Suicidal Erythrocyte Death - Regulation by the G-Protein Subunit Gαi2 and occurrence in Heart Failure and Bronchogenic Carcinoma

Suizidaler Erythrozytentod – Regulation durch die G-Protein Untereinheit Gαi2 sowie Auftreten bei Herzinsuffizienz und Bronchialkarzinom

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1 CONTENTS

2 ABBREVIATIONS

α	Alpha
ACEI	Angiotensin converting enzyme inhibitor
AHF	Acute Heart Failure
AJCC	American Joint Committee on Cancer
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
APC	Adenomatous-polyposis-coli
ARA	Aldosterone receptor antagonist
ARB	Angiotensin receptor blocker
ATP	Adenosine triphosphate
a.u.	Arbitrary units
AWB	Annexin Wash Buffer
β	Beta
BD	Becton Dickinson
BHK	Baby Hamster Kidney
BSA	Bovine Serum Albumin
°C	Celsius
C57BL6	C57 black 6
Ca^{2+}	Calcium
$CaCl_2$	Calciumchloride
cAMP	Cyclic adenosine monophosphate
CAV	Cyclophosphamide, adriamycin and vincristine
cGMP	Cyclic guanosine monophosphate
CK1α	Casein Kinase 1 alpha
CKD	Chronic Kidney Disease
Cl^-	Chloride
CNS	Central Nervous System
C-terminus	Carboxy-terminus
CVD	Cardiovascular disease
CXCL16/SR-PSOX	Chemokine (C-X-C motif) ligand 16/Scavenger Receptor that binds phos-phatidylserine and oxidized lipoprotein
DNA	Deoxyribonucleic acid
dl	Deciliter
DCF	2´7´ dichlorofluorescin
DCFDA	2´7´ dichlorofluorescin diacetate
ECL	Enhanced chemiluminescence
ED-SCLC	Extended Disease Small Cell Lung Cancer
EDTA	Ethylenediaminetetraacetic acid
e.g.	*Exempli gratia*; for example
EP	Etoposide platin

EPO	Erythropoietin
EpoR	Erythropoietin receptor
ESA	Erythropoiesis stimulating agent
FACS	Fluorescence-activated cell sorting
Fig	Figure
FITC	Fluorescein isothiocyanate
fl	Femtoliter
FL	Fluorescence channel
FLUOS	6-carboxy-fluorescein-N-hydroxysuccinimide
FSC	Forward Scatter
γ	Gamma
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
g	Gram
g	Gravity
G-protein	Guanine nucleotide-binding protein
GPCR	G-Protein coupled receptor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	Hour
Hb	Hemoglobin
HF	Heart Failure
HEPES	32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
ID	Iron deficiency
i.e.	*Id est*; that is
Ig	Immunglobuline
IL	Interleukin
IVC	Individually ventilated cage
JAK3	Janus-activated kinase 3
K^+	Potassium
KCl	Potassium chloride
kDa	Kilodalton
KH_2PO_4	Monopotassium phosphate
LC	Lung cancer
LCC	Large cell carcinoma
LD-SCLC	Limited disease small cell lung cancer
LCNEC	Large cell neuroendocrine carcinoma
LV	Left ventricular
LVEF	Left ventricular ejection fraction
LVF	Left ventricular failure
MAPK	Mitogen-activated protein kinase
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration

MCV	Mean Corpuscular Volume
$MgSO_4$	Magnesium sulfate
min	Minute
ml	Milliliter
mM	Millimolar
MSK1/2	Mitogen-and stress-activated kinases 1 and 2
n	Number
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Na_2HPO_4	Disodium phosphate
Na_3VO_4	Sodium orthovanadate
NaF	Sodium fluoride
nm	Nanometer
NSCLC	Non-small cell lung cancer
N-terminus	Amino-terminus
NYHA	New York Heart Association
PAGE	Polyacrylamide gel electrophoresis
PAK2 Kinase	p21-activated kinase 2
Pat	Patient
PBS	Phosphate-buffered saline
PGE_2	Prostaglandin E2
pH	Lat. *Pondus Hydrogenii*
PKC	Protein Kinase C
PLC	Phosphatidylinositol phospholipase C
PS	Phosphatidylserine
PVDF	Polyvinylidene fluoride
RBC	Red blood cell
Retic-COUNT	1-methyl-4[(3-methyl-2(3H)-benzothiazolylidine) methyl]-quinolinium 4-methyl benzene sulfonate (Thiazole Orange)
RGS	Regulators of G-protein signaling
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Rotations per minute
RT	Room temperature
RV	Right ventricular
RVF	Right ventricular failure
SCC	Squamous cell carcinoma
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	Standard error of mean
SPF	Specified pathogen-free
SSC	Side scatter

TBS-Tween	Tris buffered saline, with Tween
TM	Transmembrane
TNF	Tumor necrosis factor
TNM	Tumor Node Metastasis
Tris-HCl	Tris (hydroxymethyl)-aminomethane-hydrochloric acid
TRPC6	Transient receptor potential channel 6
µg	Microgram
UICC	Union International for Cancer Control
µl	Microliter
µm	Micrometer
µM	Micromolar
U/ml	Units per milliliter
VTE	Venous thromboembolism
WHO	World Health Organization

3 SUMMARY/ZUSAMMENFASSUNG

Suicidal erythrocyte death, also called “eryptosis” is a specialized type of cell death that erythrocytes can undergo prior to their senescence. Several xenobiotics, endogenous mediators and diseases trigger eryptosis, a process, characterized by morphological changes such as erythrocyte cell membrane scrambling and cell shrinkage. Mechanistically, eryptosis is triggered by increased intracellular Ca^{2+} concentration, which is, in turn, elicited by enhanced Ca^{2+} entry via non-selective cation channels. Enhanced cytosolic Ca^{2+} activity stimulates scramblases resulting in the translocation of phosphatidylserine (PS) to the erythrocyte membrane. This phenomenon also results in the activation of Ca^{2+}-activated K^+ channels leading to cellular efflux of K^+ and osmotically obliged water with subsequent erythrocyte shrinkage. On the other hand, eryptosis may be stimulated by ceramide, which can act independently of increased intracellular Ca^{2+}. Erythrocyte survival is further regulated by cellular osmotic, energy and redox balance. A wide range of signaling molecules such as kinases are decisive in the regulation of the eryptosis machinery. As eryptotic erythrocytes are rapidly cleared from the blood stream by macrophages, excessive eryptosis may contribute to the development of anemia if the loss of erythrocytes is not compensated by the production of new red blood cells. Anemia is known to impair the quality of life in patients with lung cancer (LC) and acute heart failure (AHF) and is an important indicator of prognosis in these conditions.

In the first part of the present dissertation, the hypothesis that enhanced eryptosis contributes to the development of anemia associated with LC and AHF, was tested. To this end, the present data reveal that patients with either LC or AHF showed increased reticulocytosis suggesting enhanced erythrocyte turnover. According to the present observations, erythrocyte PS exposure in LC patients is significantly more pronounced in LC patients with and without cytostatic treatment as compared to healthy controls. Erythrocytes from LC patients show enhanced forward scatter (reflective of cell volume), oxidative stress and ceramide abundance.

These effects could be attributed to components of plasma from LC patients. Similarly, AHF patients showed enhanced percentage of PS-exposing erythrocytes, which was accompanied by decreased forward scatter as well as enhanced oxidative stress. Taken together, the present data suggest that eryptosis is an important mechanism in the pathogenesis of anemia in LC and AHF patients.

In the second part of the present dissertation, the role of Gαi2, a type of G-protein coupled receptor protein, in erythrocyte survival was explored. Putative functions of the G-protein subunit Gαi2-dependent signaling in nucleated cells include ion channel regulation, cell differentiation, proliferation and apoptosis. The present data show that Gαi2 is expressed in both murine and human erythrocytes and further elucidate the influence of Gαi2 on the survival of anucleated erythrocytes. To this end, erythrocytes were isolated from Gαi2-deficient mice (*Gαi2*$^{-/-}$) and corresponding wild-type mice (*Gαi2*$^{+/+}$). Erythrocyte parameters were similar in both *Gαi2*$^{-/-}$ and *Gαi2*$^{+/+}$ mice but the mean corpuscular volume and the leukocyte count was significantly higher in *Gαi2*$^{-/-}$ mice. May Grünwald staining showed no apparent differences in erythrocyte shape from *Gαi2*$^{+/+}$ as compared to *Gαi2*$^{-/-}$ mice . Spontaneous PS exposure of circulating *Gαi2*$^{-/-}$ erythrocytes was significantly reduced in comparison to *Gαi2*$^{+/+}$ erythrocytes. PS exposure was significantly blunted in *Gαi2*$^{-/-}$ as compared to *Gαi2*$^{+/+}$ erythrocytes following *ex vivo* exposure to the pathophysiological cell stressor hyperosmotic shock (+550 mM sucrose) as well as following treatment with bacterial sphingomyelinase or C6 ceramide. Erythrocyte Gαi2 deficiency further attenuated hyperosmotic shock-elicited enhanced cytosolic Ca^{2+} activity and cell shrinkage. Moreover, *Gαi2*$^{-/-}$ erythrocytes were more resistant to osmosensitive hemolysis as compared to *Gαi2*$^{+/+}$ erythrocytes. Taken together, the present data suggest that Gαi2 deficiency in erythrocytes confers partial protection against suicidal cell death.

Zusammenfassung

Der suizidale Erythrozytentod, auch als “Eryptose” bezeichnet, ist eine besondere Form des Zelltodes, den Erythrozyten vor ihrer Seneszenz eingehen können. Verschiedene Xenobiotika, endogene Mediatoren und Erkrankungen verursachen eine Eryptose, ein Prozess, der durch morphologische Veränderungen wie z.B. durch die Umlagerung von Phospholipiden der Zellmembran („scrambling“) und Zellschwund gekennzeichnet ist. Mechanistisch wird die Eryptose durch eine erhöhte intrazelluläre Calciumkonzentration, welche wiederum durch einen erhöhten Calciumeinstrom durch nicht-selektive Kationenkanäle ausgelöst wird, eingeleitet. Die erhöhte zytosolische Calciumaktivität stimuliert Scramblasen was zur Translokation von Phosphatidylserin (PS) an die Erythrozytenoberfläche führt. Dieses Phänomen führt auch zur Aktivierung der Ca^{2+}-abhängigen Kaliumkanäle was einen Ausstrom von Kalium und Wasser und einen anschließenden Zellschwund zur Folge hat. Andererseits kann eine Eryptose auch durch Ceramid eingeleitet werden, welches unabhängig von einer erhöhten intrazellulären Calciumkonzentration agieren kann. Das erythrozytäre Überleben wird außerdem durch eine zelluläre osmotische und energetische Homöostase und einem Redoxgleichgewicht reguliert. Eine Bandbreite an Signalmolekülen wie z.B. Kinasen sind für die Regulation der Eryptosemaschinerie bedeutend. Da eryptotische Erythrozyten schnell durch Makrophagen aus der Blutbahn eliminiert werden, könnte eine übermäßige Eryptose zur Entstehung einer Anämie führen insofern der Verlust an Erythrozyten nicht durch eine Produktion neuer Erythrozyten ausgeglichen wird. Es ist bekannt, dass eine vorherrschende Anämie die Lebensqualität von Patienten mit Lungenkrebs und akuter Herzinsuffizienz beeinträchtigt. Eine vorliegende Anämie kann als wichtiger Prognosefaktor für den Verlauf dieser Erkrankungen herangezogen werden.

Im ersten Teil der vorliegenden Dissertation wurde die Hypothese, eine erhöhte Eryptose trage zur Entstehung einer mit Lungenkrebs und akuter Herzinsuffizienz assoziierten Anämie bei, untersucht. Die vorliegenden Daten zeigen, dass Patienten, die entweder an Lungenkrebs oder akuter Herzinsuffizienz leiden, eine erhöhte Retikulozytose aufweisen was auf einen erhöhten Erythrozytenumsatz hinweist.

Den vorliegenden Beobachtungen zufolge ist die erythrozytäre Phosphatidylserinexposition bei Lungenkrebs ohne und mit zytostatischer Behandlung im Vergleich zur gesunden Kontrollgruppe deutlich erhöht. Die Erythrozyten der Lungenkrebspatienten zeigen ein erhöhtes Forward Scatter (stellvertretend für das Zellvolumen) und ein erhöhtes Level an oxidativem Stress und Ceramid. Diese Effekte könnten auf Komponenten im Plasma der Lungenkrebspatienten zurückgeführt werden. In ähnlicher Art und Weise zeigen auch Patienten mit akuter Herzinsuffizienz einen erhöhten Prozentsatz an Phosphatidylserin-exponierenden Erythrozyten, begleitet von einem erniedrigten Forward Scatter und erhöhtem oxidativem Stress. Zusammengefasst lässt sich sagen, dass die vorliegenden Daten auf die Eryptose als einen bedeutenden Mechanismus in der Pathogenese der Anämie bei Lungenkrebs- und akuten Herzinsuffizienzpatienten hinweisen.

Im zweiten Teil der vorliegenden Dissertation wurde die Rolle von Gαi2, einem G-Protein gekoppelten Rezeptorprotein, im erythrozytären Überleben untersucht. Mögliche Funktionen des Gαi2-abhängigen Signalweges in zellkernhaltigen Zellen umfassen die Regulation von Ionenkanälen sowie Zelldifferenzierung, Proliferation und Apoptose. Die vorliegenden Daten belegen die Expression von Gαi2 sowohl in murinen als auch in menschlichen Erythrozyten und erläutern den Einfluss von Gαi2 auf das Überleben der kernlosen Erythrozyten. Zu diesem Zweck wurden Erythrozyten aus Gαi2-defizienten ($G\alpha i2^{-/-}$) und den dementsprechenden Wildtypmäusen ($G\alpha i2^{+/+}$) isoliert. Die Erythrozyten betreffenden Parameter waren in beiden Mäusen, $G\alpha i2^{-/-}$ und $G\alpha i2^{+/+}$, ähnlich. Das mittlere korpuskuläre Volumen und die Leukozytenzahl war allerdings in $G\alpha i2^{-/-}$ Mäusen signifikant höher. Anhand der May-Grünwald-Färbung konnten keine nennenswerten Unterschiede in der Morphologie zwischen $G\alpha i2^{+/+}$ und $G\alpha i2^{-/-}$ Erythrozyten verzeichnet werden. Das mittlere korpuskuläre Volumen war allerdings in $G\alpha i2^{-/-}$ Mäusen signifikant höher. Die spontane PS-Exposition der in der Blutbahn zirkulierenden $G\alpha i2^{-/-}$ Erythrozyten war im Vergleich zu den $G\alpha i2^{+/+}$ Erythrozyten signifikant niedriger. Die PS-Exposition war nach einer *ex vivo* Exposition gegenüber pathophysiologischen Zellstressoren wie hyperosmolarem Schock (+550 mM Saccharose) und nach Behandlung mit bakterieller Sphingomyelinase und C6 Ceramid in den $G\alpha i2^{-/-}$ Erythrozyten im Vergleich zu $G\alpha i2^{+/+}$ Erythrozyten signifikant niedriger. Die erythrozytäre Gαi2-Defizienz schwächte die durch den hyper-

osmolaren Schock erhöhte Calciumaktivität und den Zellschwund ab. Desweiteren waren die *Gαi2*$^{-/-}$ Erythrozyten gegenüber osmosensitiver Hämolyse resistenter als die *Gai2*$^{+/+}$ Erythrozyten. Insgesamt zeigen die vorliegenden Daten, dass eine Gαi2-Defizienz der Erythrozyten einen teilweisen Schutz gegenüber dem suizidalen Erythrozytentod verleiht.

4 INTRODUCTION

4.1 Physiological functions of erythrocytes

Among the different cell types in humans, erythrocytes (or red blood cells (RBCs)) are morphologically and functionally unique. They are the most abundant cell type in the human body [1] and in healthy individuals, the number of erythrocytes reaches up to 4-6 x 10^{12} per liter of blood [2]. With a diameter of about 6-8 µm and a thickness of about 2 µm, erythrocytes are smaller than most other human cells and are characterized by an outstanding formability. These features enable them to pass through small capillaries with relative ease [3, 4]. Unlike nucleated cells, erythrocytes lack important organelles such as a nucleus, mitochondria and ribosomes. The lack of these important cellular organelles enables them to store more hemoglobin, the oxygen-binding protein. Furthermore, the biconcave shape of erythrocytes is further designed for their main function i.e. the transport of oxygen and carbon dioxide by increasing the surface area for the diffusion of these gases [5]. The oxygen binding hemoglobin molecules consist of four polypeptide subunits, two Hb α and two Hb β units, each one containing an iron complex, the heme. In addition to oxygen transport, erythrocytes also play an important role in the regulation of pH in the body [6, 7] as well as in the maintenance of vascular resistance and vessel diameter due to their ability to release adenosine triphosphate (ATP) [8]. ATP is released from the erythrocytes into the surrounding environment as a response to not only different physiological stimuli including hypoxia and hypercapnia [9], decreased pH [10], membrane deformation [11] but also to pharmacological stimuli [12]. Biochemically, the only known energy source of RBCs is the anaerobic oxidation of glucose (i.e. glycolysis) [13].

4.2 Erythropoiesis

Generation of erythrocytes occurs in the fetal liver and spleen, in the embryonic yolk sac and in the fetal as well as adult red bone marrow. Erythrocyte formation from pluripotent hematopoietic stem cells in the bone marrow is stimulated by the renal hormone erythropoietin (EPO) and this process is termed as “erythropoiesis” [14].

Erythropoiesis maintains the number of circulating erythrocytes under various physiological conditions [15]. During erythropoiesis, erythrocytes undergo different stages of differentiation, from erythroblasts to reticulocytes, and finally to mature anucleated erythrocytes [1]. On average, the normal life span of a normal erythrocyte is about 120 days [1], after which they are removed by macrophages residing in the liver and spleen [16]. Aged erythrocytes expose phosphatidylserine on their membrane, which is recognized by phosphatidylserine receptors of macrophages. Subsequently, the erythrocytes are engulfed, digested and removed from the blood stream [17]. The decrease in cell volume further facilitates their engulfment [17].

4.3 Composition of the erythrocyte membrane

Being the only structural component of erythrocytes, the plasma membrane is responsible for all kinds of transport, mechanical and antigenic characteristics of these cells [3]. The erythrocyte membrane constitutes a bipolar lipid bilayer, integral membrane proteins and a membrane skeleton (cytoskeleton). Approximately 52% of the membrane mass is attributed to proteins, 40% to lipids and 8% to carbohydrates [18]. Carbohydrates are attached exclusively to the outer surface of the erythrocyte membrane. The lipid bilayer is composed of phospholipids and cholesterol [3], where the phospholipids are asymmetrically distributed [19, 20]. While sphingomyelin, phosphatidylcholine and glycolipids appear on the outer surface of this lipid bilayer, phosphatidylethanolamine, phosphatidylserine and, to a lesser extent, phosphatidylinositol are located on the inner side of this bilayer [19, 20]. This phospholipid asymmetry is generated and maintained by several phospholipid transport proteins by energy-dependent and/or energy-independent mechanisms [3, 21, 22]. The membrane skeleton is located on the inner surface of the lipid bilayer and forms a complex network that consists of structural proteins such as α and β-spectrin, actin, protein 4.1R, protein 4.9 (dematin), adducin, tropomodulin and tropomyosin [23-25]. The membrane proteins either adhere peripherally to the lipid bilayer or penetrate it (transmembrane proteins). The membrane skeleton is coupled to the lipid bilayer via transmembrane proteins [26].

The linkages are generated by ankyrin and protein 4.1R [3]. To date, more than 50 transmembrane proteins that serve different functions such as adhesion, transport or signaling, have been identified [3]. In the present study, the Gardos Channel, a Ca^{2+}-activated K^+ channel [27], is of particular relevance.

4.4 Eryptosis

Several years ago, erythrocytes were described to undergo a unique type of cell death that is analogous to apoptosis of nucleated cells [28-30]. Erythrocyte injury may jeopardize their integrity and survival in the circulation [31]. Under these circumstances, they are able to undergo a specialized form of cell death prior to their senescence i.e. the suicidal erythrocyte death, also referred to as "eryptosis" [32]. Eryptosis is a closely regulated and coordinated programmed cell death where any damage to the plasma membrane and release of intracellular content is averted [33]. Erythrocytes undergoing eryptosis show cell blebbing, cell membrane scrambling with exposure of phosphatidylserine from the inner leaflet of the membrane to the erythrocyte surface and cell shrinkage. All these aforementioned features are typical hallmarks of apoptosis in nucleated cells [28-30]. Eryptotic cells are rapidly cleared from the circulation by macrophages and therefore prevented from undergoing hemolysis, a necrosis like cell death, which is associated with the release of hemoglobin into the plasma [31, 34]. The excessive release of hemoglobin would otherwise pass the renal glomerular filter and precipitate within the acid lumen of the renal tubular system, subsequently occluding nephrons and impairing kidney function [35]. Accordingly, eryptosis is a "soft" mechanism that prevents hemolysis [34]. In contrast to apoptosis of nucleated cells, the process of eryptosis is devoid of mechanisms such as nuclear condensation, DNA fragmentation and mitochondrial depolarization [36, 37].

4.4.1 Mechanisms of eryptosis

One of the decisive mechanisms in the triggering of eryptosis is the increase of cytosolic Ca^{2+} concentration, which occurs due to the stimulation of Ca^{2+} entry into the cells [28-30]. Ca^{2+} triggers the activation of scramblases, which, in turn, mediates cell membrane scrambling and the breakdown of the cell membrane phospholipid asymmetry, whereby phosphatidylserine is translocated from the inner leaflet of the cell membrane to the erythrocyte surface [38]. In addition to cell membrane scrambling [39], Ca^{2+} further triggers vesiculation [40] and activates the cysteine endopeptidase calpain, an enzyme that causes the degradation of the cytoskeleton leading to cell membrane blebbing [41]. Enhanced cytosolic Ca^{2+} further activates Ca^{2+}-sensitive K^+ channels, also referred to as Gardos Channels, with subsequent exit of K^+. The exit of K^+ leads to hyperpolarization of the cell membrane and to an efflux of Cl^-. Water passively follows along the concentration gradient, resulting in cell shrinkage, another hallmark of eryptosis [42]. The Ca^{2+} entry may result from the activation of Ca^{2+}-permeable non-selective cation channels [43, 44]. These erythrocyte channels have not been characterized at the molecular level but it is assumed that TRPC6 channels are involved [45]. Activation of these non-selective cation channels could result from the removal of extracellular chloride [46, 47], prostaglandin E_2 (PGE_2) [48], oxidative stress [47, 49] or hyperosmotic shock [46, 49]. Eryptosis is further stimulated by energy depletion [50], an impaired antioxidant defense [51-54] and by endogenous substances as well as several xenobiotics [31]. Another important trigger of eryptosis is the formation of ceramide. Ceramide is produced from cell membrane sphingomyelin by the action of sphingomyelinase enzyme [55]. Ceramide formation sensitizes the cells to the effect of increased Ca^{2+} on phosphatidylserine exposure [34]. Both ceramide formation and increased cytosolic Ca^{2+} can also have a synergistic effect. Moreover, several kinases participate in the activation of suicidal erythrocyte death e.g. protein kinase C (PKC) [50], Janus-activated kinase JAK3 [56], casein kinase 1α (CK1α) [57], p38 MAPK [58] and caspases [59]. On the other hand, activation of other kinases results in the inhibition of eryptosis e.g. stimulation of cGMP-dependent protein kinase [60], mitogen-and stress-activated kinases MSK1 and MSK2 [61], AMP activated kinase (AMPK) [62], and PAK2 kinase [63]. Eryptosis is also triggered by the non-specific multikinase inhibitors sunitinib [64] and sorafenib [65], indicating an inhibitory effect of these kinases.

4.4.2 Eryptosis in human diseases

Besides endogenous substances and xenobiotics that stimulate eryptosis [66], enhanced suicidal erythrocyte death has also been observed in various clinical conditions including malignancy [67], chronic kidney disease [68], iron deficiency [69], and many more [70].

An overview of the mechanisms of eryptosis and diseases associated with eryptosis is illustrated in Fig. 1.

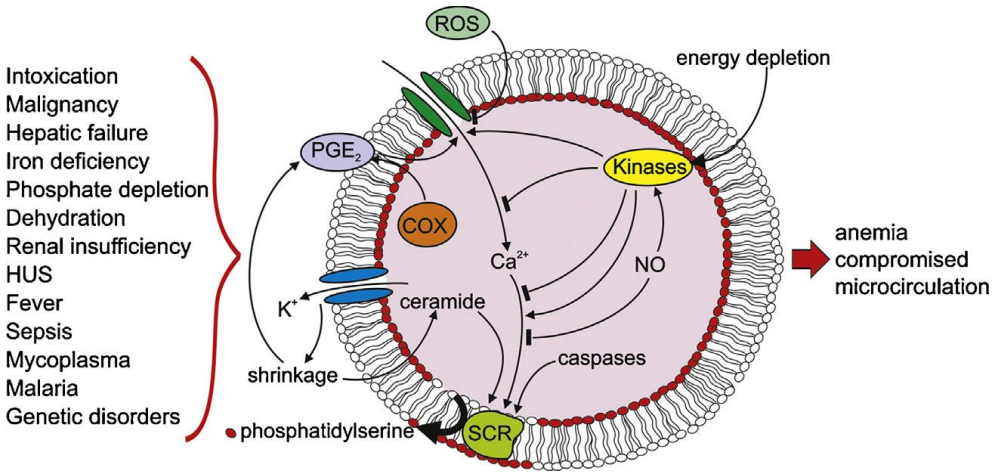

Fig. 1: Mechanisms of eryptosis in different diseases [70].

Importantly, eryptosis serves the purpose of eliminating potentially harmful defective erythrocytes from the blood stream [34]. Eryptotic erythrocytes expose phosphatidylserine on their surface, which is recognized by specific phagocyte receptors that subsequently engulf and degrade them [17, 71]. Phosphatidylserine not only binds to phagocytes but also to docking molecules such as CXCL16/SR-PSOX [72] and several other receptors expressed on the surface of endothelial cells [73, 74]. Similar to endothelial cells, CXCL16 is also expressed on platelets in an activation-dependent manner [75-77] and binding of erythrocyte phosphatidylserine to blood platelets has also been reported [75]. The microcirculation can be impaired by the binding of erythrocyte phosphatidylserine to endothelial cells of the vascular wall or to blood platelets [54]. Accordingly, eryptotic erythrocytes may play an important role in the stimulation of blood clotting and contribute to the pathogenesis of thrombosis [72, 78-80]. In theory, erythropoiesis

compensates the loss of erythrocytes by eryptosis, and the total number of erythrocytes in the circulation remains unaffected.

Increased reticulocytosis may therefore be an indicator for enhanced eryptosis, even in the presence of an apparently normal blood count [34]. However, anemia could develop if the enhanced suicidal erythrocyte death is not outweighed by a similar production of new erythrocytes [54]. Indeed, anemia is the most important manifestation of pathological changes in erythrocytes [34]. Recent epidemiological studies suggest that approximately one third of the world's population suffers from anemia [81].

4.5 Anemia

Anemia is a clinical condition, which is characterized by a decreased erythrocyte count, hemoglobin, and/or hematocrit level [82]. This results in a lower ability of the blood to supply the body tissues with sufficient oxygen. Severe anemia may potentially lead to hypoxia of different organs [83]. Anemia may result from enhanced blood loss, increased destruction of erythrocytes or decreased production of functional erythrocytes [82]. Anemia can be classified according to its pathophysiology, erythrocyte size (Mean Corpuscular Volume; MCV) and the amount of hemoglobin in each cell (Mean Corpuscular Hemoglobin Concentration; MCHC). In normochromic, normocytic anemia, the MCHC and MCV are normal (MCV: 80-100 fl). In hypochromic, microcytic anemia, MCHC and MCV are decreased (MCV: < 80 fl). In normochromic, macrocytic anemia, MCHC is normal, but MCV is increased (> 100 fl) [84]. In most cases, anemia is multifactorial and shows a diverse pathophysiology [81]. Anemia may be further subclassified into mild anemia with a hemoglobin level of 9.5-10.9 g/dl (mainly asymptomatic), moderate anemia (8.0-9.4 g/dl), severe anemia (6.5-7.9 g/dl) and life threatening anemia with a hemoglobin level of <6.5 g/dl [85]. Important markers commonly used for a more accurate characterization of anemia are the serum levels of ferritin and transferrin as well as the transferrin saturation. In the body, iron is mainly stored in the form of ferritin. Accordingly, low ferritin levels may thus be an indicator for iron deficiency (ID), a possible cause of anemia. In ID, transferrin, responsible for the transport of iron to the cells, is increased, while the transferrin saturation is decreased.

Clinical signs and symptoms of anemia include impaired cognitive function, headache, dizziness, chest pain, indigestion, menstrual problems, decreased motivation, depression, nausea, fatigue, anorexia, exertional dyspnea, cardiovascular complications, loss of libido and sleeping disorders [86].

4.6 Lung Cancer

Lung cancer can arise in virtually all parts of the lung including the trachea, bronchi or bronchioles.

Each lung section consists of a predominant cell type, which reflects a predominant subtype of cancer. Lung cancer is the main reason for cancer death worldwide, accounting for 1.6 million deaths annually [87]. In 85-90% of the cases, pathogenesis of lung cancer is directly attributable to the consumption of tobacco [88]. The mean age at death from lung cancer for both genders is similar, with a mean age of 68.6 years for males and 70.5 years for females [89]. At the time of diagnosis, approximately 70% of the patients are diagnosed with locally advanced or metastatic disease [90], which is one of the reasons for the dismal five-year survival rate with only about 15% after diagnosis [90].

4.6.1 Non-Small Cell Lung Cancer (NSCLC) and Small Cell Lung Cancer (SCLC)

According to its histopathological characterization, lung cancer can be classified into two major types: Small Cell Lung Cancer (SCLC) and Non-Small Cell Lung Cancer (NSCLC). Approximately 85% of bronchogenic carcinomas are of the NSCLC type [91]. Because of their similarity in prognosis and treatment, NSCLC is any type of epithelial cancer and can be further grouped into three main subtypes: adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Adenocarcinoma is the most frequently diagnosed histological subtype of NSCLC with a prevalence of about 50%, followed by squamous cell (epidermoid) carcinoma (~35%). Large cell carcinomas constitute approximately 15% of NSCLC cases [91]. In addition to the aforementioned types of carcinomas, a few rarer subtypes are also found [92].

In comparison to other subtypes of lung cancer, adenocarcinomas are characterized by their extensive molecular heterogeneity [93, 94]. The tumors usually originate from the smaller airways and penetrate peripheral lung tissues [95] such as the bronchioles and alveoli. They may further be classified as mucinous or nonmucinous [96]. Adenocarcinoma is the most commonly occurring type of lung cancer in general [97], is more frequently found in women [98] and is the most common type of lung cancer in nonsmokers [95, 97, 99].

Squamous cell carcinoma (SCC) tends to be more centrally located [95], peripheral SCC, however, is associated with a better prognosis and fewer symptoms as compared to central SCC [100]. The presence of cavity, an independent and unfavourable prognostic factor that is very common in SCC [101, 102], is associated with a worse clinical outcome [101]. Histologically, the most common feature of SCC is keratinization and/or the formation of intercellular bridges [103].

The criteria for the diagnosis of large cell carcinoma (LCC) vary widely. They are a heterogenous group [104] and may represent undifferentiated forms of other types of cancers [95]. In 1999, large cell neuroendocrine carcinoma (LCNEC) was introduced as a variant type of large cell carcinoma [105]. The tumor cells of LCNEC are generally large [106], lack specific histological features of NSCLC but show neuroendocrine differentiation like SCLC [105]. Thus, in some cases it can be difficult to distinguish them from SCLC [107]. At the cellular level, LCNEC shows a higher proliferative activity than classical LCC [108, 109] and, similar to SCLC [110], the survival rate is substantially worse than for other NSCLC [108].

SCLC is a high-grade neuroendocrine carcinoma [111] that mainly arises in the central airways [95, 112]. It is characterized by rapid growth and the early occurrence of widespread metastases at the time of diagnosis [113, 114]. The main location for distant metastases are liver, bones, pancreas, contralateral lung, adrenal glands, extra-thoracic lymph nodes and pleural and/or pericardial fluids [115]. Metastases to the brain are very common in this type of cancer [116, 117]. SCLC is considered to be the most aggressive type of lung cancer [118] and has the poorest five-year survival rate of all lung cancer types [119].

4.6.1.1 Lung Cancer Staging

Lung Cancer Staging enables us to classify the extent and progress of the disease. Determination of staging is performed routinely and is essential for planning of therapy. NSCLC is classified according to the AJCC (American Joint Committee on Cancer) staging system, which is based on the TNM, the “Tumor-Node-Metastasis”-system [120]. T indicates tumor size and its extension (T1-4), N describes the spread of cancer

to regional lymph nodes (N0-N3) and M indicates the occurrence of metastases (M0-M1) [120]. NSCLC can be further subdivided into stage IA & B, stage IIA & B, stage IIIA & B and stage IV. Moreover, SCLC can also be simply classified as limited disease (LD-SCLC) and extended disease (ED-SCLC) [121].

4.6.1.2 Treatment of Lung Cancer

NSCLC is relatively insensitive to radiation therapy and chemotherapy, therefore the treatment of choice is mainly surgical resection. Stage I to IIIA NSCLC is mainly treated by surgery, although stage IIIA tumors are resectable only in few cases [122]. Stage IIIB and IV are considered inoperable. For these stages, palliative chemotherapy is applied [123, 124]. Chemotherapy mainly consists of the platinum-based regimens of cisplatin [125] and carboplatin [126] that are mainly used in combination with paclitaxel [127], docetaxel [128], gemcitabine [129], pemetrexed [130], etoposide [131] and/or vinorelbine [132]. More recently, newer insights on the molecular mechanisms of NSCLC and its different mutations have enabled the development of novel therapeutic agents that have shown to improve the clinical outcome [133].

On the other hand, surgical treatment for SCLC may be considered only in selected cases such as T1-2, N0-1 stage [134]. On the basis of rapid metastasis formation, SCLC can be considered as a generalized disease and is virtually inoperable. SCLC, however, is very chemosensitive [135] and is more responsive to radiotherapy than NSCLC [105]. However, SCLC acquires drug resistance during the course of the disease [136]. In the 70s, cyclophosphamide, adriamycin and vincristine (CAV regimen) became the standard treatment for patients with limited SCLC [137, 138]. In the 80s, however, the combination of cisplatin and etoposide (EP regimen) was increasingly used in the treatment of SCLC [137, 139] and to this day is considered as gold-standard. This therapeutic regimen is often administered concomitant with thoracic radiotherapy [111]. Although a majority of patients respond to first-line therapy [140], nearly all patients with ED and about 80% with LD develop recurrent SCLC [112]. In such cases, the treatment is followed up with a second-line chemotherapy which is suitable as palliative treatment [141]. The second-line therapy comprises of a single pharmacological agent [141] and may include agents such as topotecan [142] or oral etoposide [143].

4.6.2 Anemia in Cancer

Anemia is very commonly diagnosed in cancer patients. 30-90% of cancer patients suffer from some form of anemia depending on the type and stage of cancer [144]. Patients suffering from lung cancer show a higher frequency of chemotherapy-induced anemia [145]. The etiology of anemia in cancer patients is multifactorial [82]. Malignancy *per se* can trigger anemia or may exacerbate pre-existing anemia [82]. Cancer cells may infiltrate bone marrow and thus directly subdue hematopoiesis**.** Tumor anemia can also be caused due to organ damage and chronic haemorrhages [82]. On the other hand, cancer-induced inflammatory cytokines may suppress the production of EPO and the proliferation of erythroid progenitor cells [146, 147]. Nutritional deficiencies in cancer patients could also indirectly contribute to anemia [82]. In addition, chemotherapeutic drugs negatively influence hematopoiesis as well as the synthesis of red blood cell precursors in the bone marrow [82]. Noticeably, platinum-based chemotherapeutic regimens afflict renal tubular cells, which, in turn diminish endogenous EPO production [148]. Thus, patients receiving platinum-based chemotherapy have a higher risk for developing anemia as compared to patients receiving non-platinum based chemotherapy [149].

4.6.2.1 Anemia as an independent prognostic factor

Compelling evidence suggests that anemia is an independent prognostic factor for the survival rate of cancer patients [150]. The appearance of anemia is associated with decreased survival in almost all previously studied cancer types [150]. As a matter of fact, anemia can reduce the tolerance to anticancer therapy [151]. In worse cases, anemia may lead to hypoxia, which influences tumor behaviour [146]. Tumor hypoxia is associated with resistance to both radiation therapy and chemotherapy [144]. It is further associated with angiogenesis, the growth of new capillary vessels, which supports tumor progression and tumor growth [152-155]. Anemia not only impacts tumor behaviour but profoundly affects the quality of life in cancer patients. It is associated with impaired organ function, increased risk of postoperative mortality and a higher probability of blood transfusion after chemotherapy [146]. Furthermore, anemia worsens fatigue, a cardinal symptom in cancer patients [156].

4.7 Cardiovascular diseases

Cardiovascular diseases (CVDs) remain one of the major causes of all deaths worldwide [157]. CVDs involve ailments of the heart or the blood vessels and are considered to be multifactorial with a complex pathophysiology. In 2012, approximately 17.5 million fatalities were caused by CVDs which represents around 31% of all global deaths [158]. According to the World Health Organization (WHO), CVDs will continue to remain the most common cause of mortality in the future [158].

4.7.1 Heart failure

Heart failure (HF) is one of the most common manifestations of CVDs, which afflicts 23 million people worldwide [159, 160]. HF is considered to be one of the major reasons for hospitalization in Germany [161], United States and Europe [162]. In particular, this condition has become a growing problem in the ageing population of industrialized countries [163]. The incidence of HF rises from 1.7% in the age group between 55-64 years to 9.8% in the age group between 75 and 84 years. The incidence has been reported to reach 16.8% in the octogenarian cohort [164].

Clinically, HF is defined as the inability of the heart to support the physiological and metabolic demands of the body [165, 166]. The supply of sufficient blood and oxygen is affected in the failing heart [167]. HF is considered to be a syndrome that develops as a syndrome of underlying cardiac diseases [168]. Its clinical diagnosis is often based on a constellation of different signs and symptoms [168] and thus, there is no universal consensus as to the precise definition of HF [168, 169]. Symptoms of HF often include dyspnea at rest or during exercise [170], fatigue [171], a reduced exercise capacity [172], an increased incidence of arrhythmias [173], fluid retention [174] and a diminished quality of life [175]. According to the New York Heart Association (NYHA) classification, the severity level of HF is divided into four different stages (A-D) which are based on the subjective estimate of a patient and may not necessarily correlate with the objective estimate [176].

Anatomically, HF can be further subclassified into left ventricular (LV) failure (LVF) and right ventricular (RV) failure (RVF) [177, 178], LVF being the most common type of HF [178]. Lack of adequate treatment of one type of HF may impair the normal functioning of the other ventricle, thus, leading to biventricular failure [177]. In decompensated HF, the symptoms of this condition are aggravated as a result of rapid deterioration of cardiac function. In compensated HF, however, symptoms are relatively stable [179]. There are various causes for HF which may include coronary artery disease, chronic hypertension, cardiomyopathy, valve dysfunction, cardiac arrhythmias, pericardial diseases, infections and/or diabetes mellitus [163]. Chronic anemia can also cause HF even in the absence of underlying heart disease [180, 181]. Previous studies have documented that therapeutic transfusion in anemia has a beneficial effect on HF [181, 182]. Remarkably, coronary artery disease *per se* or coexisting hypertension seems to be the most common cause for HF in developed Western countries [163]. Due to the multifactorial and complex etiology of the pathogenesis of HF, it is difficult to ascertain the precise cause in many clinical cases [163].

Depending on the duration of its manifestation, HF can be classified as either chronic or acute. Chronic HF develops over a course of several months to years, while acute heart failure (AHF) develops within few hours to days. Accordingly, AHF has been defined as "the sudden or gradual onset of the signs or symptoms of heart failure requiring unplanned office visits, emergency room visits, or hospitalization" [183]. AHF patients are further classified into patients whose chronic HF worsened or those with *de novo* AHF [183]. Irrespective of whether the HF is actively treated or is asymptomatic, it is a lethal condition and has a 60% five-year mortality rate following diagnosis [163].

4.7.1.1 Anemia in heart failure

Anemia is a very frequent comorbidity in patients suffering from either acute or chronic HF. The prevalence of anemia in HF patients is, on the one hand, dependent on the severity of the disease while, on the other hand, dependent on the exact definition of anemia. Approximately 14.4% of HF patients have a hemoglobin level lower than 11 g/dl and the percentage of HF patients with a hemoglobin level lower than 12 g/dl steeply rises to 55.6% [184-186].

Anemia in patients with HF is associated with lower blood pressure, increased edema, enhanced levels of proinflammatory cytokines, C-reactive protein and neurohormones as well as an increased use of diuretics [187]. Several studies have reported a high prevalence of anemia in HF, which is associated with a poor prognosis [180, 181, 188, 189]. Strikingly, the cause(s) of anemia in chronic and acute HF have been a subject of debate. As a matter of fact, HF can be considered as an inflammatory disease [190] and, thus, anemia could prevail due to a decreased production of erythropoietin as a consequence of enhanced inflammatory cytokine production (TNF-α, IL-6) that could, in turn, interfere with EPO gene expression [191, 192]. Furthermore, the cytokine production may reduce the activity of EPO in the bone marrow [193].

Anemia in HF can be due to a reduced erythrocyte count (true anemia) or may be the result of an increased plasma volume, also referred to as hemodilution that results in pseudoanemia [194, 195]**.** Hemodilution is a very common phenomenon encountered in patients suffering from HF and anemia, with a prevalence of about 46% [194]. It may result due to the activation of vasopressin and renin-angiotensin-aldosterone system, which consequently leads to water and sodium retention [191]. In contrast to patients with true anemia, HF patients with hemodilution may simply require an adjustment in their diuretic dosage [194]. Interestingly, pseudoanemia has been reported to have a worse clinical outcome as compared to "true" anemia [191, 194].

More recently, the role of iron deficiency (ID) in anemia associated with HF has been the subject of discussion [196-199]. Iron is crucial for optimal erythropoiesis. Around 40% of HF patients have been reported to suffer from anemia [196]. Intriguingly, approximately 60% of the patients with anemia and 40% of non-anemic patients suffer from ID [196]. Compelling evidence suggests a direct association between ID and low iron content in the heart [200, 201] and in the bone marrow [202] of HF patients. Treatment of ID by exogenous iron supplementation in HF patients had a positive impact on the quality of life and on the clinical status of these patients [203, 204]. Even in the absence of anemia, ID *per se* is associated with a worse clinical outcome in HF and individual iron status may be considered as a potentially independent and strong predictor of clinical outcome in HF [204, 205].

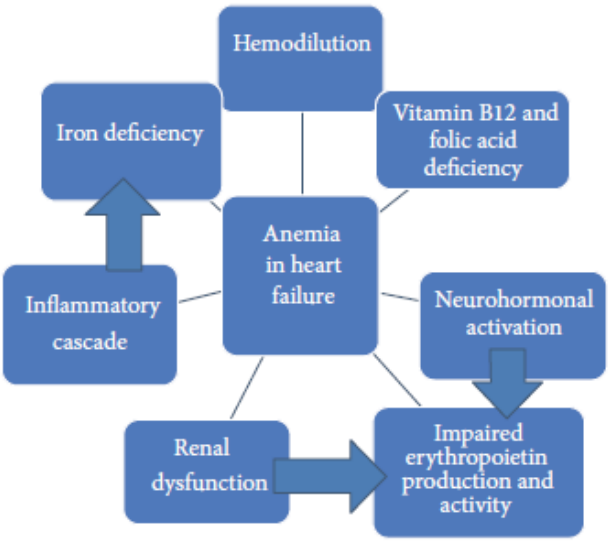

Fig. 2. Causes of anemia in heart failure [206].

Besides iron, other nutrients such as vitamin B12 and folic acid, may also negatively impact the outcome in HF patients [204]. The incidence of folic acid and vitamin B12 deficiency in HF is rather low, with a share of 8% and 6%, respectively [207]. Although both vitamin B12 and folic acid play an important role in erythropoiesis, their levels are not directly related to prognosis in HF and were further shown not to be associated with mortality [204].

HF in approximately half of the patients is associated with chronic kidney disease (CKD) that is known to cause decreased erythropoietin production in the kidney due to inadequate erythropoiesis [208, 209]. There is strong evidence that CKD is a major contributor to HF and vice versa. Remarkably, anemia further worsens CKD and HF, and all these conditions exacerbate one another, thus, establishing a vicious circle [210], the cardio-renal anemia syndrome [209]. Anemia can result in increasing the stroke volume of the heart, thereby, leading to cardiac stress, which, in turn, results in the deterioration of cardiac function [193]. Accordingly, anemia may be an underlying cause in the pathogenesis of HF [180].

Angiotensin converting enzyme inhibitors (ACEIs), beta blockers, aldosterone receptor antagonists (ARAs, e.g. spironolactone and eplerenone), angiotensin receptor blockers (ARBs), anticoagulants, vasodilators, digoxin, anti-arrhythmic drugs, diuretics and statins are commonly used in the treatment of HF [211]. Ironically, however, some of these agents are associated with the development of anemia despite improving the clinical status of HF [212].

Angiotensin, for example, is a physiological stimulator of erythropoietin and hence, the use of ACEIs has been associated with suppression of erythrocyte production subsequently resulting in anemia [213-215]. The use of carvediol, a beta-blocker, is associated with lower hemoglobin levels [216]. In particular, elderly HF patients are vulnerable to gastrointestinal bleedings and, thus, to anemia due to their advanced age and the existence of multiple comorbidities [217], increased use of anticoagulants [218] and antiplatelet agents e.g. aspirin [219, 220].

4.8 G-protein coupled receptors (GPCRs)

4.8.1 Importance of GPCRs

Receptors on cell membranes detect and transmit a variety of different molecular signals. Most of these receptors are coupled to guanine-nucleotide-binding signal transducing proteins (G-proteins) and are, therefore, referred to as G-protein coupled receptors (GPCRs). G-proteins receive the information from the receptor and subsequently transmit the signals to effector molecules [221]. Hitherto known effector molecules of G-proteins include phosphatidylinositol phospholipase C (PLC) isoforms, adenylyl cyclases, cGMP phosphodiesterase, phosphatidylinositol-3 kinases and ion channels such as potassium as well as calcium channels [222, 223].

G-protein coupled receptors (GPCRs) encompass the largest family of transmembrane receptors that are encoded by approximately 720 to 800 genes in the human genome [224]. Physiologically, they are the most important membrane proteins and play a decisive role in the regulation of different functions of almost all cells in the body [225, 226]. On the basis of their paramount physiological significance, they are categorized as the most important therapeutic targets for the pharmaceutical industry and account for about 30% of the total sales of therapeutic drugs [227, 228]. Both antagonists and agonists of GPCRs are widely used in the treatment of diseases of virtually every organ system such as the metabolic, urogenital, cardiovascular, CNS and respiratory system [229]. A study by Vassilatis et al. revealed 392 mouse and 367 human GPCRs with 343 GPCRs being common to both species [230].

4.8.1.1 Structure of GPCRs

On the basis of their structure, GPCRs are also known as “7 TM (transmembrane) receptors”, serpentine receptors [224] and heptahelical receptors [231]. Although they are diverse in their sequence homology and structure, GPCRs share a common structure that consists of a polypeptide chain with seven transmembrane helices and three intracellular as well as three extracellular loops. The C-terminus is located intracellularly, while the N-terminus is located extracellularly.

The extracellular loops and the N-terminus are responsible for the recognition of a wide variety of ligands [225]. Based on their amino acid sequence, GPCRs can be divided into five different families: the adhesion receptor family, the secretin receptor family, the glutamate receptor family, the frizzled/taste 2 receptor family and the rhodopsin family [232].

4.8.1.2 GPCR signaling

GPCRs have the ability to recognize a wide range of extracellular stimuli including light [233], amino acids [234], proteins [235], taste ligands [236], metals [237], peptides [238], fatty acids [239], nucleotides [240], biogenic amines [241], odorants [242], steroids [243], citric acid cycle intermediates [244], lipids [245] or Ca^{2+} [246]. Binding of a ligand to a G-protein coupled receptor causes a conformational change in the receptor, which, in turn, affects its interaction with heterotrimeric G-proteins that are attached on the interior surface and consist of α, β and γ-subunits. The ligand binding promotes the exchange of GDP for GTP on the α-subunit and furthermore leads to the dissociation of the α-subunit from the β γ heterodimer. Consequently, the free β γ complex and α-GTP each activate effector molecules such as ion channels or enzymes [226, 247, 248]. After the dissociation of the ligand from the receptor, the intrinsic GTPase activity of the Gα subunit results in the hydrolysis of GTP to GDP, that, in turn, leads to the reassociation of the subunits and to the rebinding of the heterotrimer to the receptor [247, 248].

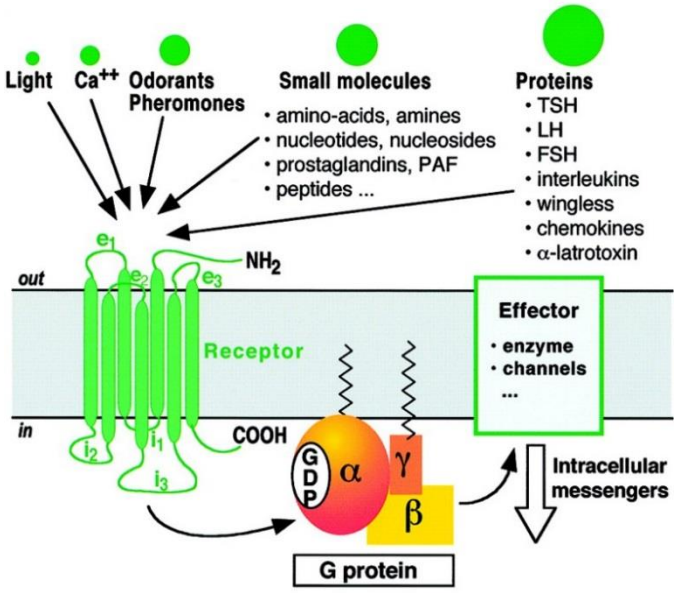

Fig. 3: Structure, activation and signaling of GPCR receptors [246].

G proteins are classified into four main families on the basis of the α subunit i.e. αq for αq containing protein, whose members activate PLC-β; Gαs for stimulatory protein, whose members stimulate adenylyl cyclase; Gα12 for α12 containing protein, whose members interact with regulators of G protein signaling (RGS) domain-containing Rho exchange factors and Gαi for inhibitory protein, whose members inhibit adenylyl cyclase [222]. The Gαi family further consists of different α subunits, namely Gαi1, Gαi2, Gαi3, Gαz, and Gα0 as well as another two α subunits i.e. Gαgust and Gαt [249]. Each family of Gαi subunits regulates specific effector proteins and is differentially expressed in various tissues [222, 249, 250]. Gαi1, Gαi2 and Gαi3 are ubiquitously expressed [249, 251] and are characterized by their sensitivity to pertussis toxin [250, 251]. These three closely related members share 85 to 95% of their amino acid sequence identities [252]. The αi2 subunit orchestrates different functions including inhibition of adenylyl cyclase, which leads to downregulation of cAMP levels [250, 253-255]. Gαi2 is the quantitatively predominant Gαi isoform [251] and is an essential regulator of endothelial [256], platelet [257] and leukocyte functions [258]. It further participates in important cellular functions such as apoptosis [259], cell proliferation, cell differentiation [260] and ion channel regulation [261-263].

4.8.1.3 GPCRs in erythrocytes

Although the functions of G-proteins in nucleated cells is widely documented, their potential role in red blood cells remains elusive. So far it has been shown that the heterotrimeric G-proteins Gαs, Gαq, Gαz, Gαi1, Gαi2 and Gαi3 are expressed in erythrocyte membranes [264-266]. Gαi2 expression was reported to be reduced in erythrocytes of humans suffering from type 2 diabetes leading to reduced accumulation of cAMP and reduced ATP release [264]. Heterotrimeric Gi proteins have been shown to play a crucial role in the release of ATP by erythrocytes [267]. These findings are consistent with data from animal models of diabetes, whereby reduced expression of the Gi subclass was observed in these animals [268, 269]. These observations suggest an important role of Gαi2 in the pathophysiology of diabetes [269]. Besides diabetes, lower levels of Gαi1, Gα12 and Gαo were observed in erythrocyte membranes of hypertensive subjects suggesting a possible role of these proteins in the regulation of blood pressure [270, 271].

Recent studies further suggest a critical role of some of the heterotrimeric G-proteins in the signaling pathway of EPO because Gαi1, Gαi2, Gαi3, Gαq and Gαs were shown to be expressed in hematopoietic cells [272, 273]. However, only Gi isoforms are present in day 10 erythroblasts [274]. Strikingly, of the Gi isoforms, only the Gαi2 isoform is potentially required for the regulation of calcium channels by erythropoietin [273].

5 OBJECTIVE OF THE STUDY

The objectives of the first part of this study are to investigate the contribution of eryptosis, the suicidal erythrocyte death, and its mechanisms in the development of anemia in two important clinical conditions i.e. lung cancer and acute heart failure. Furthermore, the present study aimed to elucidate a possible contribution of eryptosis in lung cancer-related anemia. To test this hypothesis, the exposure of phosphatidylserine on the erythrocyte surface, erythrocyte cell volume, intracellular Ca^{2+} concentration, generation of reactive oxygen species and the abundance of ceramide were determined by flow cytometry. In order to corroborate the data generated using FACS analysis, confocal microscopy was used to visualize eryptotic erythrocytes. Additionally, hematological parameters and experiments in patient plasma have been performed to elucidate the mechanisms of eryptosis associated with these conditions.

In the second section of this study, the role of Gαi2, a G-protein subunit, in the regulation of erythrocyte survival has been explored. Firstly, using immunoblotting it was shown that Gαi2 is expressed in both human and murine erythrocytes. To investigate the functional significance of Gαi2 in erythrocyte survival, erythrocytes from wild-type control mice ($G\alpha i2^{+/+}$) and Gαi2-deficient ($G\alpha i2^{-/-}$) mice were isolated and compared with one another. To this end, blood parameters were examined in these mice and flow cytometry and confocal microscopy were applied to examine the responses of erythrocytes from $G\alpha i2^{+/+}$ and $G\alpha i2^{-/-}$ mice to eryptotic stimuli *ex vivo* including hyperosmotic shock as well as treatment with bacterial sphingomyelinase and C6 ceramide. In addition to analyzing phosphatidylserine exposure, erythrocyte cell volume and intracellular Ca^{2+} concentration were analyzed both spontaneously and following exposure to pathophysiological cell stressors such as hyperosmotic shock as well as bacterial sphingomyelinase and C6 ceramide. Lastly, using spectrophotometry, the resistance of erythrocytes from $G\alpha i2^{+/+}$ and $G\alpha i2^{-/-}$ mice to osmosensitive hemolysis was examined.

6 MATERIALS & METHODS

6.1 Eryptosis in patients

6.1.1 Recruitment of patients with lung cancer

Whole blood was drawn from patients suffering from different types and stages of lung cancer (6 ♀, 11 ♂, 52-85 years). One group of patients received cytostatic treatment (n=11). Another group comprised of patients with a recent diagnosis of lung cancer where cytostatic therapy had not yet commenced (n=6). Whole blood was also drawn from age and sex matched healthy volunteers (4 ♀, 11 ♂, 41-70 years) with no known history or diagnosis of any clinical conditions.
All lung cancer patients were recruited from the Department of Internal Medicine, University of Tübingen, Germany. The healthy volunteers were blood donors at the blood bank of the University of Tübingen.

6.1.2 Recruitment of patients with acute heart failure

Whole blood was collected from patients suffering from acute heart failure (5 ♀ , 17 ♂, 33-84 years). The patients were recruited from the Department of Internal Medicine of the Charité Campus Virchow Clinic, Berlin, Germany. All patients showed signs and symptoms of heart failure during hospitalization due to an acute exacerbation, which could not be managed in an ambulatory setting. Whole blood was also drawn from an age and sex matched control group (5 ♀, 5 ♂, 35-78 years) recruited at the same clinic.

The Ethics Committee of the University of Tübingen approved both studies (184/2003V) and both patients and healthy volunteers signed a written informed consent.

6.1.3 Erythrocyte isolation, solutions and measurements

6.1.3.1 Isolation of erythrocytes

One ml Lithium-Heparin whole blood drawn from patients or healthy volunteers was added to 3 ml Ringer solution and centrifuged at 120 x g for 20 min at 23 °C, following which the platelets and leukocytes-containing supernatant was discarded and erythrocytes were transferred into a new Eppendorf tube. The subsequent measurements were performed in freshly isolated erythrocytes from healthy donors and patients or in erythrocytes from healthy donors (blood group O^-) after 24 hours (h) incubation in 500 µl plasma from patients or healthy donors at a hematocrit of 0.4%.

6.1.3.1.1 Ringer solution

Due to the lack of a nucleus and mitochondria, erythrocytes are not able to generate energy from the citric acid cycle. RBCs obtain their energy almost exclusively by glycolysis of glucose [275] and can, therefore, be stored in a relatively simple medium containing glucose as the only energy source. The Ringer solution, in which the erythrocytes were kept in this study, enables them to maintain their ion and water homeostasis. The composition of the solution is shown in Table 1.

Table 1: Composition of Ringer solution

Substance	Concentration (mM)
NaCl	125
KCl	5
$CaCl_2$	1
$MgSO_4$	1
HEPES	32.2
Glucose	5
NaOH	13

A volume of 982.798 ml H_2O was added to the different components listed in Table 1. In order to maintain physiological conditions, the pH of the Ringer solution was adjusted to 7.4 by the addition of HEPES and NaOH.

6.1.3.1.2 Annexin Wash Buffer

The Annexin Wash Buffer (AWB) is the main buffered-solution in this study, which was used to determine annexin V binding and intracellular Ca^{2+} content of erythrocytes using FACS analysis.

Since the binding of annexin V to phosphatidylserine on the erythrocyte surface is a calcium-dependent process, the AWB contains a higher amount of calcium chloride (5 mM) as compared to Ringer solution (1 mM). The composition of this buffer is illustrated in Table 2.

Table 2: Composition of "Annexin Wash Buffer"

Substance	Concentration (mM)
NaCl	140
HEPES	10
$CaCl_2$	5
NaOH	4

Subsequently, a volume of 990 ml H_2O was added to the different components listed in Table 2. The pH of the AWB was adjusted to 7.4 by the addition of HEPES and NaOH.

6.1.4 Flow Cytometry

Flow cytometry, also called FACS analysis ("Fluorescence activated cell sorting"), is a technology, which simultaneously detects multiple biochemical and physical characteristics of particles, usually cells. Measurements can be performed not only on whole cells but also on cellular components including organelles or nuclei. Flow cytometry provides information about the size of a particle, its granularity and if labelled, about its fluorescence intensity. Several thousand cells flow in a fluid stream per second and pass one or several lasers. While passing the laser, cells scatter the laser light in different directions that is captured by different detectors. Forward Scattered light (FSC) reflects the relative cell size and the cell-surface area and is displayed on the x-axis. Side-scattered light (SSC) gives information about the cell content and granularity of the cells and is shown

on the y-axis. Additionally, particles can be stained with fluorescent antibodies that are directed against specific intracellular antigens or antigens located on the cell surface. Secreted factors, however, can also be analyzed. Each fluorescent compound, e.g. FITC, absorbs light over a certain range of wavelengths, which is defined as its specific excitation or absorption spectrum. The absorbed light will be subsequently emitted again and accordingly, each fluorochrome has its specific emission spectrum. Emitted wavelengths are always longer than absorbed wavelengths because more energy is consumed at excitation (shorter wavelength). The use of such fluorescent dyes, in addition to FSC and SSC measurements, enables a more accurate characterization of several cell types and is used to differentiate cellular subpopulations.

In the present study, FACS analysis was performed on a FACS-Calibur (BD Biosciences, Heidelberg, Germany) which consists of four different laser channels (FL-1 to FL-4). Each of them detects a specific range of wavelengths. FL-1 channel for example detects green fluorescence with wavelengths between 480 and 530 nm and was the most frequently used channel in this study. The FACS-Calibur is equipped with two lasers, a 488 nm argon ion laser and a 635 nm diode laser. In this study, each of the fluorochromes was excited by the argon laser and the subsequent signal was detected in FL-1 channel. In the present study, a total of 50,000 cells were counted for the analysis of each parameter.

6.1.4.1 Measurement of annexin V binding

In order to determine the abundance of phosphatidylserine on the erythrocyte surface, freshly drawn blood was stained with Annexin V-FITC (ImmunoTools, Friesoythe, Germany). In 500 µl AWB, 2 µl erythrocytes and 2.5 µl Annexin V-FITC were added (1:200 dilution). This mixture was incubated in the dark at 37°C for 15 min. The Annexin V-FITC fluorescence was detected in FL-1 channel with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin V binding cells and control cells.

6.1.4.2 Determination of erythrocyte forward scatter

The cell volume of erythrocytes was determined by analyzing forward scatter (FSC). In this study, the forward scatter was simultaneously measured with annexin V binding. While the detection of annexin V binding requires staining with a fluorescent probe, FSC was measured without any fluorescent staining. For the determination of FSC, a dot plot of FSC vs. side scatter (SSC) was created. The measurements were performed on a linear scale for both parameters and the geometric mean of each sample was analyzed.

6.1.4.3 Estimation of intracellular Ca^{2+} of erythrocytes

One of the main hallmarks of eryptosis is the increase of the intracellular Ca^{2+} concentration of erythrocytes. For the quantification of intracellular Ca^{2+} of erythrocytes, Fluo3-AM-dependent fluorescence (Biotium, Hayward, USA) was measured. The flow cytometric analysis of intracellular Ca^{2+} in erythrocytes using Fluo3-AM has been described previously [276]. Fluo3-AM is a chemically modified version of the dye Fluo3, which has an additional acetoxymethyl group that is linked to the rest of the molecule via an ester bond. Fluo3-AM is able to penetrate the cell membrane. Intracellularly, esterases hydrolyze Fluo3-AM, which leads to the cleavage of the acetoxymethyl group. Due to this cleavage, Fluo3-AM is able to bind to calcium ions, which subsequently leads to the formation of a chelate complex. Two µl of freshly drawn blood was incubated in 500 µl AWB and 5 µM Fluo3-AM at 37°C for 30 min in the dark. Thereafter, Ca^{2+}-dependent fluorescence intensity was measured in FL-1 channel with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The geomean of Fluo3 fluorescence was individually measured for each sample.

6.1.4.4 Determination of reticulocytes

Reticulocytes are immature red blood cells and the direct precursor cells of mature erythrocytes. Similar to mature red blood cells, they have lost their nuclei but still contain ribosomes and mitochondria. These organelles contain DNA and RNA, which differentiates them from erythrocytes. Retic-COUNT is the trade name for 1-methyl-4[(3-

methyl-2(3H)-benzothiazolylidine) methyl]-quinolinium 4-methyl benzene sulfonate and is also called Thiazole Orange. The method to determine reticulocytes by Thiazole Orange was described previously by Lee et al. [277]. Thiazole is a fluorescent dye that binds to DNA and RNA, which leads to the formation of a fluorescent nucleotide-reagent complex with an absorption band at 475 nm. Reticulocytes can therefore be detected by an argon laser with 488 nm. The fluorescence emission band is at 530 nm.

In order to determine the percentage of reticulocytes, whole blood (2 µl) was added to 500 µl Retic-COUNT (Thiazole Orange) reagent from Becton Dickinson, San Jose, USA. In order to determine autofluorescence, 2 µl of whole blood was simultaneously incubated in 500 µl PBS (unstained control). After staining of the samples for 30 min at room temperature (RT) in the dark, flow cytometry was performed according to the manufacturer's instructions. The FSC and SSC were set on a logarithmic scale and the erythrocytes were gated. Subsequently, the Thiazole Orange-fluorescence intensity of the cells was determined in the FL-1 channel. The number of Retic-COUNT positive reticulocytes was expressed as the percentage of the total gated erythrocyte population. The percentage obtained from the unstained control sample was subtracted from the corresponding Retic-COUNT-stained sample.

6.1.4.5 Measurement of intracellular ceramide of erythrocytes

The observation that phosphatidylserine is still exposed on the outer leaflet of the membrane of erythrocytes even after inhibition of the cation channel by amiloride and removal of extracellular Ca^{2+} [49], raised the question of a calcium-independent pathway triggering eryptosis [55]. Finally, ceramide was identified as a mediator of calcium-independent eryptosis [55]. Ceramide is cleaved by the enzyme sphingomyelinase from sphingomyelin and enhances the sensitivity of the enzyme scramblase for the effects of calcium [55].

In order to determine the abundance of ceramide in erythrocytes, a monoclonal antibody-based assay was used with anti-ceramide antibody (clone MID 15B4) as the primary antibody [278] and polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody as the secondary antibody. Initially, 4 µl

erythrocytes were mixed in 1 ml Ringer solution. One hundred µl of the resulting cell suspension was centrifuged at 1600 rpm for 3 min at RT pelleting the erythrocytes. The supernatant was discarded and the erythrocyte pellet was subsequently stained with 1 µg/ml anti-ceramide antibody (Enzo Life Sciences, Lörrach, Germany) with a dilution of 1:10 for 1 h at 37°C in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). After two washing steps with 100 µl PBS-BSA, cells were further stained for 30 min with polyclonal fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM specific antibody (1:50 dilution; BD Pharmingen, Hamburg, Germany) in PBS-BSA. By repeated washing with 50 µl PBS-BSA, unbound secondary antibody was removed. The samples were finally resuspended in 200 µl PBS-BSA and analyzed by flow cytometric analysis in FL-1 channel at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur. The geometric mean of each sample was individually calculated.

6.1.4.6 Estimation of reactive oxygen species (ROS)

In order to determine the redox state of the RBCs and to measure the generation of reactive oxygen species (ROS), 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) was employed (Sigma Aldrich, Hamburg, Germany). DCFDA is a fluorogenic and cell-permeable dye which detects peroxyl, hydroxyl and other intracellular ROS activity. After diffusion into the cells, intracellular esterases deacetylate DCFDA to a non-fluorescent compound. It is later oxidized by reactive oxygen species into 2´7´-dichlorofluorescein (DCF), which is a highly fluorescent compound. The determination of ROS by DCFDA in red blood cells has been described previously [279].

For the determination of ROS, 4 µl of purified erythrocytes were mixed into 1 ml Ringer solution. 150 µl of the resulting cell suspension was centrifuged at 1600 rpm for 3 min at RT. The cells were stained with 10 µM DCFDA in Ringer solution at 37°C for 30 min in the dark and then washed three times in 150 µl Ringer solution. Subsequently, the DCFDA-loaded erythrocytes were resuspended in 200 µl Ringer solution. The ROS dependent fluorescence was measured in FL-1 channel at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS-Calibur. For each sample, the geometric mean was individually analyzed.

6.1.4.7 Confocal microscopy and immunofluorescence

In immunofluorescence, a microscope-based technique, specific target antigens are visualized by fluorescence-labeled antibodies [280]. In this study, phosphatidylserine on the outer membrane of erythrocytes was detected by FLUOS-labeled Annexin V. In order to visualize eryptotic erythrocytes, 20 µl erythrocytes ($1x10^6$ cells) were stained with Annexin V–FLUOS (1:100 dilution, Roche Diagnostics, Mannheim, Germany) in 200 µl AWB for 30 min in the dark at RT. The erythrocytes were washed twice and subsequently resuspended in 200 µl AWB. Forty µl were spread onto a glass slide and dried for 15 min at RT. The slides were covered with PROlong Gold antifade reagent (Invitrogen, Darmstadt, Germany). Finally, both images were taken on a Zeiss LSM 5 EXCITER confocal laser-scanning microscope and with the phase light (Carl Zeiss MicroImaging, Germany) with a water immersion Plan-Neofluar 40/1.3 NA DIC. A scale bar of 5 µm was used.

6.1.4.8 Plasma measurements

In order to determine if eryptosis is triggered by component(s) in the plasma of the patients, erythrocytes (blood group O^-) were incubated in plasma of the patients and plasma of healthy volunteers. The erythrocytes of blood group O^- were obtained from healthy volunteers of the blood bank of the University of Tübingen. In order to isolate the plasma of patients and healthy donors, whole Lithium-Heparin blood was centrifuged at 2500 rpm for 5 min at RT without brake. Subsequently, 500 µl plasma of healthy volunteers and patients was transferred to Eppendorf cups. Afterwards, 2 µl purified erythrocytes of the blood group O^- were added to the plasma and incubated for 24 h at 37°C. Finally, 150 µl cell suspension were pipetted into a 96 well plate, centrifuged for 1600 rpm for 3 min at RT and the annexin V binding (see 6.1.4.1), forward scatter (see 6.1.4.2), intracellular Ca^{2+} (see 6.1.4.3), ceramide abundance (see 6.1.4.5) and reactive oxygen species (see 6.1.4.6) were determined.

6.2 Eryptosis in mice

6.2.1 *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ mice

In order to study the participation of Gαi2-protein in the regulation of erythrocyte survival, experiments were performed in age and sex matched Gαi2 knockout mice (*Gαi2*$^{-/-}$) and their wild type littermates (*Gαi2*$^{+/+}$). The mice were generated and initially characterized on a SV129 background [281]. Mice were backcrossed on a C57BL6 background and kept under specified pathogen-free (SPF) environment in individually ventilated cages (IVC) to prolong life expectancy [257, 282]. All animal experiments were conducted according to the German law for the care and use of laboratory animals and were approved by local authorities.

6.2.1.1 Blood parameters and erythrocyte isolation

For the blood count, EDTA blood was analyzed utilizing an electronic hematology particle counter (type MDM 905 from Medical Diagnostics Marx; Butzbach, Germany) that is equipped with a photometric unit for hemoglobin determination. White blood cells, RBCs, hemoglobin, hematocrit, MCV, MCH, MCHC and platelets of *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ have been determined. For all other experiments, heparin blood was obtained from the retrobulbar plexus of the mice. Plasma erythropoietin levels were determined using a commercially available ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, USA). In order to obtain pure erythrocytes, murine erythrocytes were isolated utilizing Ficoll (Biochrom AG, Germany) and were washed two times at 2500 rpm for 5 min with Ringer solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM HEPES/NaOH (pH 7.4), 5 mM glucose and 1 mM $CaCl_2$.

6.2.1.2 Determination of reticulocytes, annexin V binding, forward scatter and Fluo3 fluorescence

The percentage of annexin V binding cells (6.1.4.1), forward scatter (6.1.4.2), intracellular Ca^{2+} (6.1.4.3) and the percentage of reticulocytes (6.1.4.4) have been measured immediately after retrieval of the blood and isolation of the erythrocytes. The percentage of reticulocytes has additionally been measured by Ter119/CD71 staining using flow cytometry. For the analysis of the intracellular Ca^{2+} content of the mouse erythrocytes, the percentage of Fluo3 positive cells instead of the geometric mean of the Fluo3 fluorescence has been determined.

6.2.2 May-Grünwald staining

May-Grünwald staining was applied to examine changes in erythrocyte shape between *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ erythrocytes. For this reason, 20 µl of erythrocytes were smeared and fixed using methanol onto a glass slide. Subsequently, the cells were stained with 5 % Giemsa Azur-Eosin (Merck Millipore, Germany) in phosphate-buffered saline for 20 mins with the following components (in mM): 1.05 KH_2PO_4, 2.97 Na_2HPO_4, 155.2 NaCl. Subsequently, images were taken on a Nikon Diaphot 300 Microscope (Nikon Instruments, Germany).

6.2.3 Triggering of suicidal death of mouse erythrocytes by different stimulators

6.2.3.1 Hyperosmotic solution

Eryptosis can be triggered by a multitude of xenobiotics, by depletion of energy, oxidative stress or hyperosmotic stress [49, 55]. In order to investigate a putative role of Gαi2 in the regulation of eryptosis, suicidal death of erythrocytes was stimulated by exposure of the cells to hyperosmotic stress. Under hyperosmotic stress, erythrocytes expose phosphatidylserine on their surface and show cell shrinkage. All events are triggered by increased cytosolic calcium activity [49]. Extracellular hyperosmotic environment was created by addition of 550 mM sucrose to Ringer solution and incubation of the erythrocytes of *Gαi2*$^{-/-}$ and *Gαi2*$^{+/+}$ mice in this solution for 30 min. 4 µl erythrocytes were incubated in 1 ml Ringer or hyperosmolar Ringer solution, respectively. After incubation, 150 µl of the respective samples were transferred onto a 96 well plate and the plate was

centrifuged at 1600 rpm for 3 min. Subsequently, the percentage of annexin V binding erythrocytes (6.1.4.1), the forward scatter (6.1.4.2) and the intracellular Ca^{2+} activity (6.1.4.3) have been determined. For the analysis of intracellular Ca^{2+} activity, the percentage of Fluo3 positive cells has been analyzed.

Table 3: Composition of hyperosmolar Ringer solution

Substance	Concentration (mM)
NaCl	125
KCl	5
$CaCl_2$	1
$MgSO_4$	1.2
HEPES	32.2
Glucose	5
Sucrose	550
NaOH	13

6.2.3.2 C6 Ceramide and Sphingomyelinase

Both C6 ceramide and sphingomyelinase have been described as powerful stimulators of eryptosis earlier [55]. Ceramide is produced from cell membrane sphingomyelin by a sphingomyelinase and thus, addition of sphingomyelinase stimulates suicidal erythrocyte death [55].

In order to further examine the role of Gαi2 in suicidal erythrocyte death, C6 ceramide and sphingomyelinase have been used to trigger eryptosis in *Gαi2*$^{-/-}$ and *Gαi2*$^{+/+}$ erythrocytes. 50 µM C6 ceramide (Enzo Life Sciences, Lörrach, Germany) have been added to 4 µl purified erythrocytes in 1 ml Ringer solution. After the incubation time of 12 h at 37°C, 150 µl of the erythrocyte suspension were centrifuged at 1600 rpm for 3 min and subsequently, phosphatidylserine exposing erythrocytes have been determined as described previously (6.1.4.1).

Another trigger of eryptosis, bacterial sphingomyelinase (Sigma Aldrich, Hamburg, Germany), at a concentration of 0.01 U/ml has been added to 1 ml Ringer solution and 4 µl erythrocytes. After incubation for 24 h at 37°C, 150 µl of the cell suspension were centrifuged at 1600 rpm for 3 min. Finally, the percentage of annexin V binding cells has been determined (6.1.4.1).

6.2.4 Confocal microscopy and immunofluorescence

In order to visualize eryptotic erythrocytes, confocal images of *Gαi2*$^{-/-}$ and *Gαi2*$^{+/+}$ erythrocytes have been taken as described above (6.1.4.7). Sections were analyzed using a Leica TCS-SP/Leica DM RB confocal laser scanning microscope. Images were subsequently processed with Leica Confocal Software LCS (version 2.61).

6.2.5 Immunoblotting

In order to examine the expression of Gαi2 in murine and human erythrocytes, western blotting was performed. The erythrocyte pellet (150 µl) was lysed in 50 ml of 20 mM HEPES/NaOH (pH 7.4). Ghost membranes were pelleted (15,000 g for 20 min at 4°C) and lysed in 200 µl lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 0.5% SDS; 1 mM NaF; 1 mM Na_3VO_4 and 0.4% β-mercaptoethanol) containing protease inhibitor cocktail (Sigma, Schnelldorf, Germany). In all cases, 60 µg of protein was solubilized in Laemmli sample buffer at 95°C for 5 min and resolved by 10% SDS-PAGE. For immunoblotting, proteins were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane and blocked with 5% nonfat milk in TBS-0.10% Tween 20 at RT for 1 h. Then, the membrane was incubated with affinity purified rabbit Gαi2 antibody (1:1000; 62 kDa; Cell Signaling, Danvers, MA, USA) at 4°C overnight. After being washed (in TBS-0.10% Tween 20) and subsequently blocked, the blots were incubated with secondary anti-rabbit antibody (1:2000; Cell Signaling) for 1 h at RT. After being washed, the antibody binding was detected with the ECL detection reagent (Amersham, Freiburg, Germany). In order to examine the expression of Gαi2 in human erythrocytes, highly purified concentrates were provided by the blood bank of the University of Tübingen. The erythrocyte concentrates contained less than 1% platelets and were virtually free of white blood cells.

6.2.6 Determination of the osmotic resistance

Two µl of blood were added to 200 µl of PBS solutions with different osmolarities (0% to 100%) on a 96 well plate. The different osmolarities were prepared by the addition of a defined volume of PBS solution to a defined volume of distilled water. The plate was incubated at 37°C for 10 min. After centrifugation for 5 min at 3000 rpm, 50 µl of the supernatant were transferred to another 96 well plate, and the absorption at 405 nm was determined as a measure of hemolysis on a spectrophotometer (BioTek Instruments GmbH, Bad Friedrichshall, Germany). Absorption of the supernatant of erythrocytes lysed in pure distilled water was defined as 100% hemolysis.

6.2.7 Statistics

All data are expressed as arithmetic means ± SEM. Student's *t*-test or Mann-Whitney test were performed to determine statistical significance between the two groups using Graph Pad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA; n denotes the number of individuals. $p<0.05$ was considered to be significant.

7 RESULTS

7.1 Eryptosis in patients

7.1.1 Eryptosis in lung cancer

In order to explore whether suicidal erythrocyte death contributes to the pathophysiology of anemia in lung cancer, whole blood was drawn from patients suffering from different stages and types of non-small cell lung cancer (n=13) or small cell lung cancer (n=4). In total, 17 patients were enrolled in the study, 11 males and 6 females, with a mean age of 65 ± 2.4 (age range: 52 to 85 years).

Non-small cell lung cancer can be further divided into three different subtypes: adenocarcinoma, squamous cell carcinoma (SCC) and large cell carcinoma (LCC). The age, sex, clinical diagnosis and the stage of disease for each patient are shown in Table 4.

In order to test the impact of the cytostatic treatment on anemia and suicidal erythrocyte death, the patients were divided into two groups. Pat. Group I did not receive cytostatic treatment (n=6, 2 ♀, 4 ♂, mean age: 68.7 ± 4.1 years) whereas Pat. Group II was under cytostatic treatment (n=11, 4 ♀, 7 ♂, mean age: 63.0 ± 2.9 years).

Whole blood was additionally taken from an age-and sex-matched healthy control group (4 ♀, 11 ♂, mean age: 57.1 ± 1.8 years, age range: 41-70 years).

Table 4: Characteristics of the lung cancer patients, without (Pat. Group I) and with (Pat. Group II) cytostatic treatment [283]

Number	Age	Sex	Clinical diagnosis	Treatment
1	63	m	Small cell lung cancer, neuroendocrine carcinoma (T4 N3 M1b, stage IV, UICC/AJCC)	Carboplatin Etoposide
2	66	m	Non-small cell lung cancer, poorly differentiated adenocarcinoma (T4 N3 M1b, stage IV, UICC/AJCC)	Cisplatin Gemcitabine Carboplatin
3	52	m	Non-small cell lung cancer, squamous cell carcinoma (cT2a cN2 cM0, stage IIIA, UICC/AJCC)	Cisplatin Vinorelbine Docetaxel
4	52	f	Non-small cell lung cancer, poorly differentiated adenocarcinoma (cT2b cN2-3 M1b, stage IV, UICC/AJCC)	Cisplatin Gemcitabine
5	62	m	Non-small cell lung cancer, poorly differentiated squamous cell carcinoma (pT3 pN0 L0 V0 Pn0 R0, stage IIB, AJCC/UICC)	Cisplatin Vinorelbine
6	56	m	Non-small cell lung cancer, adenocarcinoma (cT4 cN3 M1a, stage IV, AJCC/UICC)	Docetaxel
7	64	m	Small cell lung cancer (cT4 N3 M1b, stage IV, UICC/AJCC)	Carboplatin Etoposide Topotecan
8	66	f	Non-small cell lung cancer (stage IV, UICC/AJCC)	Carboplatin Gemcitabine
9	53	f	Non-small cell lung cancer, squamous cell carcinoma (cT3 N3 M1, stage IV, UICC/AJCC)	Carboplatin Vinorelbine Pembrolizumab
10	77	m	Non-small cell lung cancer, poorly differentiated adenocarcinoma (T4 N3 M1b, stage IV, UICC/AJCC)	Carboplatin Gemcitabine
11	81	f	Small cell lung cancer (cT2b cN2 M0, stage IIIA)	Carboplatin Etoposide
12	60	f	Small cell lung cancer (T4 N3 M1, stage IV)	No cytostatic treatment
13	71	f	Non-small cell lung cancer, adeonocarcinoma G3	No cytostatic treatment
14	74	m	Non-small cell lung cancer, poorly differentiated squamous cell carcinoma (T3 cN0-1 M0)	No cytostatic treatment
15	59	m	Non-small cell lung cancer, adenocarcinoma (cT2a pN 1-2 M0)	No cytostatic treatment
16	63	f	Non-small cell lung cancer, squamous cell carcinoma (cT4 cN3 cM0)	No cytostatic treatment
17	85	m	Non-small cell lung cancer, adenocarcinoma (cT3-4 cN0-1 M0)	No cytostatic treatment

In order to verify whether the lung cancer patients enrolled in this study suffered from anemia, the erythrocyte number, hemoglobin and the hematocrit level were determined.

Both Pat. Groups (I and II) had a significantly lower number of erythrocytes. The erythrocyte number was lower in Pat. Group II under cytostatic treatment ($3.1 \pm 0.1 \times 10^6/\mu l$) as compared to Pat. Group I ($4.2 \pm 0.2 \times 10^6/\mu l$) (Fig. 4 A).

Hemoglobin level was similarly lower in Pat. Group I (12.4 ± 0.7 g/100 ml) and was lowest in Pat. Group II (9.6 ± 0.4 g/100 ml) (Fig. 4 B).

Similar observations were made on the hematocrit level, which was significantly decreased in Pat. Group I ($37.0 \pm 1.9\%$) and reached the lowest value in Pat. Group II ($28.7 \pm 1.4\%$) (Fig. 4 C).

Thus, all patients were anemic. None of the healthy volunteers were anemic.

Although Pat. Group II showed the lowest erythrocyte number, hemoglobin and hematocrit level, they had the highest percentage of reticulocytes ($3.0 \pm 0.6\%$) as compared to the control group and Pat. Group I ($1.8 \pm 0.3\%$) indicating that the anemia was present despite enhanced production of erythrocytes (Fig. 4 D).

The mean corpuscular volume (MCV) was slightly but significantly enhanced in Pat. Group II (91.5 ± 1.0). MCV was also slightly increased in Pat. Group I (88.8 ± 1.2), the difference did, however, not reach statistical significance (Fig. 4 E).

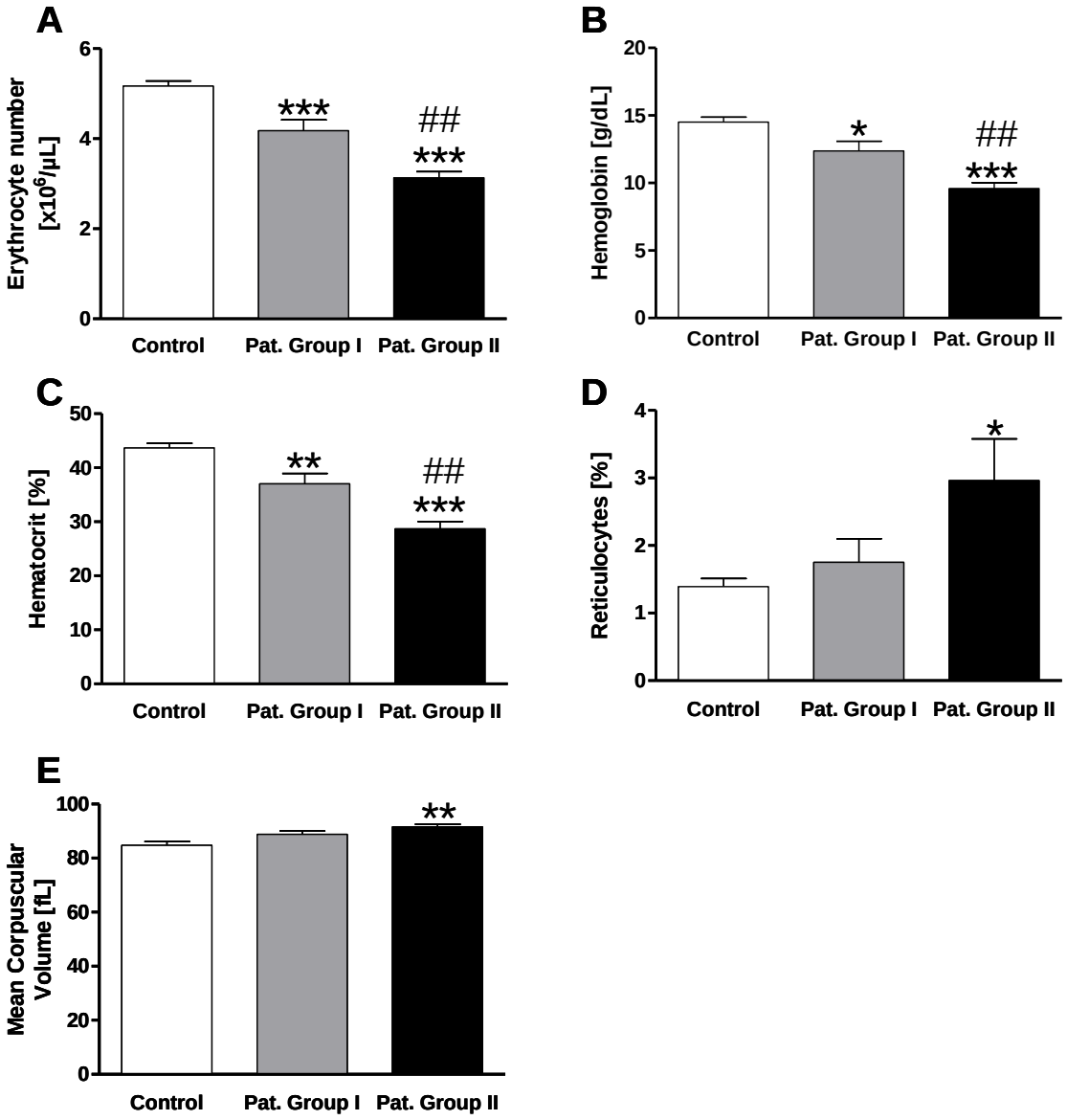

Fig. 4: Effect of lung cancer on erythrocyte blood parameters: Arithmetic means ± SEM of (**A**) erythrocyte count, (**B**) hemoglobin, (**C**) hematocrit, (**D**) percentage of reticulocytes and (**E**) mean corpuscular volume in blood drawn from healthy volunteers (n=15, white bars) and patients without (n=6; Pat. Group I, grey bars) and with (n=11; Pat. Group II, black bars) cytostatic treatment. * (p<0.05), ** (p<0.01) and *** (p<0.001) indicate significant difference from healthy volunteers (unpaired t test). ## (p<0.01) indicates significant difference from patients without cytostatic treatment (unpaired t test) [283].

The percentage of reticulocytes was inversely and significantly correlated with the erythrocyte number (Fig. 5 A), the hemoglobin level (Fig. 5 B) and the hematocrit (Fig. 5 C), which confirms that anemia prevailed despite increased reticulocyte production.

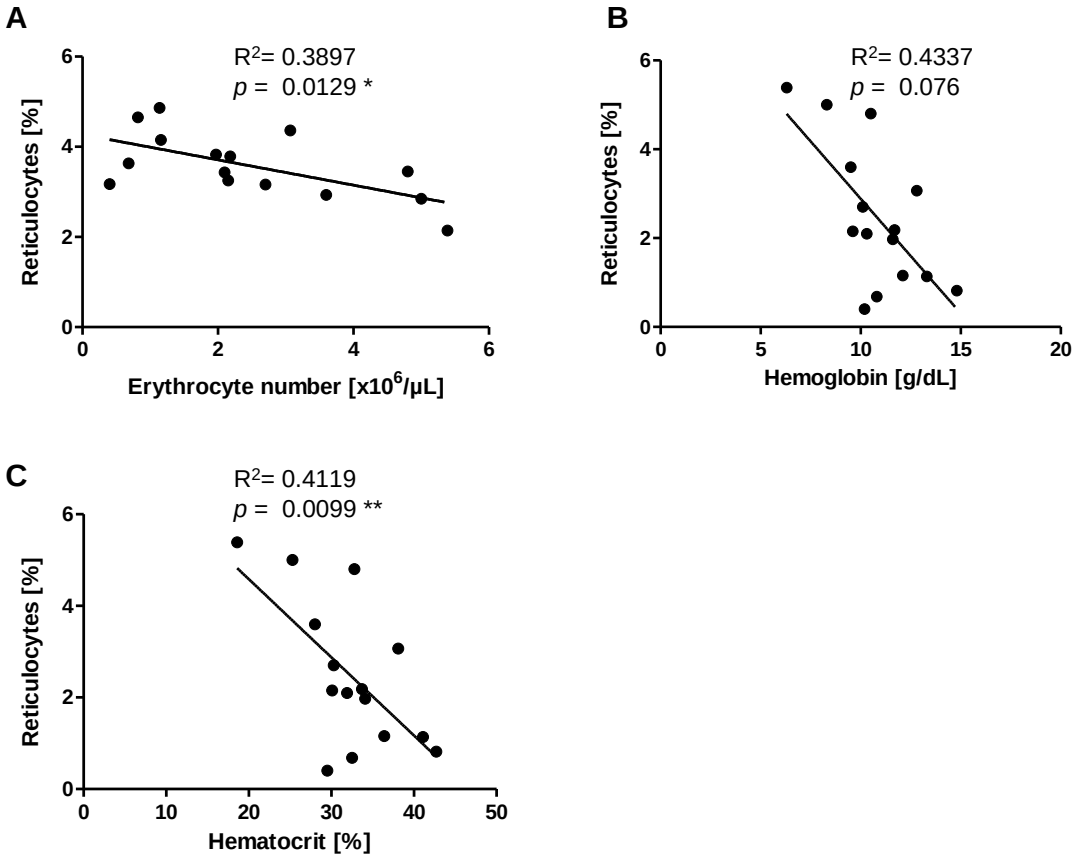

Fig. 5: Correlations of reticulocyte number with different blood parameters in LC patients. (**A-C**). Reticulocyte number as a function of (**A**) erythrocyte number (p=0.0129, R^2=0.3897), (**B**) hemoglobin concentration [g/dl] (p=0.076, R^2=0.4337) and (**C**) hematocrit (p=0.0099, R^2=0.4119) in lung cancer patients (n=17). * (p<0.05) and ** (p<0.01) indicate significant correlation. For all correlations, Spearman nonparametric analysis was used [283].

Table 5 shows several blood parameters obtained from the LC patients (Pat. Group I and II) and the healthy volunteers.

Table 5: Mean age, sex and blood parameters of lung cancer patients without (Pat. Group I) and with (Pat. Group II) cytostatic treatment (values are given as mean ± SEM). Values without SEM in this table reflect standard clinical reference values and do not represent data from healthy controls [283]

	Healthy volunteers (A)	**Patients without cytostatic treatment (Pat. Group I) (B)**	**Patients with cytostatic treatment (Pat. Group II) (C)**	**p-value**
Mean age (years)	57.1 ± 1.8	68.7 ± 4.1	63.0 ± 2.9	0.0073** (A vs. B) 0.0839 (A vs. C) 0.2722 (B vs. C)
Sex	11 males 4 females	4 males 2 females	7 males 4 females	
Plasma creatinine concentration (mg/100 ml)	0.5-0.8 (female) 0.6-1.1 (male)	0.6 ± 0.04	0.8 ± 0.07	0.1159 (B vs. C)
Plasma uric acid (mg/100 ml)	2.4-5.7 (female) 3.4-7.0 (male)	5.4 ± 0.7	5.7 ± 0.5	0.7789 (B vs. C)
Plasma ferritin concentration (µg/100 ml)	1.0-20 (female) 3.0-30 (male)	n.a.	44 ± 26.4	n.a.
Red blood cell distribution width (%)	< 14	14.0 ± 1.2	16.8 ± 0.7	0.059 (B vs C)
Erythrocytes ($x10^6$/µl)	5.2 ± 0.1	4.2 ± 0.2	3.1 ± 0.1	<0.001*** (A vs. B) <0.001*** (A vs. C) 0.001## (B vs. C)
Hematocrit (%)	43.6 ± 0.9	37.0 ± 2.0	28.7 ± 1.4	0.0018** (A vs. B) <0.001*** (A vs.C) 0.0027## (B vs. C)
Hemoglobin (g/dl)	14.5 ± 0.4	12.4 ± 0.7	9.6 ± 0.4	0.0103* (A vs. B) <0.001*** (A vs.C) 0.0029## (B vs. C)
Reticulocytes (%)	1.4 ± 0.1	1.8 ± 0.3	3.0 ± 0.6	0.381 (A vs. B) 0.0123* (A vs. C) 0.1625 (B vs. C)
Mean Corpuscular Volume (fl)	84.7 ± 1.4	88.8 ± 1.2	91.5 ± 1.0	0.0929 (A vs. B) 0.0011** (A vs. C) 0.1184 (B vs. C)
MCH (pg/erythrocyte)	27.0-34.0	29.7 ± 0.6	30.6 ± 0.3	0.175 (B vs. C)
MCHC (g/100 ml)	32.0-36.0	33.4 ± 0.4	33.4 ± 0.3	0.9094 (B vs. C)
Plasma total protein (g/100 ml)	6.5-8.5	7.4 ± 0.2	7.0 ± 0.1	0.2007 (B vs. C)
C-reactive protein (mg/100 ml)	< 0.5	4.4 ± 2.4	3.0 ± 1.1	0.5253 (B vs. C)
Leucocytes (/µl)	3800-10300	7607 ± 776.8	8839 ± 3285	0.7901 (B vs. C)
Lymphocytes (%)	20-45	19.2 ± 2.1	23.9 ± 5.4	0.5825 (B vs. C)

Monocytes (%)	2-8	8.2 ± 0.8	7.0 ± 1.1	0.4664 (B vs. C)
Thrombocytes ($x10^3/\mu l$)	150-450	317 ± 52.9	277.9 ± 43.2	0.5878 (B vs. C)

Further experiments were conducted in order to investigate, whether the anemia of the patients was paralleled by and possibly due to accelerated suicidal erythrocyte death.

In order to visually identify phosphatidylserine-exposing erythrocytes, cells of LC patients of Group I and healthy volunteers were stained with Annexin V-FLUOS and the FLUOS-dependent fluorescence was visualized by confocal microscopy. Images were as well taken with the phase light. In LC patients of Group I, the number of fluorescent cells and thus, phosphatidylserine-exposing erythrocytes was enhanced in freshly drawn blood from LC patients of Group I than in blood of healthy volunteers (Fig. 6 A).

Furthermore, the percentage of phosphatidylserine-exposing erythrocytes was quantified by FACS analysis. In order to stain PS-exposing erythrocytes, Annexin V-FITC was employed and the percentage of annexin V positive cells was determined. As depicted in Fig. 6 B and C, Pat. Group II showed the highest percentage of annexin V positive cells (1.4 ± 0.1%), followed by Pat. Group I (1.2 ± 0.3%). The percentage of phosphatidylserine-exposing erythrocytes was significantly lower in the healthy control group (0.6 ± 0.1%). As illustrated in Fig. 6 D, the percentage of annexin V binding cells was higher in lung cancer patients than in healthy volunteers irrespective of age.

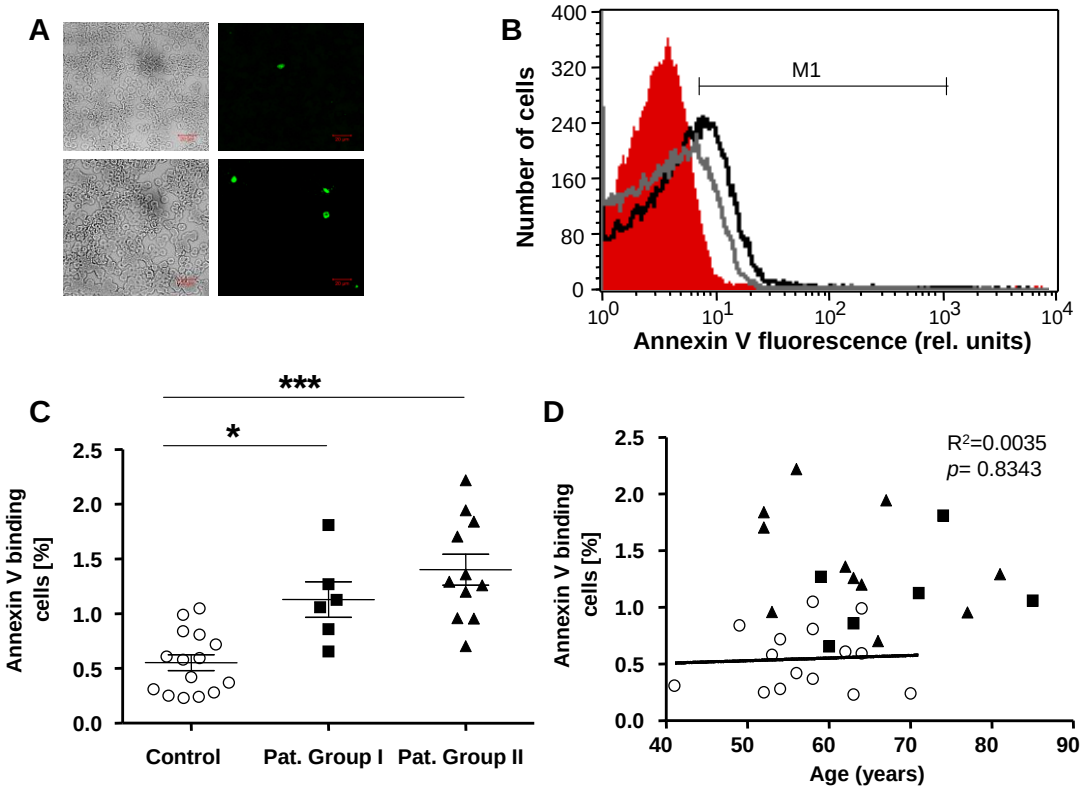

Fig. 6: Phosphatidylserine exposure of erythrocytes taken from patients without and with cytostatic treatment and healthy control group. (**A**). Light microscopy (left panel) and confocal images (right panel) of FLUOS-dependent fluorescence of human erythrocytes stained with FLUOS-conjugated annexin V. The specimens were obtained from healthy volunteers (Control, upper panels) and from lung cancer patients without cytostatic treatment (Group I, lower panels). Scale bar 20 µm. (**B**). Original representative histogram of annexin V binding of erythrocytes in freshly drawn blood from healthy volunteers (red shadow) and from patients without (Pat. Group I, grey line) and with cytostatic treatment (Pat. Group II, black line). M1 indicates the fluorescence of annexin V defining the percentage of annexin V binding erythrocytes. (**C**). Arithmetic means ± SEM of the percentage of annexin V binding erythrocytes in freshly drawn blood from healthy volunteers (n=15, control, open circles) and patients without (n=6, closed squares) and with (n=11, closed triangles) cytostatic treatment. * (p<0.05) and *** (p<0.001) indicate significant difference from healthy volunteers (Mann Whitney test). (**D**). Percentage of annexin V binding erythrocytes in healthy individuals (open circles) and lung cancer patients of group I (n=6, closed squares) and group II (n=11, closed triangles) as a function of age. The regression line shown is calculated for healthy individuals (p=0.8343, R^2=0.0035) [283].

In order to test if a component in the plasma of the LC patients triggers eryptosis, erythrocytes from healthy donors of blood group O^- were exposed to plasma of Pat. Group I and II. After a plasma exposure for 24 h, the percentage of annexin V binding erythrocytes was determined. The percentage was highest in Pat. Group II (9.3 ± 1.6%) and tended to be increased in Pat. Group I (9.0 ± 1.0%), the difference did, however, not reach statistical significance (p=0.058) (Fig. 7 B).

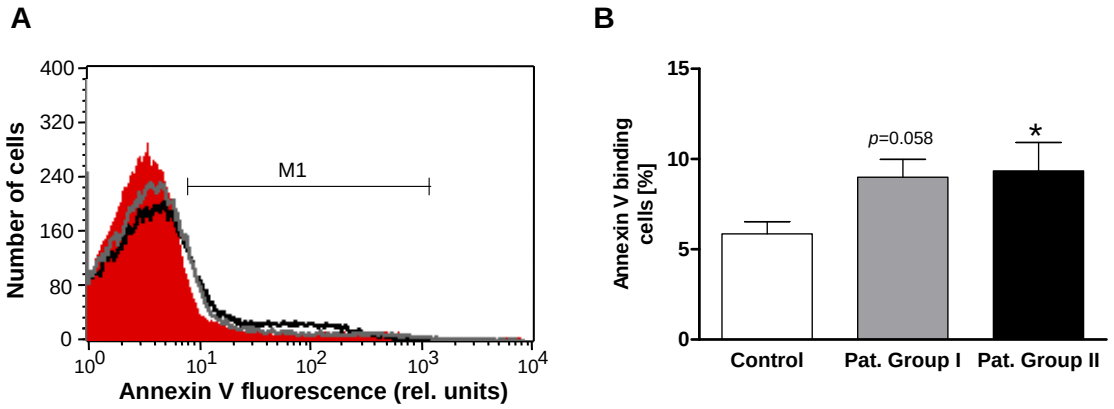

Fig. 7: Effect of patient plasma without and with cytostatic treatment on the exposure of phosphatidylserine in erythrocytes from healthy volunteers. (A). Original representative histogram of annexin V binding of erythrocytes from healthy volunteers after a 24 h exposure to plasma from healthy volunteers (red shadow) and to plasma from patients without (Pat. Group I, grey line) and with cytostatic treatment (Pat. Group II, black line). M1 indicates the annexin V fluorescence defining the percentage of phosphatidylserine binding erythrocytes. (**B**). Arithmetic means ± SEM of the percentage of annexin V binding erythrocytes from healthy volunteers after a 24 h exposure to plasma from healthy volunteers (n=15, white bar) and to plasma from patients without (n=6, grey bar) and with (n=11, black bar) cytostatic treatment. * (p<0.05) indicates significant difference from healthy volunteers (unpaired t test) [283].

One of the hallmarks of suicidal erythrocyte death is cell shrinkage, which can be estimated by forward scatter in FACS analysis. In order to verify if cell volume of cancer patients with and without cytostatic treatment is changed, forward scatter of freshly drawn blood was determined. The forward scatter tended to be lower in Pat. Group I (456.5 ± 14.1 a.u.), but was significantly increased in Pat. Group II (495.5 ± 4.1 a.u.) as compared to the control group (478.8 ± 5.2 a.u.) (Fig. 8 A and B).

Similar observations were made after the incubation of erythrocytes of blood group O^- in plasma of both LC patient groups. The cell volume in these erythrocytes was significantly enhanced after exposure to plasma of Pat. Group II (514.1 ± 19.5 a.u.), it, however, showed a slight, but not significant decrease in cell volume after exposure to plasma of Pat. Group I (414.6 ± 38.0 a.u.) as compared to the control group (450.2 ± 17.3 a.u.) (Fig. 8 C and D).

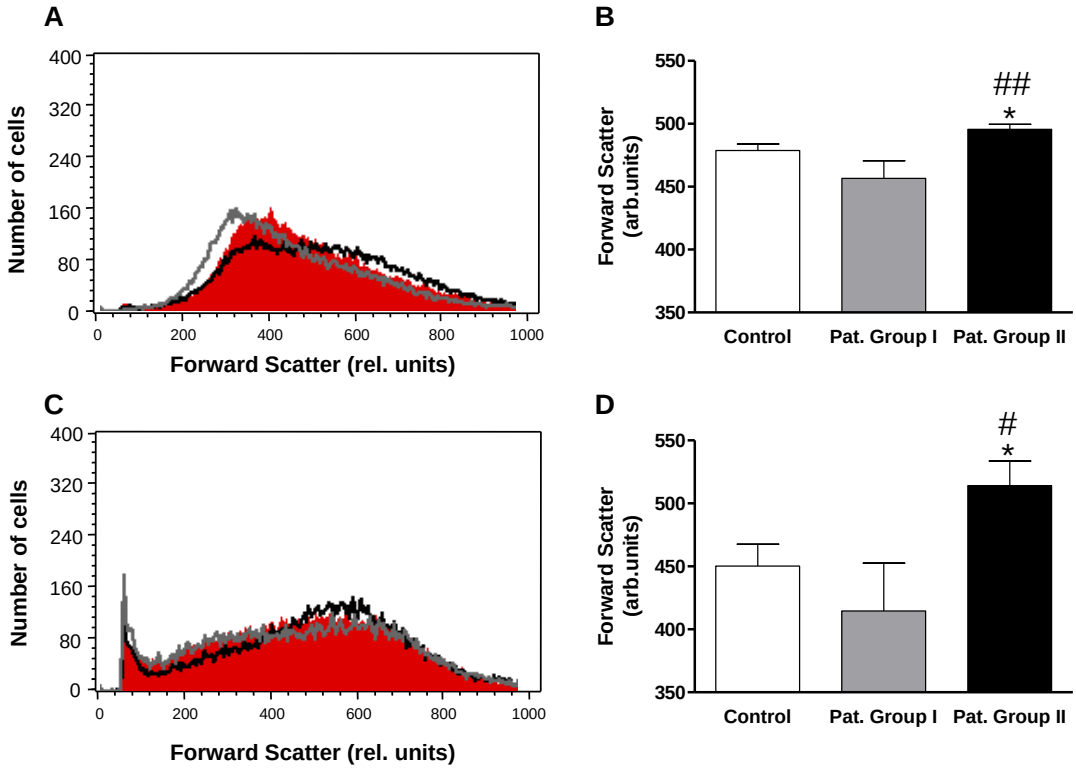

Fig. 8: Effect of lung cancer on forward scatter of erythrocytes. (**A).** Original representative histogram of erythrocyte forward scatter in freshly drawn blood from healthy volunteers (red shadow) and from LC patients without (grey line) and with cytostatic treatment (black line). (**B)**. Arithmetic means ± SEM of forward scatter geometric mean of erythrocytes in freshly drawn blood from the healthy control group (n=15, white bar) and from LC patients without (n=6, grey bar) and with (n=11, black bar) cytostatic treatment. * (p<0.05) indicates significant difference from the healthy control group. ## (p<0.01) indicates significant difference from LC patients without cytostatic treatment (unpaired t test). (**C**). Original representative histogram of erythrocyte forward scatter from healthy volunteers after a 24 h exposure to plasma from the healthy control group (n=15, red shadow) and to plasma from patients without (grey line) and with cytostatic treatment (black line) (**D)**. Arithmetic means ± SEM of forward scatter geometric mean of erythrocytes from healthy volunteers after a 24 h exposure to plasma from healthy volunteers (n=15, white bar) and to plasma from LC patients without (n=6, grey bar) and with (n=11, black bar) cytostatic treatment. * (p<0.05) indicates significant difference from the healthy control group. # (p<0.05) indicates significant difference from LC patients without cytostatic treatment (unpaired t test) [283].

An increase of the intracellular Ca^{2+} content of erythrocytes is another trigger of eryptosis that can be measured by the fluorescence of Fluo3. Fluo3 fluorescence was analyzed both in freshly drawn blood of healthy volunteers and LC patients and after exposure of healthy erythrocytes of blood group O^- to plasma of the patients and healthy volunteers for 24 h. No significant difference in Fluo3 fluorescence in freshly drawn blood between the LC patients and the healthy volunteers was observable (Fig. 9 A and B).

Furthermore, Fluo3 fluorescence in the plasma experiments was not significantly different between the respective groups (Fig. 9 C and D).

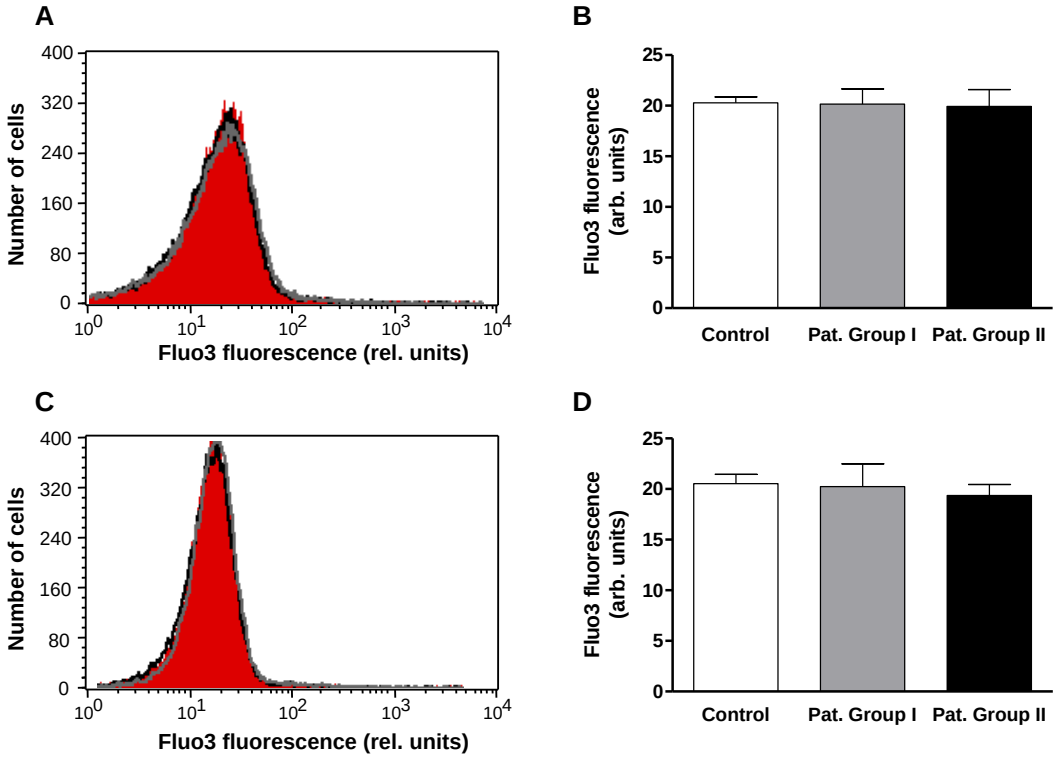

Fig. 9: Effect of lung cancer on erythrocyte intracellular Ca^{2+} activity. (**A**). Original representative histogram of erythrocyte Fluo3 fluorescence in freshly drawn blood from the control group (red shadow) and from LC patients without (grey line) and with cytostatic treatment (black line). (**B**). Arithmetic means ± SEM of erythrocyte Fluo3 fluorescence in freshly drawn blood from healthy volunteers (n=15, white bar) and from patients without (n=6, grey bar) and with cytostatic treatment (n=11, black bar). (**C**). Original representative histogram of erythrocyte Fluo3 fluorescence from healthy volunteers after a 24 h exposure to plasma from healthy volunteers (red shadow) and to plasma from patients without (grey line) and with cytostatic treatment (black line). (**D**). Arithmetic means ± SEM of Fluo3 fluorescence of erythrocytes from healthy volunteers after a 24 h exposure to plasma from healthy volunteers (n=15, white bar) and to plasma from patients without (n=6, grey bar) and with cytostatic treatment (n=11, black bar) [283].

Ceramide is another stimulator of eryptosis, which is effective even in the absence of increased intracellular Ca^{2+}. Therefore, the abundance of ceramide in freshly drawn blood was analyzed. As a result, Pat. Group I showed a slight increase of ceramide (14.5 ± 1.2 a.u.), the difference did, however, not reach statistical significance. Pat. Group II showed a significantly higher ceramide abundance (18.6 ± 1.1 a.u.) as compared to Pat. Group I and the healthy control group (13.3 ± 0.4 a.u.) (Fig. 10 A and B).

In order to analyze if a component in plasma triggers the formation of ceramide, healthy erythrocytes (blood group O^-) were incubated in plasma of both patient groups and healthy volunteers for 24 h. Erythrocytes exposed to plasma of Pat. Group II showed the highest ceramide abundance (18.6 ± 1.1 a.u.). The exposure to plasma of Pat. Group I slightly, but not significantly triggered ceramide formation (16.3 ± 2.3 a.u.) (Fig. 10 C and D). Accordingly, the results were similar to those obtained in freshly drawn blood.

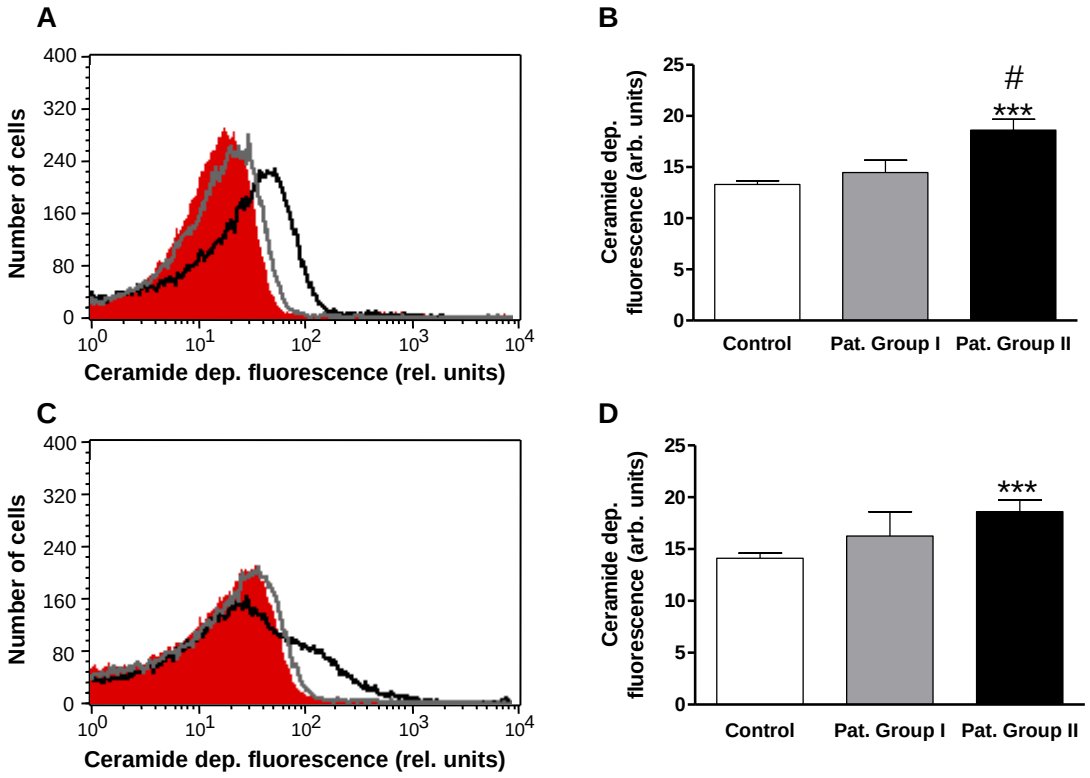

Fig. 10: Effect of lung cancer on the ceramide abundance of erythrocytes. (**A**). Original representative histogram of ceramide-dependent FITC fluorescence of erythrocytes in freshly drawn blood from the control group (red shadow) and from patients without (grey line) and with cytostatic treatment (black line). (**B**). Arithmetic means ± SEM of ceramide-dependent FITC fluorescence of erythrocytes in freshly drawn blood from the healthy control group (n=15, white bar) and from LC patients without (n=6, grey bar) and with cytostatic treatment (n=11, black bar). *** (p<0.001) indicates significant difference from healthy volunteers. # (p<0.05) indicates significant difference from Pat. Group I (unpaired t test). (**C**). Original representative histogram of ceramide-dependent FITC fluorescence of erythrocytes from healthy volunteers after a 24 h exposure to plasma from healthy volunteers (red shadow) and to plasma from patients without (grey line) and with cytostatic treatment (black line). (**D**). Arithmetic means ± SEM of ceramide-dependent FITC fluorescence of erythrocytes from healthy volunteers after a 24 h exposure to plasma from healthy volunteers (n=15, white bar) and to plasma from patients without (n=6, grey bar) and with cytostatic treatment (n=11, black bar).*** (p<0.001) indicates significant difference from healthy volunteers (unpaired t test) [283].

Another main trigger of eryptosis is the production of reactive oxygen species, a marker of cellular redox status. The production of ROS was quantified by fluorescence of 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) in freshly drawn blood. Pat. Group II showed a significantly higher DCFDA fluorescence in freshly drawn blood (14.5 ± 0.8 a.u.) as compared to Pat. Group I (12.4 ± 0.8 a.u.) and the control group (12.0 ± 0.4 a.u.) (Fig. 11 A and B).

Similar results were obtained after the exposure of healthy erythrocytes to plasma of both patient groups. Erythrocytes exposed to plasma of Pat. Group II showed the highest DCFDA fluorescence (18.5 ± 2.1 a.u.). No difference was observed between the exposure to plasma of Pat. Group I (11.2 ± 0.4 a.u.) and the healthy control group (12.1 ± 1.0 a.u.) (Fig. 11 C and D).

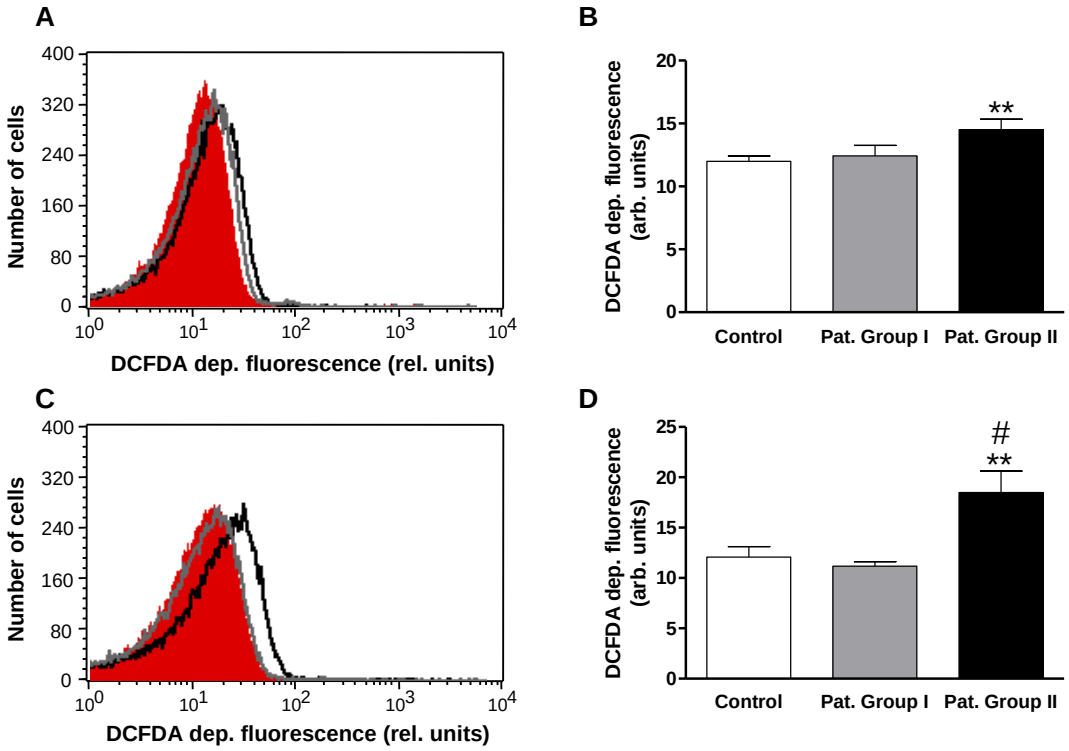

Fig. 11: Effect of lung cancer on reactive oxygen species production in erythrocytes. (**A**). Original representative histogram of DCFDA fluorescence of erythrocytes in freshly drawn blood from the healthy control group (red shadow) and from LC patients without (grey line) and with cytostatic treatment (black line). (**B**). Arithmetic means ± SEM of DCFDA fluorescence of erythrocytes in freshly drawn blood from the healthy control group (n=15, white bar) and from LC patients without (n=6, grey bar) and with cytostatic treatment (n=11, black bar). ** ($p<0.01$) indicates significant difference from the healthy control group (unpaired *t* test). (**C**). Original representative histogram of DCFDA fluorescence of erythrocytes from healthy volunteers after an exposure to plasma from healthy volunteers (red shadow) and to plasma from patients without (grey line) and with cytostatic treatment for 24 h (black line). (**D**). Arithmetic means ± SEM of DCFDA fluorescence of erythrocytes from the healthy control group after a 24 h exposure to plasma from healthy volunteers (n=15, white bar) and to plasma from patients without (n=6, grey bar) and with cytostatic treatment (n=11, black bar). ** ($p<0.01$) indicates significant difference as compared to healthy volunteers. # ($p<0.05$) indicates significant difference from patients without cytostatic treatment (unpaired *t* test) [283].

7.1.2 Eryptosis in acute heart failure

The second study in patients suffering from acute heart failure (AHF) explored whether AHF is paralleled by anemia and to what extent suicidal erythrocyte death contributes to anemia. For this reason, whole blood was drawn from patients suffering from AHF. All patients were hospitalized and had a significantly lower ejection fraction (25 ± 3%). 22 patients in total, 17 males and 5 females, were enrolled in the study, with a mean age of 65.4 ± 3.2 (age range: 33 to 84 years). The cause for the present heart failure was either ischemic (10/22) or dilated cardiomyopathy (12/22). On an average, the patients were diagnosed with heart failure for the first time around 43.5 ± 9.2 months ago.
Important clinical characteristics of the AHF patients are listed in tables 6 and 7.

Whole blood was also taken from an age and sex matched healthy control group, 5 males and 5 females in total, with a LVEF of 57 ± 2% and a mean age of 57.7 ± 4.6 years (age range: 35 to 78 years).

Table 6: Characteristics of the acute heart failure patients enrolled in the study: Age, sex and blood parameters [284]

	Control values (n=10)	**Acute Heart Failure (n=22)**	***p*-value**
Age (years)	57.7 ± 4.6	65.4 ± 3.2	0.19
Sex	5 males 5 females	5 female 17 males	
LVEF (%)	57 ± 2	25 ± 3	<0.001***
Hematocrit (%)	40.1 ± 1.0	35.6 ± 1.2	0.027 *
Hemoglobin (g/dl)	14.1 ± 0.4	11.5 ± 0.5	0.008 ***
Erythrocytes ($x10^6$/µl)	4.3 ± 0.2	4.1 ± 0.2	0.449
Reticulocytes (%)	1.1 ± 0.2	2.3 ± 0.3	0.0156 *
Mean Corpuscular Hemoglobin (pg)	31.1 ± 0.5	28.2 ± 0.5	0.0056 **
Mean Corpuscular Volume (fl)	91.0 ± 1.6	88.2 ± 1.5	0.388
Serum creatinine (mg/dl)	0.92 ± 0.06	1.44 ± 0.15	0.019 *
Leucocytes (/µl)	8.4 ± 1.9	8.5 ± 0.7	0.93
C-reactive protein (mg/100 ml)	2.7 ± 0.7	27.0 ± 7.3	0.2
Ferritin (µg/100 ml)	n.a.	238.3 ± 48.5	
Transferrin saturation (%)	n.a.	14.5 ± 2.3	

Table 7: Characteristics of the acute heart failure patients enrolled in the study: cause of heart failure, medication and time after first diagnosis [284]

	Acute Heart Failure (n=22)
Cause of Heart Failure	**Ischemic cardiomyopathy:** 10/22 (45 %) **Dilated cardiomyopathy:** 12/22 (55 %)
Medication	**ASS**: 10/22 (45%) **Clopidogrel:** 3/22 (14%) **Warfarin/NOACs:** 12/22(54%) **Beta-Blockers:** 22/22 (100%) **ACE-Inhibitors/ARB:** 18/22 (82%) **Loop diuretics:** 18/22 (82%) **Eplerenone/ Spironolactone:** 16/22 (73%)
Time after first diagnosis of Heart Failure (months)	43.5 ± 9.2

The present study explored if anemia contributes to the pathophysiology of AHF and to what extent anemia is paralleled by enhanced suicidal erythrocyte death.

As demonstrated in Fig. 12, hemoglobin level (A), hematocrit (B) and mean corpuscular hemoglobin (C) were significantly lower in the AHF patients as compared to the healthy volunteers. Accordingly, the AHF patients were anemic. Although the AHF patients had a significantly higher percentage of reticulocytes (2.3 ± 0.3%) (Fig. 12 D) than the healthy control group (1.1 ± 0.2%), their anemia still prevailed.

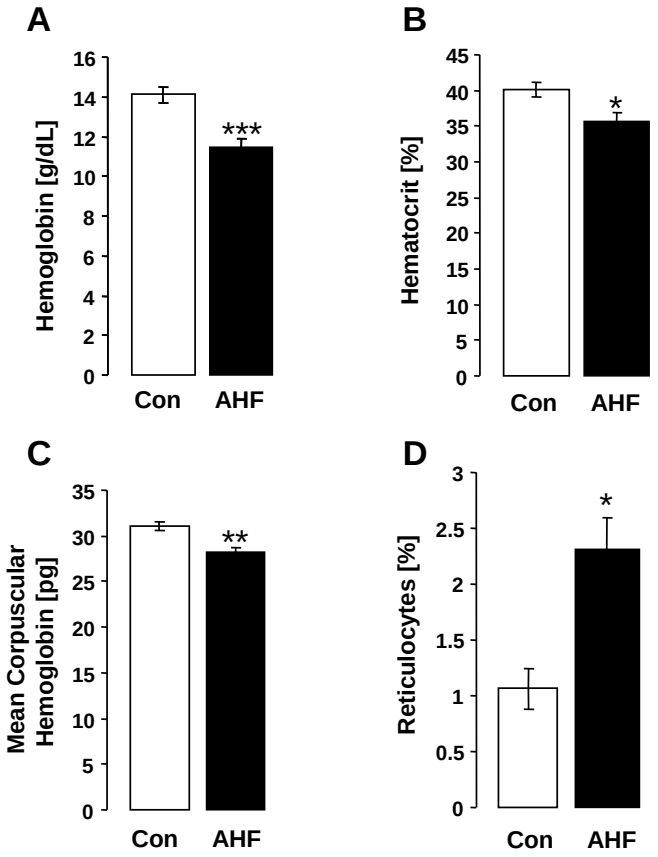

Fig. 12: Blood parameters from healthy volunteers and AHF patients. Arithmetic means ± SEM (n=10-22) of (**A**) hemoglobin level, (**B**) hematocrit, (**C**) mean corpuscular hemoglobin and (**D**) percentage of reticulocytes in blood drawn from healthy volunteers (n=10, white bars) and AHF patients (n=22, black bars). * (p<0.05), ** (p<0.01) and *** (p<0.001) indicate statistically significant difference as compared to the healthy control group (unpaired t test) [284].

A significant negative correlation was observed between the percentage of reticulocytes and the hemoglobin level (R^2= 0.6849, p<0.0001, Fig. 13 A), between the number of reticulocytes and the erythrocyte number (R^2=0.6488, p<0.0001, Fig. 13 B) and also between the reticulocyte number and the hematocrit level (R^2=0.5945, p = 0.002, Fig. 13 C). All findings suggest that the anemia of the patients occurred despite formation of new erythrocytes. Six out of the 22 patients (27%) suffered as well from iron deficiency (ID) if ID is defined by ferritin levels of lower than 100 µg per liter or between 100 and 299 µg per liter, if the transferrin saturation was <20%. The AHF patients showed a significantly higher serum creatinine level, however, none of the patients were on dialysis or taking erythropoietin.

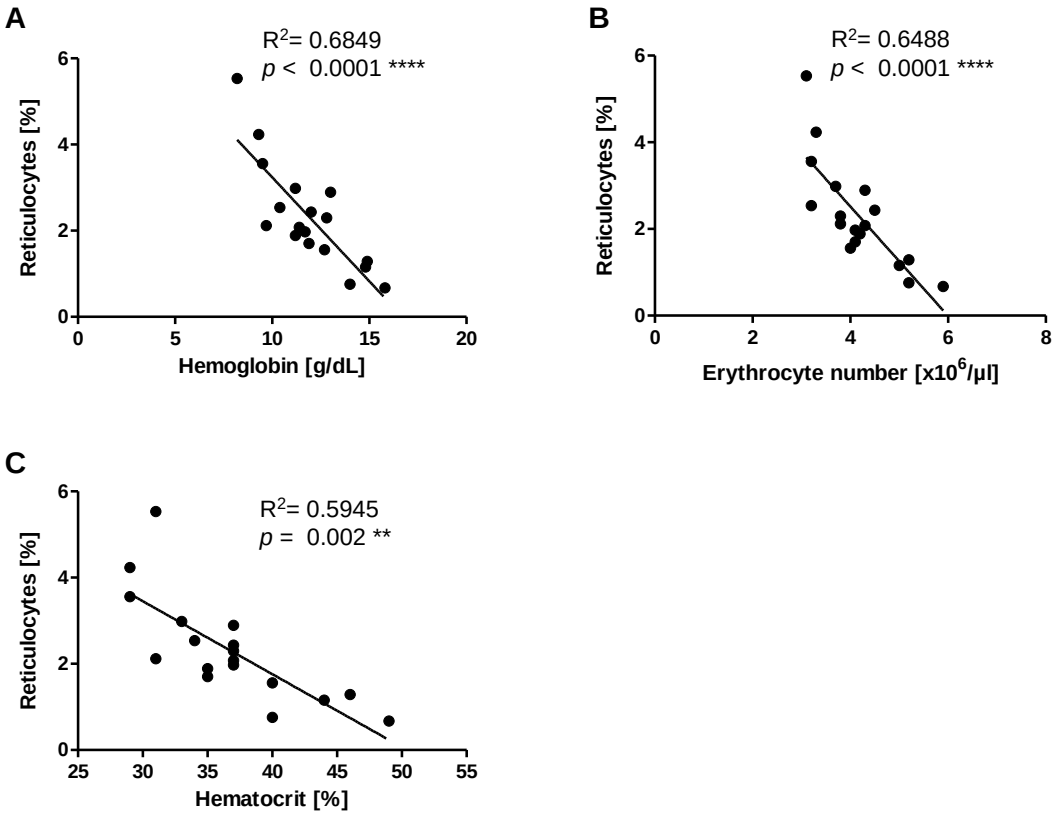

Fig. 13: (A-C). Correlations of reticulocyte number with different blood parameters in AHF patients. Reticulocyte number as a function of (**A**) hemoglobin (p<0.0001, R^2=0.6849), (**B**) erythrocyte number (p<0.0001, R^2=0.6488), (**C**) hematocrit (p=0.002, R^2=0.5945) in AHF patients. ** (p<0.01) and **** (p<0.0001) indicate significant correlation. For all correlations, Spearman nonparametric analysis was used [284].

In order to identify whether the anemia of the patients is partly due to enhanced eryptosis, phosphatidylserine-exposing erythrocytes were labeled with FITC-labeled Annexin V. As illustrated in Fig. 14 A and B, the AHF patients had a significantly higher percentage of annexin V binding erythrocytes (1.8 ± 0.1%) as compared to the control group (1.2 ± 0.2%).

In order to test whether the enhanced eryptosis of the AHF patients is stimulated by a blood-borne component, erythrocytes from healthy volunteers (blood group O^-) were incubated in plasma drawn from either, AHF patients or from healthy volunteers for 24 h. Subsequently, the percentage of annexin V binding erythrocytes was determined. As demonstrated in Fig. 14 C and D, the percentage of phosphatidylserine-exposing erythrocytes was significantly higher after an exposure to plasma from the patients (25.1 ±

3.0 %) as compared to exposure to plasma from the healthy control group (13.1 ± 2.7%).

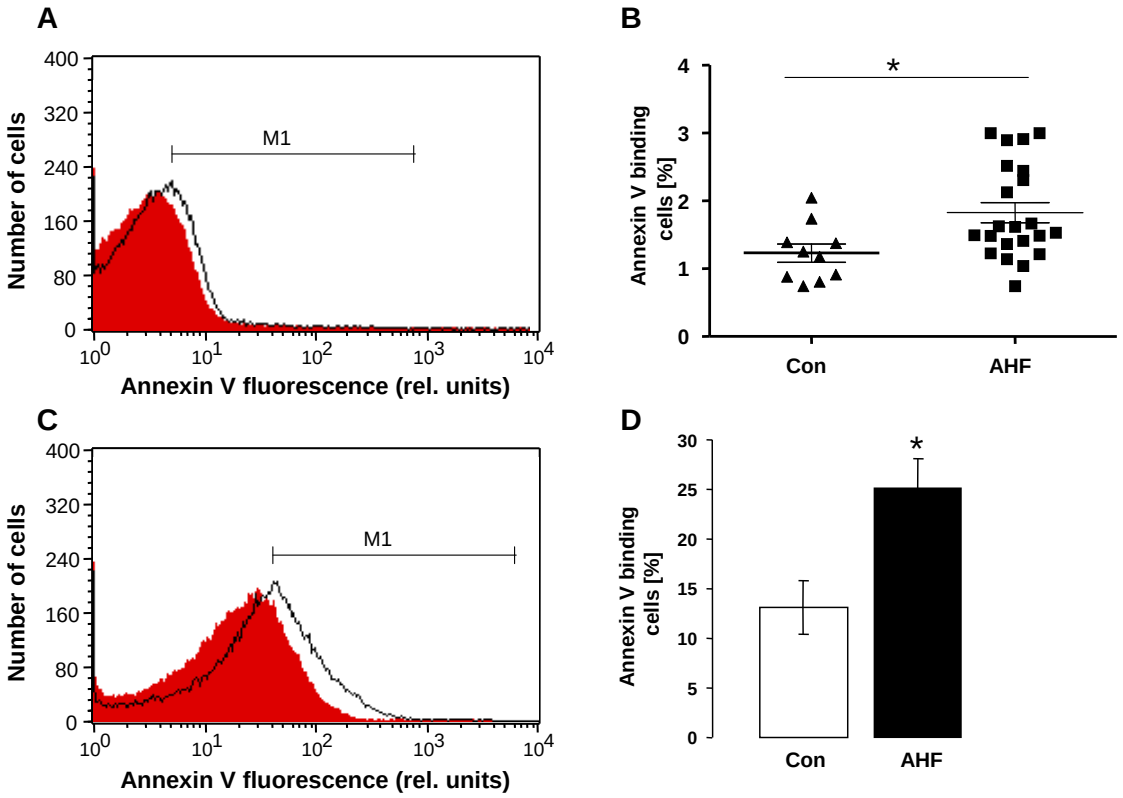

Fig. 14: Phosphatidylserine exposure of erythrocytes drawn from healthy volunteers and AHF patients (A-B) and after a 24 h exposure to plasma of the healthy control group and the AHF patients (C-D). (**A**). Original histogram of annexin V binding of erythrocytes in freshly drawn blood from the healthy control group (red shadow) and from AHF patients (black line). M1 indicates the fluorescence of annexin V defining the percentage of annexin V binding erythrocytes. (**B**). Individual values and arithmetic means ± SEM of the percentage of annexin V binding erythrocytes in freshly drawn blood from healthy volunteers (left, black triangles, n=10) and patients (right, black squares, n=22).* (p<0.05) indicates statistically significant difference between AHF patients and healthy volunteers (Mann-Whitney test). (**C**). Original histogram of annexin V binding of erythrocytes from healthy volunteers after an exposure to plasma from healthy volunteers (red shadow) and to plasma from AHF patients for 24 h (black line). M1 indicates the fluorescence of annexin V defining the percentage of annexin V binding erythrocytes**.** (**D**). Arithmetic means ± SEM of the percentage of annexin V binding erythrocytes from healthy volunteers exposed for 24 h to plasma from healthy volunteers (n=10, control, white bar) and from AHF patients (n=21, black bar). * (p<0.05) indicates statistically significant difference from exposure to plasma from healthy volunteers (unpaired t test) [284].

In order to measure the cell volume of erythrocytes, the forward scatter was determined by flow cytometry in freshly drawn blood from the control group and the AHF patients. The forward scatter of freshly drawn blood was significantly smaller in the AHF pa-

tients (484.9 ± 7.9 a.u.) as compared to the healthy control group (503.4 ± 2.5 a.u.) (Fig. 15 A and B).

The forward scatter also tended to be lower after a 24 h exposure to plasma from the patients (477.6 ± 18.5 a.u.) as compared to the healthy volunteers (518.9 ± 12.9 a.u.), the difference did, however, not reach statistical significance (data not shown).

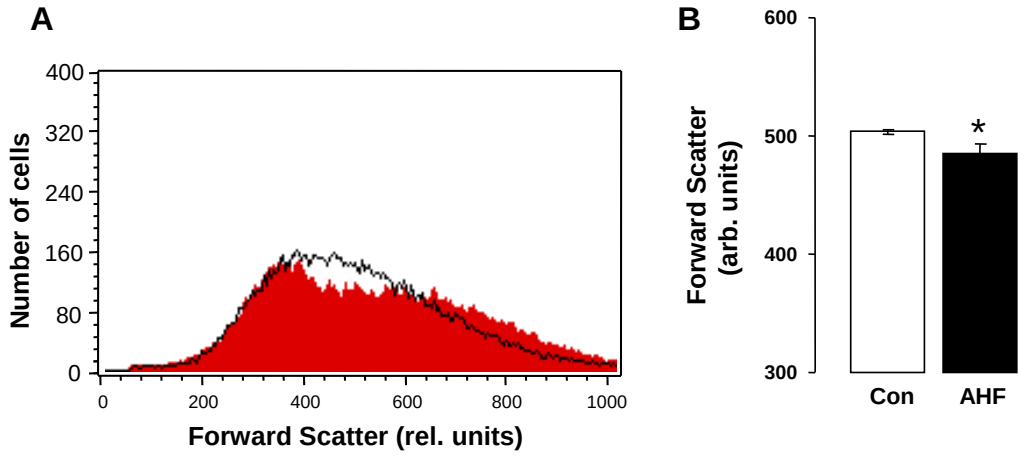

Fig. 15: Forward scatter of erythrocytes drawn from healthy volunteers and AHF patients. (A). Original histogram of forward scatter of erythrocytes in freshly drawn blood from the healthy control group (red shadow) and from AHF patients (black line). (**B)**. Arithmetic means ± SEM of the forward scatter of erythrocytes in freshly drawn blood from healthy volunteers (n=10, control, white bar) and AHF patients (n=22, black bar). * (p<0.05) indicates statistically significant as compared to healthy volunteers (unpaired t test) [284].

In order to analyze intracellular Ca^{2+}, Fluo3 fluorescence was determined in freshly drawn blood and after a 24 h plasma exposure. The Fluo3 fluorescence was similar in freshly drawn blood from the healthy volunteers (22.9 ± 1.6 a.u.) and the AHF patients (20.1 ± 1.2 a.u.). After a 24 h exposure to plasma from the patients, the Fluo3 fluorescence tended to be higher (24.5 ± 2.9 a.u.) as compared to the healthy volunteers (19.3 ± 0.7 a.u.), an observation, however, not reaching statistical significance (data not shown).

Eryptosis may further be induced by oxidative stress. In order to measure reactive oxygen species, 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) was employed. As demonstrated in Fig. 16 A and B, the DCFDA fluorescence was significantly higher in freshly drawn blood from the patients (20.2 ± 1.7 a.u.) than in the healthy control group

(14.3 ± 0.9 a.u.). In order to identify if a plasma-borne component, for example an oxidant, stimulates the eryptosis, the DCFDA fluorescence was as well determined in erythrocytes (blood group O^-) after an exposure to both, plasma from the healthy volunteers and the patients. As shown in Fig. 16 C and D, the amount of reactive oxygen species according to the 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence was significantly higher in the AHF patients (22.8 ± 2.0 a.u.) as compared to the healthy volunteers (15.4 ± 2.2 a.u.).

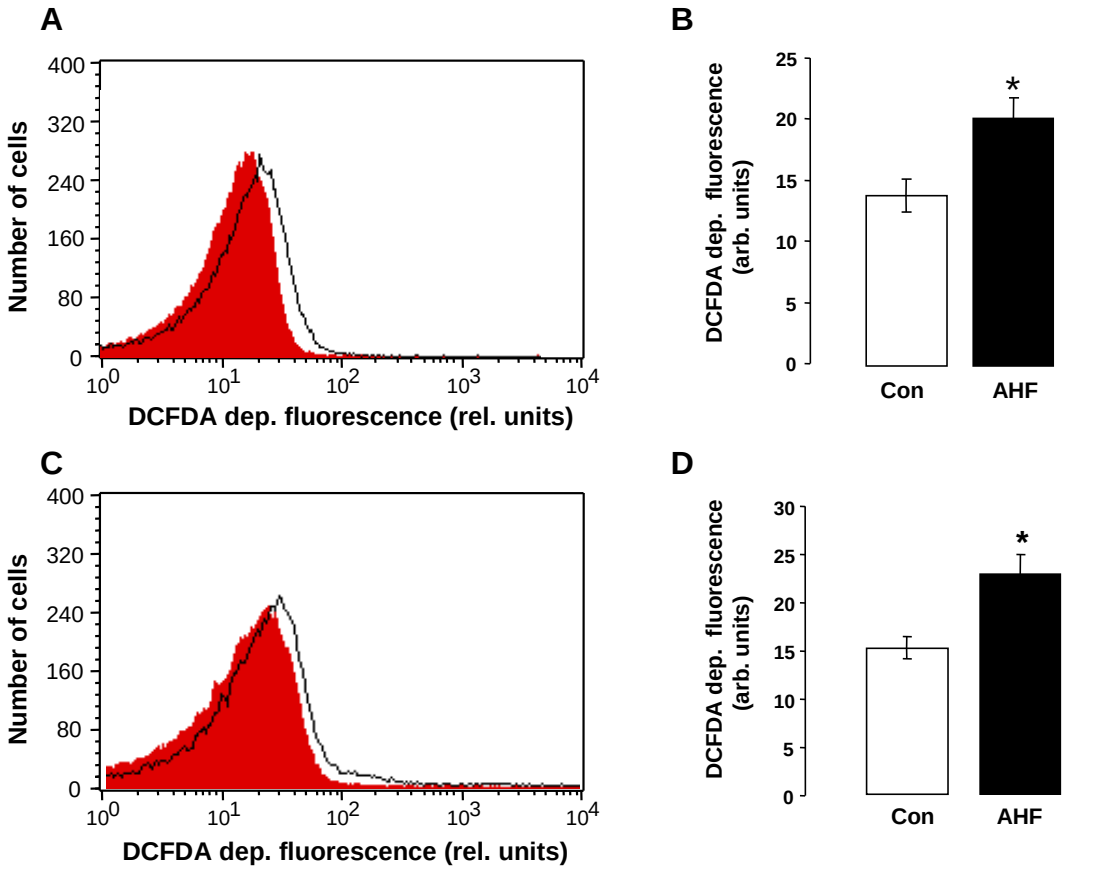

Fig. 16: Reactive oxygen species of erythrocytes drawn from AHF patients and healthy volunteers (A-B) and after an exposure to plasma from the healthy volunteers and AHF patients for 24 h (C-D). (**A**). Original histogram of 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence of erythrocytes in freshly drawn blood from healthy volunteers (red shadow) and from AHF patients (black line). (**B**). Arithmetic means ± SEM of the DCFDA fluorescence of erythrocytes in freshly drawn blood from healthy volunteers (n=7, control, white bar) and AHF patients (n=18, black bar). * (p<0.05) indicates statistically significant difference to healthy volunteers (unpaired t test). (**C**). Original histogram of 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence of erythrocytes from healthy volunteers following a 24 h exposure to plasma from healthy volunteers (red shadow) and to plasma from AHF patients (black line). (**D**). Arithmetic means ± SEM of the DCFDA fluorescence of erythrocytes from healthy volunteers exposed for 24 h to plasma from healthy volunteers (n=7, control, white bar) and from AHF patients (n=15, black bar). * (p<0.05) indicates statistically significant difference from exposure to plasma from healthy volunteers (unpaired t test) [284].

Another trigger of cell membrane scrambling without increasing intracellular Ca^{2+} is the formation of ceramide. Thus, additional experiments explored if the ceramide abundance is different in the control and the AHF patients. The abundance of ceramide was detected by FITC-labelled antibodies using flow cytometry and was similar in freshly drawn blood from the AHF patients (14.6 ± 0.4 a.u.) and the healthy volunteers (13.1 ± 0.4 a.u.). After a 24 hours plasma exposure, the ceramide abundance tended to be higher following exposure to plasma from the AHF patients (18.8 ± 3.6 a.u.) than to the healthy controls (13.3 ± 0.7 a.u.), the difference did, however, not reach statistical significance (data not shown).

7.2 Eryptosis in mice

7.2.1 Role of Gαi2 in erythrocytes

The second part of this study addressed the influence of Gαi2 on erythrocyte survival in mice. For this reason, experiments were conducted in mice lacking functional Gαi2 (*Gαi2*$^{-/-}$) and their corresponding wild type littermates (*Gαi2*$^{+/+}$).

Firstly, immunoblotting was performed in order to explore whether Gαi2 is expressed in both murine and human erythrocytes. For this reason, whole blood from human and mouse was taken and the erythrocytes were isolated and purified. Equal amounts of protein lysates were made and subsequently, immunoblotting was performed. GAPDH was used as a loading control. As demonstrated in Fig. 17A, incubation with Gαi2 specific antibodies showed a single band of 40 kDa in mouse erythrocytes from *Gαi2*$^{+/+}$ mice but not in erythrocytes from *Gαi2*$^{-/-}$ mice. Human erythrocytes displayed the same single band of 40 kDa. The bands appearing below 40 kDa may be the result of non-specific antibody binding. Densitometry analysis revealed that the Gαi2 protein is significantly less abundant in human erythrocytes as compared to murine erythrocytes (Fig. 17 B). Thus, it can be concluded that Gαi2 is expressed in both mouse and human erythrocytes.

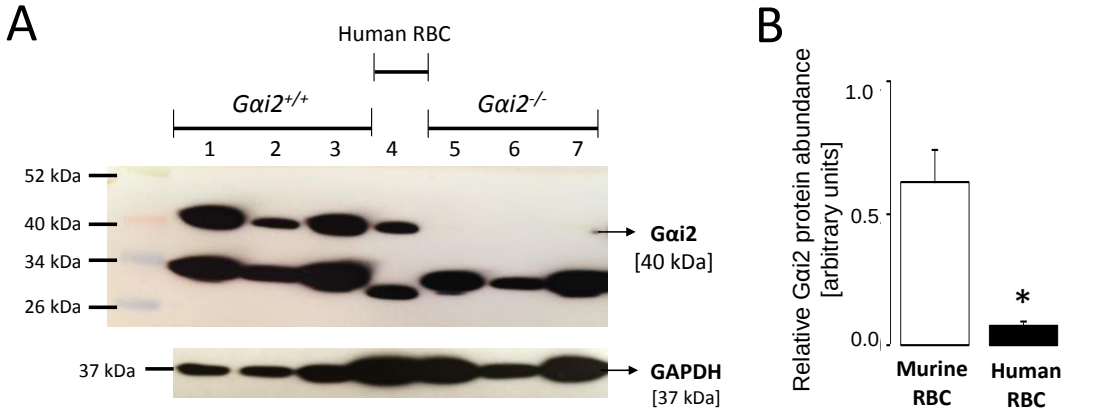

Fig. 17: Gαi2 expression in human and murine erythrocytes. (**A**). Original Western Blots showing Gαi2 (40 kDa) and GAPDH (37 kDa) expression in human erythrocytes (band 4) and in erythrocytes drawn from *Gαi2*$^{+/+}$ (bands 1-3) and *Gαi2*$^{-/-}$ (bands 5-7) mice. The Western blot demonstrates the expression of Gαi2 and GAPDH in whole blood (bands 1 and 5), diluted whole blood (1:3.7 dilution; bands 2 and 6) and isolated and purified erythrocytes (bands 3 and 7). (**B**). Means ± SEM of Gαi2 abundance in human and murine erythrocytes relative to the loading control GAPDH (n=3). * (p<0.05) indicates statistically significant difference from murine RBC [285].

Next, determination of the blood count in *Gαi2*$^{+/+}$ and *Gαi2*$^{+/+}$ mice was performed. Erythrocyte count, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and the percentage of reticulocytes were not significantly different between *Gαi2*$^{-/-}$ and *Gαi2*$^{+/+}$ mice (Fig. 18 A). The mean corpuscular volume was, however, significantly larger in *Gαi2*$^{-/-}$ mice as compared to *Gαi2*$^{+/+}$ mice (41.1 ± 0.3 fl for *Gαi2*$^{+/+}$ mice versus 42.8 ± 0.2 fl for *Gαi2*$^{-/-}$ mice, n=8, $p<0.001$). Thus, it can be concluded that *Gαi2*$^{-/-}$ erythrocytes are normochromic and moderately larger as compared to *Gαi2*$^{+/+}$ erythrocytes. May-Grünwald staining revealed no relevant changes in erythrocyte shape between *Gαi2*$^{-/-}$ and *Gαi2*$^{+/+}$ erythrocytes (Fig. 18 B). The percentage of Ter119/CD71 positive cells were similar in both mice indicating that dynamic erythrocytes maturation *in vivo* is similar (Fig. 18 C). The plasma erythropoietin (Fig. 18 D) and the platelet count (Fig. 18 F) of *Gαi2*$^{-/-}$ mice were not significantly different from *Gαi2*$^{+/+}$ mice. The *Gαi2*$^{-/-}$ mice, however, had an increased number of white blood cells (Fig. 18 E), which is consistent with a previous report showing leukocytosis in *Gαi2*$^{-/-}$ mice [286]. The leukocytosis is due to an increased production of pro-inflammatory cytokines in *Gαi2*$^{-/-}$ mice [281].

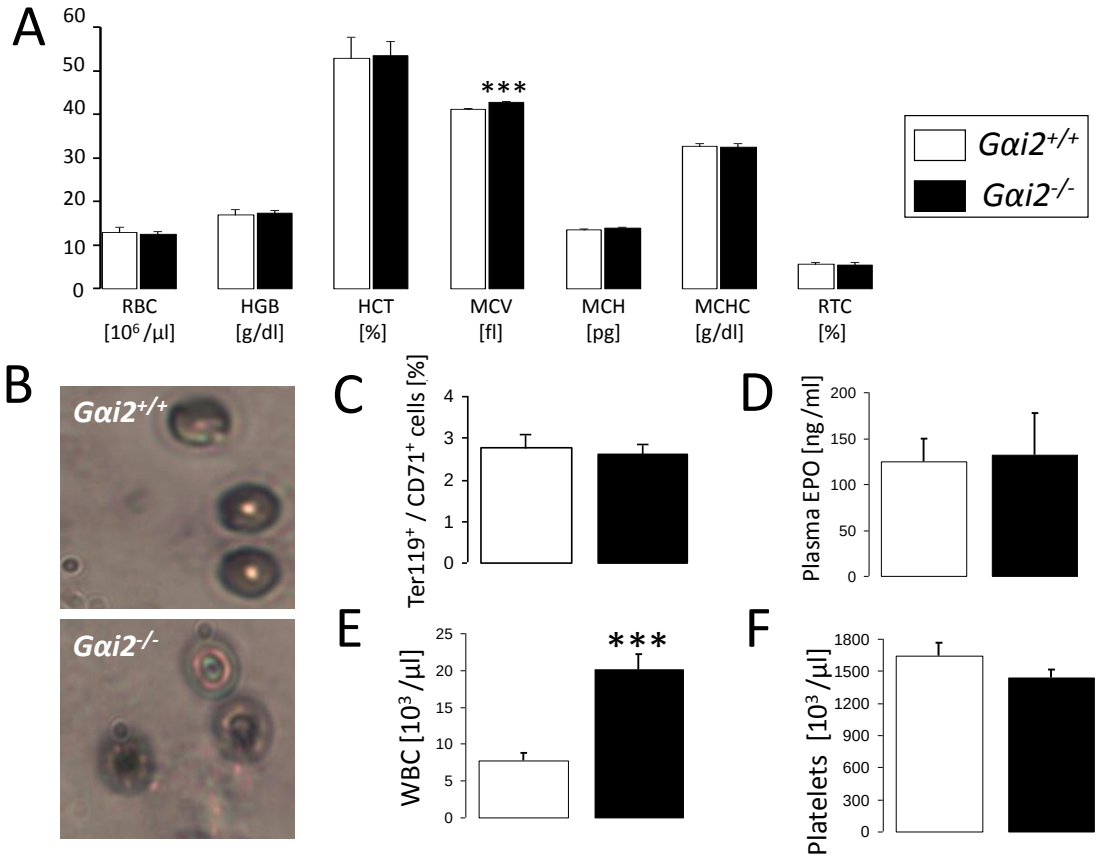

Fig. 18: Blood parameters in *Gαi2*$^{-/-}$ and *Gαi2*$^{+/+}$ mice. Means ± SEM (**A**) of erythrocyte count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and reticulocyte count (RTC, n=8) of *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ mice. (**B**). May-Grünwald staining of erythrocytes from *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ mice. (**C**). Percentage of Ter119/CD71 positive cells (n=6) of *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ mice. Means ± SEM of plasma erythropoietin (EPO) levels (**D**, n=3-4), leukocyte count (**E,** n=8) and platelet count (**F**, n=8) determined in *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ mice. *** (p<0.001) indicates significant difference from *Gαi2*$^{+/+}$ mice [285].

A next series of experiments examined whether Gαi2 deficiency influences survival of erythrocytes. For this reason, annexin V binding, forward scatter and Fluo3 fluorescence were analyzed by flow cytometry in order to determine phosphatidylserine exposure, cell shrinkage and cytosolic Ca^{2+} activity, respectively. As shown in Fig. 19A, freshly drawn erythrocytes were visualized using confocal microscopy and quantification of multiple fields revealed a decreased ratio of annexin V binding cells to total cells (observed under transmission light) per field in *Gαi2*$^{-/-}$ erythrocytes (0.028 ± 0.007, n=4) as compared to *Gαi2*$^{+/+}$ erythrocytes (0.069 ± 0.007, n=4).

Using FACS analysis, the percentage of annexin V binding cells of *Gαi2*$^{-/-}$ and *Gαi2*$^{+/+}$ erythrocytes was quantified immediately after retrieval and following incubation in Ringer solution for 12 h. Similar to the obtained results in confocal microscopy, annexin V binding was significantly lower in erythrocytes of *Gαi2*$^{-/-}$ mice (0.37 ± 0.06%) as compared to *Gαi2*$^{+/+}$ erythrocytes (0.58 ± 0.07%) measured immediately after retrieval (Fig. 19 B and C) and following 12 h incubation in Ringer solution (*Gαi2*$^{+/+}$: 2.43 ± 0.33% ; *Gαi2*$^{-/-}$: 1.52 ± 0.15%) (Fig. 19 D).

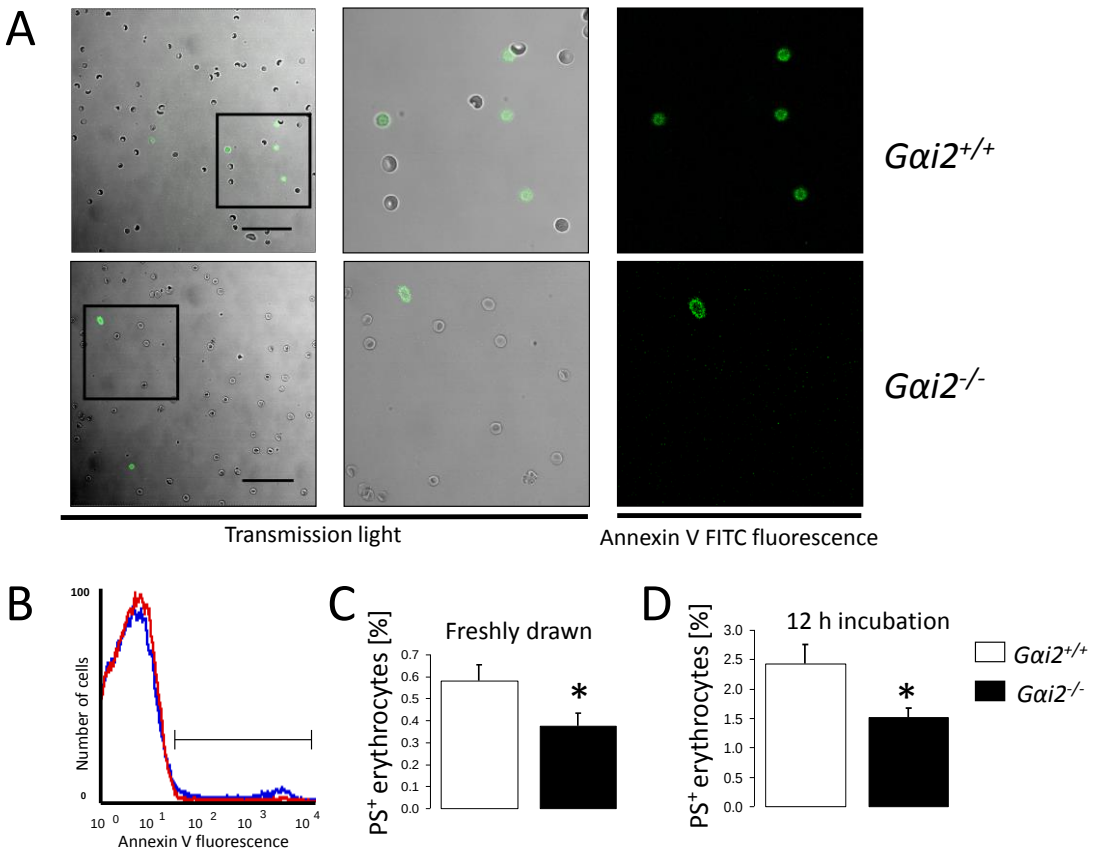

Fig. 19: Phosphatidylserine exposure in erythrocytes from *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ mice. Confocal microscopy images (**A**) of annexin V fluorescence (right panels) and transmission light (left and middle panels) in freshly drawn erythrocytes from *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ mice. Histogram overlay (**B**, blue line: *Gαi2*$^{+/+}$ and red line: *Gαi2*$^{-/-}$) and means ± SEM of annexin V binding in freshly drawn erythrocytes (**C**, n=24-40) and in erythrocytes incubated in Ringer solution for 12 h (**D**, n=11-17) retrieved from *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ mice. M1 indicates the fluorescence of annexin V defining the percentage of annexin V binding erythrocytes. * (p<0.05) indicates significant difference from *Gαi2*$^{+/+}$ mice [285].

Quantification of forward scatter indicated that cell volume was slightly, but significantly larger in *Gαi2*$^{-/-}$ erythrocytes (468.4 ± 4.17 a.u.) as compared to *Gαi2*$^{+/+}$ erythrocytes (453.8 ± 2.81 a.u.) (Fig. 20 A and B). Both cell shrinkage and exposure of phosphatidylserine are dependent on cytosolic Ca^{2+} activity. The percentage of Fluo3 positive cells was slightly, but significantly lower in *Gαi2*$^{-/-}$ erythrocytes (1.39 ± 0.33%) as compared to *Gαi2*$^{+/+}$ erythrocytes (3.3 ± 0.60%) (Fig. 20 C and D).

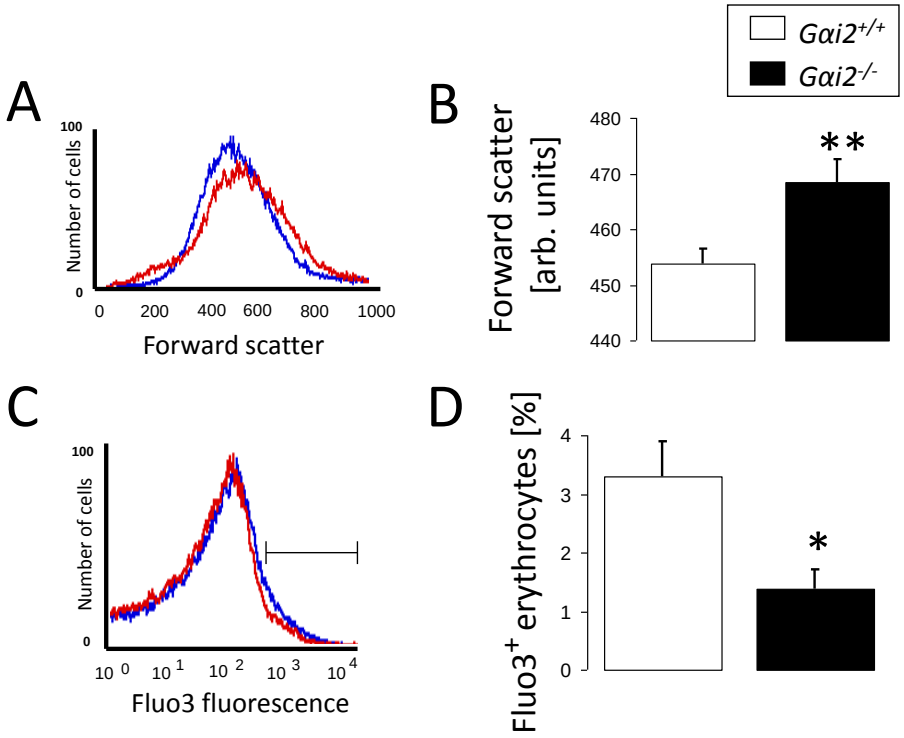

Fig. 20: Cell shrinkage and cytosolic Ca^{2+} activity in erythrocytes from *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ mice. Histogram overlay (**A** and **C**; blue line: *Gαi2*$^{+/+}$ and red line: *Gαi2*$^{-/-}$) and means ± SEM of forward scatter geomean (**B**, n=21-33) and percentage of Fluo3 positive erythrocytes (M1) (**D**, n=8-16) in freshly drawn erythrocytes retrieved from *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ mice. * (p<0.05) and ** (p<0.01) indicate significant difference from *Gαi2*$^{+/+}$ mice [285].

In further experiments, the susceptibility of *Gαi2*$^{+/+}$and *Gαi2*$^{-/-}$ erythrocytes to osmotic shock, a known stimulator of eryptosis and pathophysiological cell stressor, was addressed *ex vivo*. A 30 min exposure to hyperosmotic Ringer solution (addition of 550 mM sucrose) significantly enhanced the phosphatidylserine exposure, an effect significantly less pronounced in *Gαi2*$^{-/-}$ (21.9 ± 2.0%) as compared to *Gαi2*$^{+/+}$ erythrocytes (31.8 ± 1.5%) (Fig. 21 A and B).

In order to determine hyperosmotic shock-triggered cell shrinkage, erythrocyte forward scatter was analyzed. Forward scatter was significantly lower following exposure to hyperosmolar shock in erythrocytes of both mice, the effect was, however, significantly thwarted in *Gαi2*$^{-/-}$ erythrocytes (440.5 ± 4.8 a.u.) as compared to *Gαi2*$^{+/+}$ erythrocytes (420.0 ± 5.2 a.u.) (Fig. 21 C and D).

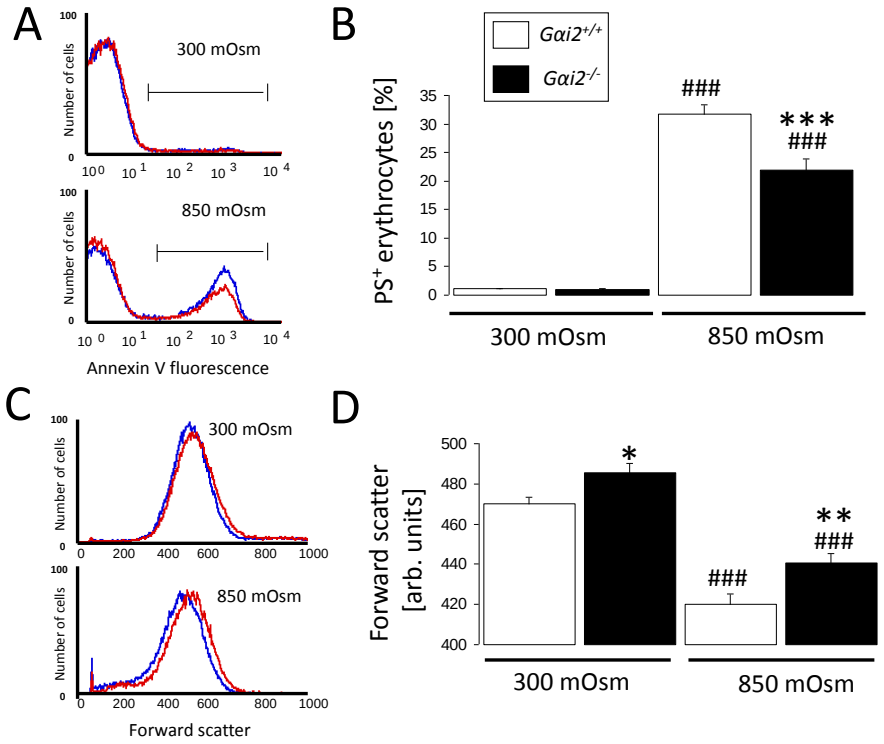

Fig. 21: Effect of hyperosmotic shock on phosphatidylserine exposure and cell shrinkage in *Gai2*$^{+/+}$ and *Gai2*$^{-/-}$ erythrocytes. Histogram overlay (**A** and **C**; red line: *Gai2*$^{-/-}$ and blue line: *Gai2*$^{+/+}$) and means ± SEM of the percentage of annexin V binding cells (**B**, n=11-14) and geomean of forward scatter (**D**, n=11-14) measured in *Gai2*$^{-/-}$ and *Gai2*$^{+/+}$ erythrocytes after incubation in isosmotic (300 mOsm) or hyperosmotic (850 mOsm) Ringer solution for 30 min. M1 indicates the fluorescence of annexin V defining the percentage of annexin V binding erythrocytes. ### (p<0.001) indicates significant difference from isosmotic Ringer solution. * (p<0.05), ** (p<0.01) and *** (p<0.001) indicate significant difference from *Gai2*$^{+/+}$ [285].

In order to elucidate the mechanism that contributed to the protective effect of Gαi2 deficiency against the eryptosis triggered by hyperosmotic shock, erythrocyte Ca^{2+} activity was determined. The percentage of Fluo3 positive cells following hyperosmotic shock was significantly increased in erythrocytes of both mice, but the effect was signif-

icantly less pronounced in *Gαi2*$^{-/-}$ erythrocytes (13.4 ± 0.9%) as compared to *Gαi2*$^{+/+}$ erythrocytes (17.6 ± 1.2%) (Fig. 22).

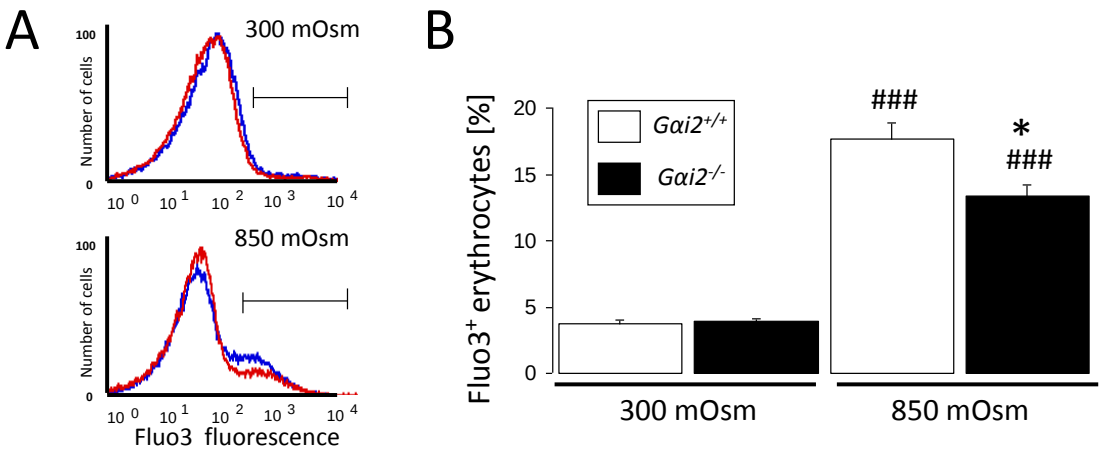

Fig. 22: Effect of hyperosmotic shock on cytosolic Ca^{2+} activity in *Gai2*$^{+/+}$ and *Gai2*$^{-/-}$ erythrocytes. Histogram overlay (**A**: Red line: *Gai2*$^{-/-}$ and blue line: *Gai2*$^{+/+}$) and means ± SEM of the percentage of Fluo3 positive erythrocytes (M1) (**B**, n=11-14) analyzed in *Gai2*$^{-/-}$ and *Gai2*$^{+/+}$ erythrocytes after incubation in isosmotic (300 mOsm) or hyperosmotic (850 mOsm) Ringer solution for 30 min. ### (p<0.001) indicates significant different from isosmotic Ringer solution. * (p<0.05) indicates significant difference from *Gai2*$^{+/+}$ [285].

Additional experiments examined whether Gαi2 deficiency protects against ceramide induced suicidal erythrocyte death. Treatment of erythrocytes from *Gαi2*$^{-/-}$ and *Gαi2*$^{+/+}$ mice with bacterial sphingomyelinase (0.01 U/ml) and C6 ceramide (50 µM) significantly enhanced annexin V binding. The effects were significantly thwarted in erythrocytes from *Gαi2*$^{-/-}$ mice as compared to *Gαi2*$^{+/+}$ erythrocytes (Fig. 23). Hence, it can be concluded that deficiency of Gαi2 has a slight effect on ceramide-induced suicidal erythrocyte death.

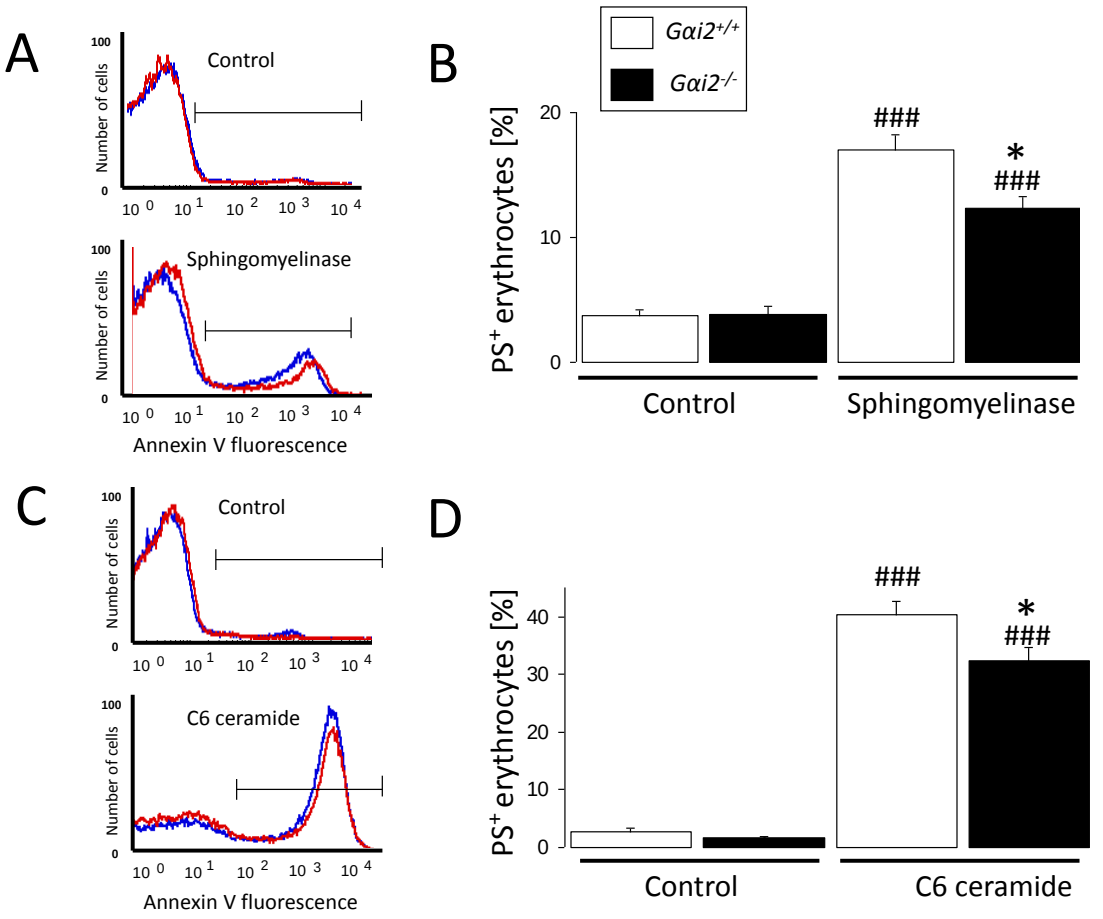

Fig. 23: Effect of sphingomyelinase and C6 ceramide on phosphatidylserine exposure of *Gai2*$^{+/+}$ and *Gai2*$^{-/-}$ erythrocytes. Histogram overlay (**A** and **C**: Red line: *Gai2*$^{-/-}$ and blue line: *Gai2*$^{+/+}$) and means ± SEM of annexin V binding determined after exposure to bacterial sphingomyelinase (**A** and **B**, 0.01 U/ml for 24 h; n=7-16) or following exposure to C6 ceramide (**C** and **D**, 50 µM for 12 h; n=11-17). M1 indicates the fluorescence of annexin V defining the percentage of annexin V binding erythrocytes. ### (p<0.001) indicates significant difference from Ringer solution. * (p<0.05) indicates significant difference from *Gai2*$^{+/+}$ [285].

Further experiments explored the resistance of *Gαi2*$^{-/-}$ and *Gαi2*$^{+/+}$ erythrocytes to different extracellular osmolarities. The resistance of erythrocytes to graded decrease of osmolarity was significantly lower in *Gαi2*$^{+/+}$ than in *Gαi2*$^{-/-}$ erythrocytes. Thus, Gαi2 deficiency counteracts the sensitivity of erythrocytes to both hyper- and hyposmotic shock (Fig. 24).

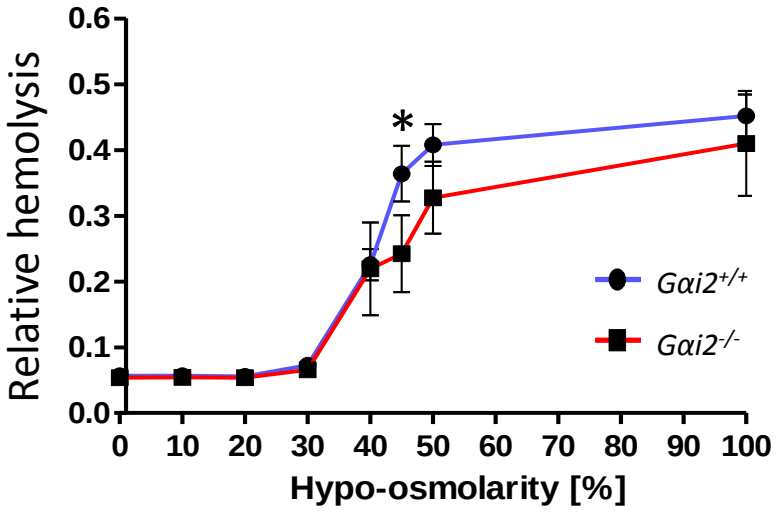

Fig. 24: Osmotic resistance of erythrocytes from *Gai2*$^{+/+}$ and *Gai2*$^{-/-}$ mice. Means ± SEM (n=3-5) of relative hemolysis as a function of extracellular osmolarity (% hyposmolar of isosmotic Ringer) determined in *Gai2*$^{+/+}$ (blue line) and *Gai2*$^{-/-}$ (red line) erythrocytes. * (p<0.05) indicates significant difference from *Gai2*$^{+/+}$ [285].

8 DISCUSSION

8.1 Eryptosis in patients

8.1.1 Eryptosis in lung cancer

The present study reveals that patients suffering from different stages and types of lung cancer (LC) suffer from anemia despite an increased number of reticulocytes. The anemia further deteriorates under cytostatic treatment. These observations indicate that the anemia in LC is not due to impaired erythropoiesis but rather due to an accelerated destruction of erythrocytes. The anemia prevails obviously despite enhanced erythropoiesis and presumably leads to upregulation of erythropoietin, which, in turn, stimulates erythropoiesis [287]. Erythropoietin has previously been shown to inhibit eryptosis [288]. Under the influence of high erythropoietin levels, however, erythrocytes are apparently generated with relatively high susceptibility to triggers of eryptosis [289]. Anemia has a profoundly negative impact on the quality of life and worsens not only fatigue of cancer patients [290] but is also associated with shorter survival rates [150] and can, thus, be considered as an independent prognostic factor for survival [150]. A patient with bronchogenic carcinoma who additionally suffers from anemia has a 19% higher relative risk of death [150]. Furthermore, the presence of anemia is associated with decreased survival in almost all cancer types studied [150]. These observations could be explained by the fact that anemia may lead to tumor hypoxia, which, in turn, is associated with enhanced tumor aggressiveness [155]. Hypoxia is also associated with resistance to both radiation therapy and chemotherapy [144].

The present observations may explain the phenomenon of accelerated erythrocyte clearance by enhanced suicidal death, which might contribute to the development of anemia in LC patients. The percentage of phosphatidylserine exposing erythrocytes in LC of both groups was significantly enhanced. Exposed phosphatidylserine at the erythrocyte surface is recognized by phagocytes (e.g. macrophages), which subsequently engulf and degrade the erythrocytes [17, 71]. If the number of erythrocytes that are cleared from circulating blood exceeds the formation of new erythrocytes, anemia develops [54].

Hence, enhanced eryptosis could substantially contribute to the anemia of LC patients. Another key hallmark of eryptosis is cell shrinkage [31]. In the present study a slight, however, not statistically significant decrease in cell volume was observed only in the patient group without cytostatic treatment. The patients with cytostatic treatment, however, showed a significantly higher cell volume, which is discordant with the decrease of cell volume that is typical for suicidal erythrocyte death. Increased $[Ca^{2+}]_i$ leads to activation of Ca^{2+}-sensitive K^+ channels that, in turn, leads to exit of K^+, hyperpolarization of the cell membrane, exit of Cl^-, and finally to cellular loss of KCl and water [49]. In the present study, the intracellular Ca^{2+} content was, however, not significantly different between erythrocytes from healthy volunteers and LC patients. Hence, the absence of cell shrinkage could be explained by the lack of increased $[Ca^{2+}]_i$. Furthermore, LC patients under cytostatic treatment *per se* had a higher MCV, which was further reflected by enhanced cell volume as shown by FACS analysis.

Oxidative stress and ceramide are powerful stimulators of eryptosis [291, 292]. In the present study it was observed that both ceramide and oxidative stress were enhanced in the erythrocytes of LC patients under cytostatic treatment determined in freshly drawn blood. Previously, an antioxidant/oxidant imbalance has been shown in the blood of cancer patients [293]. Furthermore, an enhanced ceramide formation predisposes to lung cancer [294]. After exposing healthy erythrocytes to plasma from LC patients with and without cytostatic treatment, the percentage of annexin V binding cells was significantly increased after exposure to plasma of LC patients under cytostatic treatment. The enhanced phosphatidylserine externalization following exposure to plasma of this patient group was paralleled by significantly enhanced oxidative stress and ceramide abundance but not by increased $[Ca^{2+}]_i$. These results indicate that a component in plasma (or a wide variety of them) trigger the eryptosis observed in the LC patients under cytostatic treatment, an effect which may be due to the cytostatic treatment itself. Indeed, several cytostatics have been shown to enhance suicidal erythrocyte death *in vitro*, which were used for the LC patients enrolled in this study including cisplatin [295], docetaxel [296] or topotecan [297]. As a matter of fact, it was shown that patients under platinum-based chemotherapy have a higher risk for anemia as compared to patients with non-platinum based chemotherapy [149].

Furthermore, it would be worthwhile to investigate if other cytostatics used in this study e.g. etoposide, gemcitabine, vinorelbine and pembrolizumab have a similar effect on erythrocytes.

Venous thromboembolisms (VTEs) are known to commonly occur in cancer patients [298]. Patients with LC show especially high incidence rates of VTE [299, 300]. Mechanisms that lead to the development of venous thrombosis in LC patients are poorly understood but the etiology is probably multifactorial [298]. It is assumed that cancer itself increases the risk for thromboembolic events. Additional risk factors for these events are surgery, chemotherapy, bed-rest, hormone therapy, venous catheters, infections and radiotherapy [298]. Eryptosis may not only contribute to anemia but is also assumed to interfere with the microcirculation [54]. Phosphatidylserine-exposing erythrocytes not only bind to CXCL16/SR-PSOX [72] but also to other receptors expressed on endothelial cells [73, 74]. Hence, the enhanced eryptosis in LC patients could, thus, contribute to the development of thromboembolic events. Moreover, the susceptibility to eryptosis is further enhanced by iron deficiency [69], which may play a key role in the anemia of patients with malignancy [301]. In the present study, however, transferrin levels have not been determined. Thus, the contribution of iron deficiency to the observed stimulation of suicidal erythrocyte death requires further investigation.

In a previous study, enhanced suicidal erythrocyte death has been shown in mice carrying a mutation in the *APC* (adenomatous-polyposis-coli, $apc^{Min/+}$) gene [67] resulting in development of multiple intestinal tumors [302, 303]. The animals were also shown to be anemic [67, 304]. Similar to the observations in the present study, the anemia in those mice prevailed despite enhanced reticulocyte numbers indicating an accelerated erythrocyte turnover, which was shown to result from enhanced eryptosis [67].

Despite the known negative effects of anemia, anemia is still not optimally treated in cancer patients [305]. In the management of cancer, mild anemia has not been considered important enough to be improved [144] and hence, only moderate to severe anemia is corrected [306]. If the anemia is not due to iron deficiency, red blood cell transfusions and the application of Erythropoiesis Stimulating Agents (ESAs) are used to improve the level of hemoglobin [82].

ESAs increase the production of red blood cells in the bone marrow by the activation of the erythropoietin receptor (EpoR) on erythrocyte progenitor cells [207]. The three different recombinant erythropoietins, epoetin alfa, epoetin beta and darbepoietin alfa are commonly used for the treatment of anemia [307]. They all show a similar clinical efficacy [307-309]. Erythropoietin not only prevents apoptosis of the erythrocyte progenitor cells [310] but also counteracts eryptosis by inhibition of the Ca^{2+}-permeable cation channels [34, 311].

The main advantage of the use of ESAs is that the complications of red blood cell transfusion can be avoided [82] and their use decreases the transfusion rates in cancer patients [312-314]. However, the application bears risks as it is associated with a decreased survival rate and an increased mortality rate [314-316]. There is also controversy regarding the correlation between their use, enhanced tumor progression [317, 318] and the occurrence of cardiovascular complications [319]. Cancer patients are known to be at a higher risk for developing thrombosis even without receiving recombinant erythropoietin [320, 321] and may be at an even higher risk with the use of ESAs [82].
Prior to the use of erythropoietin, the transfusion of red blood cells was the only option for the treatment of severe anemia in cancer patients [307] and continues to be a mainstay for the treatment at present [322]. Transfusion provides a rapid increase of the hematocrit and hemoglobin levels and is, thus, the only option for patients who require an immediate correction of their anemia [82]. However, studies analyzing the benefits of transfusions have observed conflicting findings [323]. Potential complications include transfusion reactions, lung injury [324], allo-immunization, over-transfusion and transmission of infectious diseases [307]. Transfusion has even been linked with an increased long-term risk for developing cancer [325-327]. Other studies have also observed a correlation between transfusion and thrombosis [323, 328] and even decreased survival [323, 329]. Furthermore, patients often prefer to avoid blood transfusions [330].

Indeed, the present study shows for the first time that anemia in lung cancer is at least in part due to enhanced suicidal erythrocyte death. In view of this fact, anemia in these patients could possibly be diminished by inhibition of eryptosis, which could counteract anemia.

Several natural components and drugs have been shown to be effective against eryptosis *in vitro*: resveratrol [331], zidovudine [332] or thymol [333]. It would be worthwhile to investigate if any of these compounds may be used in the treatment of anemia in cancer patients or other diseases. However, the possibility must be kept in mind that, at least theoretically, inhibitors of eryptosis may similarly counteract apoptosis of tumor cells and, thus, interfere with cytostatic treatment. On the other hand, correction of anemia would be expected to lower release of erythropoietin that has previously been shown to promote tumor angiogenesis and lymphangiogenesis [334].

8.1.2 Eryptosis in acute heart failure

The present study discloses that patients with acute heart failure (AHF) suffer from enhanced suicidal erythrocyte death. The findings confirm the observations of Mahmud et al. who previously showed an increased percentage of phosphatidylserine-exposing erythrocytes and a decreased cell volume in rodents with AHF [335].

Phagocytes recognize and eliminate phosphatidylserine-exposing cells from the blood stream [17, 71]. Thus, enhanced eryptosis might lead to anemia if the loss of erythrocytes is not compensated by the formation of new erythrocytes [33]. Consistent with the observations in rodents [335], the AHF patients suffered from anemia despite a higher percentage of reticulocytes as compared to the control group indicating that the anemia resulted from an enhanced destruction of erythrocytes rather than an alleviated production of new erythrocytes.

Furthermore, the present study investigated the mechanisms contributing to enhanced eryptosis in AHF patients. In contrast to the observations in the animal model showing an increased $[Ca^{2+}]_i$ in erythrocytes after treatment with oxidative stress, hyperosmotic shock and energy depletion [335], $[Ca^{2+}]_i$ was not significantly different between the healthy control group and the AHF patients in this study. Moreover, the enhanced erythrocyte cell membrane scrambling observed in AHF patients was not due to increased ceramide abundance, another important trigger of eryptosis [54]. However, the enhanced cell membrane scrambling was paralleled by a significant higher level of oxidative stress, which is known to stimulate eryptosis [292]. An enhanced cell membrane scrambling after treatment with the oxidant *tert*-butylhydroperoxide has also been shown in five patients suffering from AHF [335]. The present study particularly shows that phosphatidylserine exposure and oxidative stress are enhanced after exposing healthy erythrocytes to plasma from AHF patients as compared to healthy volunteers. This observation indicates that a component in plasma of the patients triggers eryptosis. Another possibility is that the enhanced eryptosis might be due to an altered concentration of a variety of components in the plasma. The observations do, however, not rule out altered properties of the erythrocytes of AHF patients.

The AHF patients showed significantly lower MCH values and 27% of the AHF patients enrolled in the study suffered from iron deficiency (ID). Previously, it has been shown that anemia due to ID does not only result from reduced erythropoiesis [336, 337] but is also the result of a shortened lifetime of the iron-deficient erythrocytes [338]. The decreased lifespan of these erythrocytes might be explained by enhanced eryptosis [69]. In the present study, however, patients without ID suffered from anemia and as well as enhanced eryptosis, which excludes the possibility that ID *per se* accounts for suicidal erythrocyte death in these patients.

The AHF patients in this study also had significantly higher creatinine values, which indicate worsened renal function. Patients on dialysis in end stage renal disease show enhanced suicidal erythrocyte death [68]. However, none of the patients enrolled in this study were taking erythropoietin or were on dialysis.

Suicidal erythrocyte death is increased in several clinical conditions that are commonly associated with patients suffering from chronic and acute heart failure such as diabetes [339, 340], metabolic syndrome [341] and hepatic failure [342]. Thus, eryptosis in AHF is most probably multifactorial.

Eryptosis is also triggered by a wide variety of xenobiotics [66]. Taking into account that these xenobiotics could worsen the enhanced suicidal erythrocyte death and the anemia in the AHF patients, their use should be avoided in the treatment of cardiac failure.

No study has hitherto demonstrated that the use of ESAs in the treatment of anemia in acute heart failure improves survival rate [343]. Furthermore, the use of ESAs is associated with severe adverse effects and studies report the occurrence of thromboembolic complications after treatment of AHF by the administration of ESAs [344]. However, heart failure *per se* is associated with thrombosis [345] and it is recommended that oral anticoagulation should be routinely used [346]. As phosphatidylserine-exposing erythrocytes adhere to the vascular wall [72] and trigger blood clotting [79], the thromboembolic events with and without administration of erythropoietin in AHF might be, at least partially, explained by enhanced eryptosis.

The enhanced eryptosis in AHF may be a target for therapy. Because the increased suicidal erythrocyte death in these patients seems to be mainly triggered by increased levels of reactive oxygen species, antioxidants could be used in the treatment of these patients. Several antioxidants have been shown to counteract eryptosis in *in vitro* studies [331, 347, 348]. In the animal model, the antioxidant thymol, has indeed been shown to enhance hematocrit levels and thus counteracts anemia in the rodents [335].

The present study shows that patients suffering from AHF suffer as well from anemia, which could, at least partly, be attributed to enhanced suicidal erythrocyte death. As anemia in those patients is associated with a worse prognosis [189], treatment of the anemia by inhibition of suicidal erythrocyte death could be beneficial for these patients [335] and may decrease the enhanced morbidity and mortality associated with anemia in AHF [189]. It is suspected that an underlying anemia may even be the primary cause for the genesis of HF [180]. As eryptosis contributes to anemia, suicidal erythrocyte death might even account for the development of HF and its inhibition gains even more importance in the treatment of anemia.

8.2 Eryptosis in mice

8.2.1 Role of Gαi2 in eryptosis

The present study reveals the expression of Gαi2 in both murine and human erythrocytes and further discloses that Gαi2 deficiency is associated with a partial protection against eryptosis. The present data show that the percentage of phosphatidylserine exposing cells in the blood stream is significantly lower in *Gαi2*$^{-/-}$ mice as compared to their wild type littermates, *Gαi2*$^{+/+}$ mice. Although the difference between the percentage of annexin V binding cells in *Gαi2*$^{+/+}$ and *Gαi2*$^{-/-}$ mice is subtle, it is yet statistically significant. In previous studies it was shown that spontaneous PS-exposure does not exceed 1% of the total number of erythrocytes circulating in the blood stream [31]. Furthermore, no changes in the eryptosis-related blood parameters e.g. erythrocyte count, reticulocyte number, hemoglobin and hematocrit level, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and plasma erythropoietin level were detectable. The mean corpuscular volume was, however, significantly higher in *Gαi2*$^{-/-}$ mice. The impact of Gαi2 deficiency is unveiled in the presence of cell stressors such as C6 ceramide, bacterial sphingomyelinase or hyperosmolar shock. Under these stress conditions *ex vivo*, suicidal erythrocyte death is significantly less pronounced in *Gαi2*$^{-/-}$ erythrocytes as compared to *Gαi2*$^{+/+}$ erythrocytes.

C6 ceramide and sphingomyelinase are strong stimulators of eryptosis [55]. In erythrocytes, ceramide is produced by the breakdown of sphingomyelin, a process catalyzed by the enzyme sphingomyelinase [55]. In this study, the effect of C6 ceramide and sphingomyelinase on eryptosis is attenuated in *Gαi2*$^{-/-}$ mice as compared to *Gαi2*$^{+/+}$ mice. Ceramide sensitizes red blood cells to the eryptotic effect of enhanced intracellular Ca^{2+} concentration [55] and may be enhanced without appreciable upregulation of cytosolic Ca^{2+} activity [349]. Furthermore, ceramide modifies the interaction of the erythrocyte membrane with the cytoskeleton, thereby increasing membrane fragility [350]. In accordance to the blunted effect of C6 ceramide and sphingomyelinase in *Gαi2*$^{-/-}$ mice, the eryptosis was significantly less pronounced in *Gαi2*$^{-/-}$ following exposure to hyperosmotic stress, another trigger of eryptosis that leads to the formation of ceramide [55]. Exposure of erythrocytes to hypertonic extracellular environment *in vitro* simulates the

osmotic conditions encountered in the kidney medulla [55]. In conditions such as acute renal failure, erythrocytes may enter eryptosis due to their entrapment in the kidney medulla. Gαi2 deficiency may blunt eryptosis and thus favorably influence the respective clinical condition. The observations lead to the conclusion that Gαi2 may additionally mediate hyperosmotic shock-induced eryptosis by influencing ceramide signaling. In addition to diminishing the exposure of phosphatidylserine, the present data show that *Gαi2*$^{-/-}$ erythrocytes showed enhanced resistance to cell shrinkage after exposure to hyperosmotic shock. Accordingly, the mean corpuscular cell volume was significantly larger in *Gαi2*$^{-/-}$ erythrocytes. Along these lines, it is intriguing to speculate that Gαi2 influences cell volume regulatory ion channels in erythrocytes. Furthermore it was observed that following hyperosmotic shock of erythrocytes, Gαi2 deficiency fosters a slight but significant decrease of cytosolic Ca^{2+} entry, which, in turn, diminishes the exposure of phosphatidylserine. Gαi2 is an essential regulator of Ca^{2+} signaling in nucleated cells and, thus, it is possible that the inhibitory effect of Gαi2 deficiency on erythrocyte death is at least partially mediated by its influence on cytosolic Ca^{2+} activity.

Suicidal erythrocyte death is also inhibited by several catecholamines including dopamine [351]. Particularly interesting is the observation that the dopamine-dependent signaling involves pertussis toxin-sensitive Gαi2 [352]. In BHK cells, it was previously shown that Gαi2 induces caspase-3 activation and hence stimulates apoptosis [259]. Caspase-3 activation is involved in the suicidal erythrocyte death that is induced by certain stimuli [28]. Whether caspase-3 mediated eryptosis signaling in erythrocytes is influenced by Gαi2, however, remains to be elucidated. As eryptosis is stimulated by several xenobiotics [66] and diseases [70], *Gαi2*$^{-/-}$ mice could be resistant to several of these triggers and impairment of the microcirculation could be significantly less pronounced in these mice. Accordingly, pharmacological targeting of Gαi2 may further provide a new possibility in the treatment of conditions which are associated with anemia resulting from increased eryptosis [31]. Additionally, the modulation of Gαi2 may serve as a novel target for the treatment of malaria [353].

In conclusion, Gαi2 is expressed in both murine and human erythrocytes and participates in the regulation of erythrocyte survival.

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11 LIST OF PUBLICATIONS

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