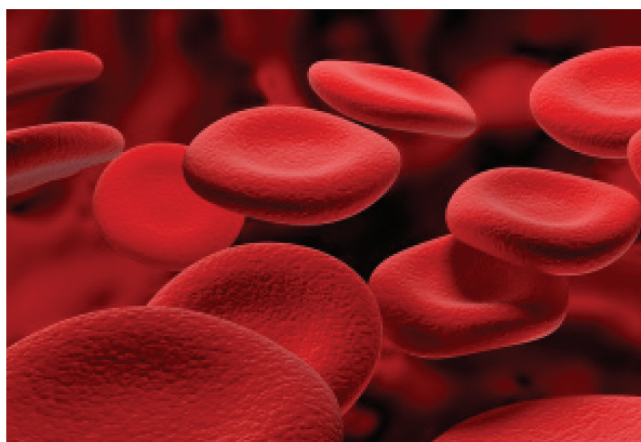


# Investigations on Storage-Induced Changes of the Red Blood Cell Lipidome



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## Introduction

Lipid biomarkers for red blood cell (RBC) storage lesions could potentially be used to develop direct detection strategies for autologous blood doping. Within this study, two complementary approaches for the analysis of the RBC lipidome were developed by using high performance liquid chromatography/time-of-flight mass spectrometry (HPLC/TOF-MS) and gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS). The applicability of both approaches was demonstrated by analyzing an exemplary set of four blood samples, which were stored *ex vivo* for a period of 42 days.

## Materials and methods

Blood donations were conducted to collect whole blood samples from healthy volunteers that met the international criteria for blood donation (Figure 1). RBC concentrates were prepared and stored according to standard blood bank conditions for the maximum length of 42 days.<sup>1</sup> For lipid extraction,<sup>2</sup> a total of 800  $\mu$ L of cold chloroform/methanol (2:1, v/v) were added to 200  $\mu$ L of RBC lysate, and the mixture was subjected to ultrasonication (10 minutes). Following centrifugation (5 minutes, 16,000  $\times$  g), the lower lipophilic phase was removed and dried under reduced pressure (30  $^{\circ}$ C).

### Approach 1

#### High performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry

For LC-TOF analysis, the dried lipid extracts were dissolved in acetonitrile/methanol (50/50, v/v). High resolution/high accuracy ESI-MS was conducted using an Agilent 6550 iFunnel Q-TOF LC/MS instrument (Waldbronn, Germany) equipped with a Dual AJS electrospray ion source operated at 25  $^{\circ}$ C and 3,500 V. The mass spectrometer was calibrated using the manufacturers' protocol, allowing mass accuracies <3 ppm for the period of analysis. The fragmentor voltage was set to 365 V. Separation of lipids was achieved using an Agilent 1290 Infinity LC equipped with an

Agilent Eclipse Plus C18 RRHD column (2.1  $\times$  50 mm, 1.8  $\mu$ m). The solvents were A = methanol/water (50/50, v/v) and B = methanol/acetonitrile (50/50, v/v). The gradient started with 70% A, then decreased to 0% A in 45 minutes, held for 15 minutes with subsequent re-equilibration for 5 minutes. The flow was constant at 0.3 mL/min and the injection volume was 5  $\mu$ L.

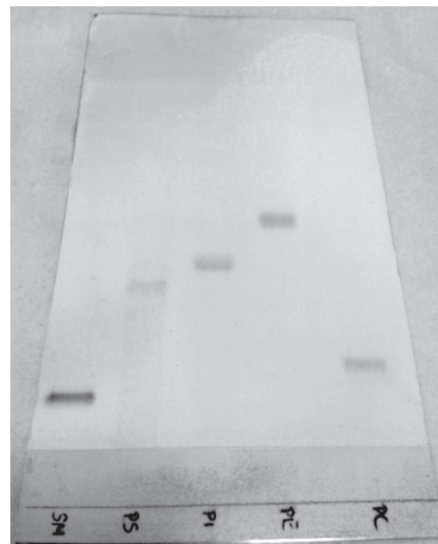
### Approach 2

#### Thin-layer chromatography (TLC) and gas chromatography/electron ionization time-of-flight mass spectrometry

Prior to GC-TOF analysis, the dried lipid extracts were reconstituted in 100  $\mu$ L of chloroform and separated by means of TLC using concentrating zone plates (Merck, Darmstadt, Germany) and a solvent mixture of chloroform/ethanol/triethylamine/water (30/35/35/7, v/v). The assignment of the separated spots to the different lipid classes was achieved by comparison to corresponding lipid standards (Sigma-Aldrich, Steinheim, Germany). To analyze the fatty acid composition of the lipids, they were extracted from the scraped silica gel with 600  $\mu$ L of chloroform/methanol (2:1, v/v; 10 minutes at 1,000 rpm followed by 10 minutes ultrasonication), saponified (1 M KOH/MeOH; 2 hours 40  $^{\circ}$ C), extracted with N-hexane, and finally derivatized to trimethylsilyl derivatives (80  $\mu$ L ethyl acetate, 20  $\mu$ L of MSTFA, 1 hour 60  $^{\circ}$ C) before analysis.

High resolution/high accuracy mass spectrometry following electron ionization was conducted on an Agilent 7890A GC interfaced to a 7200 Accurate-Mass Q-TOF analyzer. The GC was equipped with an Agilent J&W Ultra 1 column column (17 m, inner diameter 0.2 mm, film thickness 0.11  $\mu$ m), and instrument temperatures were 280  $^{\circ}$ C for transfer line and injector and 230  $^{\circ}$ C for the ion source. Helium (purity grade 4.6) was used as carrier gas; injection was conducted in pulsed split mode (5  $\mu$ L) with a split ratio of 5:1. The GC temperature was programmed from 60  $^{\circ}$ C (1.5 minutes) to 120  $^{\circ}$ C at 40  $^{\circ}$ C/min, then at 10  $^{\circ}$ C/min to 325  $^{\circ}$ C, where the temperature was maintained for another 2 minutes. MS data were acquired in full scan mode at 2 GHz from  $m/z$  50 to 800.

All data were statistically evaluated using Agilent Mass Profiler Professional and Principal Component Analysis (PCA).

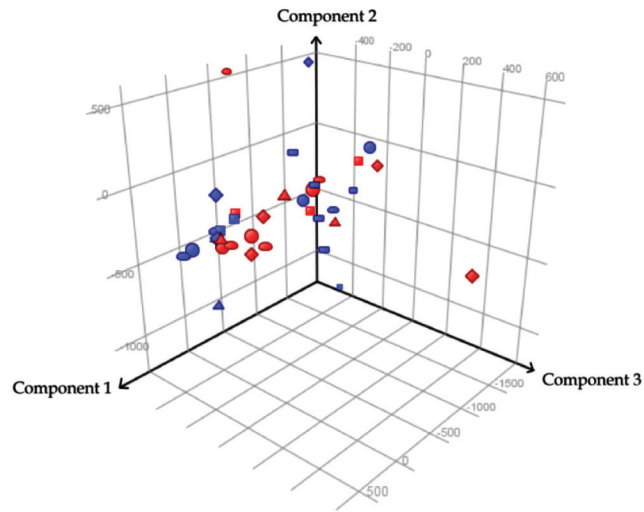


**Figure 1.** TLC plate with separated lipid standards (SM = Sphingomyelin, PS = Phosphatidylserine, PI = Phosphatidylinositol, PE = Phosphatidylethanolamine, PC = Phosphatidylcholine)

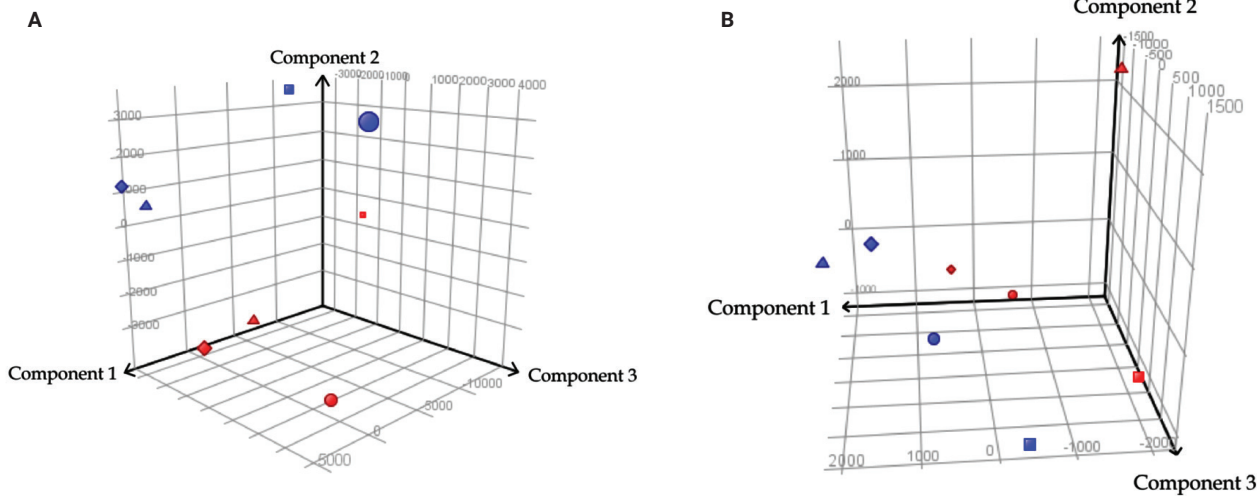
## Results and discussion

The analysis of the storage-induced changes of the RBC lipidome by means of HPLC/TOF-MS yielded highly significant differences between fresh blood and RBCs stored for 42 days ( $p < 0.01$ , t-test). In Figure 2, the PCA plots of untargeted results obtained by LC-TOF measurements in positive and negative mode are depicted, and the samples of subjects 1 to 4 stored for 0 (red) and 42 days (blue) are clearly separated.

However, for GC measurements, no significant differences could be confirmed for any of the investigated lipid sub-classes (Figure 3).



**Figure 3.** PCA plot of untargeted results obtained by GC-TOF measurements after TLC separation. Fraction of PC represented by squares, PE by triangles, PI by circles, PS by diamonds and SM by ovals. Red color represents fresh blood, blue color 42 days of storage.



**Figure 2.** PCA plot of untargeted results obtained by LC-TOF measurements in positive mode. Subject 1: squares, subject 2: circles, subject 3: triangles, subject 4: diamonds. Red represents fresh blood, blue represents 42 days of storage.

In the last years, several studies focused on the characterization of RBC storage lesions and successfully described alterations of both membrane and cytosolic proteins.<sup>1,3-7</sup> Thus, storage-induced changes of the RBC lipidome were to be expected. Both approaches presented in this study proved to be suitable for the analysis of the RBC lipidome and further investigations of the identified lipids will have to show if the observed alterations are sufficient for doping control purposes.

## Conclusion

Within this study, two complementary approaches for the analysis of the RBC lipidome were presented and successfully used for the analysis of the RBC lipidome. By using HPLC/TOF-MS, highly significant differences between the lipidome of RBCs stored for 0 and 42 days were detected. Further evaluation of the data might result in the identification of lipid biomarkers for RBC storage lesions that could be used to provide direct evidence for the misuse of ABT following reinfusion in elite athletes.

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