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ORIGINAL ARTICLE

Distribution and dynamic changes of sphingolipids in blood in response to platelet activation

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Summary. *Background:* Sphingolipids are signaling molecules in a range of biological processes. While sphingosine-1-phosphate (S1P) is thought to be abundantly stored in platelets and released upon stimulation, knowledge about the distribution and function of other sphingolipids in blood is lacking. *Objectives:* To analyze the sphingolipid content of blood components with special emphasis on dynamic changes in platelets. *Methods:* Blood components from mice and humans were prepared by gradient centrifugation and analyzed by liquid chromatography–mass spectrometry. Additionally, murine platelets were activated *in vitro* and *in vivo*. *Results:* Isolated non-activated platelets of mice were devoid of S1P, but instead contained dihydrosphingosine-1-phosphate (dhS1P), along with a high concentration of ceramide. Activation of platelets *in vitro* led to a loss of dhS1P and an increase in sphingosine, accompanied by a reduction of ceramide content. Platelet activation *in vivo* led to an immediate and continuous rise of dhS1P in plasma, while S1P remained stable. The sphingolipid distribution of human blood was markedly different from mice. Human platelets contained dhS1P in addition to S1P. *Conclusions:* Mouse platelets contain dhS1P instead of S1P. Platelet activation causes loss of dhS1P and breakdown of ceramide, implying ceramidase activation. Release of dhS1P from activated platelets might be a novel signaling pathway. Finally, the sphingolipid composition of mouse and human blood shows large differences, which must be considered when studying sphingolipid biology.

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Introduction

Sphingolipids are a complex class of lipids named for their mysterious, sphinx-like character. While they were first described as cell membrane constituents, knowledge about their function has dramatically expanded over the last decades. Not only are they involved in dynamic membrane functions, e.g. signal transduction by membrane microdomains, but they can also act as first and second messengers in a variety of signaling pathways (reviewed in [1]). Ceramide, the simplest sphingolipid, is composed of sphingosine and a fatty acid, and serves as the precursor for the more complex sphingolipids such as sphingosine-1-phosphate (S1P). Ceramide can be formed by the breakdown of sphingomyelin or de novo synthesis, and is associated with cellular senescence and death [2]. Sphingosine, the backbone molecule of ceramide, can be phosphorylated by sphingosine kinases 1 and 2 [3] to produce S1P, which binds to S1P receptors 1–5 ($S1P_{1-5}$, also called Edg-1, -5, -3, -6, and -8, respectively). S1P influences many biological processes, such as vascular development [4], carcinogenesis [5], chemotaxis [6] and proliferation [7].

While the ceramide molecule lacking the trans double bond (i.e. dihydroceramide) does not share the ability of ceramide to induce apoptosis, very little is known about the function of the corresponding saturated sphingosine, dihydrosphingosine. Dihydrosphingosine is also metabolized by sphingosine kinases [3], yielding dihydrosphingosine-1-phosphate (dhS1P). There is scant knowledge about the function of dhS1P, although it does bind to several S1P receptors [8]. The first description of differential effects of S1P and dhS1P was recently published [9].

Interest in sphingolipid biology has been enhanced by the recent development and clinical application of the new immunosuppressive agent FTY720, which causes lymphocyte sequestration by targeting S1P receptors [10]. While S1P is thought to be abundantly stored in platelets and released upon stimulation [11,12], knowledge about the distribution and

function of other sphingolipids in blood is lacking. In an organism, blood is the central distributor of many signaling molecules. Therefore, we set out to analyze the sphingolipid content of blood components *in vivo*, with special emphasis on platelet biology.

Material and methods

Reagents

Histopaque-1077, Histopaque-1083, Histopaque-1119, bovine serum albumin (BSA), murine thrombin, $CaCl_2$ and $MgCl_2$ were from Sigma-Aldrich (Buchs, Switzerland). Fetal calf serum (FCS) was from PAA Laboratories (Pasching, Austria).

Animal studies

All animal experiments were approved by the veterinary office of the canton of Zurich and were carried out according to institutional guidelines. Balb/c mice (Harlan Laboratories, The Netherlands) were kept under controlled environmental conditions with a 12-h light–dark cycle. *In vivo* platelet activation was induced by i.p. injection of 1 mg kg^{-1} anti-CD41 antibody (MWReg30; BD Biosciences, San Jose, CA, USA, 553847) in 200 μL phosphate-buffered saline (PBS). Control mice were treated by i.p. injection of an equivalent amount of non-immune immunoglobulin G2 (BD Biosciences). Group sizes were $n = 5$ at all time points. Blood was drawn under isoflurane anesthesia from the inferior vena cava and anticoagulated with ethylenediaminetetraacetic acid (EDTA) at a final concentration of 6 mM. Blood cell counts were determined with a Coulter AcT Diff counter (Beckman Coulter, Nyon, Switzerland). Immunohistochemical staining of activated platelets was performed using the rabbit polyclonal anti-CD61 antibody (#4702; Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. Digital images of liver sections at 400× magnification were acquired on an Axioplan 2 microscope (Carl Zeiss, Feldbach, Switzerland) using the Axiocam HrC digital camera (Carl Zeiss).

Isolation of blood components

All procedures were performed at room temperature. Murine blood was diluted 1:3 in calcium-free PBS, overlaid on Histopaque-1119 and Histopaque-1083 and centrifuged at $700 \times g$ for 30 min, yielding a separation into five components. The uppermost, component 5 (plasma), was removed and stored at −80 °C. Components 1 (erythrocytes) and pooled components 3 plus 4 (platelets and leukocytes) were diluted with PBS-FCS 2%, overlaid on PBS–FCS 2%–BSA 20% and centrifuged for 15 min at $300 \times g$. This step was repeated for the pellet of component 1 (i.e. erythrocytes), the pellet of components 3 + 4 (i.e. leukocytes) and the supernatant of components 3 + 4 (i.e. platelets). The supernatant of components 3 + 4 was then centrifuged at $1500 \times g$ for 15 min to pellet the platelets. Finally, all components were washed in PBS and pelleted at $1500 \times g$. After removing aliquots for cell counting and fluorescence-activated cell sorter (FACS) analyses, the samples were lyzed in RIPA buffer and stored at −80 °C for mass spectrometry analysis.

Human studies were approved by the ethical committee of the University Hospital Zurich. EDTA-anticoagulated blood was drawn from five volunteers (non-smoking Caucasians, 30–35 years of age) after informed consent had been obtained. Human blood components were isolated according to the manufacturer's instructions (Sigma-Aldrich).

Fluorescence-activated cell sorter (FACS) analyses

Blood cell fractions were analyzed for different cell populations using size (forward scatter) and granularity (side scatter) in FACS. FACS analyses were performed on a Becton Dickinson FACScan (Becton Dickinson, San Jose, CA, USA). Blood cell fractions were stained for 20 min with fluorescently labeled antibodies and fixed in 1% paraformaldehyde. The platelet population was identified by forward and side scatter, as well as by staining with phycoerythrin (PE)-labeled Armenian hamster antimouse CD61 monoclonal antibody (mAb) (2C9.G2; BD Biosciences, 553347). Platelet activation was assessed with fluorescein isothiocyanate (FITC)-labeled rat antimouse CD62P mAb (RB40.34; BD Biosciences, 553744). Thresholds for CD62P-positivity were set according to isotype controls. Platelets were activated for 20 min with 0.5 U mL^{-1} murine thrombin, 2 mM $CaCl_2$ and 1 mM $MgCl_2$. B lymphocytes were identified with FITC-labeled rat antimouse B220 mAb (RA3-6B2, 553088), and PerCP-labeled Armenian hamster antimouse CD3e mAb (145-2C11; BD Biosciences, 553067) was used to identify T lymphocytes. To stain macrophages and granulocytes, PE-labeled rat antimouse CD11b mAb (M1/70; BD Biosciences, 553311) and allophycocyanin-labeled rat antimouse Gr1 antibody (RB6-8C5; BD Biosciences, 553129) were used. The amounts of cells in different cell fractions were quantified using calibration beads (BD Biosciences, 340486). Background was determined by amount of debris.

Lipid analysis

Liquid chromatography–mass spectrometry (LC-ESI/MS/MS) analysis of endogenous ceramide, sphingosine bases and their phosphates was performed in the Lipidomics Core of the Medical University of South Carolina on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer, operating in a Multiple Reaction Monitoring positive ionization mode. Briefly, the blood components were fortified with the internal standards, and lipids were extracted and analyzed as described [13]. Results were normalized to protein content measured by the detergent compatible (DC) protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Reinach, Switzerland).

Results

Isolation procedure

The procedure allowed us to isolate four blood compartments from each mouse (plasma, erythrocytes, leukocytes and platelets). The relative recoveries of erythrocytes, leukocytes and platelets in their corresponding blood cell fractions were 40%, 35% and 25%, respectively. Analysis of these cell fractions with automated cell counting showed no contamination, which was also confirmed by FACS analysis. The leukocyte fraction was composed of 84.6% lymphocytes, 10.5% granulocytes and 4.9% monocytes.

Platelet activation was assessed at each stage of the isolation procedure by analyzing the expression of CD62P. Directly after drawing blood from mice, platelets showed a low degree of activation (10%, not shown). The pure platelet fraction at the end of the procedure contained only 2% activated platelets (Fig. 1A). Importantly, the remaining platelets could be activated by thrombin stimulation (Fig. 1B).

Sphingolipid composition of mouse blood compartments

The distribution of sphingolipids in the different blood compartments from mice is depicted in Fig. 2. S1P was present in leukocytes and plasma, but to our surprise no S1P was detectable in platelets (Fig. 2A). In contrast, mouse platelets contained copious amounts of dhS1P. In addition, dhS1P was abundantly present in leukocytes, and to some extent in erythrocytes. Free sphingosine was seen in leukocytes and platelets but not in erythrocytes or plasma. There was very little dihydrosphingosine in these compartments.

The majority of circulating sphingolipids in mice were ceramide species, the bulk of which were C_{24} and $C_{24:1}$ ceramides (Fig. 2B). The highest relative concentration of ceramides was found in platelets, followed by leukocytes. Interestingly, the relative distribution of ceramide species was very similar in all blood components.

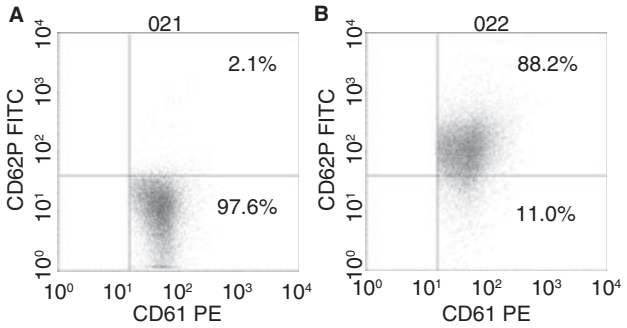

Fig. 1. Activation state of isolated mouse platelets assessed by expression of CD62P in fluorescence-activated cell sorter analysis. (A) Platelets at the end of the isolation procedure. (B) Platelets were isolated and then activated with thrombin. A representative example is shown ($n = 5$).

Effects of platelet activation on their sphingolipid composition in mice

Human platelets have previously been shown to release S1P upon activation [11,12]. As mouse platelets contained only the related compound dhS1P, we hypothesized that murine platelets lose dhS1P when activated. Therefore, for the next experiment, purified mouse platelets were activated with thrombin, and activation was monitored by CD62P expression. While platelets lost nearly all of their dhS1P following activation, they tremendously increased their pool of sphingosine and dihydrosphingosine (Fig. 3A). Concomitantly, ceramide content dropped by a factor of 4 (Fig. 3B). Therefore, platelet activation in mice resulted in dramatic changes of sphingolipid content.

Mouse platelet activation in vivo

After showing that mouse platelet activation induces loss of dhS1P *in vitro*, we assumed that platelet activation *in vivo* would concomitantly lead to an increase in dhS1P plasma levels. As thrombin injection *in vivo* is associated with severe systemic side-effects, such as widespread coagulatory activation, systemic vasodilatation and severe arterial hypotension [14], we instead injected mice with anti-CD41 antibody, which was previously shown to activate and aggregate platelets [15]. As depicted in Fig. 4, platelet aggregation in the liver, a major

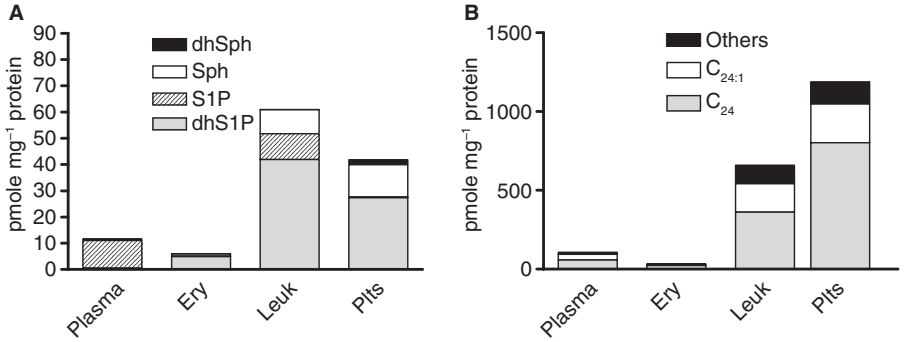

Fig. 2. Distribution of sphingolipids in mouse blood at baseline. Plasma, erythrocytes, leukocytes and platelets were isolated and subjected to liquid chromatography and mass spectrometry. Results were normalized to protein content. (A) Distribution of sphingosine, dihydrosphingosine, sphingosine-1-phosphate and dihydrosphingosine-1-phosphate. (B) Distribution of C_{24}-ceramide, $C_{24:1}$-ceramide and other ceramides. Data are mean values for $n = 5$.

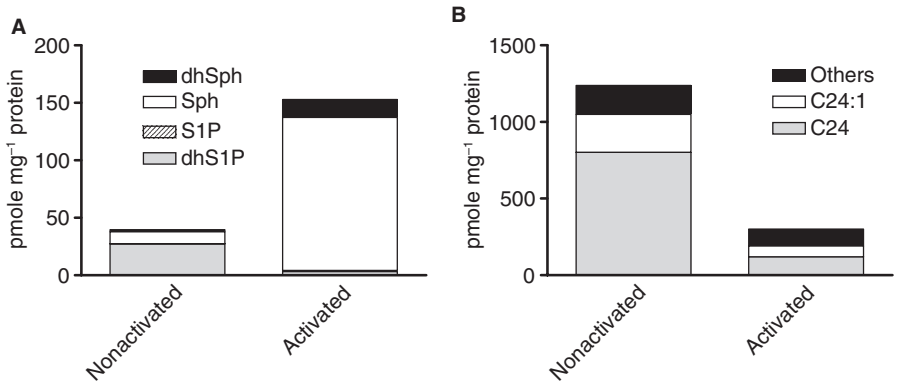

Fig. 3. Activation-induced changes in the sphingolipid composition of mouse platelets. Platelets were isolated, activated with thrombin and subjected to liquid chromatography and mass spectrometry. Results were normalized to protein content and compared with non-activated platelets. (A) Distribution of sphingosine, dihydrosphingosine, sphingosine-1-phosphate and dihydrosphingosine-1-phosphate. (B) Distribution of C_{24}-ceramide, $C_{24:1}$-ceramide and other ceramides. Data are mean values for $n = 5$.

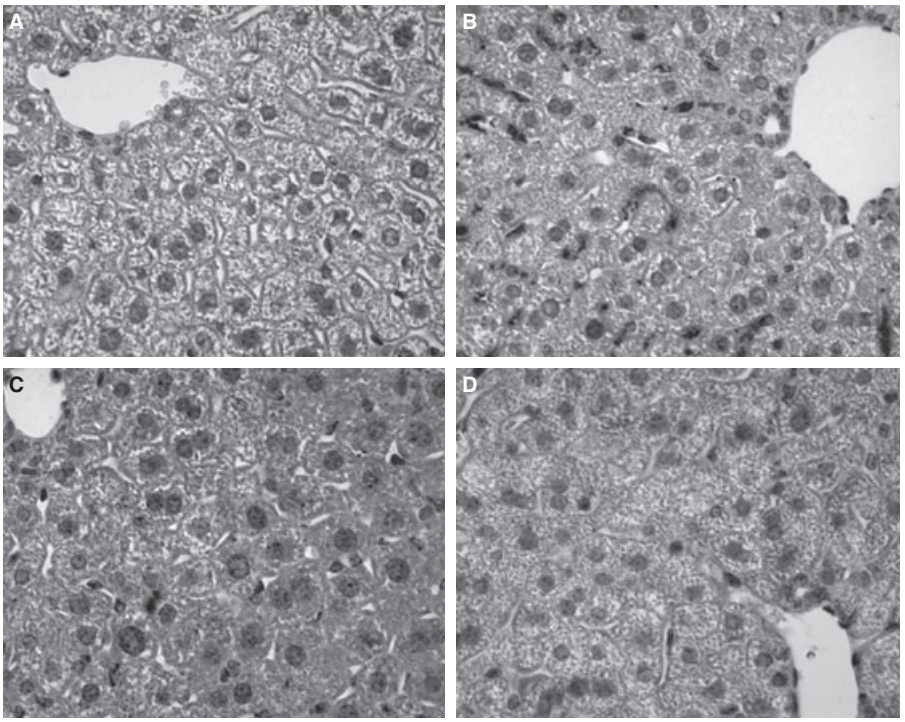

Fig. 4. Time-course of anti-CD41-induced platelet activation. Representative photomicrographs of liver sections immunostained for CD61. (A) Baseline; (B) 1 h after antibody injection; (C) 6 h after antibody injection; (D) 24 h after antibody injection.

component of the reticuloendothelial system, peaked at 1 h after injection. Platelet activation *in vivo* led to a steady increase of plasma dhS1P levels (Fig. 5A). Levels of S1P, which was the most abundant plasmatic sphingoid species at baseline, remained stable after activation (Fig. 5B).

Sphingolipid distribution in human blood

The surprising sphingolipid composition of mouse platelets stood in contrast to previously published human data [11,12]. Therefore, we hypothesized that the sphingolipid composition of the remaining blood compartments would also reveal interspecies differences. The relative distribution of sphingolipid species in humans (Fig. 6) is strikingly different from that in mice (Fig. 2). Human plasma contained not only S1P but also sizeable amounts of dihydrosphingosine (Fig. 6A). Besides storing S1P, as previously shown, human platelets also contained a non-negligible amount of dhS1P. In addition, human platelets contained considerably less ceramide, albeit with a similar distribution of ceramide species.

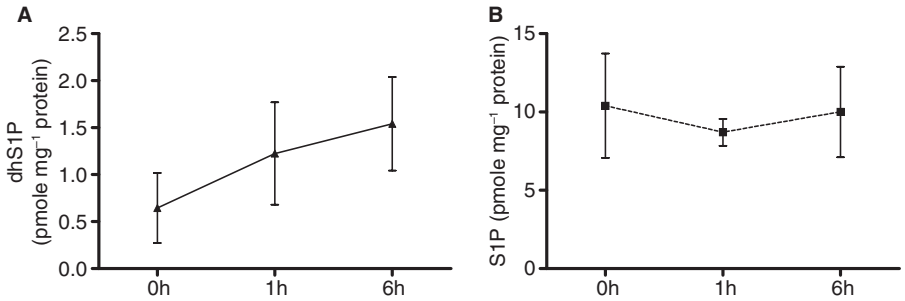

Fig. 5. Changes in dihydrosphingosine-1-phosphate (dhS1P) and sphingosine-1-phosphate (S1P) plasma levels after anti-CD41-induced platelet activation. Plasma was subjected to liquid chromatography and mass spectrometry at the indicated time points and results were normalized to protein content. (A) Plasma levels of dhS1P. (B) Plasma levels of S1P. Data are mean ± SD for $n = 5$.

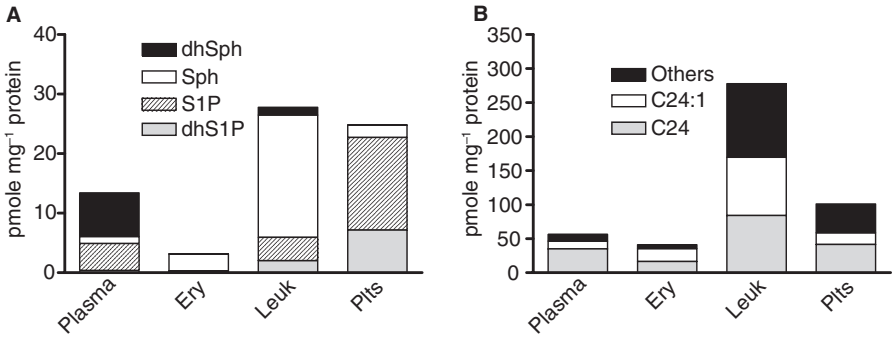

Fig. 6. Distribution of sphingolipids in human blood. Plasma, erythrocytes, leukocytes and platelets were isolated and subjected to liquid chromatography and mass spectrometry. Results were normalized to protein content. (A) Distribution of sphingosine, dihydrosphingosine, sphingosine-1-phosphate and dihydrosphingosine-1-phosphate. (B) Distribution of C_{24}-ceramide, $C_{24:1}$-ceramide and other ceramides. Data are mean values for $n = 5$.

Discussion

This study represents a detailed mass-spectrometric analysis of the selected sphingoid bases, their phosphates and ceramide species in different blood compartments in mice, as well as the changes associated with platelet activation *in vitro* and *in vivo*. To this end, we developed a procedure that allowed all blood components to be isolated from a single mouse. This technique was well-controlled at all steps, and it yielded pure fractions of plasma, erythrocytes, leukocytes and platelets with minimal contamination. As the volume-distribution within the different blood cell fractions was not altered by any isolation step, we are confident that no selection of cellular subpopulations occurred. Importantly, isolated platelets were in an inactive yet viable condition, as they could still be activated by thrombin stimulation.

Platelets are known to take up sphingosine and to convert it to S1P [11,16,17]. Upon activation, the high amounts of stored S1P are released [12]. This understanding is based on *in vitro* studies with human platelets, using the thin-layer chromatography approach, a technique that might not be able to accurately differentiate between closely related sphingolipids.

A surprising finding was the complete lack of S1P in mouse platelets. Instead, platelets were found to contain its saturated analog dhS1P. This prompted us to question whether dhS1P plays a similar biological role in mouse platelets compared with the human situation. In analogy to S1P in humans, dhS1P was secreted from activated mouse platelets. This was confirmed *in vivo*, as plasma levels of dhS1P rose immediately after the injection of activating antibodies. The use of different activation protocols *in vitro* and *in vivo* was due to technical considerations and might represent a limitation of the study, but can also be interpreted to support the fact that platelet activation does indeed lead to a loss of dhS1P. In mice, plasma S1P did not seem to be derived from platelets, as illustrated by its absence in platelets and by the kinetics of plasma levels after activation *in vivo*: dhS1P rose continuously, while S1P levels remained stable. Although S1P and dhS1P can bind to the same receptors [8], the biological effects of dhS1P have hardly been explored. Differential effects of S1P vs. dhS1P were recently described, leading the authors to postulate a putative dhS1P receptor [9].

Platelets are known to be rich in ceramides [18]. This is corroborated by the current study, where we show C_{24} and $C_{24:1}$ ceramides to make up the bulk of platelet ceramides in mice and humans. Interestingly, activation led to the breakdown of ceramides and an accumulation of sphingosine in murine platelets, suggesting the activation of a ceramidase. Because acid ceramidase has been found in platelets, this enzyme emerges as a likely candidate, although thrombin stimulation did not enhance its activity in a previous study [19]. While short thrombin activation (1 min) of platelets did not affect ceramide levels [20], longer exposure has previously been shown to reduce platelet ceramide content [21].

We show for the first time a detailed and comparative analysis of sphingolipids in mouse and human blood compartments. The sphingolipid composition showed major interspecies differences. In contrast to mice, human platelets contain S1P and also sizeable amounts of dhS1P. Furthermore, human plasma contains not only S1P but also sphingosine and dihydrosphingosine. Finally, in humans the highest ceramide content can be found in leukocytes, not in platelets.

In conclusion, our results show the sphingolipid composition of mouse blood compartments in unprecedented detail, along with the dynamic alterations induced by platelet activation *in vitro* as well as *in vivo*. Importantly, distinct interspecies differences must be considered when studying sphingolipid biology *in vivo*. This is illustrated by differences in the sphingolipid constituents such as S1P and dhS1P, which might exert differential biological effects.

Authors' contributions

F. Dahm and A. Nocito designed the study, performed experiments, analyzed data and wrote the paper. A. Bielawska performed experiments, analyzed data and co-wrote the paper. K. S. Lang, P. Georgiev and J. Bielawski performed experiments and analyzed data. L. M. Asmis and J. Madon contributed key techniques and analyzed data. Y. A. Hannun contributed to study design, data analysis and interpretation, and co-wrote the paper. P.-A. Clavien supervised the research.

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Disclosure of Conflict of Interests

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