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Investigations on storage-induced changes of the red blood cell lipidome

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Abstract

Since more than forty years, athletes use blood doping techniques to improve the oxygen delivering capacity of the blood by artificially increasing the number of circulating red blood cells (RBCs). While the misuse of recombinant human erythropoietin can be directly detected in blood and urine specimens by using different analytical strategies, only indirect approaches such as the monitoring of blood parameters or the detection of phthalates are currently available to provide sufficient evidence for autologous blood transfusions (ABT).
Generally, RBCs have a comparably simple structure and can be readily obtained in large amounts. Metabolic and structural changes are an ideal starting point for preventive doping research as biomarkers for RBC storage lesions might be used to provide direct evidence for the misuse of ABT following reinfusion in elite athletes.
The long-term aim of this preliminary study was to identify new lipid biomarkers that can potentially be used to develop direct detection strategies for autologous blood doping and complement the already existing approaches. For that purpose, the RBC lipidome of four healthy subjects and its alterations in the course of 42 days of *ex vivo* storage were investigated using both liquid chromatography-high resolution mass spectrometry (LC-TOF) and gas chromatography-high resolution mass spectrometry (GC-TOF).
While for LC-TOF measurements no extensive sample preparation besides a liquid-liquid-extraction was necessary as all lipid classes can directly be investigated here, samples for GC-TOF investigations were prepared by thin layer chromatography followed by saponification and finally derivatization.

Introduction

Since the introduction of a doping control method for the detection of EPO in 2001, the misuse of blood transfusions in sports is increasing continuously [2]. While homologous blood transfusions can be directly detected on the basis of specific erythrocytic antigens and DNA profiles, only indirect analytical approaches are currently available to provide evidence for autologous blood doping. Thus, lipid biomarkers for RBC storage lesions could be potentially 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 HPLC/TOF-MS and 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.

Experimental

Blood donations were conducted to collect whole blood samples from healthy volunteers that met the international criteria for blood donation. RBC concentrates were prepared and stored according to standard blood bank conditions for the maximum length of 42 days [4], a total of 800 µL of cold chloroform/methanol (2:1, v/v) were added to 200 µL of RBC lysate and the mixture was subjected to ultrasonication (10 min). Following centrifugation (5 min, 16000 x *g*), the lower lipophilic phase was removed and dried under reduced pressure (30 °C).

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Approach 1: High performance liquid chromatography/electrospray ionization time-of-flight mass spectrometry

For LC-TOF analysis, the dried lipid extracts were solved 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 equipped with a Dual AJS electrospray ion source operated at 290 °C and 3500 V. The mass spectrometer was calibrated using the manufacturers' protocol allowing for 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 C^{18} RRHD column (1.8 µm, 2.1 x 50 mm). 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 min, held for 15 min with subsequent re-equilibration for 5 min. The flow was constant at 0.3 mL/min and the injection volume was 5 µL

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 µL of chloroform and separated by means of TLC using concentrating zone plates 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 (Figure 1).

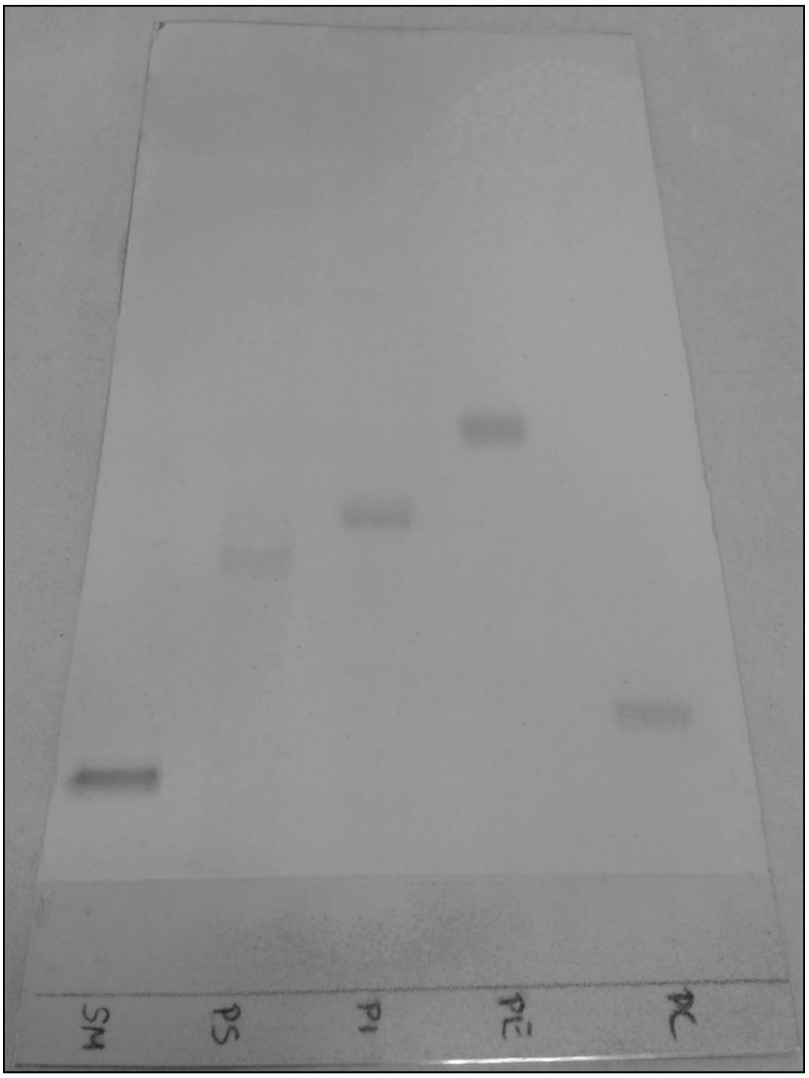

Figure 1: TLC plate with separated lipid standards (SM = Sphingomyelin, PS = Phosphatidylserine, PI = Phosphatidylinositol, PE = Phosphatidylethanolamine, PC = Phosphatidylcholine)

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In order to analyze the fatty acid composition of the lipids, they were extracted from the scraped silica gel with 600 µL chloroform/methanol (2/1, *v/v*; 10 min at 1000 rpm followed by 10 min ultrasonication), saponified (1 M KOH/MeOH; 2h 40 °C), extracted with N-hexane and finally derivatized to trimethylsilyl derivatives (80 µL ethyl acetate, 20 µL MSTFA, 1h 60 °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 HP-ULTRA 1 column (17 m, inner diameter 0.2 mm, film thickness 0.11 µm), and instrument temperatures were 280 °C for transfer line and injector and 230 °C for the ion source. Helium (purity grade 4.6) was used as carrier gas; injection was conducted in pulsed split mode (5 µL) with a split ratio of 5:1. The GC temperature was programmed from 60 °C (1.5 min) to 120 °C at 40 °C/min, then at 10 °C/min to 325 °C, where the temperature was maintained for another 2 min. MS data were acquired in full scan mode at 2 GHz from m/z 50 to 800.
All data was statistically evaluated using Mass Profiler Professional (Agilent) and Principal Component Analysis (PCA).

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 Figures 2 and 3, the PCA plots of untargeted results obtained by LC-TOF measurements in positive and negative mode are depicted and the samples of subjects 1-4 stored for 0 (red) and 42 days (blue) are clearly separated.

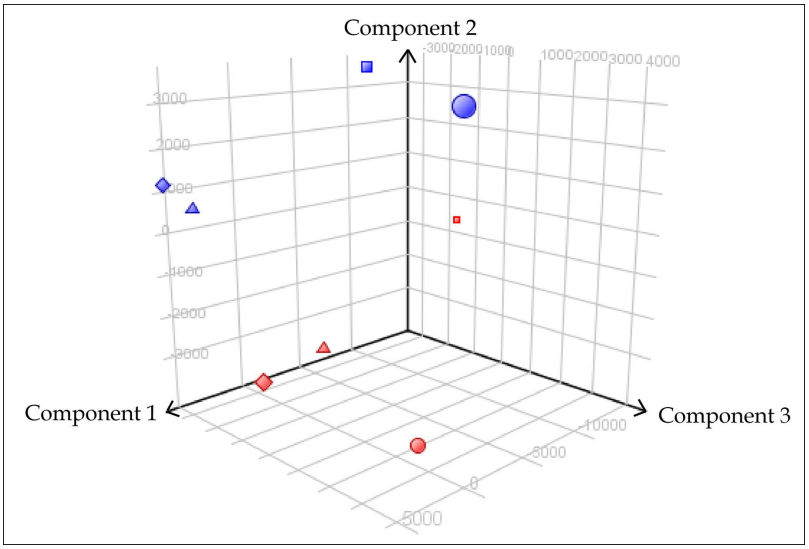

Figure 2: PCA plot of untargeted results obtained by LC-TOF measurements in positive mode. Subject 1: squares, subject 2: circles, subject 3: triangles, subject 4: diamonds. Red color represents fresh blood, blue color 42 days of storage.

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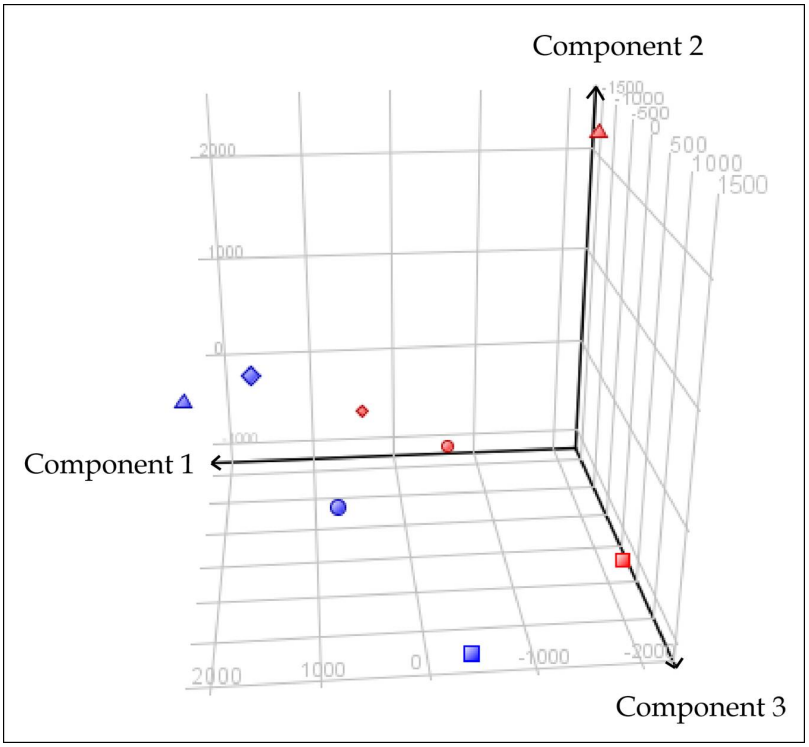

Figure 3: PCA plot of untargeted results obtained by LC-TOF measurements in negative mode. Subject 1: squares, subject 2: circles, subject 3: triangles, subject 4: diamonds. Red color represents fresh blood, blue color 42 days of storage.

However, for GC measurements no significant differences could be confirmed for any of the investigated lipid sub-classes (Figure 4).

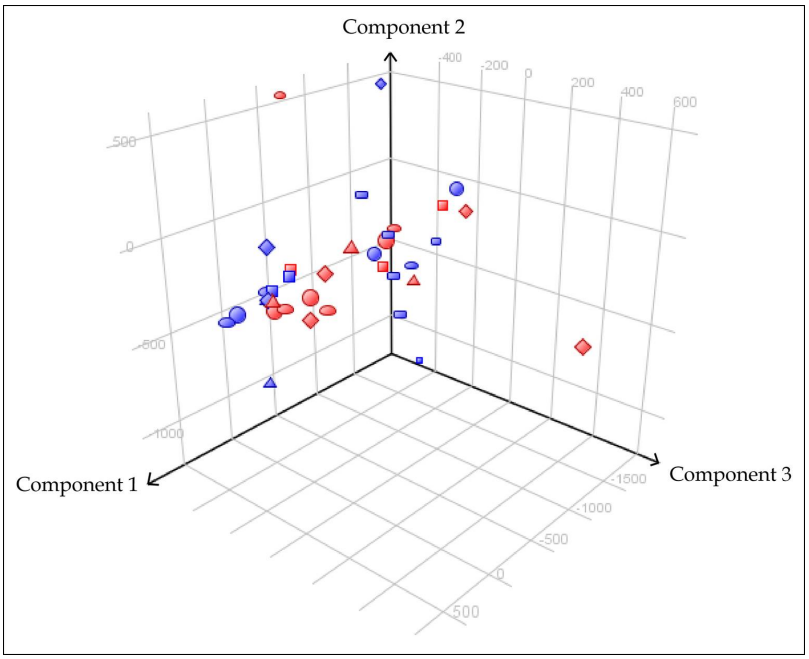

Figure 4: PCA plot of untargeted results obtained by GC-TOF measurements after TLC separation. Fraction of PC represented by squares, PE by triangles, PI by circles, PS by diamonds and SM by ovals. Red color represents fresh blood, blue color 42 days of storage.

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In the last years, several studies focused on the characterization of RBC storage lesions and successfully described alterations of both membrane and cytosolic proteins [5-9]. 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.

Conclusions

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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