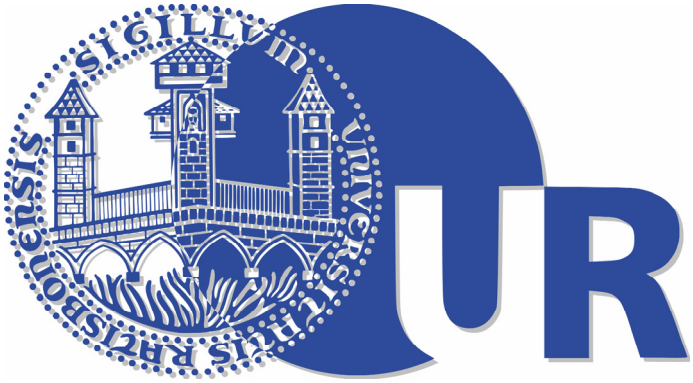
Lipidomic analysis of circulating human blood cells



Dissertation

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Table of contents

Index of figures

Index of tables

List of abbreviations

Apo	apolipoprotein
CE	cholesteryl ester
Cer	ceramide
DihSM	dihydrosphingomyelin
ESI-MS/MS	electrospray ionization tandem mass spectrometry
FA	fatty acid
FC	free cholesterol
GC	gas chromatography
GlcCer	glycosylceramide
LASS	longevity assurance homolog
LacCer	lactosylceramide
LCAT	lecithin-cholesterol-acyltransferase
LC-MS/MS	liquid chromatography tandem mass spectrometry
LPC	lysophosphatidylcholine
MS	mass spectrometry
OCS	open canalicular system
PBMC	peripheral blood mononuclear cells
PC	phosphatidylcholine
PC O	ether-phosphatidylcholine
PE	phosphatidylethanolamine
PE-pl	PE based plasmalogen
PG	phosphatidylglycerol
PI	phosphatidylinositol
PRP	platelet rich plasma
PS	phosphatidylserine
RBC	red blood cells
S1P	sphingosine-1-phosphate
SA1P	sphinganine-1-phosphate
SM	sphingomyelin
SOAT1	sterol O-acyltransferase
SPA	sphinganine
SPC	sphingosylphosphorylcholine
SPH	sphingosine

1. Introduction

1.1 Lipidomics

The ‘omics’ revolution has stimulated the concept of molecular profiling in biological systems. Although having been an intensive area of research already in the 1960s, lipid research has recently gained prominence with the emergence of lipidomics (1). Lipidomics can be defined as the system-level analysis and characterization of lipids and their interaction partners (2;3). A lipidome is the comprehensive and quantitative description of a set of lipid species e.g. constituting a cell. Lipidomics can be viewed as a sub-discipline of metabolomics (Figure 1). Furthermore, lipidomics can be subdivided into membrane, organelle, metabolism and mediator lipidomics (4) which address either the comprehensive and quantitative description of membrane lipid constituents, or the structural characterization and quantification of low abundant bioactive lipid species, respectively.

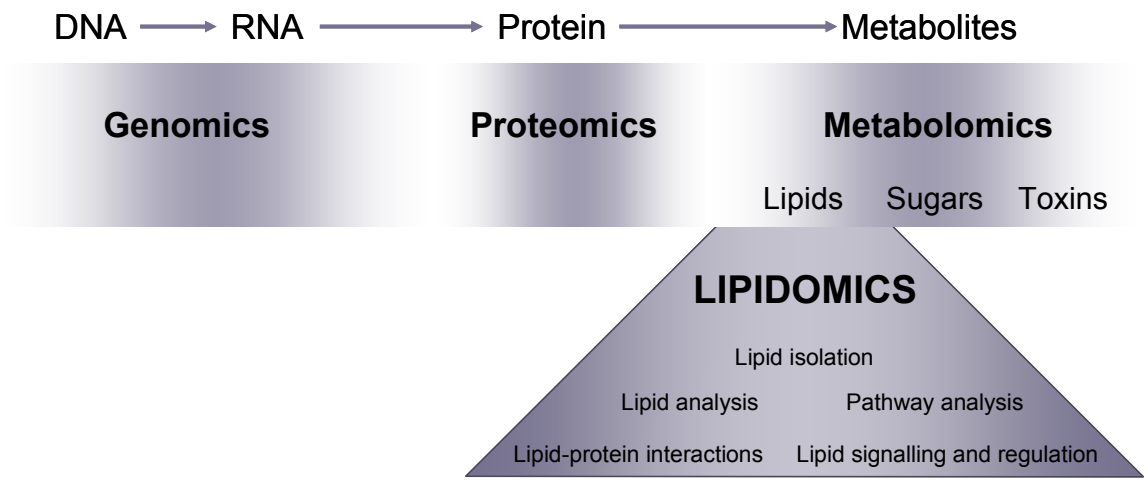


Figure 1: Lipidomics – system level scale analysis of lipids and their interaction partners
Genes encode proteins that collectively together with environmental factors, lead to the metabolite inventory of a cell, tissue or body fluid. Novel approaches now allow for qualitative and quantitative measurements at each level on global scales (genomics, proteomics and metabolomics). Lipidomics is a subgroup within the field of metabolomics (3).

1.1.1 Lipidomic development and goals

Over the past 30 years basic cell biology has been a major driver of lipid research not only with respect to cholesterol homeostasis. Elegant experiments in model organisms including yeasts, worms, flies and mice have provided deep insight into lipid metabolism in living organisms. Using modern genetic approaches in combination with functional assays, these studies addressed the roles of many lipid enzymes, including lipases, kinases, phosphatases and transferases. Common to these studies is their focus on mechanistic aspects which is necessary to understand the biology of lipids at the molecular level.

The major goal in lipidomics is the identification of metabolic pathways which are activated or deactivated during development of an organism or when a cell is shifted from an established physiological condition to another physiological or pathological condition (metabolic learning). A better understanding of the regulation of underlying metabolic pathways is necessary to design novel strategies for intervention (3;5). Lipids in biomembranes and others which are circulating as signaling molecules in our blood reflect physiological states during a given point in time. Side-by-side analysis of lipid and protein levels (overlay of lipidomics with proteomics) will help us understand better the molecular significance of the temporal relationships of metabolite and enzyme/effector fluctuations, and the careful analysis of lipids is likely to yield more refined and defined biomarkers (3;6).

1.1.2 Classification and role of lipids

For the future studies ranging from an overview of the entire lipidome to precise quantitative determination of rare bioactive lipid species will be necessary to reveal distinct lipid changes. To comprehend the entity of the whole lipidome several groups

of the lipidome have to be studied (Figure 2) (7). The inherent chemical diversity present in biological samples is achieved through multiple discrete covalent assemblies of a lipid backbone which is typically glycerol with linear combinations of various aliphatic chains alone (cholesteryl esters or triacylglycerols) or in conjunction with a high variety of polar head groups like spingolipids or phospholipids (8). This biological diversity facilitates the specific tailoring of cellular responses to alterations in cellular nutrient status, metabolic history, and signaling events.

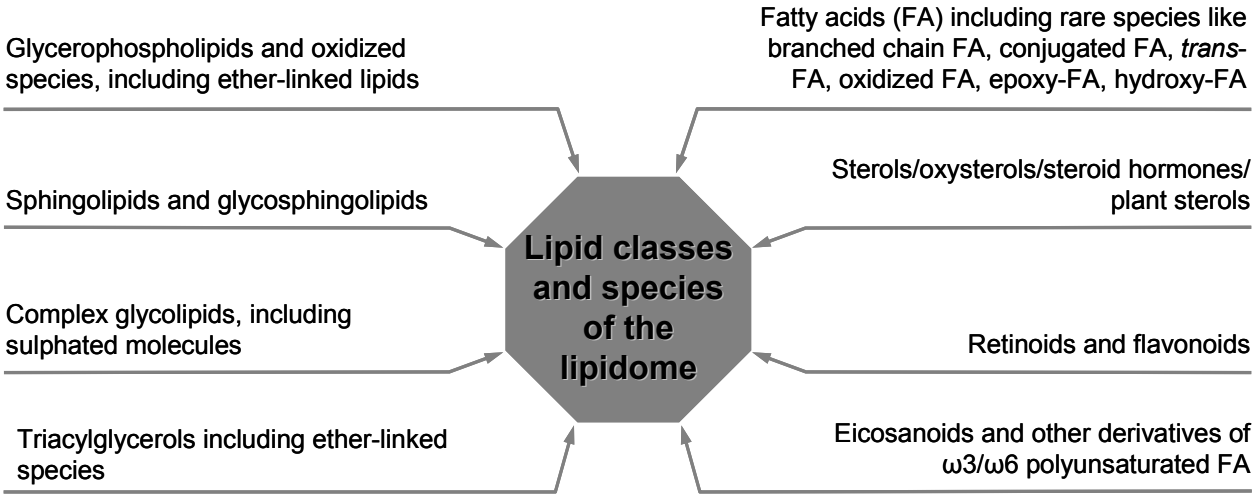
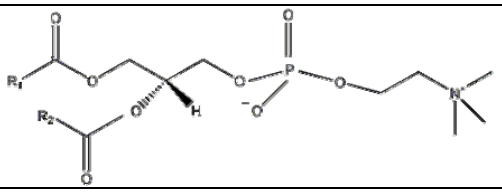
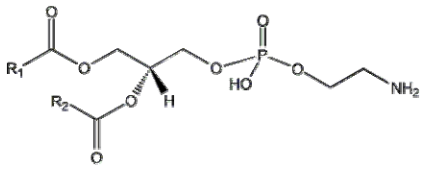
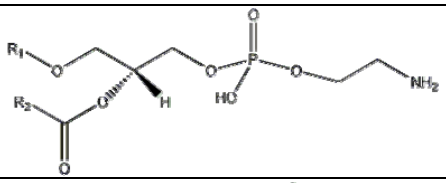
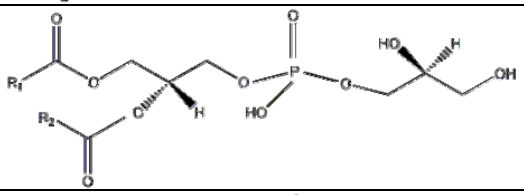
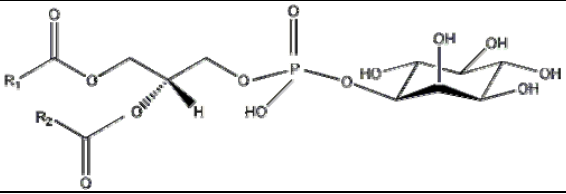
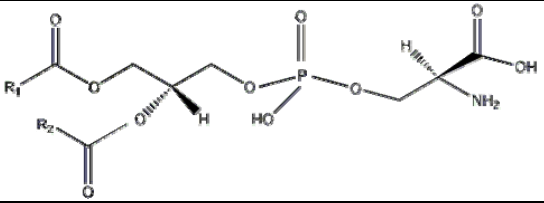
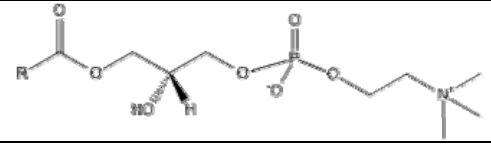


Figure 2: Lipid groups of the lipidome

Pertinent to this thesis work are the three main classes of membrane lipids: glycerophospholipids, sphingolipids and sterols.

Glycerophospholipids are the major components of biological membranes (9) and have inherent biological activities by acting as second messengers themselves or as precursors for the generation of second messengers. The principal feature of glycerophospholipids is the presence of a polar head group attached to the end of the glycerol backbone via a phosphodiester bond (10). They are chiral molecules divided into subclasses according to different head groups (Table 1).

Table 1: Chemical structures of glycerophospholipids and lyso-glycerosphospholipids

Name	Short name	Chemical structure
Phosphatidylcholine	PC	
Phosphatidylethanolamine	PE	
PE based plasmalogens	PE-pl	
Phosphatidylglycerol	PG	
Phosphatidylinositol	PI	
Phosphatidylserine	PS	
Lysophosphatidylcholine	LPC	

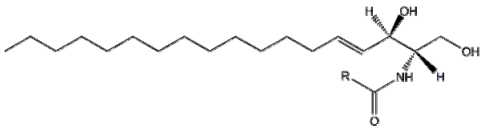
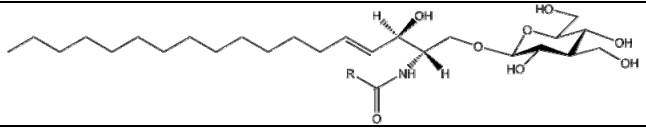
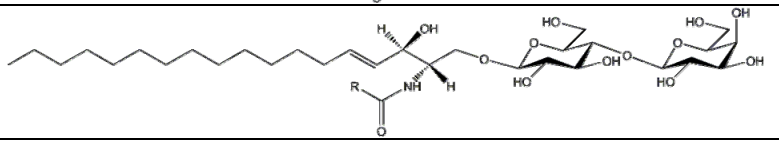
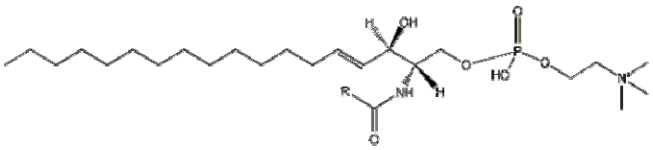
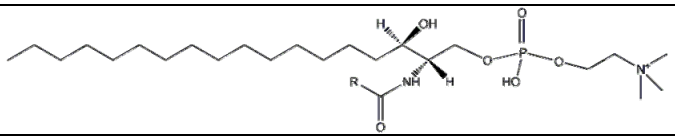
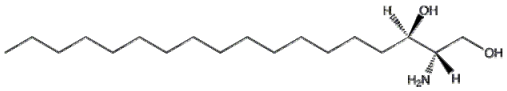
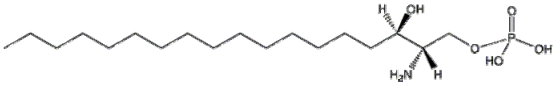
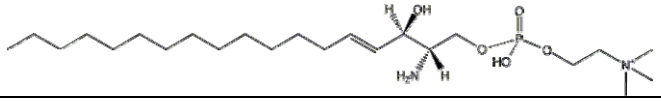
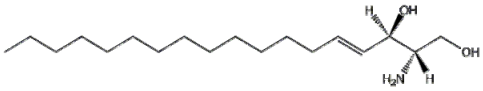
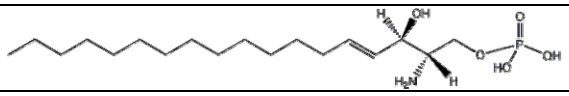
Various fatty acids (FA) are linked to the glycerol backbone at *sn*-1 and *sn*-2 positions via either two acyl linkages or one acyl and one alkyl linkage (10). Glycerophospholipids with an ether linked fatty alcohol moiety at the *sn-1* position are subdivided into plasmalogen species (11). In mammalians, ether linkages occur predominantly in phosphatidylcholine (PC) and phosphatidylethanolamine (PE). For this study only PE-based plasmalogens were measured. Lysophospholipids have

only one FA moiety attached to the glycerol phosphate backbone. Their role as intracellular signaling molecules beside the function of membrane phospholipid metabolites is increasingly appreciated (12). They regulate a wide variety of cellular activities including proliferation, wound healing, smooth muscle contraction and tumor cell invasiveness (13). The cellular glycerophospholipids are compartmentalized and different compartments possess markedly different glycerophospholipid species which undergo differential transfer and turnover (14).

Sphingolipids which are found in many living organisms (from yeast, plants, to humans) and in virtually all cell types, comprise more than 300 species (15); and this does not include the structural heterogeneity of the ceramide backbone. Besides playing structural roles in cellular membranes, sphingolipid metabolites act as bioactive signaling molecules involved in the regulation of cell growth, differentiation, senescence, and apoptosis (16). Bioactive sphingolipids are induced by several agonists, and, in turn, they regulate several downstream targets that mediate their various effects on cell function (16). Sphingolipids have also been reported to form in a dynamic cluster with sterols membrane microdomains which function as hubs for effective signal transduction and protein sorting (17). The common structural features of all sphingolipids are sphingoid bases (also termed long chain bases) composed of a hydrophobic moiety and a hydrophilic head group. Sphingolipids can be divided into two groups. The first group is termed sphingophospholipids and the second one glycosphingolipids which comprise of lacto-, globo- and gangliosides (15). The relevant sphingolipids for this study are shown in Table 2. It should be noted that the cellular levels of the various bioactive sphingolipids exhibit great differences. Concentrations of ceramide (Cer), sphingosine (Sph), and sphingosine-1-phosphate

(S1P) differ approximately by an order of magnitude, with Cer presenting the highest and S1P the lowest level (16).

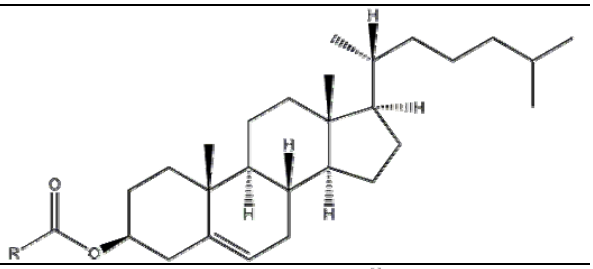
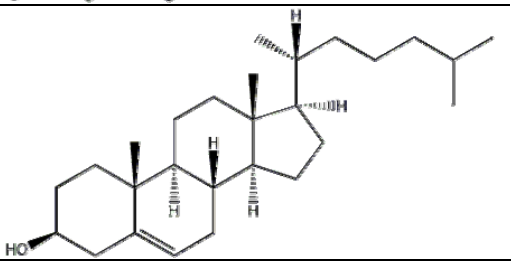
Table 2: Chemical structures of common sphingolipids

Name	Short name	Chemical structure
Ceramide	Cer	
Glycosylceramide	GlcCer	
Lactosylceramide	LacCer	
Sphingomyelin	SM	
Dihydrosphingomyelin	DihSM	
Sphinganine	SPA	
Sphinganine-1-phosphate	SPA1P	
Sphingosylphosphorylcholine	SPC	
Sphingosine	SPH	
Sphingosine-1-phosphate	S1P	

Sterols like cholesterol and its derivates are the most widely studied lipids for decades (18). Sterols are an important component of membrane lipids and a determinant of membrane organization (18). Mammals have developed sophisticated mechanisms to keep cellular sterol levels precisely controlled by regulation of

synthesis, influx and efflux (19;20). This structurally complex lipid class is implicated in numerous physiological functions including digestive processes (bile acids), hormonal actions (steroid hormones), tissue development (21) and regulation of cell membrane permeability (22). The major sterol cholesterol is mainly found in its “free” form or esterified to FA acyls (cholesteryl esters) (Table 3). Further prominent members of sterol lipids are bile salts and secosteroids (Vitamins D2, D3 and derivatives) (23) which were not under focus for this study.

Table 3: Chemical structures of common sterol lipids

Name	Short name	Chemical structure
Cholesteryl ester	CE	
Free cholesterol	FC	

The enormous structural diversity of chemically distinct molecular lipid species arises from various combinations of different FA and functional headgroups linked with backbone structures (24). A large number of studies indicated that the number of different FA species found in mammalian lipids is typically in the order of 30–60 (3). It was estimated that the number of possible molecular lipids in a given biological system is in the order of 200,000 (1). However, many of these theoretically possible species do not exist in nature or exist in very low abundance. In crude tissue extracts

approximately ~100 molecular species are observed experimentally in a given class of glycerophospholipids (3).

Additionally, lipids undergo extensive enzymatic as well as non-enzymatic modifications, which change their properties and functions. General enzyme classes are indicated in Figure 3. Many of these enzymes are tightly linked to signaling and implicated in a wide variety of processes including membrane trafficking, cell activation and many others (25).

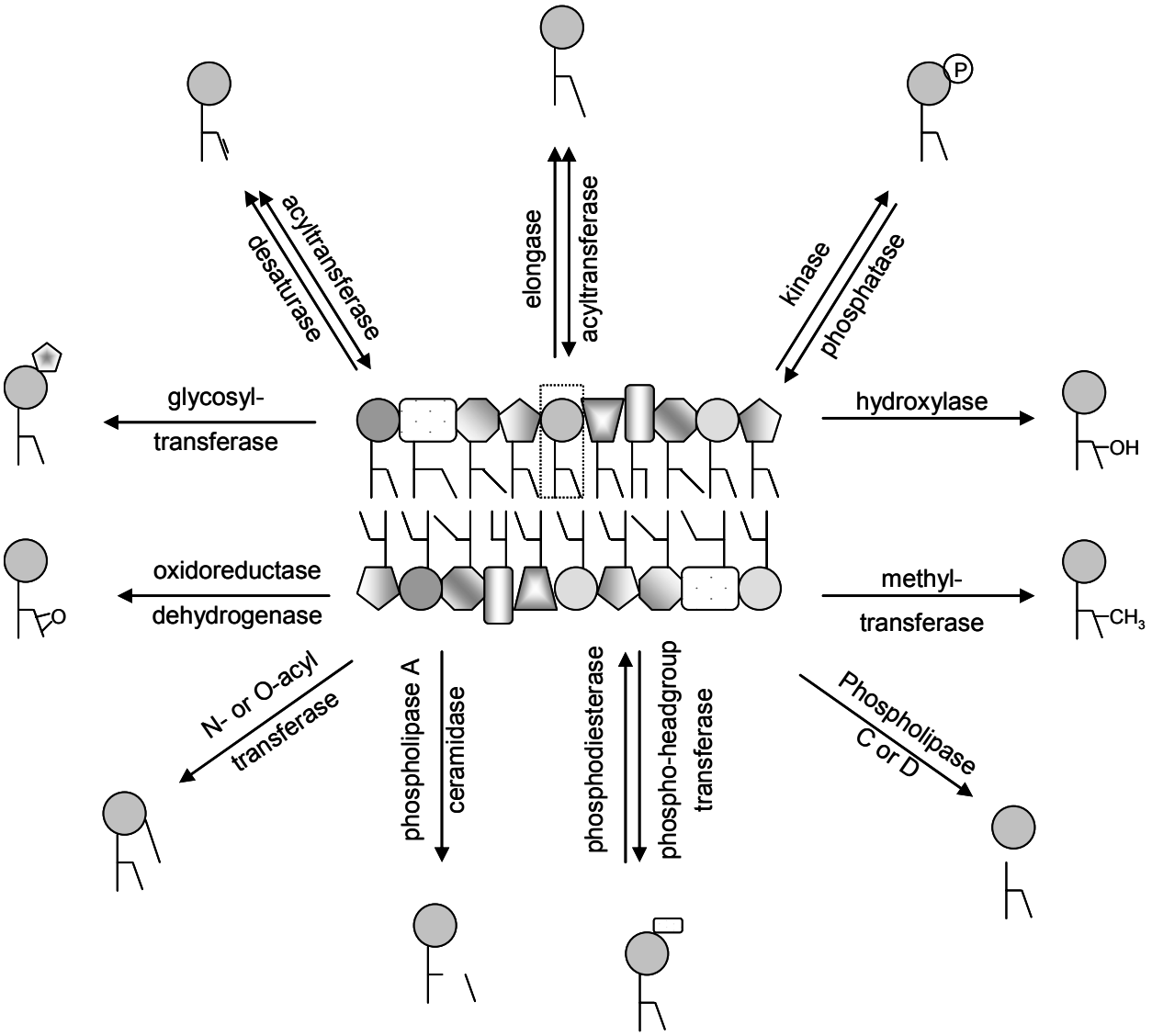


Figure 3: Enzymatic modifications of lipids

The crucial role of lipids in cell, tissue and organ physiology is evident by

- their unique membrane organizing properties that provide cells with functionally distinct subcellular membrane compartments and organelles (e.g. plasma membrane, endoplasmatic reticulum, Golgi apparatus, secretory vesicles, etc.),
- their functional role in cell signaling (e.g. ceramide and sphingosine-1-phosphate in cellular events including growth arrest, differentiation, and apoptosis),
- their essential role for energy production, metabolism and storage,
- and their endocrine actions (e.g. steroid hormones) (26).

The critical role of lipids in cell, tissue and organ physiology is already demonstrated by many human diseases involving the disruption of lipid metabolic enzymes and pathways. Examples of such diseases include diabetes, cancer, neurodegenerative disorders and infectious diseases (3;27).

1.2 Enabling technologies for the emerging field of lipidomics

So far, the explosion of information in the fields of genomics and proteomics has not been matched by a corresponding advancement of knowledge in the field of lipids. This was largely due to the complexity of lipids because nature is synthesizing an enormous number of chemically and structurally distinct lipid species. Another reason was the lack of powerful tools for lipid analysis. Novel analytical approaches now allow for systems level analysis of lipids and their interacting partners. Two core technologies have emerged as the workhorses for lipid analysis: nuclear magnetic resonance (NMR) spectroscopy and tandem mass spectrometry (MS/MS) (28). NMR spectroscopy, which is used almost exclusively for analyzing small biochemicals in

the blood, requires relatively little sample preparation and is non-destructive, allowing further analysis. However, the method tends to have low sensitivity and can detect only highly abundant analytes. Lipid analysis is traditionally performed by gas chromatography–mass spectrometry (GC–MS), however, the recent development of electrospray ionization (ESI) (29) and matrix-assisted laser desorption/ionization (MALDI) (30) has significantly expanded the range of lipids that can be analyzed by MS in unfractionated materials (shotgun lipidomics). The coupling of ESI-MS to liquid chromatography (LC) has greatly increased the number of lipid classes studied in a single experiment. The Nobel Prize for John B. Fenn in chemistry 2002 also emphasized the importance of mass spectrometry for developments in this area.

An overview of the different technologies used in lipidomic research is presented in Table 4 and was already nicely reviewed by Wenk (3). Furthermore, instruments capable of performing MS/MS provide the detailed structural information necessary for characterization of novel lipids, not previously possible, and the selectivity required for the determination of individual lipid species (28). MS/MS allows to determine analyte masses with such high precision and accuracy that lipids can be identified unambiguously in complex mixtures, therefore applicable to a wide range of biological samples (31). The developments for detection of phospholipid species in different cell types by high sensitivity and high throughput methods have especially contributed a lot to novel findings in lipidomics (32-35). The area of mass spectrometric quantitative analysis continues to emerge as an important application for biology and medicine because new lipid molecules continue to be discovered and some have profound action on cellular biochemistry (36). Therefore it can be stated that the success of lipidomics is primarily a result of technological advances in MS. The advantage of lipid MS is that it combines high-throughput analysis with high

sensitivity and resolution together with the ability of automated sample and data handling (37).

Table 4: Technologies for lipidomic research (3)

Technology	Studied lipids	Advantages	Disadvantages
Mass spectrometry			
MALDI	many lipids	TLC combination possible, direct detection by *m/z*	Matrix backgrounds, ionization suppression
ESI	many lipids	LC combination possible, direct detection by *m/z*, high sensitivity and selectivity, high turnover	Absolute quantification involves significant effort, ionization suppression
NMR			
^{1}H	all lipids	Non-destructive, direct measurement	Low sensitivity, spectra dominated by very abundant lipids
^{31}P	phospholipids	Non-destructive, direct measurement, quantitative	Low sensitivity
Chromatography			
Gas chromatography	non-polar compounds	Detection by mass spectrometry, mainly used for FA	Requires derivatization of polar lipids or volatile compounds
High-performance liquid chromatography	many lipids	Quantitative, easy automation	Detection by mass detector or refractive index
Thin-layer chromatography	many lipids	Technically easy, minor instrumental investment	Low sensitivity and resolution drawbacks
Biochemistry			
Photoactivatable lipids	few	Identification of lipid-binding proteins	Limited number of probes, specificity
Lipid antibodies	very few	Study of cell biology	Antibody specificity
Immobilized lipids in assays	many lipids	Identification of ligand-lipid interactions	Automating and immobilization technically difficult
Soluble lipids in assays	many lipids	Quantitative binding studies, high turnover	Optimization challenging, technically difficult

However, the biggest challenge of lipidomics remains the elucidation of important pathobiological phenomena from the integration of the large amounts of new data becoming available. Computational and informatics approaches allow studying lipid molecular profiles in the context of known metabolic pathways and established

pathophysiological responses. Lately computational background is available for automated calculations, where each compound entry is linked to the available information on lipid pathways and contains the information that can be utilized for its automated identification from high-throughput based lipidomic techniques (38).

The new analytical tool for lipid detection combined with molecular imaging and modern information technology are able to revolutionize the understanding of the complex interaction networks in a functioning cell and how lipids together with genes and proteins determine cellular functions in health and disease.

1.3 Human blood cells

The entire blood volume recirculates throughout the body every minute, delivering oxygen and nutrients to every cell and transporting products from and towards all different tissues (39). The two main blood compounds are plasma and blood cells whereof the latter represents approximately 45% of the blood volume (40). Blood cells form a heterogenous mixture which continuously responds and adapts to the smallest changes in physiology. Consequently, blood cell analysis are of prime interest for both medical and science applications, and hold a central role in the diagnosis of many physiologic and pathologic conditions, localized or systemic (40).

1.3.1 Types of blood cells

Each type of blood cells is quite distinct in appearance and biological function (Figure 4) (41):

- **Granulocytes** and **monocytes** are myeloid cells which can exit from the blood compartment and migrate into many tissues and organs. Both cell types play a key role in innate immunity, inflammation and phagocytosis.

- **Lymphocytes** mediate specific immunity against microorganisms and other sources of foreign macromolecules. A distinction is drawn between B cells and T cells. B lymphocytes confer humoral immunity through the production of specific, membrane bound and soluble antibodies (humoral immune response), while T lymphocytes direct a large variety of immune functions, including killing of cells that bear foreign molecules on their surface membranes (cellular immune response).
- **Platelets** are anucleate, very small cells that contain molecules stored in specific granules that are required for haemostasis. These cells provide haemostasis through their ability to adhere, aggregate, and by offering a surface for coagulation reactions.
- **Red blood cells** (RBC) are anucleate, bioconcave discoid cells filled with haemoglobin, the major oxygen-binding protein. RBC transport beside oxygen also the respiratory gas carbon dioxide.

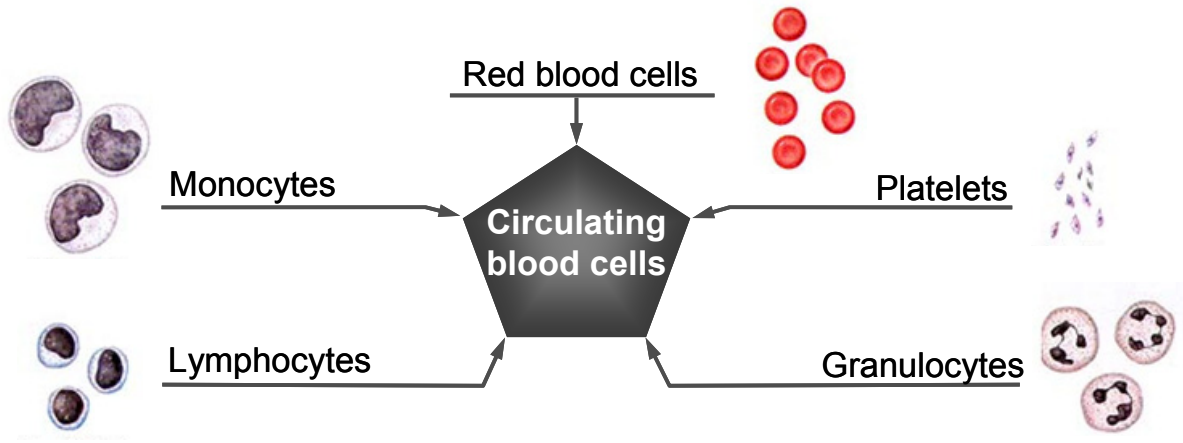


Figure 4: The five types of circulating human blood cells

Despite these extreme structural and functional differences among the blood cells, all of them share progeny of a single type of cell, the hematopoetic stem cell.

1.3.2 Origin and lifetime of blood cells

In the bone marrow the hematopoietic stem cells differentiate into myeloid or lymphoid precursor cells (42) (Figure 5). The granulocyte-monocyte progenitors and the megakaryocyte-erythrocyte progenitors derive from the common myeloid precursor cell (43). The common lymphoid precursor cell differentiates to lymphocytes, the megakaryocyte-erythrocyte progenitors to megakaryocytes and red blood cells and the granulocyte–monocyte progenitors to granulocytes and monocytes (44). Monocytes can further differentiate into dendritic cells or macrophages after recruitment into peripheral tissues (44).

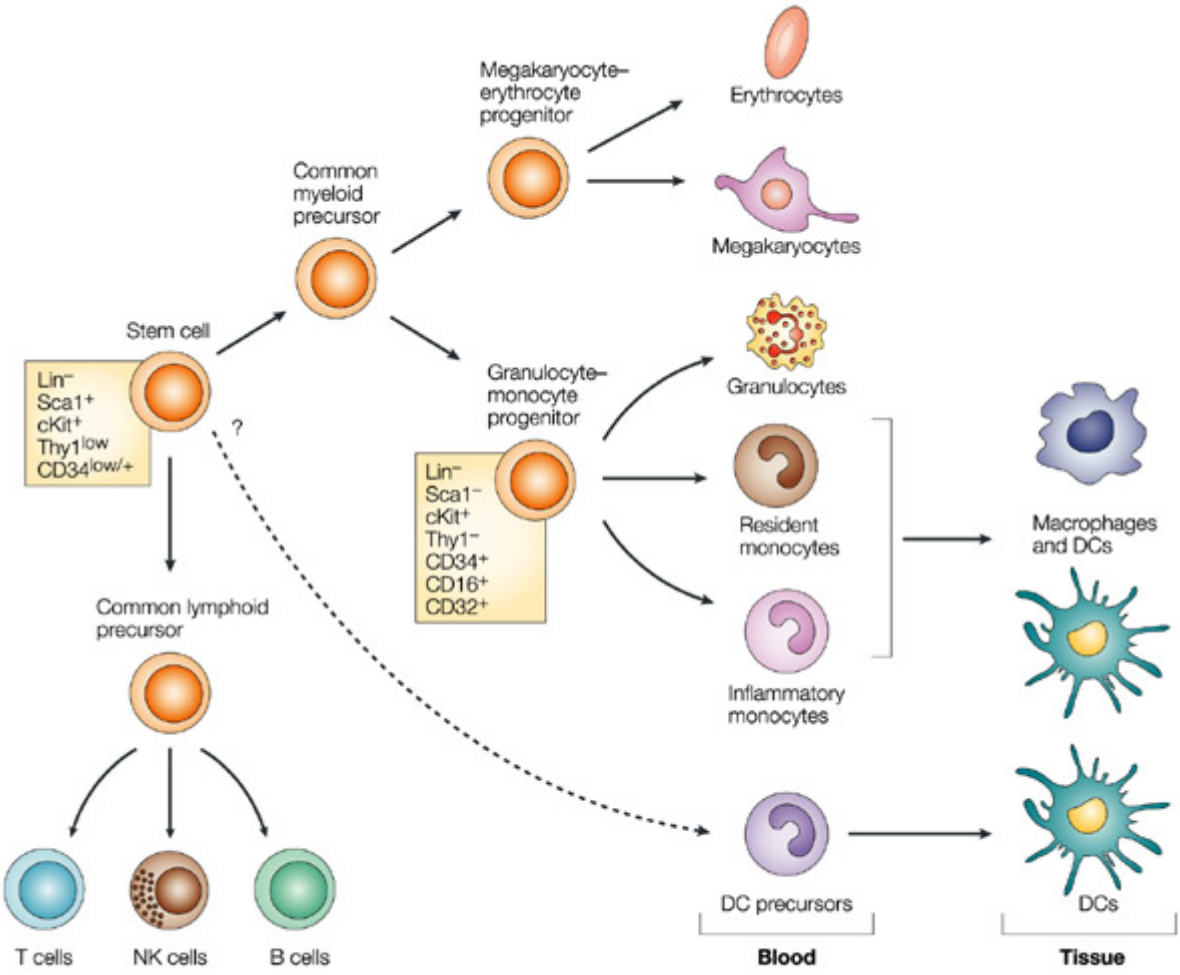


Figure 5: Origin and haematopoietic differentiation of myeloid antigen-presenting cells published by Imhof and Aurrand-Lions (44).

Abbreviations: DC: dendritic cell, NK: natural killer

The turnover and the replenishment of the hematopoietic system continues throughout life in contrast to organ systems that form in early life and are not continually replaced (41). In general the blood cell production is an enormously complex process. It can be calculated that each day an adult human produces $2x10^{11}$ erythrocytes, $1x10^{11}$ platelets and $1x10^{11}$ leukocytes based on the adult blood volume (five liters) and the circulatory half-life of each of the blood cell types (45). However, the numbers of the various blood cell types are normally kept in relatively constant ranges because the blood cells have finite lifespans and the system of production, differentiation and elimination is strongly controlled (Table 5). While granulocytes and platelets circulate only a few days, red blood cells can exist for several months and some lymphocytes even exist for years as memory cells (41;46).

Table 5: Absolute and relative number of cell populations and subpopulations and their lifetime in normal blood (adapted from (40))

Cell type	**Average/µL**	**Percent of leukocytes**	**Lifetime**
Granulocytes			~12 hours (47)
- Neutrophils	4000–8000	40%–66%	
- Eosinophils	50–300	1%–3%	
- Basophils	0–100	0%–1%	
Lymphocytes	1000–4000	20%–40%	weeks to years (48;49)
Monocytes	200–800	4%–8%	8-70 hours (48)
Platelets	200,000–500,000		7-10 days (47)
Red blood cells	5,000,000		~120 days (50)

1.3.3 Blood cell storage and transfusion

Worldwide, over 75 million units of blood are estimated to be donated every year (51). The ability to collect and store blood *ex vivo* in its liquid state is restricted to transfusion medicine institutions and blood banks (52).

The reasons for blood transfusions can be the following:

- massive blood loss due to trauma (53)
- replacement of blood loss during surgery (54)
- treatment of severe anaemia or thrombocytopenia caused by a blood cell disease (55)
- treatment of patients with hemophilia, sickle-cell disease or other hemorrhagic disorders (56)

Modern medical practice commonly uses only components of the blood. For this reason aphereses are performed where the blood of a donor or a patient passes through an apparatus which separates one particular constituent and returns the remainder back to the circulation. The separation of blood components is based on centrifugation by weight, filtration by size or a combination of both (57).

It can be differentiated between four categories of component collections obtained from healthy donors (58):

- Plasmapheresis: collection of blood plasma
- Leukapheresis: collection of white blood cells
- Plateletpheresis: collection of platelets
- Erythrocytapheresis: collection of red blood cells

Pertinent for this thesis were only two types of aphereses. The first one was leukapheresis which was utilized to obtain the different blood cell types (monocytes, lymphocytes, granulocytes and platelets) for subsequent lipidomic characterization.

The second one was plateletpheresis which was used in this work for subsequent lipidomic studies of platelet aging during storage.

The storage conditions for different apheresis products with focus on time spent between donation and transfusion, are displayed in Table 6.

Table 6: Storage conditions of apheresis products (59)

Apheresis category	Storage time	Storage temperature
Erythrocytapheresis	28-49 days	4°C±2°C
Plasmapheresis	36 months	-30°C±3°C
Leukapheresis	1 day	22°C±2°C
Plateletpheresis	4 days under agitation (60)	22°C±2°C

The storage of blood components in preservative medium is associated with metabolic, biochemical and molecular changes collectively referred as storage lesion (52). The storage lesion can eventually result in irreversible damage and reduced post-transfusion survival (52). Traditionally the term storage lesion has been restricted to corpuscular damage, but it was shown that a number of bioreactive substances accumulate in the medium during storage (52;61).

2. Aim of the thesis

The blood cell compartment can be regarded as a liquid organ and blood cells are easily accessible compared to solid organs and tissues. Therefore the study of the blood cell compartment is a major interest of medical research especially the study of lipids which provide additional information of cellular function beside genomic, transcriptomic and proteomic knowledge. To determine lipid species in blood cells with high sensitivity the recent developments in electrospray ionization tandem mass spectrometry (ESI-MS/MS) opened new analytical options. The major aim of this thesis was to analyze the detailed lipid pattern of human blood cells by MS and reveal their lipid-specific features in healthy donors and related blood products.

Firstly the different circulating human blood cells (monocytes, lymphocytes, granulocytes, platelets and red blood cells) of healthy donors were isolated by leukapheresis followed by counterflow centrifugation elutriation. Lipid extraction was performed according to Bligh and Dyer and the different lipid classes and species analyzed by ESI-MS/MS. Striking differences between the individual blood cell lipids were elaborated.

In a second extensive study the reasons for the specific lipid composition of a single blood cell type should be elucidated. The cells of interest were the platelets because they revealed an interesting cholesteryl ester profile observed in the analysis described above. Therefore 50 platelet apheresis concentrates were stored for five days at 22°C under agitation and the cells and the plasma were daily isolated by gel-filtration. The aim of this work was to study the influence of storage on the lipid pattern of platelet apheresis products and the effect of the plasma lipid environment on the cells.

3. Materials and Methods

3.1 Materials

3.1.1 Technical equipment

Apheresis machine Trima Accel system	CaridianBCT, Lakewood, CO, USA
Autoclave Steam Sterilizer Varioklav	Thermo Scientific, Waltham, MA, USA
Autosampler HTS and HTC PAL	CTC Analytics, Zwingen, Switzerland
Biofuge 15R	Heraeus, Hanau, Germany
Cell Separator Spectra	CardianBCT, Lakewood, CO, USA
Chromatography HILIC silica column	Interchim, Montlucan, France
Clinical Chemistry System ADVIA 1800	Siemens Healthcare Diagnostics GmbH, Bad Nauheim, Germany
FACS Canto	Becton Dickinson, Heidelberg, Germany
Hematology analyzer ADVIA 120	Siemens Healthcare Diagnostics GmbH, Bad Nauheim, Germany
Horizontal Shaker GFL-3016	GFL, Großburgwedel, Germany
Ice Machine Scotsman AF-100	Progen Scientifc, London, United Kingdom
Incubator B 6120	Heraeus, Hanau, Germany
Lumi Imager F1	Boehringer, Mannheim, Germany
Mass Spectrometer Quattro Ultima Triple Quadropole	Micromass, Manchester, UK
Mass Spectrometer API 4000 Q TRAP	Applied Biosystems, Darmstadt, Germany
Megafuge 1.0 R	Heraeus, Hanau, Germany
Microplate Reader Sunrise™	Tecan, Crailsheim, Germany
Mini Protean-3 Electrophoresis Cell	BioRad, Munich, Germany
MiniSpin Plus Centrifuge	Eppendorf, Hamburg, Germany
Mini-Sub Cell GT Electrophoresis	BioRad, Munich, Germany
Pipetting Robot Genesis RSP 150	Tecan, Maennedorf, Switzerland
pH-Meter pH537	WTW, Weilheim, Germany
Precision Balance Sartorius MD BA 200	Sartorius, Göttingen, Germany

Power Supply PAC 300	BioRad Laboratories, Munich, Germany
Pumps (binary) Agilent 1100 and 1200	Agilent, Waldbronn, Germany
Shaking Water Bath Julabo SW-20C	Julabo, Seelbach, Germany
Ultrasonic Water Bath Bandelin Sonorex	Progen Scientific, London, UK
Ultrasonic Disintegrator Soniprep 150 MSE	Beun de Ronde, Abcoude, The Netherlands
Ultracentrifuge Optima Max XP	Beckman Coulter, Krefeld, Germany
Ultracentrifuge Optima TLX	Beckman Coulter, Krefeld, Germany
Vacuum Concentrators Christ RVC	Wolf Laboratories Limited, York, UK
Vortex-Mixer REAX 2000	Heidolph, Kelheim, Germany
Water Purification System Milli-Q	Millipore, Eschborn, Germany

3.1.2 Consumables

Cell culture flasks (Nunc)	Thermo Fisher Scientific, Langenselbold, Germany
Columns PD-10	GE Healthcare, Freiburg, Germany
Cups (0.5, 1.5, 2.0ml)	Eppendorf, Hamburg, Germany
ECL Hyperfilm Amersham	GE Healthcare, Freiburg, Germany
ELISA 96 MicroWell plates	Nunc, Langenselbold, Germany
Falcon tubes (15, 50ml)	Sarstedt, Nümbrecht, Germany
Filter tips (2, 10, 20, 200, 1000µl)	Eppendorf, Hamburg, Germany
Latex gloves	Hartmann, Heidenheim, Germany
PVDF membranes	Pall Filtron GmbH, Dreieich, Germany
Sterile filter	PALL, USA
X-Ray films Biomax	Kodak, Rochester, NY, USA

3.1.3 Reagents

Acetyl chloride (puriss. p.a.)	Sigma, Taufkirchen, Germany
Adenosine diphosphate (ADP)	DiaAdin, DiaMed, Cressier, Switzerland
Ammonium acetate (SigmaUltra)	Sigma, Taufkirchen, Germany
Bovine serum albumin (BSA, lipid-free)	Sigma, Taufkirchen, Germany

Cholesterol and cholesteryl ester standards	Sigma, Taufkirchen, Germany
[25,26,26,26,27,27,27-D_7]-cholesterol	Cambridge Isotope Laboratories, Andover, USA
Chloroform (HPLC grade)	Merck, Darmstadt, Germany
Complete mini protease inhibitor cocktail	Roche, Mannheim, Germany
EDTA (di-sodium)	GE Healthcare, Freiburg, Germany
Glucose	Merck, Darmstadt, Germany
Hepes	Merck, Darmstadt, Germany
Lipid standards	Avanti Polar Lipids, Alabaster, USA Toronto Research, North York, Canada
Methanol (HPLC grade)	Merck, Darmstadt, Germany
NuPAGE® LDS 4x sample buffer	Invitrogen, Karlsruhe, Germany
NuPAGE® MOPS SDS running buffer	Invitrogen, Karlsruhe, Germany
Phosphate buffered saline (PBS) w/o Ca^{2+}/Mg^{2+}	Gibco BRL, Berlin, Germany
Protein standard precision plus (all blue)	BioRad Laboratories, Munich, Germany
Ready gels, 4-20%	Invitrogen, Karlsruhe, Germany
Sepharose 2B	Sigma, Taufkirchen, Germany
Sodium dodecylsulfate (SDS)	Roth, Karlsruhe, Germany
Thrombin receptor-activating peptide 6 (TRAP-6)	Bachem, Weil, Germany

All other chemicals and solvents were from Sigma, Taufkirchen, Germany

3.1.4 Antibodies

Western Blot:

ApoA-I (rabbit, polyclonal)	Calbiochem, Darmstadt, Germany
ApoA-IV (rabbit, polyclonal)	Gift of Prof. Dieplinger (Division of Genetic Epidemiology University of Innsbruck, Austria)
ApoC-I (clone 10F4) (mouse, monoclonal)	MP Biomedicals, Solon, USA
ApoE (goat, polyclonal)	Calbiochem, Darmstadt, Germany
CD36 (rabbit, polyclonal)	Acris Antibodies, Herford, Germany

Goat (rabbit, polyclonal)	Jackson Immuno Research (Hamburg, Germany)
Mouse (goat, polyclonal)	Jackson Immuno Research (Hamburg, Germany)
Rabbit (goat, polyclonal)	Jackson Immuno Research (Hamburg, Germany)
Transferrin (rabbit, polyclonal)	Acris Antibodies, Herford, Germany

Flow cytometry:

FITC-anti-CD62P	Beckmann Coulter, Krefeld, Germany
FITC-anti-CD63	Coulter-Immunotech, Marseilles, France
PE-anti-CD41	Beckmann Coulter, Krefeld, Germany
PE-anti-CD36 BD	Biosciences Pharmingen, Heidelberg, Germany
PerCP-anti-CD61	Becton Dickinson, Heidelberg, Germany

3.1.5 Kits

BCA assay kit	Uptima-Interchim, Montluçon, France
ECL plus western blotting analysis system	GE Healthcare, Freiburg, Germany

3.1.6 Analysis software

FACSDiva software	Becton Dickinson, Heidelberg, Germany
MassLynx software including the NeoLynx tool	Micromass, Manchester, UK
Microplate reader software Magellan™	Tecan, Crailsheim, Germany
SPSS 15.0 software for Windows	SPSS Inc., Chicago, USA

3.2 Methods

3.2.1 Blood cell isolation

3.2.1.1 Isolation of red blood cells (RBC)

RBC were isolated according to Breitling-Utzmann et al. (62) with slight modifications. Briefly, 4ml venous blood was collected from healthy donors into sterile EDTA tubes and blood cells were pelleted by centrifugation at 2700 *x g* for 30min at 4°C. Plasma and buffy coat were removed. The packed red cells were washed three times with PBS by subsequent centrifugation (2700 *x g* for 4min at 4°C). Erythrocyte cell number and purity was analyzed by an ADVIA 120 cell counter (Siemens Healthcare Diagnostics GmbH, Bad Nauheim, Germany). In parallel a blood smear was performed as control to evaluate the shape of erythrocytes. Only erythrocyte samples without fragmentocytes were used for further examination. After washing with PBS, the cell pellets were stored at -80°C.

3.2.1.2 Isolation of monocytes, lymphocytes, granulocytes and platelets from leukapheresis products

Blood cells were collected by leukapheresis in a Spectra cell separator (CardianBCT, Lakewood, CO, USA), followed by subsequent counterflow centrifugation elutriation as described elsewhere (63). In brief, cells were elutriated in the order platelets, lymphocytes, monocytes and granulocytes. Aliquots of the different cell fractions were analyzed for cell purity with a BD FACSCanto (Becton Dickinson, Heidelberg, Germany) determining the cell size with linear forward scatter and granularity and cell shape with logarithmic sideward scatter format of the BD FACSDiva Software. The cell numbers were determined by an ADVIA 120 cell counter (Siemens Healthcare

Diagnostics GmbH, Bad Nauheim, Germany). After washing with PBS, the cell pellets were stored at -80°C.

3.2.1.3 Aging and isolation of platelets and plasma from platelet apheresis products

Platelet concentrates were obtained after platelet apheresis on an Amicus (Fenwal, Mont Saint Guibert, Belgium) or a Trima Accel system (CaridianBCT, Lakewood, CO, USA). Any additive solution except acid citrate dextrose solution A (ACDA) was added to the platelet concentrates. After collection platelet concentrates were stored for five days at 22°C under continuous horizontal agitation. The platelet concentrates were opened under sterile conditions and approximately 5ml of platelet rich plasma (PRP) was removed each day from day 0 (day of collection) until day five. After removal of PRP from platelet apheresis products they were sealed under sterile conditions and were kept for further 24h at 22°C under continuous shaking. Platelets were separated from plasma components using gel-filtration. The platelet concentrates were opened under sterile conditions and 2ml of PRP layered onto a column (column volume: 13.5ml) (GE Healthcare, Freiburg, Germany) filled with Sepharose 2B in calcium-free Tyrode-Hepes buffer (138mM NaCl, 3mM KCl, 12mM $NaHCO_3$, 0.4mM NaH_2PO_4, 1mM $MgCl_2$, 5mM glucose, 10mM Hepes, 10mM EDTA, 0.5% (w/v) BSA), pH 7.4, sterile-filtrated. Dead volume of 2.5ml was discarded and fractions 3-5 (elution volume: 2.5-5.0ml) collected and pooled together (Figure 6). The cell number of the separated platelets was quantified by the ADVIA 120 hematology analyzer (Siemens Healthcare Diagnostics GmbH, Bad Nauheim, Germany). Platelets were disrupted by sonication on ice (Soniprep 150, Beun de Ronde, Abcoude, The Netherlands). To obtain plasma, 2ml of PRP were centrifuged

sequentially twice at 1500 *x g* for 10min and the supernatant harvested. Platelets and plasma were stored at -80°C before lipid extraction.

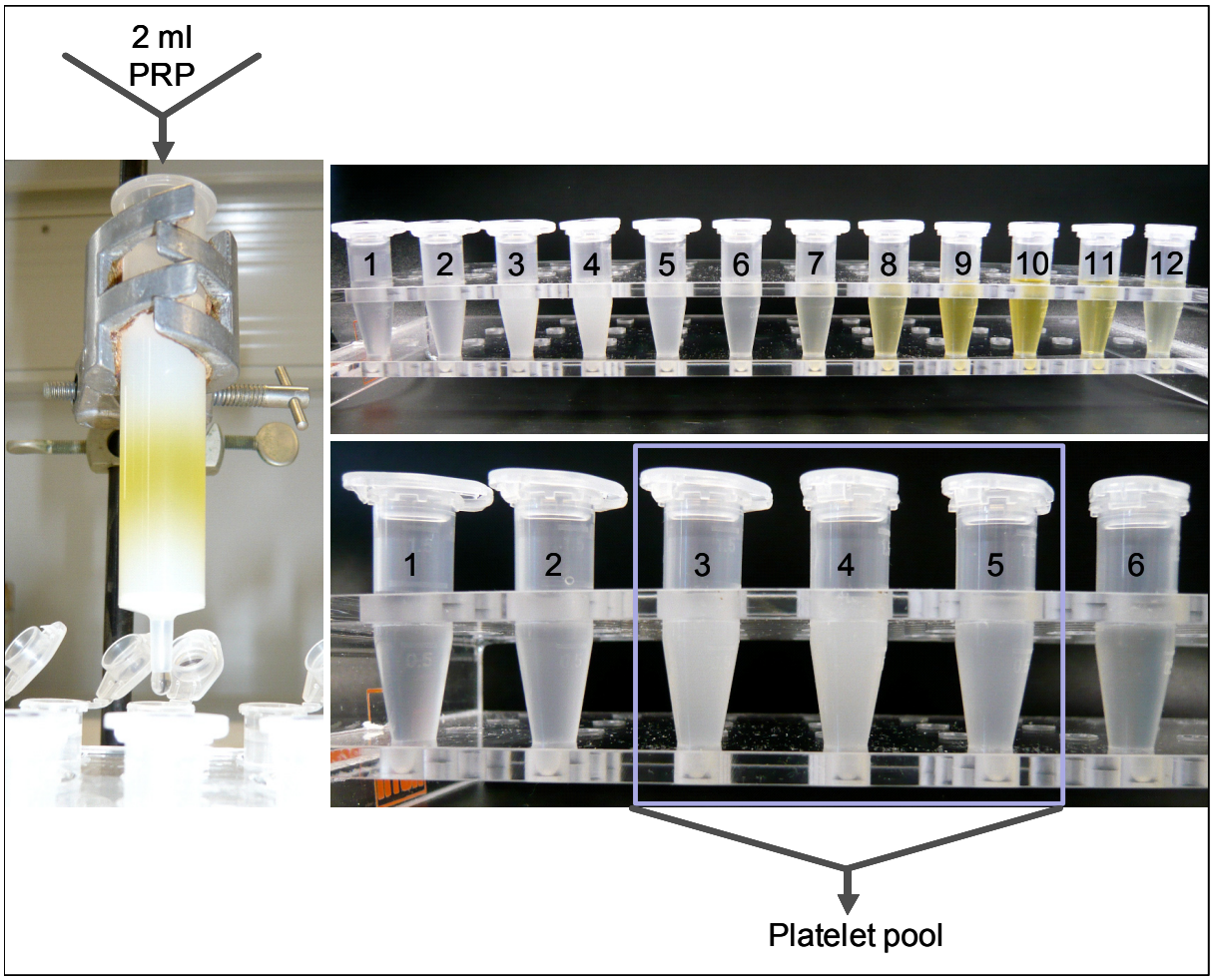


Figure 6: Platelet separation by gel-filtration procedure
Two millilitres of platelet rich plasma (PRP) was layered onto a column and the platelet fractions 3-5 were collected and pooled for analysis while fraction 6 was discarded. The rising plasma content in the fractions was nicely visible by stronger yellowish colouring.

3.2.2 Flow cytometry

Platelet activation was examined by anti-CD62P, -CD63, -CD41, -CD36 and -CD61 antibody expression analysis before and after gel-filtration isolation in a BD FACSCanto (Becton Dickinson, Heidelberg, Germany) flow cytometer equipped with BD FACSDiva Software as previously described (64). Briefly 10µl of platelet samples

(10x dilution) were incubated for 10min with 10µl of PBS acting as control, 30µM thrombin receptor-activating peptide 6 (TRAP-6) (Bachem, Weil, Germany) and 5µM adenosine diphosphate (ADP) (DiaAdin, DiaMed, Cressier, Switzerland), respectively. After activation 10µl of the appropriate platelet antibody was added and incubated for 5min before resuspension in PBS and subsequent flow cytometric analysis. For each measurement data from 5,000 platelets were acquired.

3.2.3 Proteomics

3.2.3.1 Protein determination

Protein concentrations were measured according to Smith et al. (65) using BCA Assay of Uptima-Interchim (Montluçon, France) with bovine serum albumin as standard. The haemoglobin content of the RBC was quantified by the ADVIA 120 system (Siemens Healthcare Diagnostics GmbH, Bad Nauheim, Germany).

3.2.3.2 SDS-PAGE

45µl of each fraction gained by gel-filtration separation were mixed with 15µl NuPAGE® LDS 4x sample buffer (Invitrogen, Karlsruhe, Germany) and incubated for 10min at 70°C in the presence of 50 mM DTT. Samples were run on 4–12% Bis-Tris gels (Ready Gels, Invitrogen, Karlsruhe, Germany) with NuPAGE® MOPS SDS running buffer (Invitrogen, Karlsruhe, Germany) at 200V per gel. Proteins separated within the gels were subsequently transferred to a PVDF membrane. Incubation with antibodies against CD36, transferrin, ApoA-I, ApoA-IV, ApoC-I or ApoE was performed in 1% non-fat dry milk in PBS and 0.1% Tween-20. The immune complexes were detected with an ECL plus Western blot detection system (GE Healthcare, Freiburg, Germany).

3.2.4 Lipidomics

3.2.4.1 Sample preparation for mass spectrometry analysis

Cell pellets were dissolved in deionised water (Millipore, Eschborn, Germany) and disrupted by sonication on ice (Soniprep 150, Beun de Ronde, Abcoude, The Netherlands). For lipidomic comparison of blood cells an aliquot corresponding to 100µg of cellular protein was used for mass spectrometric lipid analysis. For the later platelet senescence study 100µl of the pooled platelet fractions 3-5 were used to analyze the different sphingolipids. For glycerophospholipid and sterol measurements an aliquot of 300µl of the pooled platelet fractions 3-5 and 20µl of plasma was used. Lipid extraction was preformed according to the method of Bligh and Dyer (66) in the presence of non-naturally occurring lipid species as internal standards. The following lipid species were added as internal standards: PC 14:0/14:0, PC 22:0/22:0, PE 14:0/14:0, PE 20:0/20:0 (di-phytanoyl), PS 14:0/14:0, PS 20:0/20:0 (di-phytanoyl), PG 14:0/14:0, PG 20:0/20:0 (di-phytanoyl), PI 16:0/16:0 or PI 17:0/17:0, LPC 13:0, LPC 19:0, Cer 14:0, Cer 17:0, D_7-FC, CE 17:0 and CE 22:0. The chloroform phase was dried in a vacuum centrifuge and dissolved in 10mM ammonium acetate in methanol/chloroform (3:1 vol/vol). Free cholesterol (FC) was analyzed after selective derivatization using acetyl chloride (35).

For the sphingolipids except SM and Cer a butanolic extraction procedure according to Baker et al. (67) was used with the following internal standards: [$^{13}C_2D_2$]S1P, SPH C17:1, SPC C17:1, GluCer C12:0 and LacCer C12:0. The butanol phase was evaporated to dryness under reduced pressure and the dried sample dissolved in ethanol.

3.2.4.2 Lipid mass spectrometry

Lipid species were quantified by electrospray ionization tandem mass spectrometry (ESI-MS/MS) using methods validated and described previously (35;37;68-71). In brief, samples for the glycerophospholipid and sterol analysis were analyzed by direct flow injection on a Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Manchester, UK) by direct-flow injection analysis using a HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland) and an Agilent 1100 binary pump (Waldbronn, Germany) with a solvent mixture of methanol containing 10 mM ammonium acetate and chloroform (3:1, v/v). A flow gradient was performed starting with a flow of 55µl/min for 6s followed by 30µl/min for 1.0min and an increase to 250µl/min for another 12s.

The sphingolipid analysis except for SM and Cer was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS) with an API 400 Q-Trap mass spectrometer equipped with a Turbo V source ion spray used for detection (Applied Biosystems, Darmstadt, Germany). The HPLC equipment consisted of an Agilent 1200 binary pump (G1312B), a 1200 series isocratic pump (G1310A) and a degasser (G1379B) (Waldbronn, Germany) connected to an HTC Pal autosampler (CTC Analytics, Zwingen, Switzerland). Gradient chromatographic separation was performed on an Interchim hydrophilic-interaction chromatography silica column (50 x 2.1 mm) (Montlucan, France) with a 2.2µm particle size and equipped with a 0.5µm pre-filter (Upchurch Scientific, Oak Harbor, WA, USA). For GlcCer, LacCer, SPH, SPA, S1P and SPC detection the Applied Biosystems TurboIonSpray source was operated in positive-ionization mode with an analysis time of 4.5 min per sample while S1P and SA1P were detected in negative-ionization mode with an analysis time of 2.2 min per sample. The mobile phase consisted of water containing 0.2% formic acid and 200mM ammonium formate for measurement in positive-ionization mode

and 90mM ammonium formate for measurement in negative-ionization mode (eluent A), respectively. Eluent B consisted of acetonitril containing 0.2% formic acid. The gradient elution for detection of GlcCer, LacCer, SPH, SPA, S1P, SA1P and SPC was performed with 100% B for 0.1 min, a step to 90% B until 0.11 min, a linear increase to 50% B until 2.5 min, 50% B until 3.5 min and reeqilibration from 3.5 to 4.5 min with 100% B. In case of detection of S1P and SA1P a gradient elution was performed with 5% A for 0.7 min, a linear increase to 25% A until 1.5 min, 50% A until 1.7 min, and reequilibration from 1.7-2.5 min with 5% A. The flow rate was set to 800µl/min (71). All analytes were monitored in the multiple reaction monitoring (MRM).

A precursor ion scan of m/z 184 specific for phosphocholine-containing lipids was used for phosphatidylcholine (PC), sphingomyelin (SM) (70) and lysophosphatidylcholine (LPC) (37). Neutral loss scans of m/z 141 and m/z 185 were used for phosphatidylethanolamine and phosphatidylserine (PS), respectively (68). PE based plasmalogens (PE-pl) were quantified according to the principles described by Zemski Berry et al. (72). In brief, fragment ions of m/z 364, 380 and 382 were used for PE p16:0, p18:1 and p18:0 species, respectively. Ammonium-adduct ions of phosphatidylglycerol (PG) and phoshatidylinositol (PI) were analyzed by neutral loss scans of m/z 189 and 277, respectively (73). FC and CE were quantified using a fragment ion of m/z 369 (35). Sphingosine (d18:1) based ceramides (Cer) were analyzed by product ion of m/z 264 similar to a previously described method (69). After identification of relevant lipid species, selected ion monitoring analysis was performed to increase precision of the analysis for the following lipid classes: PE, PE-pl, PG, PI, PS, LPC, Cer, CE. In case of S1P and SA1P the product ion spectra showed only a single intense fragment of m/z 79 used for analysis. Fragment ions of

m/z 282, 284 and 184 were used for SPH, SPA and SPC, respectively. Precursor ion scans of m/z 264 could be attributed to the glycosphingolipids GlcCer and LacCer.

Quantification was achieved by calibration lines generated by addition of naturally occurring lipid species to cell and plasma homogenates respectively. All lipid classes were quantified with internal standards belonging to the same lipid class, except SM (PC internal standard) and PE-based plasmalogens (PE internal standards). Calibration lines were generated by adding the following naturally occurring species PC 34:1, 36:2, 38:4, 40:0 and PC O 16:0/20:4; SM 16:0, 18:1, 18:0; LPC 16:0, 18:1, 18:0; PE 34:1, 36:2, 38:4, 40:6 and PE p16:0/20:4; PS 34:1, 36:2, 38:4, 40:6; Cer 16:0, 18:0, 20:0, 24:1, 24:0; FC, CE 16:0, 18:2, 18:1, 18:0, GlcCer 16:0, LacCer 16:0, 24:0 and the naturally occurring sphingolipid classes S1P, SA1P, SPA and SPC. These calibration lines were also applied for not calibrated species, as follows: Concentrations of saturated, monounsaturated and polyunsaturated species were calculated using the closest related saturated, monounsaturated and polyunsaturated calibration line slope, respectively. For example PE 36:2 calibration was used for PE 36:1, PE 36:3, PE 36:4; PE 38:4 calibration was used for PE 38:3 and PE 38:5 and so on. Ether-PC species were calibrated using PC O 16:0/20:4 and PE-based plasmalogens were quantified independent from the length of the ether-linked alkyl chain using PE p16:0/20:4.

The quantitative values were related to the plasma volume, protein amount or cell number of the sample, respectively. Correction of isotopic overlap of lipid species as well as data analysis was performed by self-programmed Excel Macros for all lipid classes according to the principles described previously (70).

3.2.4.3 Phosphorus assay

In order to compare the phospholipid content determined by ESI-MS/MS a phosphorus assay was performed according to Bartlett and Lewis (74). Briefly 500µl of 70% perchloric acid was added to phosphate standards (0 - 100nmol) and samples and was incubated for 1h at 100°C. The samples were cooled to room temperature, before adding 1ml of 1% ammonium molybdate and 4% ascorbic acid and incubation for 2h at 37°C in a water bath. Finally the standard and samples were measured at 700nm in a microplate reader (Sunrise, Tecan, Crailsheim, Germany).

3.2.5 Statistical analysis

All data are expressed as mean ± S.D. and mean with range in parentheses, respectively. Statistical significance was determined by a student's t-test for normally distributed data. Correlation analysis was performed using a bivariate correlation analysis with Pearson correlation coefficient. For significant results a linear regression analysis was performed to determine the correlation coefficient (R) and the coefficient of determination (R^2). P-values <0.05 (*), <0.01 (**) and <0.001 (***) were considered statistically significant. Significance is depicted in the respective figures. All analyses were performed with a commercially available statistics computer program (SPSS 15.0 for Windows, SPSS GmbH Software, Munich, Germany).

4. Results

4.1 Lipidomic analysis of circulating human blood cells

The lipids of circulating blood cells are an important target to study in detail. The composition and organization of blood cell lipids and membranes is tightly controlled. Alteration leads to the loss of specific functions or early demise of cells in circulation (75).

So far the analysis of blood cell lipid metabolism in human subjects has been largely confined to variations in metabolic and vascular diseases. Admittedly, most investigations have dealt with changes of defined lipids, cholesterol being probably the most intensively studied. For blood cells most of the lipid analyses in terms of lipid classes and mainly FA composition concentrated on red blood cells (RBC) (76-81), however, there are also studies which were carried out for platelets (82-85) and peripheral blood mononuclear cells (PBMC) containing a mix of monocytes and lymphocytes (86). In most cases only one blood cell type was analyzed, rarely comparisons between FA pattern of blood cells were shown (77). Due to technological limitations, little attention was paid to a detailed examination of phospholipid species. The latest developments in ESI-MS/MS have opened new analytical possibilities to determine phospholipid species in different cell types by high sensitivity and high throughput methods (32-37;69;70). Recent publications showed already lipid patterns of blood cells using the new methodological approaches for RBC (87;88), PBMC (89), leukocytes (90) and granulocytes (91). However, there is so far no study of the different blood cells available which shows a detailed comparison of blood cell lipid species.

Therefore, a comprehensive analysis of most abundant lipid classes and their species was performed in circulating blood cells (monocytes, lymphocytes, granulocytes, platelets and RBC) of healthy donors to highlight specific features of the individual blood cells. The detailed lipid pattern of circulating blood cells may provide new insights into cellular lipid abnormalities in diseases such as atherosclerosis, obesity and type-2-diabetes and will help to understand how lipids affect pathologic states.

4.1.1 Characteristics of cell donors and isolated blood cells

Blood samples were obtained from nine healthy normo-lipidemic volunteers recruited from blood donors of our transfusion medicine laboratory with ApoE3/E3 phenotype. The clinical and laboratory phenotype of the blood donors are presented in Table 7. The blood donors were gender-balanced (5 male and 4 females) with a mean age of 28 and normo-lipidemic with triglycerides and LDL below 150 mg/dl. The glucose values represent fasting level. After blood cell separation the purity levels of the blood cell fractions were determined and were shown to be higher than 95% for monocytes and platelets, higher than 85% for lymphocytes and granulocytes and above 98% for RBC.

Table 7: Characteristics of blood cell donors (n=9)[1]

Parameters	Value[2]
Gender	5 males / 4 females
Age (years)	28 (19-46)
Total cholesterol (mg/dL)	182 (156-203)
Triacylglycerols (mg/dL)	88 (53-138)
HDL (mg/dL)	63 (42-75)
LDL (mg/dL)	101 (85-145)
Glucose (mg/dL)	88 (80-97)

[1] Nine healthy donors volunteered for the study. Displayed are their lab parameters which were analyzed by the ADVIA 1800 system (Siemens Healthcare Diagnostics GmbH, Bad Nauheim, Germany)

[2] All values are mean, range in parentheses

4.1.2 Lipid class composition of blood cells

In order to get an overview of the lipidome of circulating blood cells, cell homogenates corresponding to 100µg protein were subjected to lipid analysis by ESI-MS/MS analyzing the following lipid classes: PC, LPC, PE, PE-pl, PG, PI, SM, Cer, FC and CE. Moreover, the reproducibility of the presented data was shown by a second independent analysis. The coefficient of variation (CV) for the lipid classes was below 6% and for the lipid species in general below 10%. Only for a few species a CV up to 21% was observed. An additional gender dependent analysis did not reveal significant differences (data not shown).

To evaluate the total lipid content (sum of the lipid classes analyzed) of the different blood cells a lipid to protein ratio as well as lipid content related to cell number was calculated. Total lipid content of the different blood cells varied between 41 to 179 nmol/mg protein or 0.56 to 5.50 nmol/10^6 cells, respectively (Table 8). The highest mean content of total lipids was measured for platelets followed by RBC, monocytes, granulocytes, and the lowest in lymphocytes. The phospholipid content analyzed by ESI-MS/MS corresponded to the results measured by a phosphorus assay (Table 9).

The lipid class composition showed a low variation between different donors and characteristic pattern were observed for all cell types (Figure 7A). PC and unesterified cholesterol represented the predominant lipid fractions with a range of about 55 mol% for leukocytes and platelets and 63 mol% of RBC. The molar PC/FC ratio was 1.9 in monocytes, 1.3 in lymphocytes, 1.1 in granulocytes, 0.8 in platelets and 0.3 in RBC, respectively. The CE content in platelets of 2.5 mol% was at least four-fold higher than in the other analyzed blood cells.

Table 8: Total lipid content of all circulating blood cells

Illustrated are the total lipids of the different blood cells (monocytes, lymphocytes, granulocytes, platelets and red blood cells) which were normalized to same protein content (100µg) and analyzed by ESI-MS/MS. Total lipids represent all measured phospholipids (PC, SM, PE, PE-pl, PG, PI, PS, LPC, Cer, CE and FC)

Blood cells	**Total lipid content (nmol/mg protein)**[1]	**Total lipid content (nmol/10^6 cells)**[1]
Monocytes[2]	118.7 ± 16.7	5.50 ± 0.93
Lymphocytes[2]	40.8 ± 10.2	3.28 ± 0.75
Granulocytes[2]	84.1 ± 22.2	4.41 ± 0.89
Platelets[2]	179.4 ± 38.9	0.48 ± 0.10
Red blood cells[3]	154.2 ± 31.2	0.56 ± 0.11

[1] Values are mean±SD from nine different donors

[2] Total protein content of white blood cells and platelets was detected by a BCA assay

[3] Total protein content of red blood cells was related to the haemoglobin concentration

Table 9: Comparison between the lipid content of blood cell samples analyzed by ESI-MS/MS and by phosphorus assay

Samples	ESI-MS/MS (nmol/mg protein)	Phosphorus-Assay (nmol/mg protein)
monocyte 1	95,4	101,3
monocyte 2	100,5	90,4
monocyte 3	84,4	86,9
monocyte 4	100,2	98,8
monocyte 5	78,1	83,0
lymphocyte 1	28,6	30,8
lymphocyte 2	35,7	36,7
lymphocyte 3	39,1	31,1
granulocyte 1	50,3	54,0
granulocyte 2	58,7	52,9
granulocyte 3	66,8	66,1
granulocyte 4	61,9	67,1
granulocyte 5	76,1	64,5
platelet 1	120,3	117,9
platelet 2	145,0	150,4
platelet 3	112,5	115,0
platelet 4	154,1	146,1
platelet 5	141,2	144,8
platelet 6	109,4	104,8
RBC 1	90,3	93,1
RBC 2	102,3	99,9
RBC 3	84,6	85,7
RBC 4	114,5	112,7

In order to assess the glycerophospholipid and sphingolipid content, the lipid pattern without FC and CE (Figure 7B) were used. Interestingly, granulocytes showed the highest PE-pl proportion with 21 mol% combined with a significantly lower PE fraction of 11 mol% compared to other leukocytes. The proportion of Cer in granulocytes was with 3.7 mol% 6-fold higher compared to other leukocytes and platelets (~3-fold compared to RBC). RBC differed substantially from other blood cells: A decreased PC and increased SM proportion resulted in a PC/SM ratio of 1.1, whereas the other cell types range from 2.4 to 3.5. Moreover, RBC were characterized by the lowest PE-pl and PI (< 1/3) as well as the highest PS proportion of all cell types analyzed. PG was for all cell types close to the limit of detection (therefore no evaluation of species profiles was shown below). A statistical analysis of the lipid classes for the analyzed blood cells is depicted in Table 10.

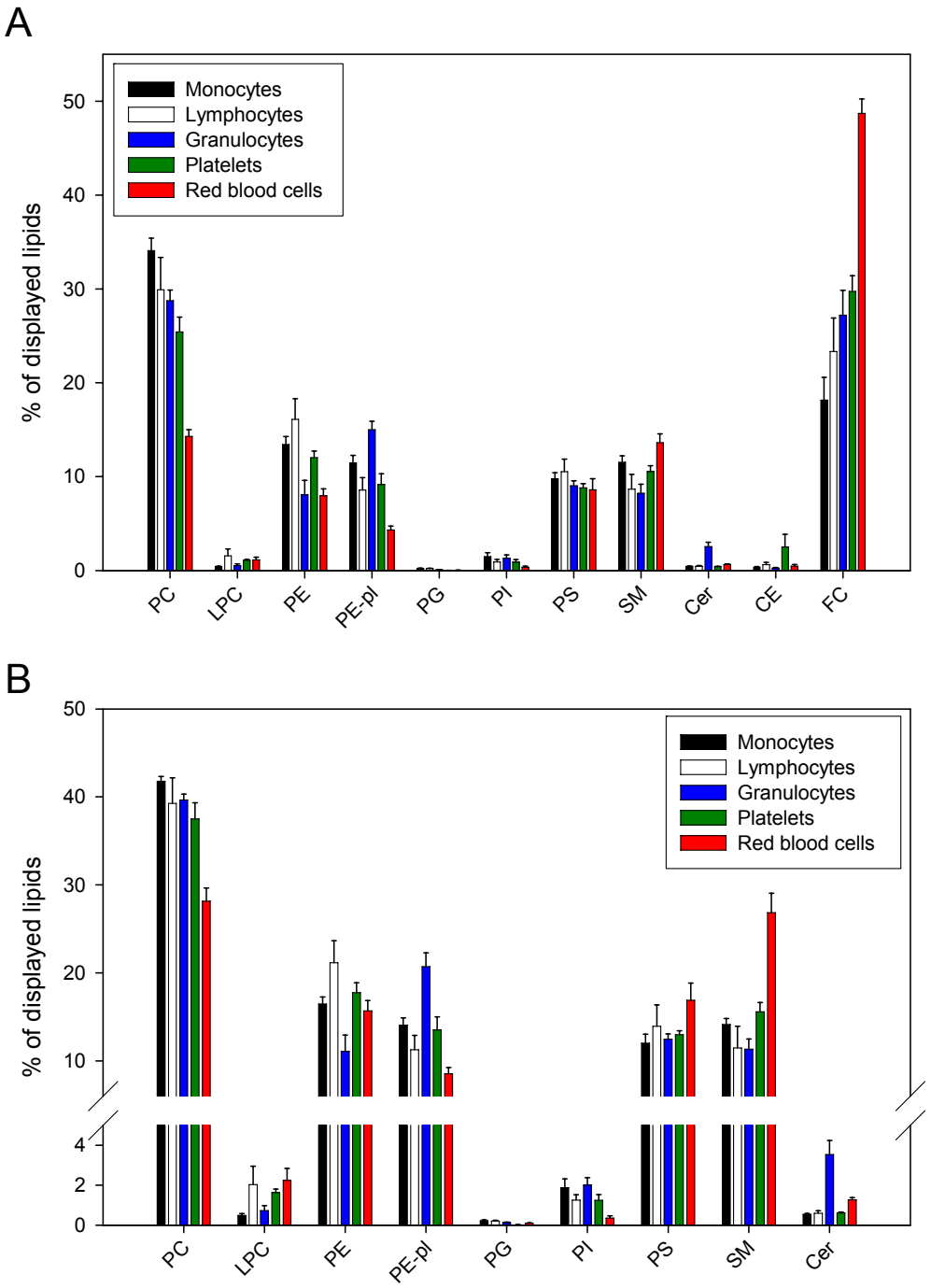


Figure 7: Lipid composition of monocytes, lymphocytes, granulocytes, platelets and red blood cells

Leukocytes and platelets were separated by leukapheresis followed by counterflow centrifugation elutriation. Red blood cells were washed by repetitive centrifugation steps. The different lipid fractions were quantified by ESI-MS/MS. The displayed values are percent of the respective lipid class of all analyzed lipids (A) and glycerophospholipid/sphingolipids (B) for monocytes (black bars), lymphocytes (white bars), granulocytes (blue bars), platelets (green bars) and red blood cells (red bars), respectively. Apart from cholesteryl esters (CE) and free cholesterol (FC), the shown lipids are phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylethanolamine (PE), PE-based plasmalogens (PE-pl), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), lysophosphatidylcholine (LPC) and ceramide (Cer). Values are mean ± SD from nine different donors. Statistic calculations are shown in Table 10.

Table 10: Statistical significances of different lipid classes among the analyzed blood cells

PC	Monocytes	Lymphocytes	Granulocytes	Platelets
Monocytes				
Lymphocytes	NS			
Granulocytes	***	NS		
Platelets	***	**	**	
RBC	***	***	***	***
LPC	Monocytes	Lymphocytes	Granulocytes	Platelets
Monocytes				
Lymphocytes	**			
Granulocytes	NS	*		
Platelets	***	NS	***	
RBC	***	NS	***	NS
PE	Monocytes	Lymphocytes	Granulocytes	Platelets
Monocytes				
Lymphocytes	NS			
Granulocytes	***	***		
Platelets	**	**	**	
RBC	***	***	NS	***
PE-pl	Monocytes	Lymphocytes	Granulocytes	Platelets
Monocytes				
Lymphocytes	**			
Granulocytes	***	***		
Platelets	***	NS	***	
RBC	***	***	***	***
PG	Monocytes	Lymphocytes	Granulocytes	Platelets
Monocytes				
Lymphocytes	NS			
Granulocytes	***	**		
Platelets	***	***	***	
RBC	***	***	**	**
PI	Monocytes	Lymphocytes	Granulocytes	Platelets
Monocytes				
Lymphocytes	**			
Granulocytes	NS	NS		
Platelets	*	NS	NS	
RBC	***	***	***	**
PS	Monocytes	Lymphocytes	Granulocytes	Platelets
Monocytes				
Lymphocytes	NS			
Granulocytes	NS	NS		
Platelets	**	NS	NS	
RBC	NS	NS	NS	NS
SM	Monocytes	Lymphocytes	Granulocytes	Platelets
Monocytes				
Lymphocytes	**			
Granulocytes	***	NS		
Platelets	**	NS	**	
RBC	***	***	***	***
Cer	Monocytes	Lymphocytes	Granulocytes	Platelets
Monocytes				
Lymphocytes	NS			
Granulocytes	***	***		
Platelets	*	NS	***	
RBC	***	***	***	***
FC	Monocytes	Lymphocytes	Granulocytes	Platelets
Monocytes				
Lymphocytes	*			
Granulocytes	***	*		
Platelets	***	**	NS	
RBC	***	***	***	***
CE	Monocytes	Lymphocytes	Granulocytes	Platelets
Monocytes				
Lymphocytes	**			
Granulocytes	NS	**		
Platelets	**	**	**	
RBC	***	NS	***	**

Abbreviations: NS =non-significant; * =p<0.05; ** = p<0.01; *** = p<0.001

4.1.3 Lipid species composition of blood cells

Lipid profiles related to the total content of each respective lipid class were calculated for the evaluation of lipid species of the different blood cells (Figure 8-12). The performed mass spectrometric analysis only allows the determination of the total number of carbon atoms and double bonds in the FA moiety for lipid classes containing two FA esterified to the glycerol-backbone (PC, PE, PG, PI, PS). For example a PC 34:1 may represent different combinations of FA such as 18:0/16:1, 16:0/18:1, etc. Moreover, the assignment to a bond type (acyl or ether) is based on the assumption that FA with odd-numbered carbon atoms constitute a negligible fraction. Lysophospholipids (LPC), sphingolipids (SM, Cer) and CE contain one FA denominated by the species nomenclature.

The PC species pattern of the blood cells (Figure 8A and B) showed a broad distribution with PC 34:1 representing the major species beside PC 34:2 in RBC. A decreased level of highly polyunsaturated PC species (more than three double bounds) of 6.5 mol% was observed in granulocytes compared to monocytes (22 mol%), lymphocytes (26 mol%), platelets (23 mol%) and erythrocytes (10 mol%). The amount of ether-phosphatidylcholine (PC O) species in granulocytes (about 40% of total PC) was clearly elevated compared to other circulating blood cells (12 to 24 %) (Figure 8B). For all PC O species granulocytes exhibited an increased proportion compared to other blood cell types, except highly polyunsaturated species where monocytes revealed the highest proportion. The LPC species profile (Figure 8C) displayed a dominance of saturated LPC species 16:0 and 18:0 in RBC and platelets, whereas leukocytes especially monocytes contained a high fraction of polyunsaturated LPC.

Cellular PE species profiles (Figure 9A) showed a strikingly high percentage of PE 38:4, most likely a combination of 18:0/20:4, in monocytes, lymphocytes and

platelets. Similarly, their predominant PE-pl species (Figure 9B) were arachidonic acid containing PE p16:0/20:4 and PE p18:0/20:4. Only granulocytes showed a higher content of monounsaturated species for PE as well as PE-pl species. Interestingly, granulocytes contained PE 36:1 and PS 36:1 (Figure 10B) as species with the highest abundance.

Compared to other glycerophospholipid classes, for PI only a reduced number of species was detected (Figure 10A). PI 38:4 was the dominant lipid species for all analyzed cell types (>50 mol%), except for RBC where PI 36:2 composed nearly 50 mol% at the expense of PI 38:4. PS species pattern (Figure 10B) showed two prominent species depending on the cell type: PS 36:1, particularly for granulocytes, and PS 38:4 for platelets and RBC. A chain length dependent analysis of glycerophosholipids (Table 11) demonstrated a striking shift for RBC compared to other cell types. Thus, for PC, PE, PI an increased fraction of species with shorter chain length was observed in RBC, whereas PS showed an elevated proportion of long chain species. SM species were assigned based on the assumption that di-hydroxy C18:1 is the major sphingoid base in all blood cells (92). Also characteristic cellular SM species pattern (Figure 11A) with a dominant proportion of SM 16:0 particularly in leukocytes were found. Compared to all analyzed cell types, platelets showed a unique SM species distribution, most prominent a more than 3-fold increased fraction of SM 20:0 and 22:0. Longer chain SM species (SM 24:1 and SM 24:0) were significantly elevated for RBC in comparison to other blood cells ($p<0.05$). In principle the Cer species pattern (Figure 11B) was comparable to the SM pattern. One exception was a high percentage of Cer 16:0 in granulocytes of 65 mol%.

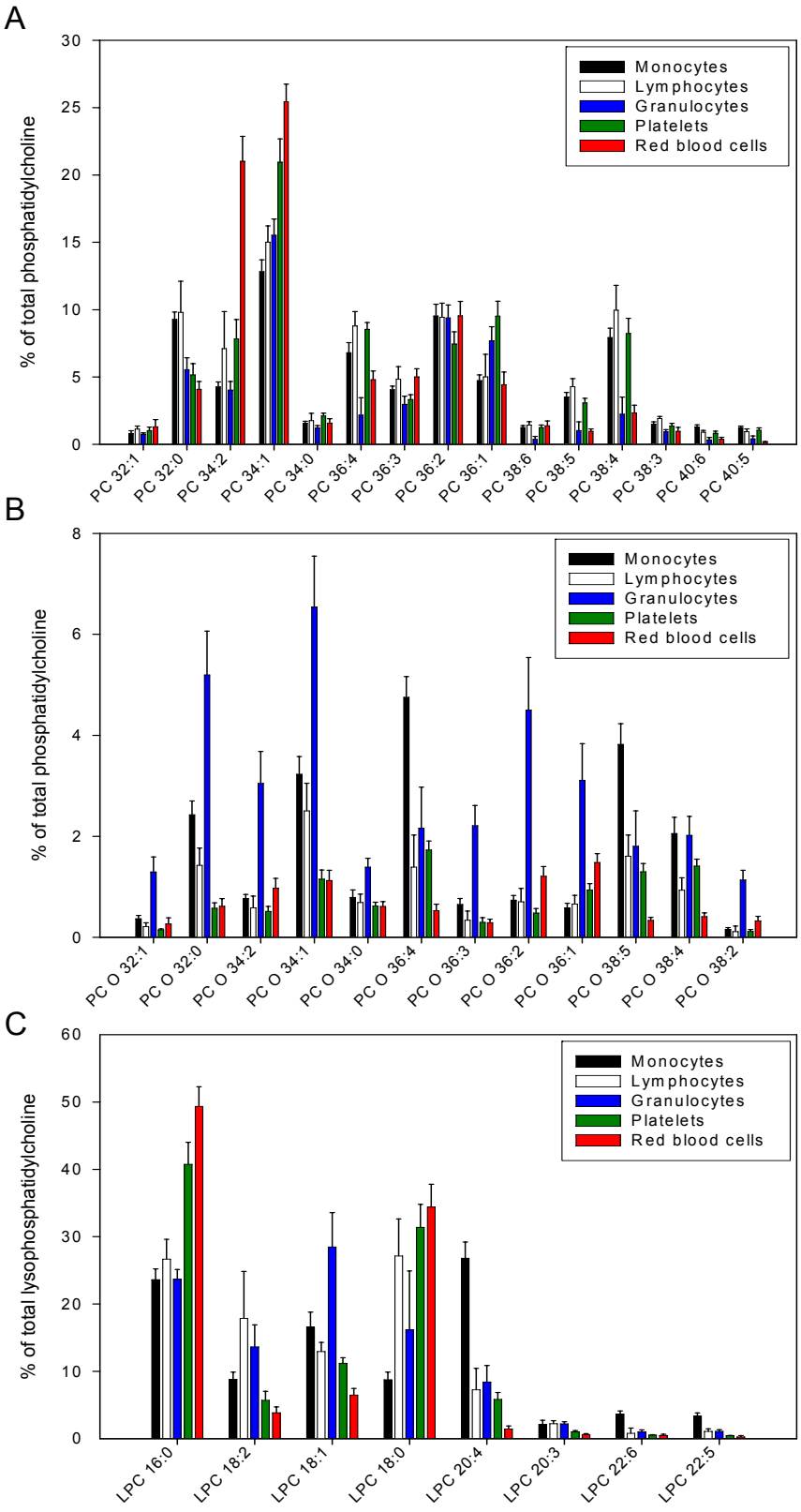


Figure 8: Phosphatidylcholine (PC), ether-phosphatidylcholine (PC O) and lysophosphatidylcholine (LPC) species composition of blood cells

Cells were obtained and lipids extracted and analyzed as described in the legend to Figure 7. Displayed are mol% of PC (A) and PC-O (B) species related to total PC and LPC species (C) related to total LPC for monocytes (black bars), lymphocytes (white bars), granulocytes (blue bars), platelets (green bars) and red blood cells (red bars). Values are mean ± SD from nine different donors.

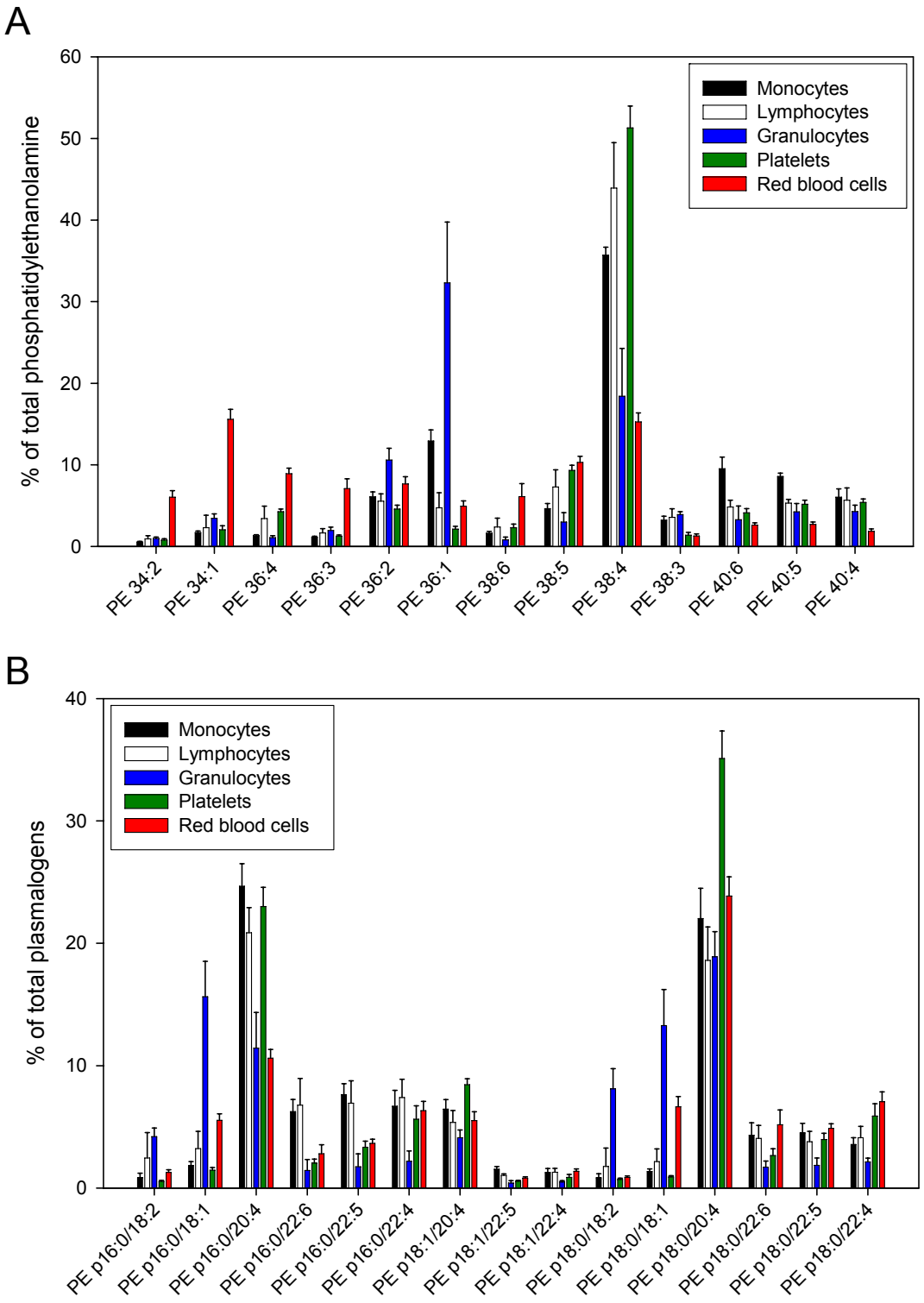


Figure 9: Phosphatidylethanolamine (PE) and PE-based plasmalogens (PE-pl) species composition of blood cells

Blood cells were handled as described in the legend to Figure 7. Panel A shows PE species and panel B PE-pl species in mol% of the total lipid class. Monocytes are illustrated by black bars, lymphocytes by white bars, granulocytes by blue bars, platelets by green bars and red blood cells by red bars. Values are mean ± SD from nine different donors.

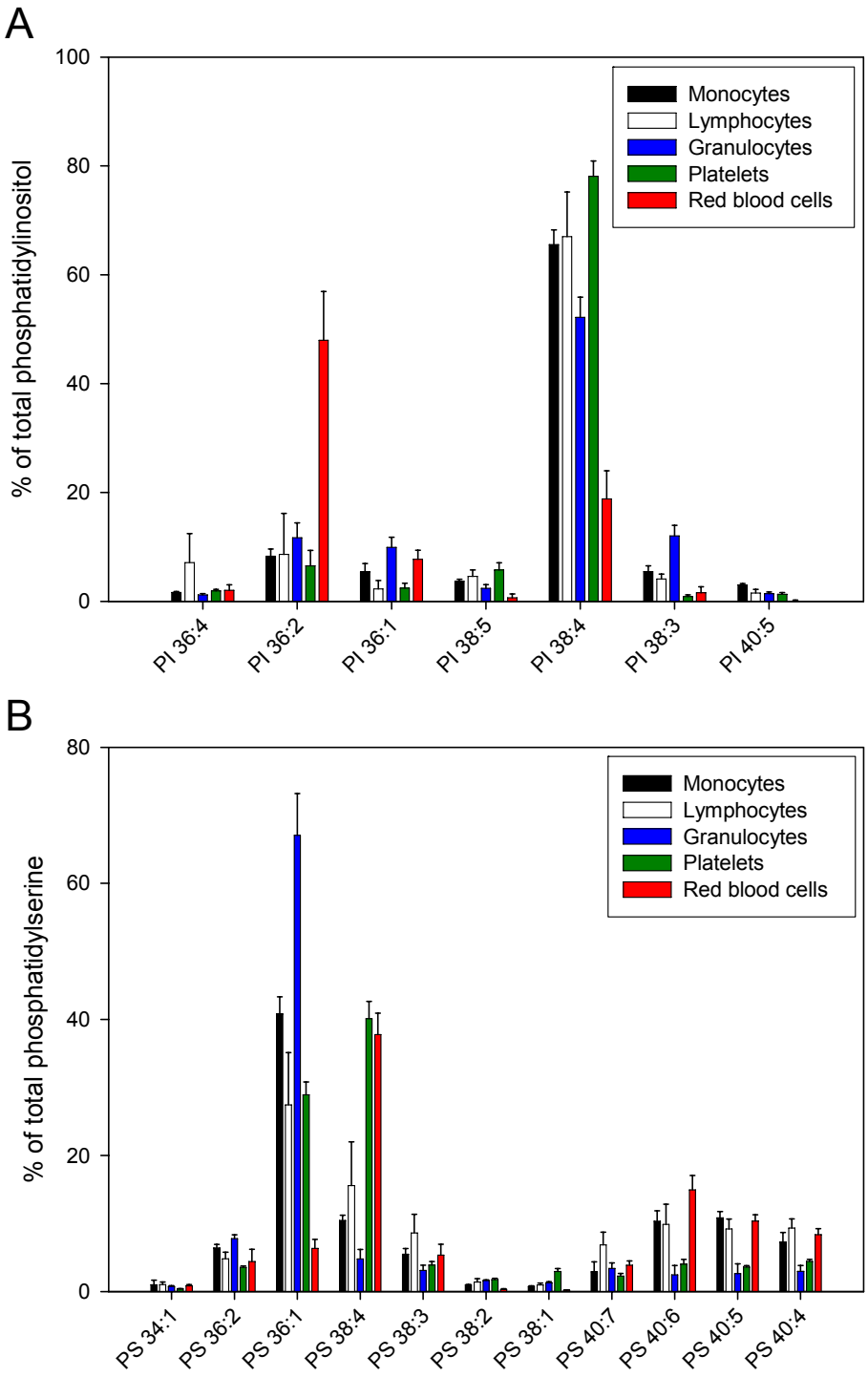


Figure 10: Phosphatidylinositol (PI) and phosphatidylserine (PS) species composition of blood cells
Displayed are mol% of total PI (A) and PS (B) lipid species of monocytes (black bars), lymphocytes (white bars), granulocytes (dark grey bars), platelets (light grey bars) and red blood cells (red bars). Values are mean ± SD from nine different donors.

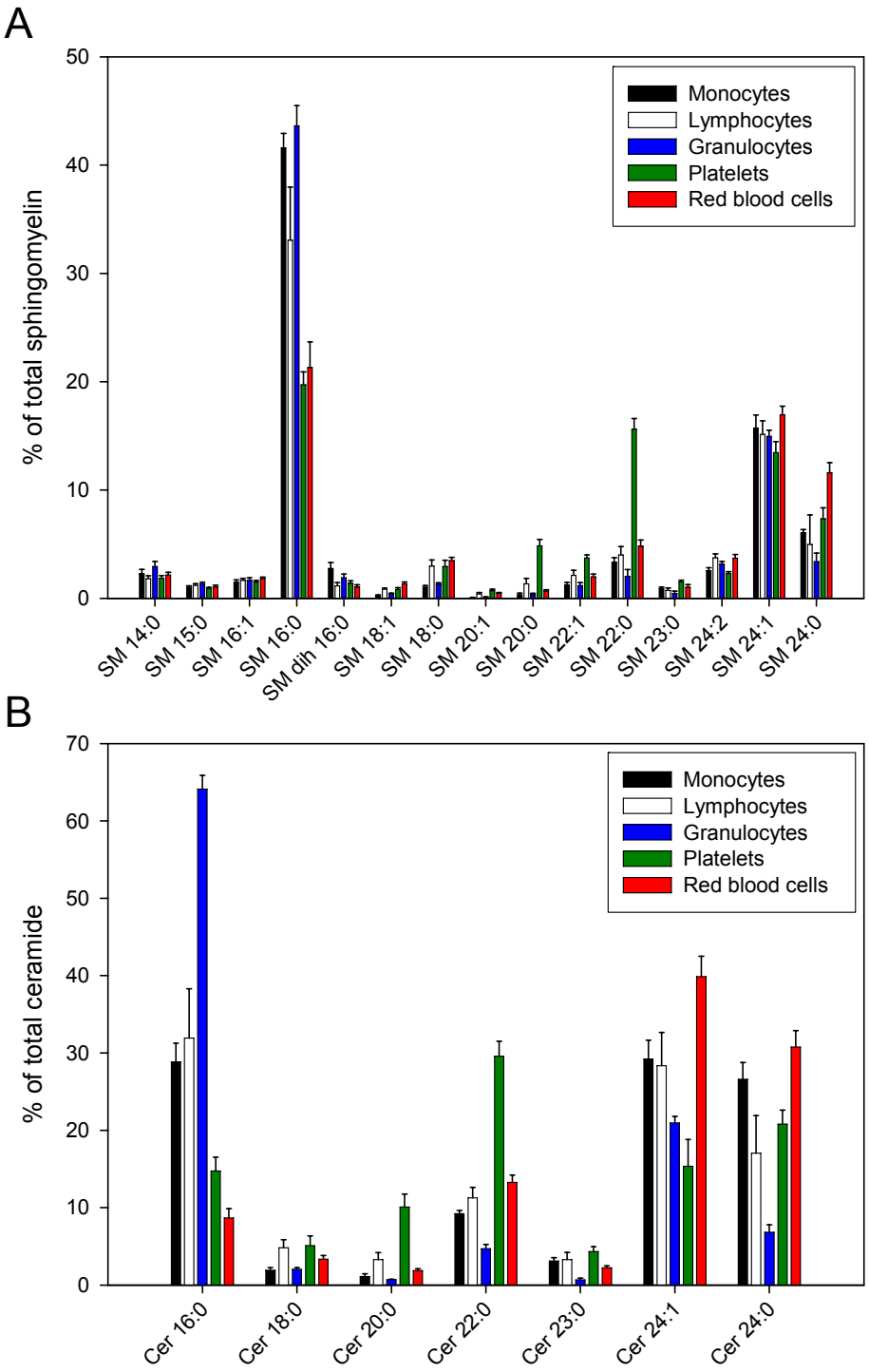


Figure 11: Sphingomyelin (SM) and ceramide (Cer) species composition of blood cells

The sphingolipids of monocytes (black bars), lymphocytes (white bars), granulocytes (dark grey bars), platelets (light grey bars) and red blood cells (red bars) are displayed in mol% of total analyzed lipid class. SM species are shown in panel A. Cer species in panel B. Values are mean ± SD from nine different donors.

Table 11: Glycerophospholipids add up according to number of C-atoms

PC	30	32	34	36	38	40	≤ 34	>34	≤ 36	>36
Monocytes	0.7%	10.2%	18.9%	25.6%	1.4%	4.1%	29.8%	31.0%	55.4%	5.4%
Lymphocytes	0.9%	11.0%	24.2%	28.7%	1.0%	3.1%	36.1%	32.8%	64.8%	4.1%
Granulocytes	0.7%	6.4%	21.0%	22.5%	1.4%	1.7%	28.0%	25.6%	50.5%	3.1%
Platelets	0.5%	6.3%	31.3%	30.0%	1.6%	4.0%	38.2%	35.6%	68.2%	5.6%
Red blood cells	0.8%	5.6%	48.7%	24.9%	1.4%	1.5%	55.1%	27.8%	80.0%	2.9%

PC-O	30	32	34	36	38	40	≤ 34	>34	≤ 36	>36
Monocytes	0.2%	2.8%	4.8%	7.6%	6.4%	1.5%	7.8%	15.4%	15.4%	7.9%
Lymphocytes	0.1%	1.6%	3.9%	3.5%	2.9%	0.5%	5.6%	6.9%	9.1%	3.4%
Granulocytes	0.4%	6.6%	11.3%	12.7%	6.7%	1.6%	18.3%	21.0%	31.0%	8.3%
Platelets	0.1%	0.7%	2.4%	4.4%	3.1%	0.7%	3.2%	8.2%	7.6%	3.8%
Red blood cells	0.2%	0.9%	3.2%	4.2%	1.8%	0.4%	4.3%	6.4%	8.5%	2.2%

PC + PC-O	30	32	34	36	38	40	≤ 34	>34	≤ 36	>36
Monocytes	0.9%	13.0%	23.7%	33.2%	7.8%	5.5%	37.6%	46.5%	70.7%	13.3%
Lymphocytes	1.0%	12.7%	28.1%	32.2%	3.9%	3.6%	41.8%	39.7%	73.9%	7.5%
Granulocytes	1.1%	12.9%	32.3%	35.2%	8.1%	3.3%	46.3%	46.6%	81.5%	11.4%
Platelets	0.6%	7.1%	33.7%	34.4%	4.8%	4.7%	41.4%	43.8%	75.7%	9.5%
Red blood cells	1.0%	6.5%	51.9%	29.1%	3.2%	1.9%	59.4%	34.2%	88.6%	5.1%

PE	30	32	34	36	38	40	≤ 34	>34	≤ 36	>36
Monocytes	/	0.2%	2.4%	21.6%	46.6%	24.3%	2.6%	92.5%	24.2%	70.9%
Lymphocytes	/	0.2%	3.4%	15.5%	58.2%	16.0%	3.6%	89.7%	19.2%	74.2%
Granulocytes	/	0.3%	4.7%	46.1%	31.0%	12.3%	5.0%	89.5%	51.1%	43.4%
Platelets	/	0.1%	2.9%	12.4%	65.6%	14.9%	3.0%	92.9%	15.4%	80.5%
Red blood cells	/	1.2%	22.2%	29.3%	34.1%	7.4%	23.5%	70.7%	52.8%	41.4%

PE-pl	30	32	34	36	38	40	≤ 34	>34	≤ 36	>36
Monocytes	/	0.2%	3.7%	28.7%	50.3%	17.1%	3.9%	96.1%	32.6%	67.4%
Lymphocytes	/	0.5%	7.7%	28.3%	47.3%	16.2%	8.2%	91.8%	36.5%	63.5%
Granulocytes	/	0.3%	20.8%	40.2%	31.4%	7.2%	21.2%	78.8%	61.3%	38.7%
Platelets	/	0.1%	2.5%	26.3%	56.2%	14.9%	2.6%	97.4%	28.9%	71.1%
Red blood cells	/	0.3%	9.4%	23.5%	45.6%	21.2%	9.7%	90.3%	33.2%	66.8%

PS	30	32	34	36	38	40	≤ 36	>36
Monocytes	/	0.1%	1.7%	47.5%	18.5%	31.6%	49.2%	50.2%
Lymphocytes	/	0.2%	1.6%	33.4%	28.0%	35.6%	35.2%	63.7%
Granulocytes	/	0.1%	1.2%	75.3%	11.2%	11.7%	76.6%	22.9%
Platelets	/	0.0%	0.5%	32.7%	50.9%	14.8%	33.3%	65.8%
Red blood cells	/	0.2%	2.3%	11.9%	47.0%	37.8%	14.3%	84.8%

PI	30	32	34	36	38	40	≤ 36	>36
Monocytes	/	/	1.2%	16.4%	75.1%	7.3%	17.6%	82.4%
Lymphocytes	/	/	0.9%	19.0%	76.0%	4.0%	20.0%	80.0%
Granulocytes	/	/	2.0%	24.1%	68.3%	5.6%	26.1%	73.9%
Platelets	/	/	0.5%	11.1%	85.3%	3.1%	11.6%	88.4%
Red blood cells	/	/	17.0%	60.7%	21.1%	1.1%	77.7%	22.3%

CE FA species were also characterized in detail (Figure 12). Interestingly, the CE 18:2 (linoleic acid) fraction in platelets was with 55 mol% nearly 4-fold higher than in other circulating blood cells. An at least 2-fold higher content of CE 20:4 was measured in monocyte samples.

In summary, lipid species analysis by ESI-MS/MS of the major circulating blood cell types from nine individuals demonstrated characteristic lipid class and lipid species patterns for each different cell type.

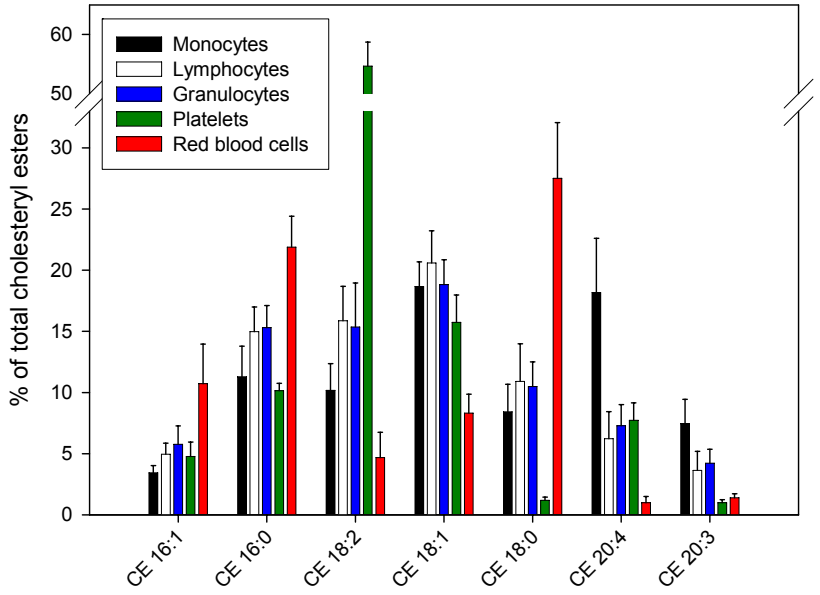


Figure 12: Cholesteryl ester (CE) species composition of blood cells

CE species of monocytes (black bars), lymphocytes (white bars), granulocytes (dark grey bars), platelets (light grey bars) and red blood cells (red bars) are displayed in mol% of total analyzed CE. Values are mean ± SD from nine different donors. Only species above 5% related to total CE content of at least one blood cell type were shown.

4.2 Lipidomic analysis of platelet senescence

Currently the factors controlling the lifespan of platelets both *in vivo* and *in vitro* are poorly understood. It is known that older platelets *in vivo* are less haemostatically active than younger ones and that senescent platelets have a decreasing ability to respond to physiological agonists and adhere to collagen (93). When platelet concentrates are stored *ex vivo* for purpose of transfusion a phenomenon called platelet storage lesion occurs which represents a non-physiological form of platelet death resulting in a loss of platelet function (94) and decrease of post-transfusion survival (95). Previously described changes can be classified into three broadly defined categories: metabolic alterations in the cytosol and mitochondria; platelet activation; and platelet senescence (96).

Alterations during storage (97;98) already highlight the importance of cytoskeletal and lipid reorganization. The involvement of lipids in the development of the platelet storage lesion is also likely because of their hemostatic and structural importance and their chemical and physical lability (99). Hamid et al. (100) and Okuma et al. (101) found a loss of both cholesterol and phospholipids during platelet storage. The mechanisms behind the lipid loss are mainly microvesiculation and lipid peroxidation (99). Lipids also play a role in clearance of platelets from the circulation by the reticuloendothelial system of the spleen. Platelet aging *in vivo* is triggered by loss of membrane phospholipid asymmetry most important by phosphatidylserine (PS) exposure on the outer leaflet which is an important signal for ingestion by spleen macrophages (102). The loss of phospholipid asymmetry was also shown in an *ex vivo* study of stored platelets by Gaffet et al. (103). Furthermore mitochondria which are key organelles in the regulation of apoptosis decrease their membrane potential during platelet aging *in vivo* (104). In this context Leytin and Freedman (97) indicated

that the intrinsic (mitochondria-mediated) pathway may be involved in platelet apoptosis during platelet storage while the role of the extrinsic (death receptor-death ligand-dependent) pathway needs to be further elucidated. Beside “platelet apoptosis“ (“plateletptosis”) of this anucleate cells it seems that platelet activation is almost equally involved in the deterioration of platelets obtained by apheresis (105). The association between platelet lipid alterations and platelet activation was already demonstrated (106;107).

Platelet lipid composition is also influenced by plasma lipoproteins whose interactions with platelets are crucial in atherosclerosis and thrombosis (108). LDL and HDL rapidly deliver SM, PC and PE to platelets (109) and also VLDL can transfer phospholipids to platelets (110). The phospholipid transfer from LDL to platelets is independent of high affinity binding of LDL to platelets (111). LDL sensitizes platelets via receptor-mediated signaling and lipid exchange (108). Related to these findings, LDL also directly contributes to the formation of platelet eicosanoids by supplying arachidonic acid either phospholipid bound or free (112).

However, in context of platelet senescence the plasma and platelet lipid interactions were not studied so far. Therefore the changes of platelet lipids in platelet apheresis products were characterized and a correlation analysis between plasma and platelet lipids performed in this work.

4.2.1 Characteristics of cell donors and collected platelet apheresis products

The clinical and laboratory phenotype of the 50 healthy blood donors participating in the study are presented in Table 12. The blood donors, who routinely visited the department of transfusion medicine for platelet apheresis, were gender-balanced with

a mean age of 29. Platelet apheresis products of the study fulfilled all criteria for transfusion acceptance.

Table 12: Characteristics of platelet donors (n=50)[1]

Parameters	**Value**[2]
Gender	28 males / 22 females
Age (years)	29 (19-51)
Total cholesterol (mg/dL)	173 (114-241)
Triacylglycerols (mg/dL)	95 (37-161)
HDL (mg/dL)	64 (33-100)
LDL (mg/dL)	83 (55-114)
VLDL (mg/dL)	26 (8-39)
Platelets (/nL)	228 (177-319)

[1] Fifty healthy donors volunteered for the study. Displayed are their lab parameters which were analyzed by the ADVIA 1800 system (Siemens Healthcare Diagnostics GmbH, Bad Nauheim, Germany).

[2] All values are mean, range in parentheses.

4.2.2 Validation of platelet gel-filtration separation

Recent publications propose Sepharose chromatography for *in vitro* and *in vivo* assays to study platelet function (113;114) because platelet isolation by density gradient centrifugation leads already to significant platelet activation. Pre-tests were carried out to establish optimal platelet isolation conditions by gel-filtration. The collected fractions were analyzed for cell number, protein and lipid content (Figure 13A). A high platelet count could only be detected in fractions 3-5 where also an elevation of the protein and lipid content was observed. To determine the purity of the collected fractions different platelet and plasma specific proteins were analyzed by blotting from the different fractions (Figure 13B). The thrombospondin receptor CD36

was detected in fractions 3-6. The plasma proteins transferrin and apolipoprotein A-I, A-IV and C-I raised after fraction 5. Only ApoE seemed to be also present in the platelet fraction. In order to exclude contamination of lipoproteins, only fractions 3-5 were used for further platelet analysis. Platelet activation was tested by flow cytometry giving evidence of nearly any activation during gel-filtration isolation (data not shown).

4.2.3 Total lipid content of senescent platelets

To monitor changes in the lipidome of platelet apheresis products during five days of storage lipid analysis was performed by ESI-MS/MS covering the following lipid classes: PC, LPC, PE, PE-pl, PG, PI, SM, Cer, FC and CE. The time limit was chosen because regulations restricted the use of platelet concentrates older than five days in transfusion medicine.

During storage time of five days the total lipid content of platelets decreased by 10.3% from 5,306 to 4,759 nmol/10^{10} platelets ($p<0.05$, paired t-test) (Figure 14) due to an 8.9% loss of phospholipids and an 18.1% loss of cholesterol. Vice versa the total lipid content of plasma increased by 5.2% from 5,323 to 5,600 nmol/ml plasma ($p<0.001$, paired t-test).

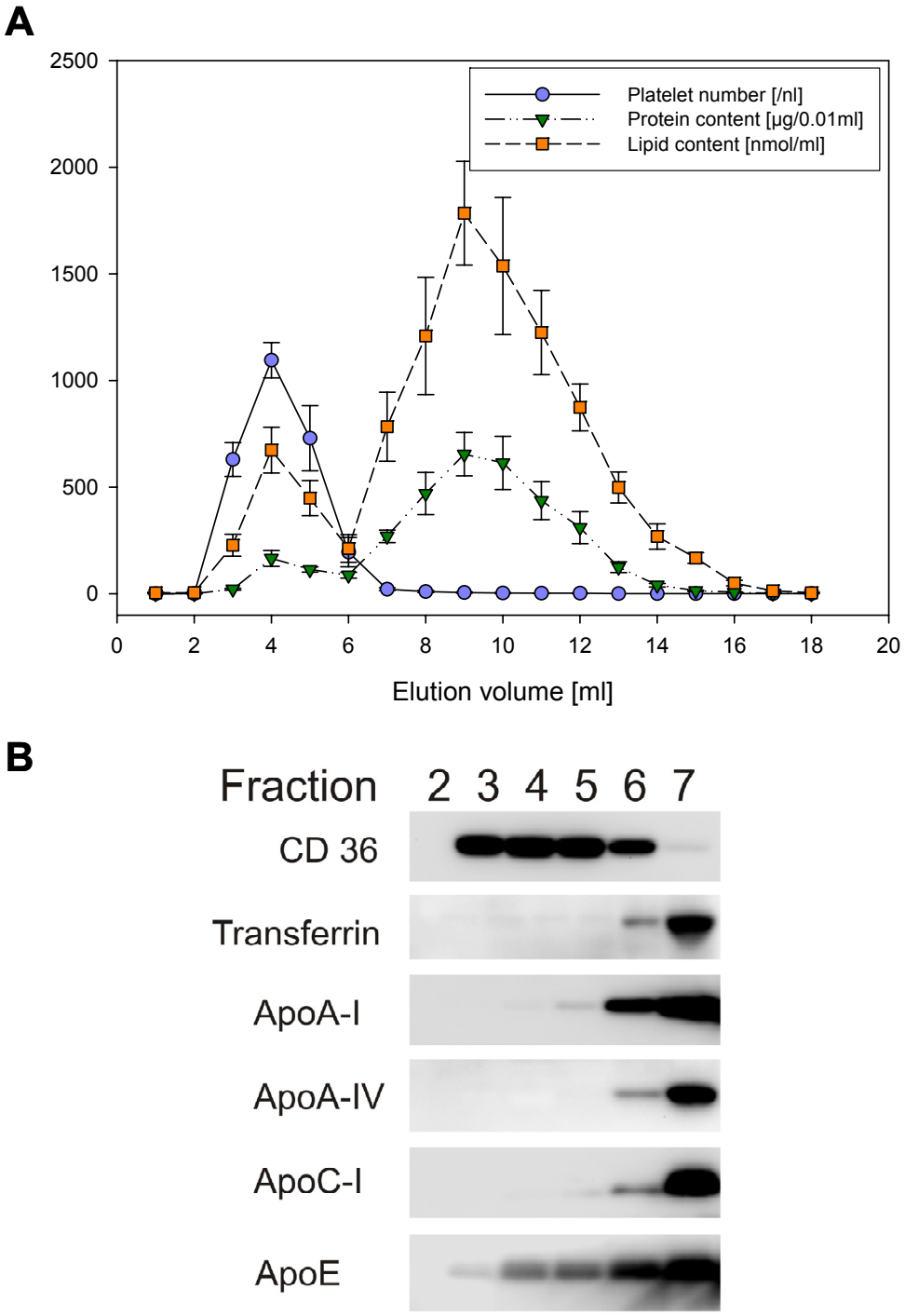


Figure 13: Validation of gel-filtration isolation.

Panel A shows the counted platelet cell number per fraction of the gel-filtration separation procedure which was measured by ADVIA 120 system (Siemens Healthcare Diagnostics GmbH, Bad Nauheim, Germany). It also displays the protein content (in µg/10nl) of each fraction measured by a BCA assay as well as the total lipid content (in nmol/ml) per fraction analyzed by ESI-MS/MS. All three displayed units are adapted to a similar exponent on the y-axis. Values are mean±SD from 10 different donors. Panel B displays the total lipid content of the different fractions during gel-filtration isolation measured by ESI-MS/MS as described in materials and methods. Panel B shows a SDS-PAGE of different platelet (CD36, transferrin) and lipoprotein specific proteins (ApoA-I, ApoA-IV, ApoC-I, ApoE) per gel-filtration isolated fraction.

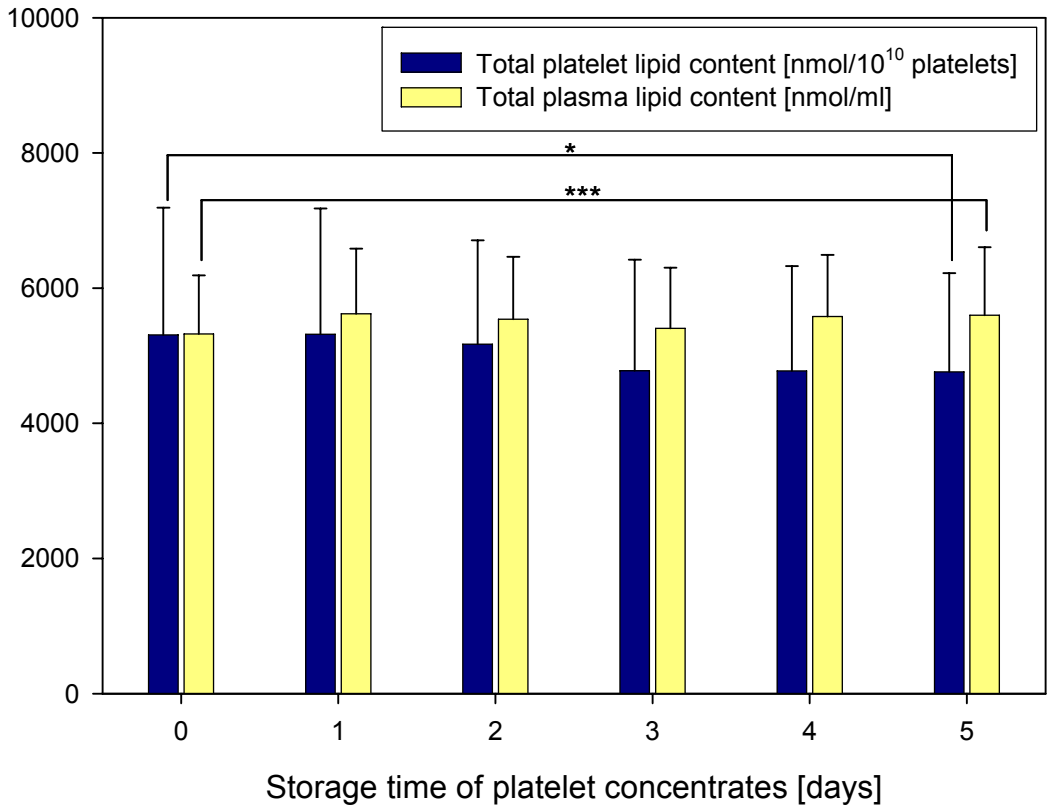


Figure 14: Total lipid content of senescent platelet concentrates.
Platelets were isolated by gel-filtration procedure and plasma by centrifugation. The different lipid fractions were quantified by ESI-MS/MS. Displayed is the total lipid content of platelets (in nmol/10^{10} platelets) (blue bars) and plasma (in nmol/ml plasma) (yellow bars) during an aging period of five days after apheresis. Significant changes between day 0 and day 5 are calculated by a donor specific, paired t-test. Values are mean ± SD from 50 different donors.

4.2.4 Lipid class composition of senescent platelets

The lipid class composition showed a low variation between different donors except for CE and characteristic pattern were observed for platelets (Figure 15A) and plasma (Figure 15B). PC and FC represented the predominant lipid fractions with nearly 50 mol% for platelets considering all measured lipid classes. During five days of platelet storage significant increases of 69% for Cer, 32% for LPC, 8% for SM and 49% for CE were found as well as a decrease of 10% for FC fractions related to day 0. The platelet PC fraction did not change significantly. In plasma significant changes during storage were detected in LPC (increase of 43%), PC (decrease of 20%) and

FC (decrease of 24%) fractions related to day 0. The dominating plasma lipid class was CE which increased from 52 to 59 mol% from day 0 to day 5.

To better understand the significant increase of Cer during platelet aging the whole sphingolipid pattern was further analyzed by MS (Figure 16A-C). Beside the major sphingolipids SM and Cer (Figure 15A) DihCer and LacCer were found above 3 mol% of all analyzed sphingolipids (Figure 16A*).* LacCer showed as expected a ten times higher ratio (Figure 16C) and percentage of displayed sphingolipids (Figure 16A) compared to GlcCer in platelets. During storage a significant increase of GlcCer from 0.17 to 0.27 mol% was detected while the LacCer content did not change significantly (Fig. 16A). Due to the significant increase of Cer and GlcCer during storage of platelet concentrates significant changes of the Cer/LacCer and GlcCer/LacCer ratios were obtained between day 0 and day 5 (Figure 16C). For all other sphingolipids except SM (Figure 15A) and DihSM highly significant decreases of 63% for SPH, 78% for SPA, 68% for SPC, 55% for S1P and 89% for SA1P related to collection day were observed (Figure 16A). Thus, highly significant changes of ratios for SPH, SPA, SPC, S1P and SA1P in relation to rising Cer were obtained during the storage time of five days (Figure 16B).

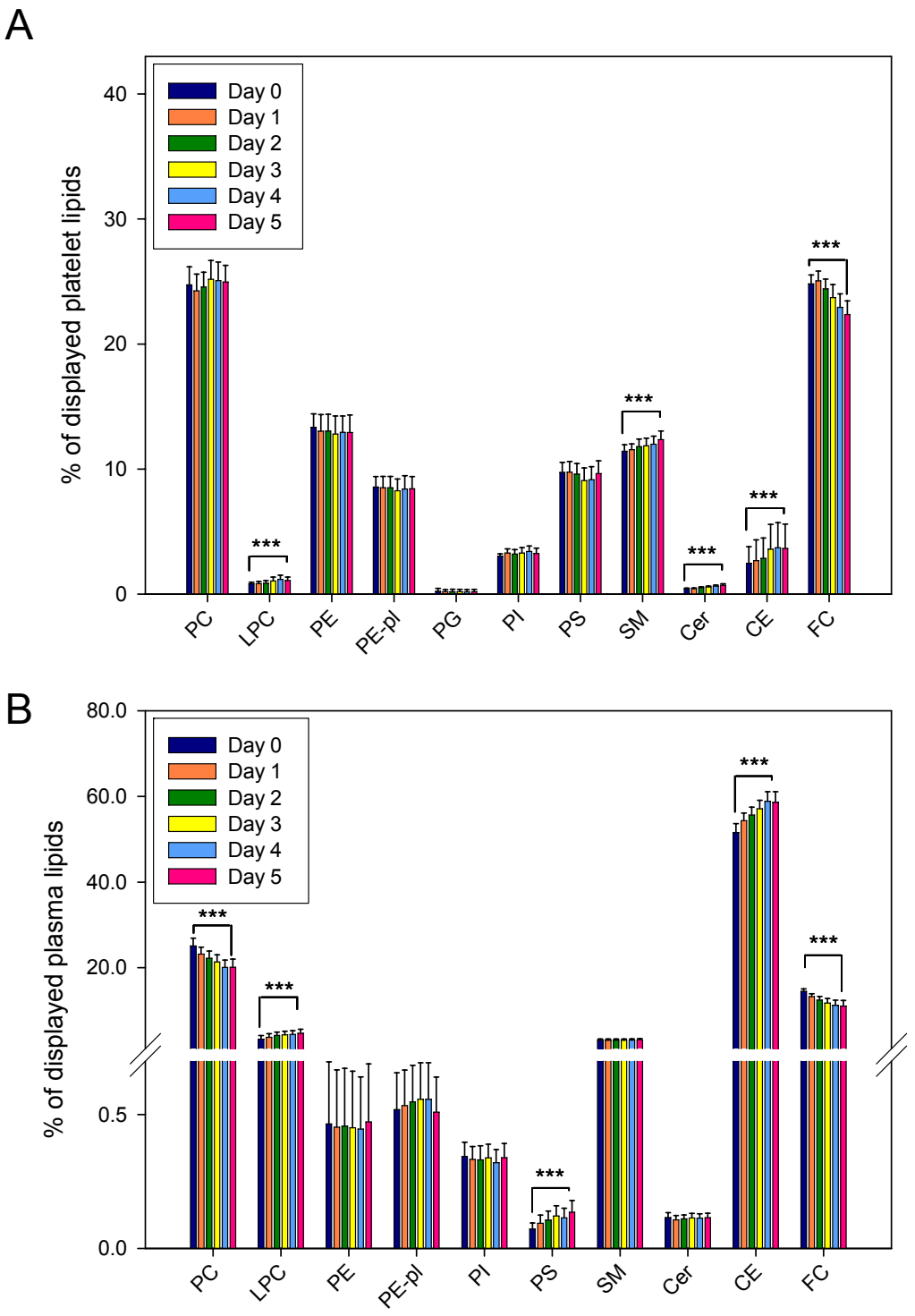


Figure 15: Lipid composition of senescent platelet concentrates.

Platelets and plasma were prepared as described in the legend to Figure 14. The displayed values are percent of the respective lipid class of all analyzed lipids in platelets (A) and appropriate plasma samples (B) during the platelet age of 5 days in different colours: day 0 (dark blue bars), day 1 (orange bars), day 2 (green bars), day 3 (yellow bars), day 4 (blue bars), day 5 (pink bars). Apart from cholesteryl esters (CE) and free cholesterol (FC), the shown lipids are phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylethanolamine (PE), PE-based plasmalogens (PE-pl), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), lysophosphatidylcholine (LPC) and ceramide (Cer). Values are mean ± SD from 50 different donors.

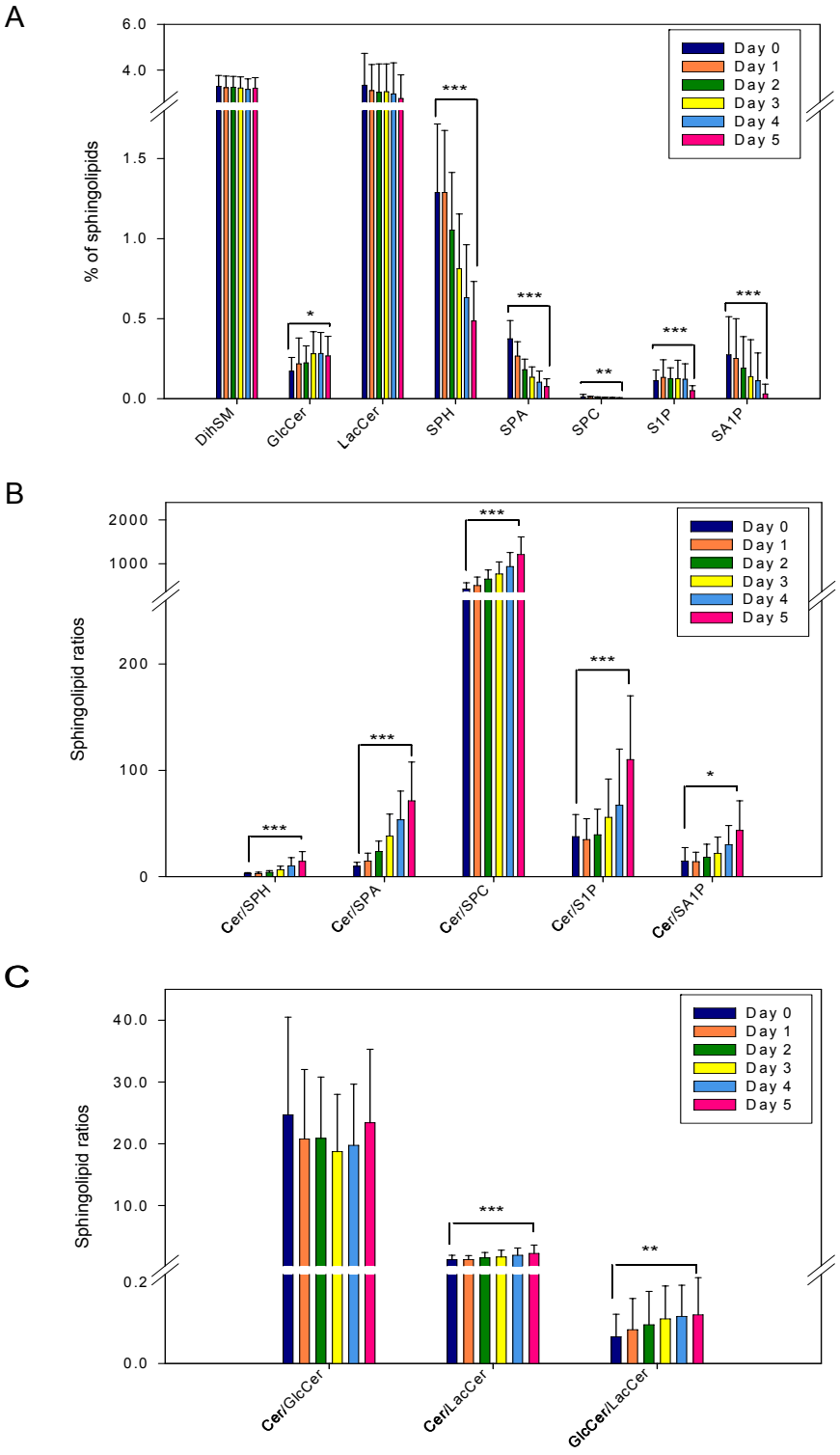


Figure 16: Sphingolipid composition and ratios of senescent platelets

Platelets were handled as described in the legend to Figure 14. Displayed are mol% of the respective sphingolipid classes of all analyzed sphingolipids in platelets stored for 5 days (A). Panel B displays the ratios of Cer to SPH, SPA, SPC, S1P and SA1P. Panel C illustrates the ratios of Cer to GlcCer and LacCer and of GlcCer to LacCer. For calculation of lipid ratios the quantitative values of respective sphingolipids in nmol/10^8 platelets were used. The lipid values for each day of storage are shown in different increments (day 0 (dark blue bars), day 1 (orange bars), day 2 (green bars), day 3 (yellow bars), day 4 (blue bars), day 5 (pink bars)). Values are mean ± SD from 50 different donors.

4.2.5 Lipid species composition of senescent platelets

Lipid species profiles related to the total content of the respective lipid class were determined for all collected platelet concentrates (Figure 17 and 18). The performed mass spectrometric analysis only allows the determination of the total number of carbon atoms and double bonds in the FA moiety for lipid classes containing two FA esterified to the glycerol-backbone. For example a PS 36:1 may represent different combinations of FA such as 18:0/18:1, 16:0/20:1, etc. Moreover, the assignment to a bond type (acyl or ether) is based on the assumption that FA with odd-numbered carbon-atoms constitute a negligible fraction. LPC, Cer and CE contain one FA denominated by the species nomenclature. Lipid species pattern which are significantly changed during platelet aging were PS (Figure 17A), LPC (Figure 17B) and Cer (Figure 17C). The composition of remaining lipid species which are not significantly altered during platelet storage was already shown in the first part of this thesis (4.1.3) (115). The major PS species were PS 36:1 and PS 38:4 with more than 60 mol% of total PS. During storage a shift from shorter, less unsaturated to longer, more unsaturated PS species was observed. Regarding LPC an elevation of 7.1 mol% occurred for saturated LPC 18:0. Cer species showed a clear increase of Cer 18:0 from 3 to 7 mol% as well as Cer 20:0 from 9 to 14 mol% while the longer Cer chains (Cer 23:0, Cer 24:1 and Cer 24:0) declined in total about 10 mol%. For plasma samples of platelet concentrates significant PS (Figure 18A) and LPC (Figure 18B) lipid species shifts were detected during storage. The three major plasma PS species showed a species specific relative increase for PS 36:1 of 19% and PS 38:4 of 90% and a decrease of PS 40:7 of more than 50% between collection and storage of five days (Figure 18A). In case of LPC the saturated species LPC 16:0 and LPC 18:0 increased both about 27% and 51% among the species, respectively. In contrast, the disaturated LPC 18:2 decreased from 28 to 16 mol% (Figure 18B).

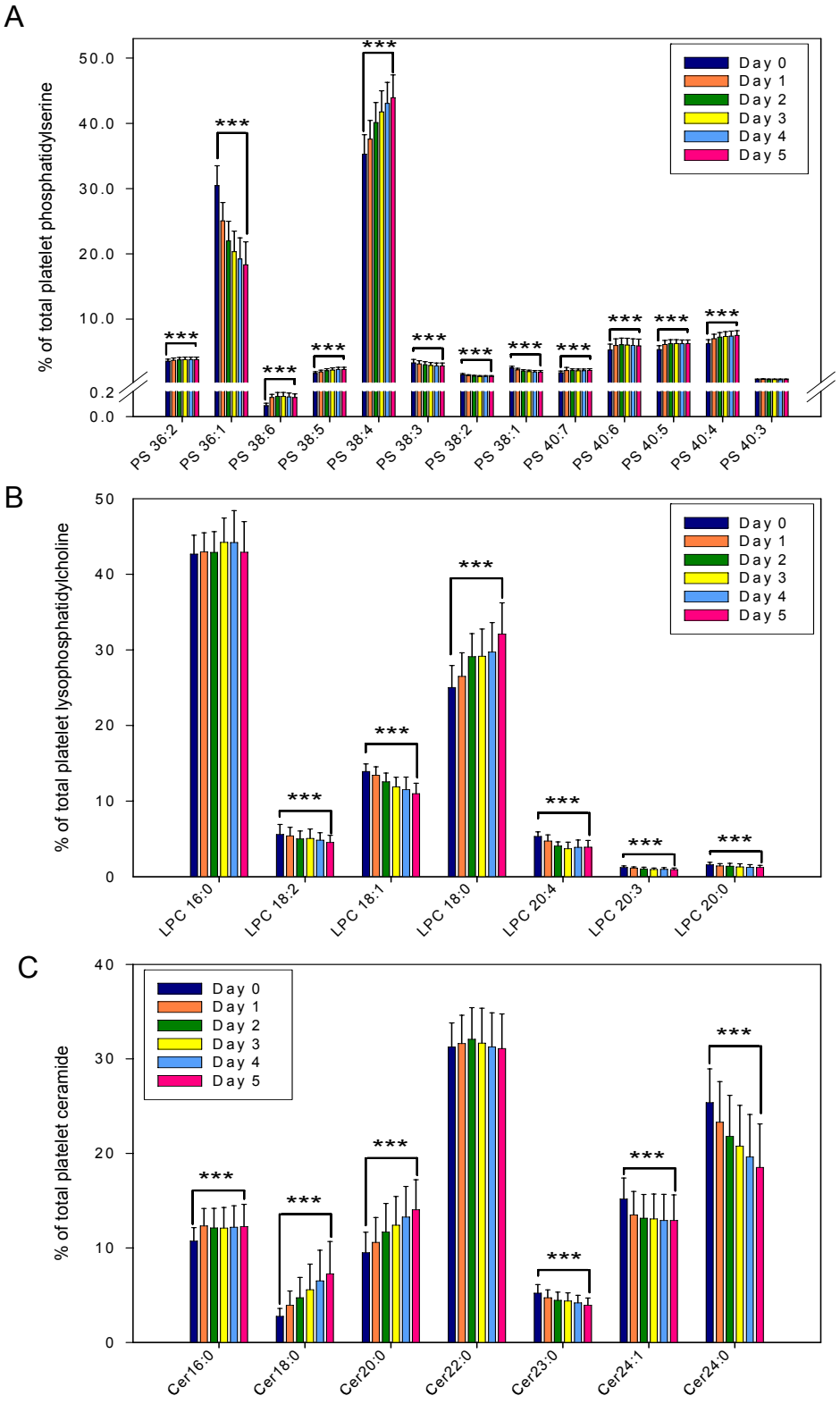


Figure 17: Lipid species profiles of senescent platelets.

Platelets were treated as described in the legend to Figure 14. Displayed are mol% of phosphatidylserine (PS) (A), lysophosphatidylcholine (LPC) (B) and ceramide (Cer) (C) species related to total lipid content of the appropriate lipid class for senescent platelets stored for 5 days. The lipid values for each day of storage are shown in different increments (day 0 (dark blue bars), day 1 (orange bars), day 2 (green bars), day 3 (yellow bars), day 4 (blue bars), day 5 (pink bars). Values are mean ± SD from 50 different donors.

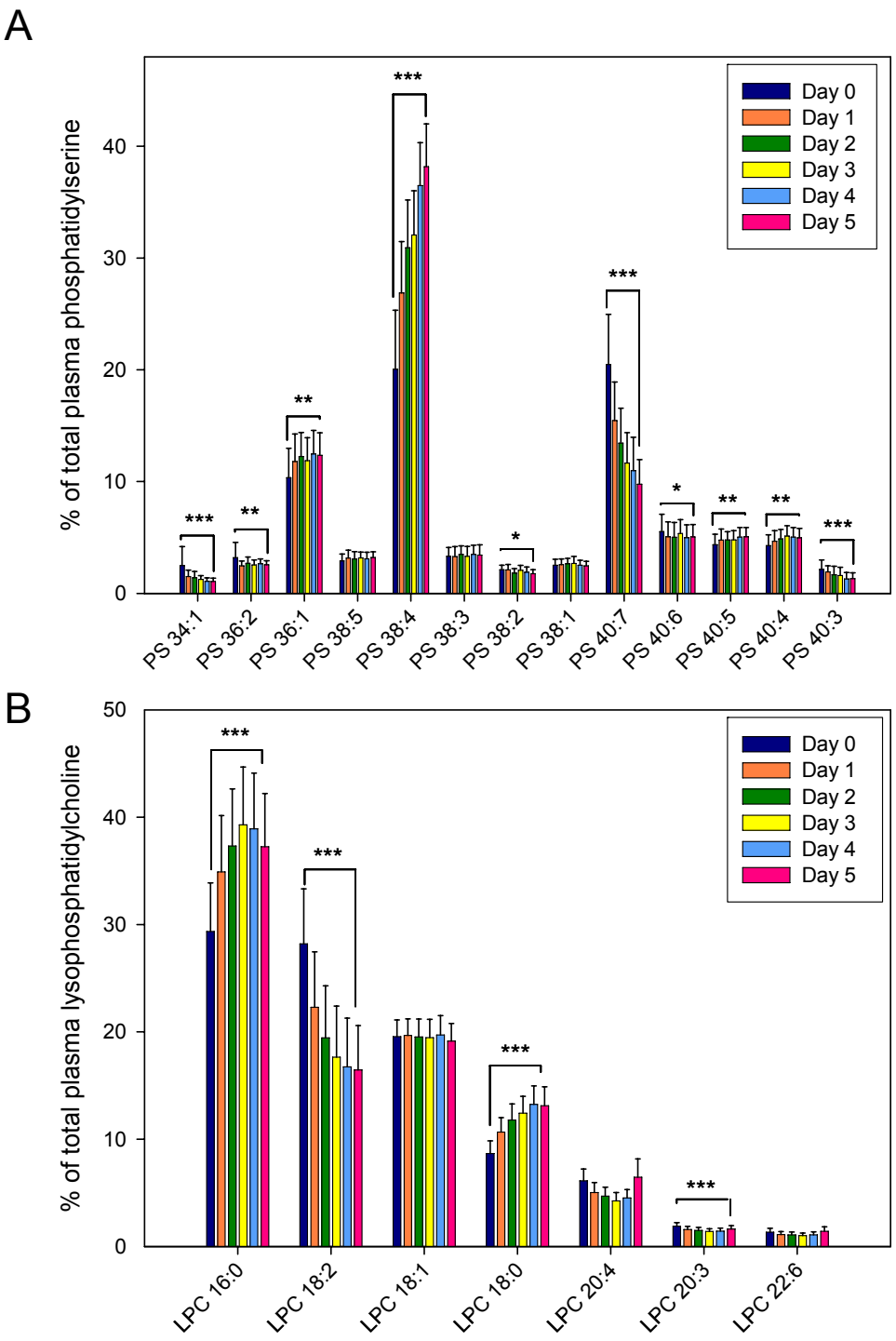


Figure 18: Lipid species profiles of plasma used for platelet concentrate storage.

Plasma was obtained and lipids extracted as described in the legend to Figure 14. Panel A shows phosphatidylserine (PS) species and panel B lysophosphatidylcholine (LPC) species in mol% of total lipid class. The different bars illustrate the age of plasma between plasma collection (day 0) and end of platelet concentrate storage (day 5). Values are mean ± SD from 50 different donors.

Regarding the CE lipid species pattern of platelets and plasma an identical distribution was observed together with a similar trend during storage of platelet concentrates with no significant changes (Figure 19).

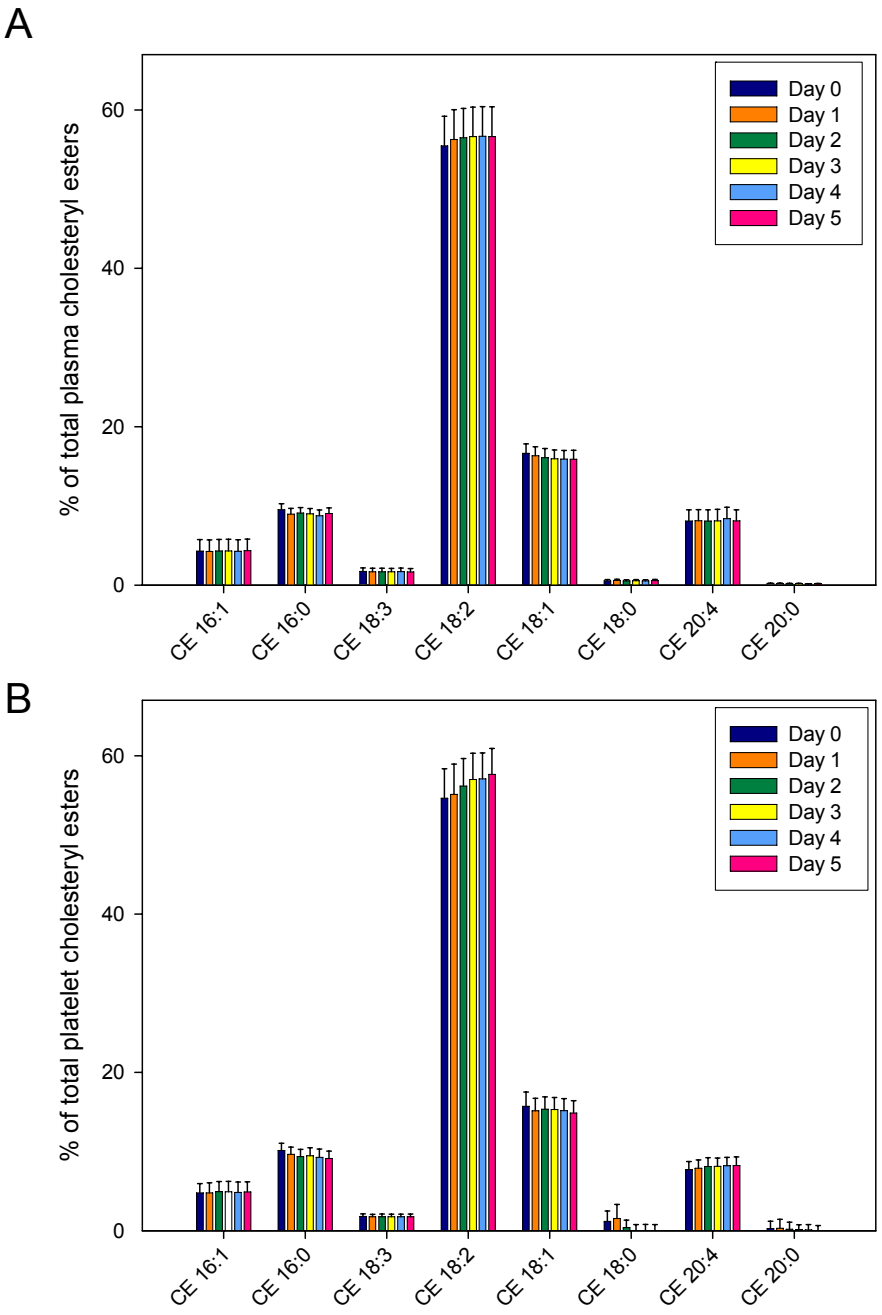


Figure 19: Cholesteryl ester (CE) species composition of plasma and platelets of apheresis products. CE species of day 0 (dark blue bars), day 1 (orange bars), day 2 (green bars), day 3 (yellow bars), day 4 (blue bars) and day 5 (pink bars) altered plasma (A) and platelets (B) are displayed in mol% of total analyzed CE. Values are mean ± SD from 50 different donors.

4.2.6 Correlation analysis of lipid changes during storage

In order to evaluate changes in the lipid profiles during storage of platelet apheresis products correlation analysis was performed. Normalization of the data was achieved by calculation of ratios day 1 to 0 and day 5 to 0 of lipid fractions, respectively. Since the changes observed for the plasma samples may be related to lecithin-cholesterol-acyltransferase (LCAT) activity, PC and LPC (Figure 20A) as well as FC and CE changes (Figure 20B) were correlated with each other. In both cases the changes revealed significant correlation with negative regression supporting the hypothesis of an ongoing esterification reaction of FC to CE.

As a next step it was asked how changes in the plasma influenced platelet lipid composition. Therefore, correlation of lipid changes in plasma and platelets was analyzed. Except for PC (Figure 21A) significant correlations with positive regression for LPC (Figure 21B), FC (Figure 21C) and CE (Figure 21D) were detected indicating a direct link between certain plasma and platelet lipids. Taken together these data indicate that LCAT activity changes lipid composition in the plasma of platelet concentrates which consequently affects platelet LPC, FC and CE composition.

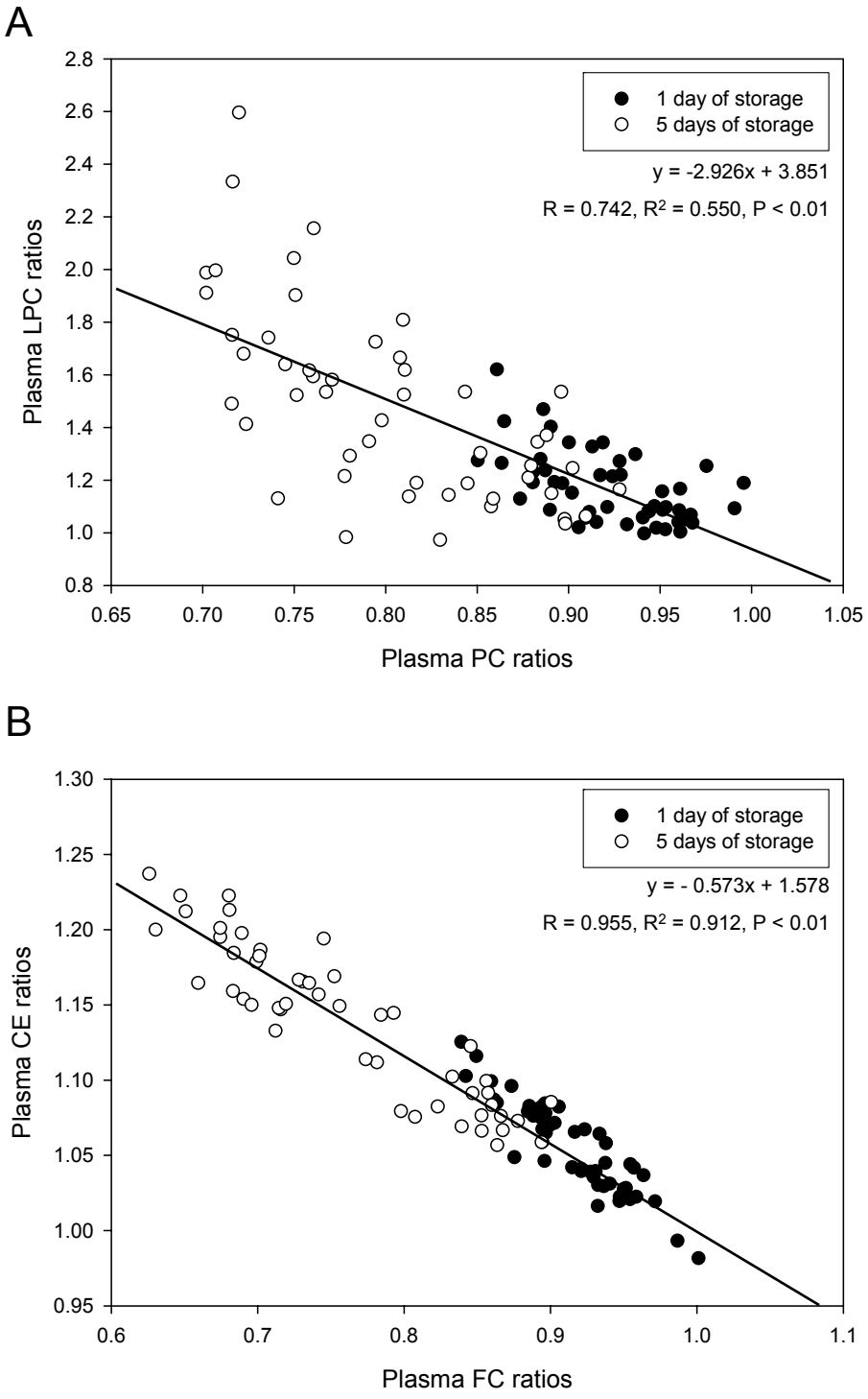


Figure 20: Correlation analysis between plasma lipids of apheresis products.

Displayed are the bivariate correlation analyses with Pearson correlation coefficient between the ratios day1/day0 (black circles) and day5/day0 (white circles) of different plasma lipids. The lipid ratios after one and five days of storage compared to collection day were calculated from 50 different donors. Panel A shows the correlation between plasma phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) ratios and panel B the correlation between plasma free cholesterol (FC) and cholesteryl esters (CE). A linear regression analysis was performed to determine the correlation coefficient (R) and the coefficient of determination (R^2).

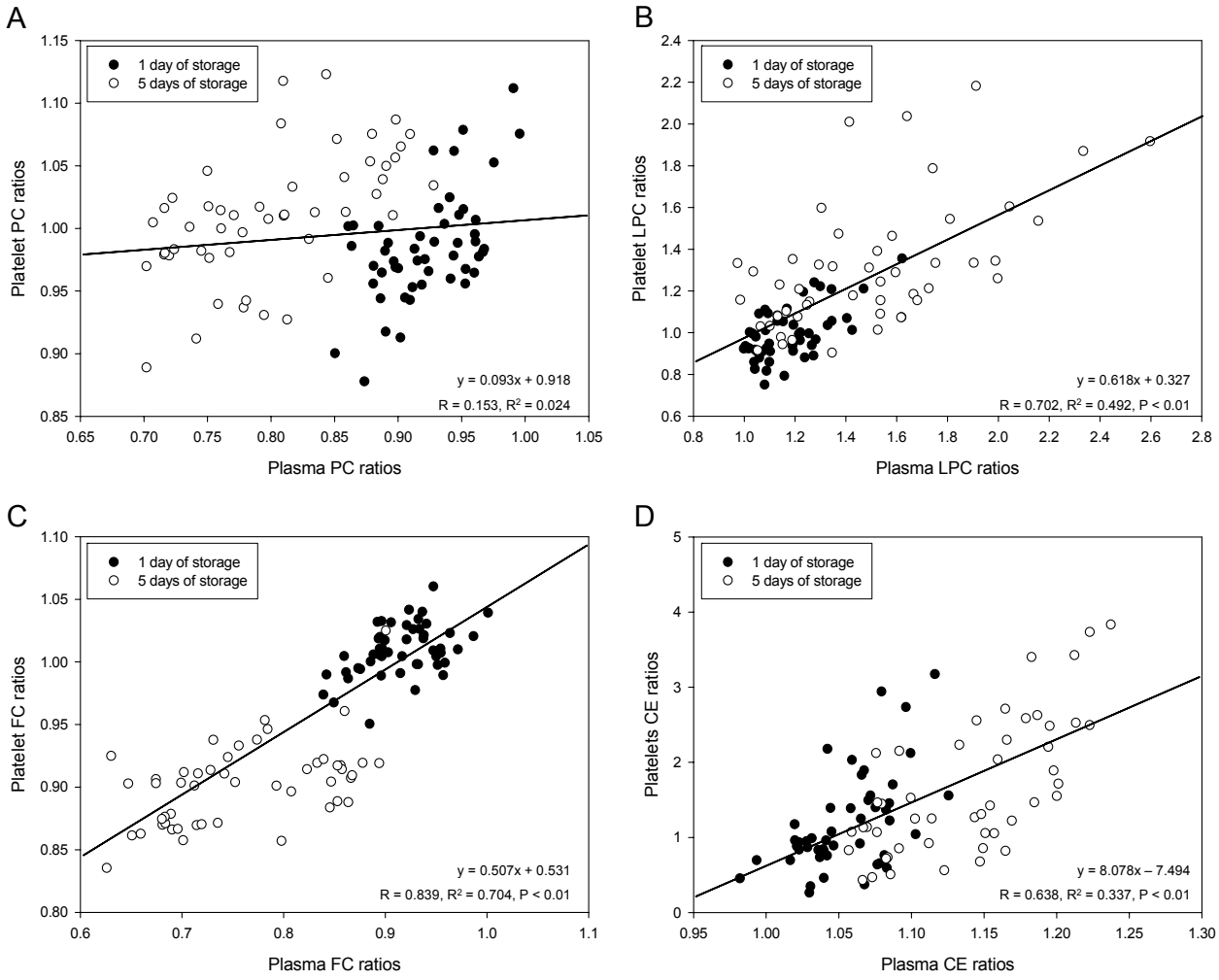


Figure 21: Correlation analysis between platelet and plasma lipids of apheresis products.

Bivariate correlation analyses with Pearson correlation coefficient combined with a linear regression analysis was performed between platelet and plasma lipids. The ratios of one (black circles) and five (white circles) day aged platelet apheresis products in relation to collection day (day 0) were analyzed for the following lipids in plasma and platelets: phosphatidylcholine (PC) (A), lysophosphatidylcholine (LPC) (B), free cholesterol (FC) (C) and cholesteryl esters (CE) (D). The values are obtained from 50 different donors.

5. Discussion

5.1 Lipidomic analysis of circulating human blood cells

The blood cell compartment can be regarded as a liquid organ. To identify novel cellular biomarkers, blood cells are easily accessible as compared to tissues and solid organs. The composition of lipid species of different human blood cells appears to be unique and cell specific and any deviation may disrupt cellular homeostasis (116).

In general the data showed a good correlation to previous studies presenting mostly lipid class analysis in these cells. Phospholipid classes of platelets (82) as well as their detailed phospholipid species composition (83) correspond to the obtained results. PC, PE, PE-pl, PS and SM phospholipid species of PBMC determined by a normal-phase LC–MS/MS method (89) also correlated to the results for the major phospholipid species of monocytes and lymphocytes. A study by Postle et al. (90) investigated the dynamics of the PC metabolism and observed a similar species pattern of lymphocytes and neutrophils as in the present study. Moreover, the distribution of lipid classes including PC/SM and PC/FC ratios of RBC fitted well to measured data (76;79).

The most interesting differences of the lipid species profile were found for granulocytes, platelets and RBC. More than 100 billion granulocytes are produced everyday and enter the blood (46). Neutrophils, the main granulocyte cell type, display about 50-70% of leukocytes. They have a short life time of only 8-20 hours. If they are not activated and constitutively undergo apoptosis (117). Hence, an elevated Cer content of granulocytes (predominantly Cer 16:0, Figure 11B), which was at least three-fold higher compared to other analyzed blood cells (Figure 7B),

could be a sign of pro-apoptosis of neutrophils. In addition, granulocytes are characterized by a decreased level of highly polyunsaturated FA species (≥ three double bounds) in the main phospholipid classes and an elevated PE-pl lipid content. It is well known that neutrophils produce superoxide to kill pathogens like bacteria. Because plasmalogens are described as cellular antioxidants (118), both a low content of polyunsaturated FA, as a major target of free radical attack (119), and a high content of plasmalogens may be protective mechanisms of granulocytes against oxidative stress. Additionally, a high PE-pl content may reflect a high peroxisomal activity, since essential steps of the plasmalogen biosynthesis reside in peroxisomes (11;120).

Another interesting observation was the high CE content of platelets (Figure 7A) with CE 18:2 as main species (Figure 12). CE 18:2 also represents the main CE species in plasma (35) while the cellular FC esterifying enzyme sterol O-acyltransferase (SOAT1) preferentially uses oleoyl-CoA (18:1) (121). Therefore the question arises whether the high CE content of platelets originates from plasma lipoproteins. To answer this question an additional study is required analyzing in more detail the effect of plasma lipids on platelet lipids (see 4.2).

The membrane fluidity of blood cells is mainly determined by their phospholipid/cholesterol molar ratio (79) and seems to be adapted to the cellular function. The PC/FC ratio of platelets was 0.8 and clearly lower in comparison to leukocytes (1.9 in monocytes, 1.3 in lymphocytes and 1.1 in granulocytes) (Figure 7A). The increased amount of FC relative to phospholipids in platelets reflects more rigid and non-fusogenic membrane features which may be necessary to prevent premature clot formation. In contrast, the highest PC/FC ratio in monocytes may relate to their migratory capability into the endothelial space and phagocytic activity, which require a fluidic membrane. RBC have to be able to repeatedly pass through

capillaries four times smaller than their own size (122) and to keep their biconcave shape to provide a large surface for gas exchange (123). This may be reflected by a low PC/SM ratio together with the highest cholesterol content in RBC (Figure 7A) indicating a less fluid membrane (79).

In summary, different circulating blood cells of healthy human donors are characterized by unique lipid class and species pattern. The lipid composition of blood cells matched with their functional requirements concerning blood cell shape and size in the vascular system. The current study provides for the first time a detailed overview of lipid species in circulating blood cells generated using ESI-MS/MS as a single platform. Moreover, this study may be a reference for the search of novel lipid biomarker in circulating blood cells in patients with blood cell-related disorders, vascular and metabolic diseases, thrombosis and systemic inflammation.

5.2 Lipidomic analysis of platelet senesence

The present study identified significant changes in lipid profiles of platelets and plasma during storage of platelet concentrates. In agreement with previous studies a loss of total lipids in platelets was found (100;101) that is accompanied by an increase of plasma lipids (Figure 14) providing evidence for lipid cession due to microparticle shedding during platelet senescence.

The data showed a good correlation to previous studies concerning the lipid class distribution of platelets (82;115) and the total lipid content (115). This total lipid loss with a decline of 9% for total phospholipids and 18% for free cholesterol matched well with the results detected by Hamid et al. where a loss of 15% total cholesterol and a loss of up to 11% for phospholipids was observed for platelets stored at 20°C (100). Okuma et al. found a loss of 30% for cholesterol and 15% for phospholipids during

storage at 4°C (101). The higher difference compared to the latter study was probably due to isolation and storage of platelets at 4°C. Another explanation may be related to the preparation of platelets since previous studies use washing and several centrifugation steps. In contrast to theses studies the platelets in this study were separated by gel-filtration which was already recommended for analysis of platelet function (113;114). Additionally, it was shown that this approach provided negligible platelet activation and a clear separation of lipoprotein and platelet fractions to exclude lipid contamination in the platelet analysis (Figure 13).

The question arises whether lipid alterations observed in this study are associated with storage lesion and apoptosis? In non-nucleated red blood cells a process called “eryptosis” occurs due to energy or antioxidant depletion causing a loss of membrane PS asymmetry, with consequent exposure of PS at the red blood cell surface (124). Similarly, senescent, activated platelets also loose membrane asymmetry (125). Leytin et al. (105) observed an increase of 13.3% PS exposure after five days storage of platelet apheresis products which may be related to PS species shifts both in platelets and plasma (Figure 17A and 18A). Another potential explanation may also be connected to the release of PS-rich microvesicles which are able to re-associate with the platelet membrane (126). Besides changes of PS species, one novel finding of this study was a relative increase of 69% of the Cer fraction (Figure 15A) as well as a shift in the Cer species profile (Figure 17C) during platelet storage. Although platelets are anucleate cells and do not show cell growth, reticulated platelets seem to undergo terminal differentiation (127) and programmed cell death during senescence (128;129), biological processes in which sphingolipids play an important role (130). Previous studies already mentioned the function of certain Cer species in apoptosis. In this context Cer 16:0 (131;132) and Cer 18:0 (133) were highlighted. It was also described that Cer generation and oxidative stress are

connected to cellular death (134). However, no significant amounts of oxidized lipid species were observed in the mass spectrometry data. It is tempting to speculate that observed lipid changes for PS and Cer are probably signs of rising apoptosis playing a minor role during conventional storage with more impact after platelet storage prolongation.

Further sphingolipid metabolites were analyzed by LC-MS/MS because the sphingolipid metabolism in platelets has special features (Figure 22). Platelets are deficient in de novo sphingolipid biosynthesis starting from condensation of L-serine and palmitoyl-CoA by serine palmitoyl CoA transferase (SPTLC) (135). Platelets also lack S1P lyase (SGPL) activity (136). Yang et al. (137) found out that exogenously added [^{3}H]SPH and [^{3}H]C_6-Cer were rapidly converted to [^{3}H]S1P while the conversion to [^{3}H]SM was very weak (138) indicating a persistently active spingosine kinase (SPHK) activity (130). However, the maximum time points for [^{3}H]SPH and [^{3}H]C_6-Cer stimulation in this study ranged between 15 and 180 min, respectively (137). In contrast the platelet senescence in this thesis was studied until day five of storage revealing an increase of Cer with an associated decrease of SPH, SPA, SA1P, S1P and SPC (Figure 16A/B). It seems that the high SPH kinase activity decreases during aging, moving towards a more Cer dominated the equilibrium (Figure 22). That means, distinct from the well-known SM breakdown via acid sphingomyelinase (SMPD) to Cer, that the presence of a transmembrane cycling pathway in platelets starts with S1P phosphatase (SGPP) (130). The conversion of SPH to Cer occurs via intracellular Cer synthase (LASS) (139). The different LASS homologs synthesize Cer with a different FA composition (140). In this study Cer 16:0, Cer 18:0 and Cer 20:0 significantly increased during aging (Figure 17C) indicating a main activity of LASS1, 4 and 5 (140). However, no isoform specificity for sphingolipid enzymes in platelets has been described so far. To fully understand the

sphingolipid metabolism during aging, it will be necessary to measure the distinct enzyme activities and trace the pathways of certain sphingolipid metabolites after longer incubation of labeled SPH and SPA.

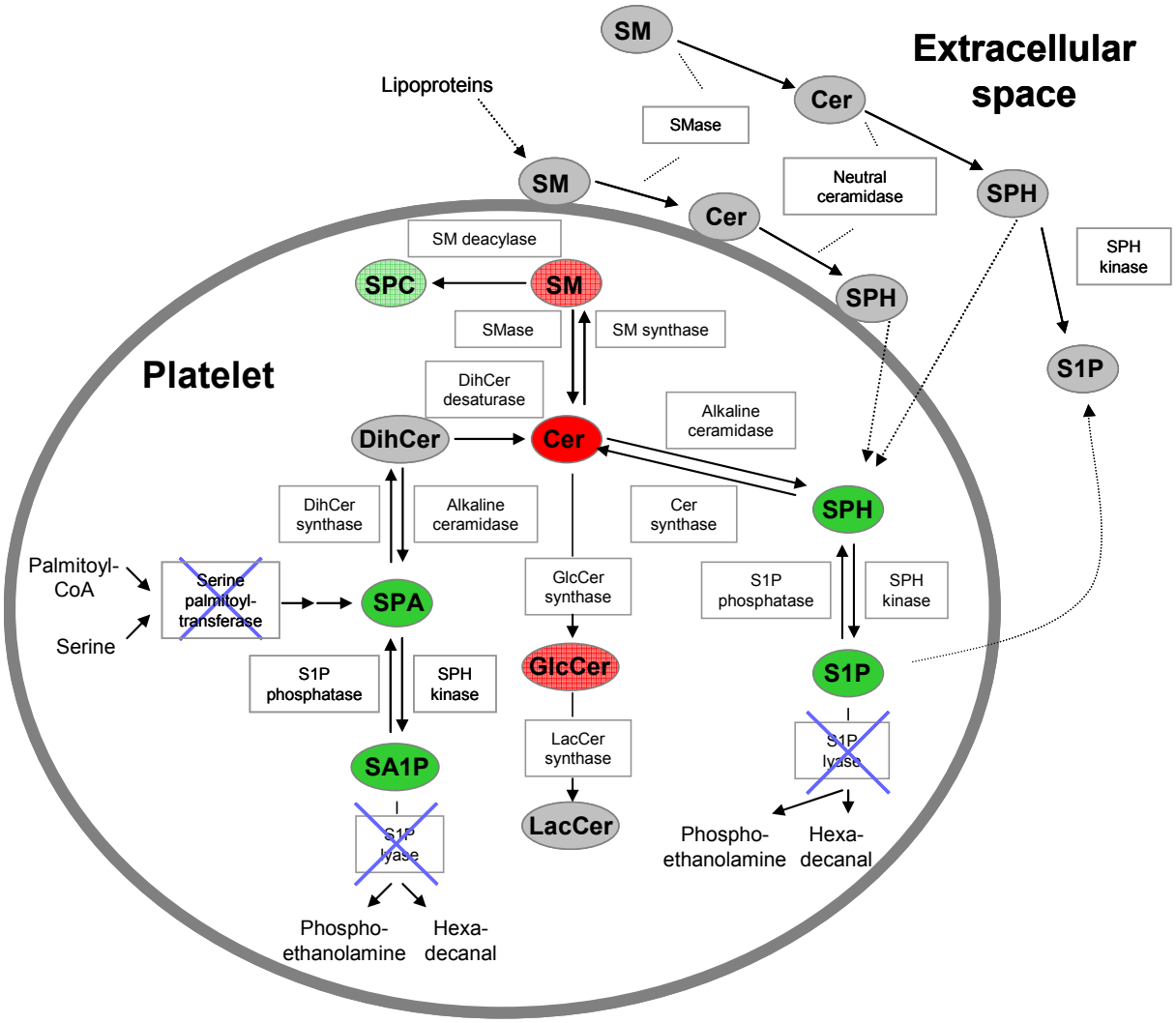


Figure 22: Sphingolipid metabolism in platelets during senescence
The first step of *de novo* sphingolipid synthesis in platelets, the condensation of L-serine and palmitoyl-CoA, is blocked in platelets (135). Platelets are also the only cell type known where no S1P lyase activity was detected (141). Platelets use SPH at the outer leaflet of the plasma membrane and in the extracellular space (135;142). Cer stands in the center of the whole sphingolipid metabolism. During aging SM, Cer and GlcCer were increased (marked in red) while SPH, SPA, S1P, SA1P and SPC were decreased (marked in green) (adapted from (143;144)).

Another interesting observation of the present study is the trend of plasma lipid changes in platelet concentrates during storage. Decreases of PC and FC were

accompanied with increases of LPC and CE (Figure 15B). These changes can be explained by FC to CE conversion due to action of LCAT. In order to substantiate the hypothesis, a correlation analysis of the lipid composition changes observed in plasma was performed and significant correlations for PC and LPC and notable for FC and CE were found, respectively (Figure 20 A and B).

Since this study analyzed plasma and platelets of 50 platelet concentrates, it was possible to study the influence of lipid changes in plasma on the platelet lipid composition by correlation analysis. This way evidence was provided that increases of LPC and CE as well as the decline of FC fractions in platelets were caused by changes in the plasma compartment during storage (Figure 21B-D). An explanation why any correlation between the changes of plasma and platelet PC fractions (Figure 21A) was observed may be that platelets metabolize and do not simply “store” PC. The exceptional high content of CE in platelets compared to other blood cells was already shown in Figure 7A (115). Further evidence for a CE plasma uptake came from the similarity of plasma and platelets CE species profiles (Figure 19). This is also substantiated by a specific substrate preference of the different esterifying enzymes. Intracellular sterol O-acyltransferase (SOAT1) preferentially uses oleoyl-CoA (18:1) for storage in lipid droplets while CE 18:2, the main CE species in platelets (Figure 19), is a specific product of the LCAT reaction in plasma (121). Taken together these data clearly argue for the uptake of lipoprotein lipid components into platelets during storage. These changes due to LCAT activity may be of relevance for transfusion of platelet concentrates since LPC, generated during storage, was shown to activate NADPH oxidase, potentially playing a role in severe complications of transfusion therapy (145;146).

The question arises how plasma lipoproteins are “stored” within the platelets? There are three major mechanisms explaining the lipid transfer from the plasma

compartment to the platelets: (i) internalization of lipoprotein particles into the cells (e.g. by endocytosis or trapping in the open canalicular system (OCS)), (ii) total (specific and unspecific) binding of lipoproteins to the platelet surface and (iii) selective uptake of phospholipids by cellular membranes. Concerning the first mechanism (i) most reports agree that endocytosis of lipoprotein particles into platelets is unlikely (147). However, the uptake and trapping of plasma components within the OCS seem to be rather possible. Platelets enhance their cell surface by an OCS with tubular invaginations (148) constituting up to 25% of total platelet volume (149). Measurements of OCS diameter of platelet electron microscopic pictures revealed a range between 70-160 nm in resting platelets, therefore large enough to incorporate even a VLDL particle (30-80 nm) (Figure 23). During platelet storage the total volume fraction of the OCS increased from 7% at day 1 to 17% at days 5 and 8 (149). Concerning the two other described mechanisms (ii) and (iii) Engelmann et al. (109) pointed out that both yielded comparable proportions of lipoprotein particles bound to the platelets. This group also mentioned that the incorporation of lipoprotein-derived phospholipids into platelet membranes may become more and more independent of lipoprotein binding to the cells after prolonged incubation of lipoproteins with platelets. This may be particularly relevant under *in vitro* conditions during storage of platelet concentrates for several days. In this context the incorporation of plasma components into platelet membranes would play a minor role compared to trapping in a dilated OCS. However, the hypothesis of engulfment of protein and lipid plasma components in the OCS has to be further elucidated by labeling experiments.

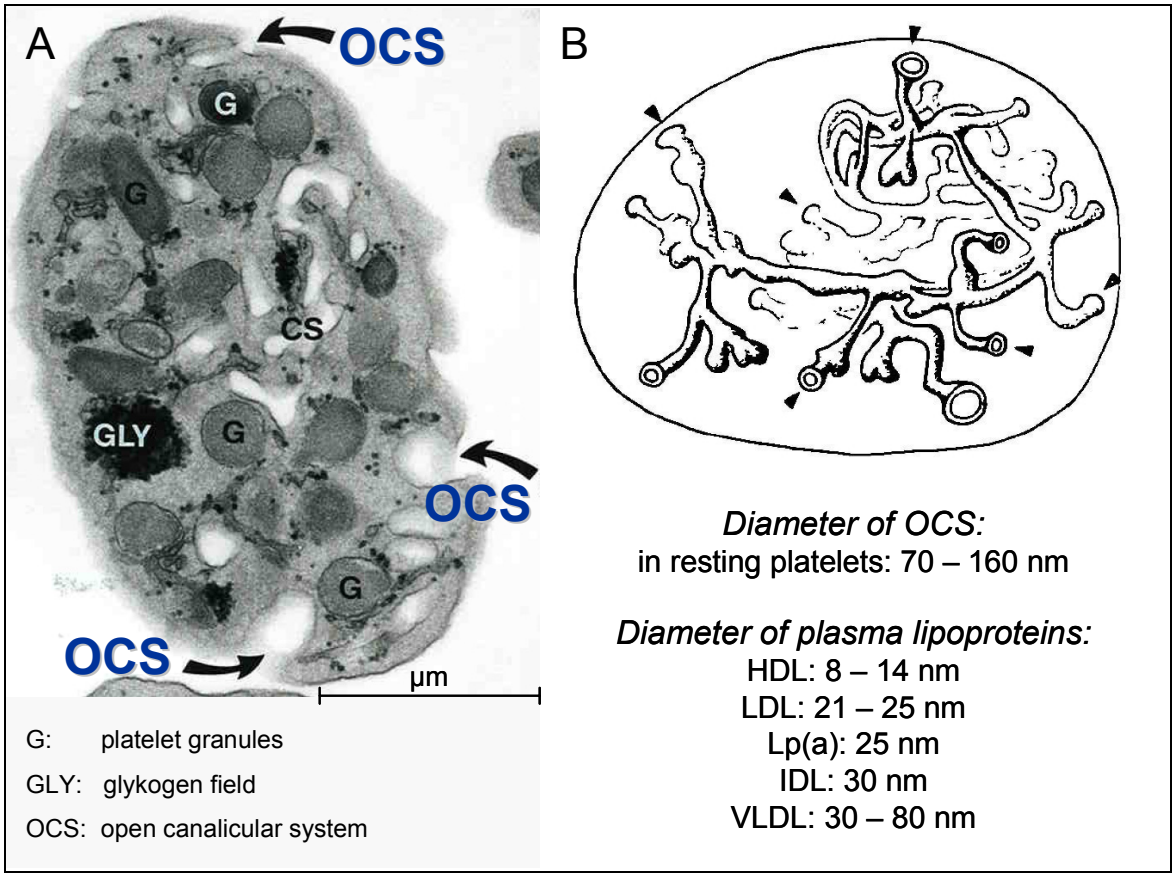


Figure 23: The open canalicular system (OCS) of the platelets

Panel A illustrates the ultrastructure of resting human platelets (equatorial plane) taken by electron microscopy. Panel B shows a schematic drawing of the three-dimensional reconstruction of the OCS in a human platelet which is distributed throughout the cytoplasm and consists of one continuous tubular system inside the cell, although it has many braches and protrusions (150). The arrowheads indicate the orificium of the OCS.

In summary, the current study provides for the first time a detailed overview of lipid species shifts in senescent platelets and plasma analyzed by ESI-MS/MS. Moreover, clear evidence for LCAT-mediated changes in plasma and platelet lipid compositions were shown. However, further investigations have to show how much these lipid changes contribute to poor post-transfusion survival and haemostatic function of platelets. The current study could contribute to enhance our understanding of cellular senescence and platelet storage.

6. Conclusion

The blood cells and plasma harbours a massive amount of information about the functioning of all tissues and organs in the body (40). Therefore an accurate, fast, and affordable analysis of the cellular components of blood is of prime interest for laboratory and transfusion medicine and clinical research. The field of lipidomics adds an additional layer of information to data derived from proteomics and genomics providing additional knowledge of blood cell function. Therefore the aim of this thesis was to compare the individual blood cell lipid pattern of healthy donors and to highlight specific differences of the individual blood cells in relation to function.

In the first part of the study the different blood cells, monocytes, lymphocytes, granulocytes, platelets and red blood cells (RBC), of healthy human individuals were analyzed using electrospray ionization tandem mass spectrometry (ESI-MS/MS). Striking differences among the examined blood cells were detected. It was shown that the different blood cells were characterized by unique lipid class and species patterns. The predominant lipid classes were phosphatidylcholine (PC) and free cholesterol (FC) with cell type specific PC/FC ratios as markers of membrane fluidity which were 1.9 in monocytes, 1.3 in lymphocytes, 1.1 in granulocytes, 0.8 in platelets and 0.3 in RBC, respectively. Beside a 3-fold elevated ceramide (Cer) level of 2.6 mol%, granulocytes revealed the highest percentage of phosphatidylethanolamine-based plasmalogens and a decreased fraction of highly polyunsaturated (≥ 3 double bonds) species compared to other cell types. RBC also showed a remarkable shift of glycerophospholipid chain length. In platelets a nearly 4-fold increase of the cholesterol ester (CE) 18:2 (linoleic acid) fraction (55 mol% of total CE) together with the highest content of total CE in all analyzed blood cells was found. Furthermore a nearly identical distribution of CE lipid species pattern between platelets and plasma was observed.

For this reason platelets were chosen as the blood cell type to be analyzed in more detail in related, therapeutic blood products. In this second part of the work plasma

and platelet lipids of 50 platelet apheresis concentrates stored under agitation for five days at 22°C were studied. This limit was chosen because legal regulations restrict the use of platelet concentrates older than five days in transfusion medicine. During storage time the lipid content decreased by 10% in platelets and increased by 5% in plasma. A more detailed analysis of additional sphingolipids revealed significant decreases of 63% for sphingosine, 78% for sphinganine, 68% for sphingosylphosphorylcholine, 55% for sphingosine-1-phosphate and 89% for sphinganine-1-phosphate and an increase of 69% for Cer between lipid fractions of fresh and five days aged platelets providing novel information about the sphingolipid metabolism in platelets in relation to senescence. In case of glycerophospholipids and sterols the following changes in lipid fractions relative to day of preparation were found: Increases of 32% lysophosphatidylcholine (LPC) and 49% CE and a decrease of 10% FC in platelets; elevation of 43% LPC and 14% CE and a decline of 20% PC and 24% FC in plasma. Significant lipid species shifts were also observed for phosphatidylserine, Cer and LPC. Really interesting results concerning lipid transfer between the plasma and the cell compartment arose from calculations of lipid correlations. The data provided clear evidence for lecithin-cholesterol acyltransferase (LCAT) mediated esterification of FC and generation of CE and LPC in the plasma of platelet concentrates. These lipid changes also correlated between plasma and platelets for LPC, FC and CE fractions. It could be concluded that plasma lipid changes impact the platelet lipids, in particular CE species, leading to the characteristic platelet lipid distribution observed in the first part of this work.

The whole study perfectly demonstrates that the application of ESI-MS/MS for lipidomic analysis leads to novel, interesting results useful for a better understanding of the blood cell compartment. The obtained results could serve as a reference for further studies with blood cell material of patient cohorts, opening new opportunities in drug and biomarker development for blood cells, in particular at the various stages in the preclinical and early clinical categories.

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8. Publications

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Review articles

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9. Eidestattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet. Bei der Auswahl und Auswertung folgenden Materials haben mir bereits aufgeführte Personen in der Danksagung in der jeweils beschriebenen Weise unentgeltlich geholfen. Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, den 01. Dezember 2009

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(Katharina Rübsaamen)