or representatives of the sponsor for purposes of monitoring or auditing, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

5. I agree to my GP being informed of my participation in the study. ☐

6. I agree to take part in the above study and have been given a copy of this form. ☐

7. OPTIONAL: This is optional. Please indicate below if you (or your child) agree to the gift of a blood sample to the researchers. This blood sample will be stored and used for testing for indicators that may influence the course of Sickle Cell Anaemia.

[ ] YES, I AGREE to have my (my child's) blood gifted and stored for future

sickle cell studies that are related to this research study.

[ ] YES, I AGREE to have my (my child's) blood gifted and stored for future sickle cell studies that are not related to this research study.

[ ] YES, I AGREE to have my (my child's) gifted blood sample to be shared for sickle cell research done by investigators who are related to this research study.

[ ] YES, I AGREE to have my (my child's) gifted blood sample to be shared for sickle cell research done by other investigators who are not related to this research study.

[ ] NO, I do NOT AGREE to have my (my child's) blood gifted and stored for future sickle cell research

If subject is 18 years or older:

_____ _____ _____

Name of Adult participant Date Signature

If subject is less than 18 years old:

_____ _____ _____

Relationship to child Date Signature of parent/guardian

_____ _____ _____

Child/Adolescent's name Date Signature of child/adolescent

If child/ adolescent participant's signature is not obtained, please specify why.

_____ _____ _____

Name of person taking consent Date
Signature
(if different from PI)

When completed: 1 for participant; 1 for research site file; 1 (original) to be kept in medical notes.

## Appendix 4

Healthy volunteer control Information Sheets

**GENERIC HEALTHY VOLUNTEER INFORMATION SHEET V3 12.2.13**
**PROTOCOL V5 12.2.13**
**UCL PROJECT ID: 10/0270**
**NHS REC REFERENCE: 10/H0715/61**
**TITLE OF PROJECT: MACS STUDY: MICROVESICLES AND COAGULATION IN SICKLE CELL ANAEMIA**

**PARTICIPANT INFORMATION:**

**PARTICIPANT STUDY NUMBER:**

### PART 1:

### Why am I being asked to be in this Research Study?

We are inviting you or your child to take part in a research study.
Research is a way of attempting to gain new knowledge. A person who participates in research study is called a "subject". Before you decide we would like you to understand why the research is being done and what it would involve for you. **One of our team will go through the information sheet with you and answer any questions you have.** We would suggest this should take at least 30 minutes. Talk to others about the study if you wish.
Part 1 tells you about the purpose of the study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study. Ask us if there is anything that is not clear.

### What is the purpose of this study?

The purpose of the research study is to understand the role of microvesicles in Sickle Cell Anaemia. Sickle cell diseaseis an inherited blood disorder commoner in people who are of African ethnicity or from other countries where malaria is prevalent. Microvesicles are broken bits of cells that circulate in the blood. Research done so far, shows that these microvesicles are sticky and this may be important in Sickle cell diseasewhere we know that many of the problems are caused by cells becoming sticky and blocking the blood vessels. We will measure the levels of the microvesicles and related aspects of blood stickiness in the blood and see if that helps identify people who have more problems with their Sickle Cell Anaemia. We hope in the future these measures may be used to predict who may get complications of their disease or to guide doctors when to start or stop treatments for the complications. We will publish the results of the study and present it at meetings. We will provide the study centres with a leaflet explaining the outcome of the study that they can distribute to participants.

### Why have I (my child) been invited?

You (or your child) are being asked to take part in this study because you (or your child) do(es) not have Sickle Cell Anaemia. This study will simply involve a blood test and does not involve any treatment changes. It is expected that a total of approximately 60 non-sickle cell participants will participate in this study from UCLH, The Whittington Hospital and Guys and St Thomas' Hospital.

This research information sheet explains why this study is being done, what is involved in participating in the research study, the possible risks and benefits of the research study, and your rights as a research subject. The decision to participate or not is yours. Your participation in this research study is voluntary and you can stop being in this study at any time. Leaving the research study will not affect your medical care in any way. If you decide to participate, please sign and date at the end of the consent form. We will give you a copy so that you can refer to it while you are involved in this research study.

**Do I have to take part?**

It is up to you to decide to join the study. We will describe the study and go through the information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

**What is involved in the research study?**

The study doctors and nurses will make sure that you (or your child) meet the study requirements to take part in the study. After your study doctor determines that you (or your child) are eligible to participate in the study then you (or your child) will have a blood test. This will be in the form of an additional blood bottle that in small children takes about a teaspoon of blood and in a larger child or adult, five teaspoons of blood. If you are having an apheresis procedure, we will take the left over blood that is usually diSCDrded also. Children will not have an extra needle for their study, they will only take part if they are having a blood test in any case. Healthy adult volunteers may participate even if they are not due a blood test other than for the study. If the adult does not know their sickle cell status we will need to check that also. That will involve an extra sample. We will tell you the results of your sickle cell test (see consent form). The samples will be taken to the laboratory and prepared for later analysis at University College London, University College Hospital London or the National Blood Service (NHSBT). We will also be comparing the results of this test with your other blood results and measurements taken in clinic.

**Will there be any cost or compensation for my participation?**

You (or your child) will not be paid for participating in this trial. Participation in the study will not involve any additional cost to you.

**What are the possible disadvantages and risks of taking part?**

There are no drugs involve in this study. The possible risks related to the study are listed below.

Risks associated with blood tests: Risks include a possible bruise or infection at the needle site. Suitable precautions will be taken to minimize risk. Children will be offered local anaesthetic cream (EMLA / Ametop) or cold-spray to numb the skin prior to the blood test. The blood is being taken when other blood tests are being taken as a matter of normal care in any case. No extra needle will be needed. There will be one further sample taken at that time for the study (one teaspoon in a small child, five teaspoons in a larger child or adult).

**What are the possible benefits of taking part?**

Taking part in this research study may or may not provide direct benefit to you (or your child). We hope the information learned from this research study will help the research doctors learn more about your (or your child's) future treatment and that of other patients with Sickle Cell Anaemia.

**Will my participation in the study be stopped?**

Your (or your child's) participation in the study may be stopped by your (or your child's) doctor, even without your consent, for medical reasons. The sponsor of this study, UCL (University College London) can end the study at any time, for any reason.

**What if there is a problem?**

The risks of participation in an observational study are minimal. However, in the event of an injury resulting directly from your (or your child's) participation in this research study, medical treatment will be provided. Provision of such medical care does not imply any negligence or other wrongdoing on the part of Hospital or any of the physicians or other personnel involved in the study. The NHS indemnity scheme will apply and the sponsor will provide insurance for negligent harm.

**Will my taking part in the study be kept confidential?**

Yes. We will follow ethical and legal practice and information about you will be handled in confidence. The details are included in Part 2.

This completes part 1

**Part 2**

**What if relevant new information becomes available?**

Sometimes we get new information about the investigation being studies. If this happens and it may affect your care, we will let you know.

**What are my rights as a study participant?**

You (or your child) are entitled to certain rights. These rights include, but are not limited to, your (or your child's) right to have information about the purpose and nature of the research, the procedures and any treatment, any discomfort or other risks, any benefits to you (or your child) or to others and available medical care if complications occur. You (or your child) have the right to receive answers to all of your questions, as best as your doctors can explain them to you (or your child).

If you decide that you want to withdraw/destroy any of your (or your child's) blood samples during the study, please notify the Chief Investigator or study staff members at your facility in writing (their contact information is on the first page of this form). Since the blood samples stored in the repository (storage facility) and the clinical database will have all your identifying information removed at the end of the study, you (your child) will not be able to withdraw/destroy your (or your child's) samples once the database has been sealed and all identifiers and means of linking your samples back to you have been removed at the end of the study. Blood tests completed before you request that the sample be withdrawn/destroyed will be kept by the study.

**What if I have more questions? What if there is a problem?**

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your question. You can contact the Principle or Chief Investigators on the numbers above.
For Complaints please contact the Patient advisory Liaison Service (PALS) on …….. or the complaints department at this site on …….

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for legal action for compensation against UCL or your hospital trust, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

**Will my study records be kept confidential?**

General Principles: All our procedure for handling, processing, storage and destruction of their data adhere to the Caldecott principles, the Data Protection Act (1998) and the NHS Data Protection Charter. If you join the study, some parts of your medical records and data collected for the study will be looked at by authorised persons form the sponsor (UCL). They may also be looked at by authorized people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty. Data collected during this study may be sent to associated researchers to countries where the law's don't protect your privacy to the same extent as the law in the UK but all reasonable steps will be taken to protect your privacy.

Medical Records: Medical information collected during this study will become part of your (or your child's) hospital record only if it is determined to be pertinent to the care you (or your child) receive at University College London / The Whittington / Guy's and St Thomas' Hospitals. Medical records are considered permanent records; therefore, materials cannot be deleted from the record. Medical records are available to health care professionals at University College London / The Whittington / Guy's and St Thomas' Hospitals and may be reviewed by Hospital staff in their course of carrying out their responsibilities; however, they are required to maintain confidentiality in accordance with applicable laws and Hospital policies.

Research Records: Information collected during the study that does not become part of your (or your child's) medical record will be stored in separate research files maintained by the investigator and other research staff. The records will be stored on an encrypted memory stick and backed up on the encrypted component of a designated computer hard-drive. Any transfer of information will be encrypted, also. All data will be managed in accordance with the Data Protection Act. The data will be pseudoencrypted. That is, it will have most personal data removed, but the study team will be able to trace back whose sample was whose, if the need arose. Records are kept for 20 years at the first instance. This is standard procedure.

**Storage and use of study specimens**

Blood will be collected at the hospital and then tested at University College London (UCL). Some of the sample will be tested within the week, others at a later date. The sample will be stored at University College London (UCL). Samples will be kept for 5 years at the first instance. The samples will be used for the purpose of the study. If you have chosen to "gift" your sample, then that will give us permission to use any left-over sample for a future study (please see consent form).

**Involvement of the General Practitioner / Family Doctor (G.P.)**

We will inform your G.P. that you have been involved in a research study. We will ask for your permission for this on the consent form.

**What will happen to the samples I give?**

Your sample will be taken to your NHS laboratory where it will be prepared and stored for later transfer to UCL for analysis. Those working the departments have access to the samples. Your (or your child's) blood sample will not be labeled with your (or your child's name) or other direct personal information. On the consent form you will be able to tell us whether we can use any sample left over at the end of the planned testing for other research or whether it must be destroyed. This is known as "gifting" your sample for future research.

**What will happen to the results of the research study?**

We hope to publish the research in peer reviewed journals and at national and international meetings. We will present the findings to the UK forum for Haemoglobin Disorders, to the Sickle Cell Society and to the teams in the centres taking part in the study.

**Who is organising/funding the research?**

The cost is being covered by the NHS and the Chief Investigator. UCL is the research Sponsor.

**Who has reviewed this study?**

All research in the NHS is looked at by an independent group of people called a Research Ethics Committee, to protect your interest. This study has been reviewed and given favourable opinion by Central Research Ethics Committee 4.

## Appendix 5

Healthy volunteer control Consent form

**HEALTHY VOLUNTEER CONSENT FORM V4 12.2.13**
**PROTOCOL V5 12.2.13**
**UCL PROJECT ID: 10/0270**
**NHS REC REFERENCE: 10/H0715/61**
**TITLE OF PROJECT:**
**MACS STUDY: MICROVESICLES AND COAGULATION IN SICKLE CELL ANAEMIA**
**CENTRE NUMBER:**
**PARTICIPANT INFORMATION:**

**PARTICIPANT STUDY NUMBER:**

**Please initial box:**

1. I confirm that I have read and understand the information sheet dated 12.2.13 (version 5) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I confirm that I have had sufficient time to consider whether or not I want to be included in the study. ☐

3. I understand that my participation is voluntary and that I am free to withdraw my consent at any time, without giving any reason, without my medical care or legal rights being affected. ☐

4. I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by responsible individuals from regulatory authorities, from the NHS trust or representatives of the sponsor for purposes of monitoring or auditing, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

5. I agree to my GP being informed of my participation in the study. ☐

6. I agree to take part in the above study and have been given a copy of this form. ☐

7. I understand that if I do not know my sickle cell status, then I shall be tested and the results given to me via (please mark preference and give contact details). ☐
   [ ] address
   [ ] telephone number

8. OPTIONAL: This is optional. Please indicate below if you (or your child) agree to the gift of a blood sample to the researchers. This blood sample will be stored and used for testing for indicators that may influence the course of Sickle Cell Anaemia.

   [ ] YES, I AGREE to have my (my child's) blood gifted and stored for future sickle cell studies that are related to this research study.

   [ ] YES, I AGREE to have my (my child's) blood gifted and stored for future sickle cell studies that are not related to this research study.

[ ] YES, I AGREE to have my (my child's) gifted blood sample to be shared for sickle cell research done by investigators who are related to this research study.

[ ] YES, I AGREE to have my (my child's) gifted blood sample to be shared for sickle cell research done by other investigators who are not related to this research study.

[ ] NO, I do NOT AGREE to have my (my child's) blood gifted and stored for future sickle cell research

If subject is 18 years or older:

| | | |
|---|---|---|
| Name of Adult participant | Date | Signature |

If subject is less than 18 years old:

| | | |
|---|---|---|
| Relationship to child | Date | Signature of parent/guardian |
| Child/Adolescent's name | Date | Signature of child/adolescent |

If child/ adolescent participant's signature is not obtained, please specify why.

| | | |
|---|---|---|
| Name of person taking consent (if different from PI) | Date | Signature |

When completed: 1 for participant; 1 for research