Effects of anticoagulants and storage conditions on clinical oxylipid levels in human plasma

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ABSTRACT

Metabolomics and lipidomics are of fundamental importance to personalized healthcare. Particularly the analysis of bioactive lipids is of relevance to a better understanding of various diseases. Within clinical routines, blood derived samples are widely used for diagnostic and research purposes. Hence, standardized and validated procedures for blood collection and storage are mandatory, in order to guarantee sample integrity and relevant study outcomes. We here investigated different plasma storage conditions and their effect on plasma fatty acid and oxylipid levels. Our data clearly indicate the importance of storage conditions for plasma lipidomic analysis. Storage at very low temperature (−80 °C) and the addition of methanol directly after sampling are the most important measures to avoid ex vivo synthesis of oxylipids. Furthermore, we identified critical analytes being affected under certain storage conditions. Finally, we carried out chiral analysis and found possible residual enzymatic activity to be one of the contributors to the ex vivo formation of oxylipids even at −20 °C.

1. Introduction

Clinical metabolomics and lipidomics can contribute to personalized health care as they allow for a detailed molecular assessment of individual metabolite patterns and concentrations. Due to the fact that blood is one of the most widely used bio-fluids in clinical studies, it is not surprising that it has been extensively applied in clinical metabolomics and lipidomics studies [1,2]. A specific sub-field of lipidomics analysis, which is at present rapidly moving towards the clinic, is the analysis of oxylipids, including bioactive lipid mediators (LM) [3,4]. Although several interesting studies in this field have already been published, a surprisingly limited number of investigations have yet studied the influence of sample handling and storage on study outcomes [5]. Plasma, collected using different anticoagulants, and serum are the most often used blood components for lipidomics and metabolomics studies. As these blood products are often stored for different lengths of time before analyses, it is highly relevant to identify the proper storage conditions to avoid storage artifacts, i.e. ex vivo modification or formation of lipids. Other pitfalls and challenges in lipidomic research are the use of different anti-oxidants, the intrinsic problem of radical-induced lipid autoxidation [6], as well as storage stability [7]. All these factors should be systematically studied in order to guarantee high quality data and allow for correct biological interpretation of the results.

Oxylipid analysis is usually carried out using liquid chromatography coupled to tandem mass spectrometry with electrospray ionization (LC-ESI-MS/MS). Matrix effects in LC-ESI-MS/MS are known to affect quantitation of analytes in plasma [8,9] and different anticoagulants can change the physical properties of the plasma and thus affect the matrix effect. Despite the inevitable need for storage of samples in clinical studies, as of yet there is no general consensus on which anticoagulant is best suited for oxylipid analysis. Moreover, to our knowledge, only one study has addressed the effect of anticoagulants on plasma phospholipids and triglycerides so far [10].

Oxylipids are, chemically speaking, oxidized products of polyunsaturated fatty acids (PUFAs) which contain one or several oxygen functionalities when compared to their PUFA precursor. The molecular oxygen insertion can occur either via enzymatic or non-enzymatic pathways. The non-enzymatic pathways, also referred to as autoxidation and peroxidation due to radical reactions, are suspected to take place in vivo in many diseases [11]. However, these processes are undesirable ex vivo, as they are the main contributors to storage artifacts. Several antioxidants such as vitamins A and E, or butyLATED...
hydroxytoluene (BHT) [12] are commonly used to prevent ex vivo oxidation and to maintain sample integrity. Their usage includes the coating of blood collection containers [7] and/or addition to the samples [13]. It has been known for >25 years that ex vivo radical reactions and peroxidation of PUFAs are of considerable concern when samples are stored at elevated temperatures, or even at −20 °C [11,14]. Despite that, no dedicated studies have been performed on how such parameters affect oxylipids. The possibility of oxygen insertion via residual enzymatic activity should in this respect also be considered. It is furthermore of crucial importance to assess which timeframe between sample collection/preparation and freezing is considered acceptable, since daily clinical routine samples are usually not processed immediately.

All of the above-mentioned points were addressed in the present study, in which the effects of different storage temperatures, storage time, sample preparation as well as use of two of the most widely used anticoagulants [15] on levels of oxylipids and PUFAs in human plasma samples were determined. The anticoagulants compared in this study are ethylenediaminetetraacetic acid (K₂EDTA, from here on referred to as EDTA) and sodium heparin (from here on referred to as heparin). As we had to focus our efforts to some extent we chose EDTA as a representative of the ion chelator class of anti-coagulants and heparin as example of a thrombin inhibitor. We believe that our study is of fundamental importance for the further integration of oxylipid analysis in clinical research as it lays the fundament for proper sample handling and storage, which is critical for the generation of consistent clinical data.

2. Materials and methods

2.1. Chemicals and materials

K₂EDTA (EDTA) and sodium heparin vacutainers were from BD (Mississauga, ON, Canada). LC-MS grade methanol (MeOH), glacial acetic acid pro analytis (p.a.), and LC-MS grade water, were from Sigma Aldrich (Schnelldorf, Germany). Ethanol p.a. was from Merck (Darmstadt, Germany). Acetonitrile (ACN) and butylated hydroxytoluene (BHT) were from Sigma Aldrich. All substances used as standards were from Cayman Chemicals (Ann Arbor, MI, USA), except 17-hydroxydocosatetraenoic acid (17-HDTE) which was made in-house as described in [3]. Sample tubes (1.5 mL) were from Eppendorf (Hamburg, Germany). Autosampler vials, caps and inserts were from Agilent Technologies (Waldbrohn, Germany). The internal standard (IS) solution used for oxylipid analysis consisted of LTB₄-d₄, 15(S)-HETE-d₈, PGE₂-d₄ 50 ng/mL each and 500 ng/mL DHA-d₅ in MeOH. The IS solution was prepared beforehand in sufficient amount, aliquotted and the aliquots were stored at −80 °C and used for this purpose alone.

2.2. Preparation of plasma samples and storage

Blood was collected from 4 healthy, non-fasting volunteers, 2 females and 2 males upon written informed consent. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center. All methods were performed in accordance with the relevant guidelines and regulations outlined by the Medical Ethical Committee of the Leiden University Medical Center. Two EDTA and 2 heparin tubes of blood from each volunteer were collected. The blood was spun at 2100 × g for 10 min in order to obtain platelet-poor plasma. Subsequently a heparin and EDTA plasma pool were prepared and the plasma divided into 200 μL aliquots.

After aliquotting, samples were treated in one of four different ways. 1) Proteins were precipitated with 3-fold volume MeOH w/ 40.8 g/mL BHT. 2) 4 μL ETOH w/ 15 mg/mL BHT was added. 3) Proteins were precipitated with 3-fold volume MeOH. 4) no additives. For condition 1) this resulted in a final BHT concentration of 30.8 μg/mL and for condition 2) 294 μg/mL, respectively. After treatment, argon was blown over the samples before storing the aliquots at 4 different storage temperatures, for different amounts of time, see Fig. 1, resulting in 134 samples.

A baseline for each anticoagulant was established by working-up samples immediately after aliquotting.

2.3. Targeted lipidomics

Targeted lipidomics analysis of the plasma was carried out as described previously [3] with some modifications: To plasma aliquots that were stored without prior protein precipitation, 600 μL of MeOH was added, 5.4 μL IS was added to all samples and after vortexing, samples were stored at −20 °C for 20 min. The samples were then centrifuged at 4 °C for 10 min at 16,100 × g. From each sample 270 μL MeOH extract was transferred to two glass autosampler vials and dried under a gentle stream of N₂ before reconstituting with 36 μL MeOH, vortexing and sonicating and adding 54 μL H₂O₂ giving two technical replicates for each sample. After reconstitution, the samples were placed in the autosampler at 6 °C for direct analysis.

Liquid chromatography combined with mass spectrometry (LC-MS/ MS) analysis was carried out as previously published [3] with some modifications. Briefly: A QTrap 6500 mass spectrometer was used in MRM mode in negative ESI mode (Sciex, Nieuwerkerk aan den IJssel, The Netherlands), coupled to a LC system employing two LC-30AD pumps, a SIL-30AC autosampler, and a CTO-20AC column oven. (Shimadzu, ’s-Hertogenbosch, The Netherlands). The employed column was a Kinetex C₁₈ 50 × 2.1 mm, 1.7 μm, protected with a C8 precolumn (Phenomenex, Utrecht, The Netherlands), kept at 50 °C. The following binary gradient of water (A) and MeOH (B) with 0.01% acetic acid was used: 0 min 30% B, held for 1 min, then ramped to 45% at 1.1 min, to 53.5% at 2 min, to 55.5% at 4 min, to 90% at 7 min, and to 100% B at 7.1 min, held for 1.9 min. The injection volume was 40 μL and the flow rate 400 μL/min. The MS was operated under the same conditions as in [3]. In addition to the mass transition used for each analyte (see Supplementary Table 1), relative retention times (RRTs) were used for
identification. For quantification at baseline, calibration lines, made with standard material for each analyte (see Supplementary Table 1 for range), were used and only peaks with a signal to noise (S/N) > 10 were quantified. For analytes where no calibration line was used, area ratios were used and S/N > 3 was used as a detection limit. The LC-MS/MS method used does not discriminate between alpha-linolenic acid (ALA) and gamma-linolenic acid (GLA), and therefore the detected fatty acid(s) is listed as ALA/GLA.

2.4. Chiral analysis

Chiral analysis was carried out after initial fractionation on our RPLC platform. 300 μL of MeOH extract was dried down, reconstituted in 44 μL of MeOH, vortexed and 66 μL of water added. This sample was then injected on the RP C-18 platform and fractions collected, 6.5–7.5 min for LM analysis and 7.5–8.5 min for HETE analysis. Fractions were dried down under a flow of N₂, LM fractions were reconstituted in 28 μL MeOH and 42 μL water and HETE fractions in 40 μL MeOH and 60 μL water. The chiral LC-MS/MS analysis was carried out with the same Shimadzu LC system as the RP analysis, injecting 40 μL on a Chiralpak® AD-RH (Daicel, Tokyo, Japan) at room temperature with water (A) and ACN (B) with 0.01% acetic acid at 200 μL/min. For HETE analysis an isocratic run with 70%B for 10 min was used. For LM analysis the following gradient was used: 60%B kept constant for 4 min and then linearly increased to 70% at 10 min, kept constant for another 2.5 min.

2.5. Data analysis

Peaks were integrated with manual supervision using MultiQuant™ software (Sciex, MA, USA).

3. Results

Oxylipid analysis was carried out using LC-ESI-MS/MS according to published protocols [3,16]. In order to prevent any possible influence of an extended sample preparation procedure we opted for protein precipitation using MeOH. For every condition an aliquot of pooled plasma obtained from four healthy individuals (two female, two male) was analyzed in technical duplicate. Corrected areas for all analytes in all samples can be found in Supplementary Figs. S2–S61. The effects of the following key parameters were studied: i) the anticoagulant, EDTA and heparin; ii) plasma storage temperature, including short-term storage at room/refrigerated temperature and long term storage when frozen; iii) the addition of either the anti-oxidant BHT or immediate protein precipitation using MeOH; and iv) the combined effect of BHT, MeOH and storage temperature. A detailed study design is shown in Fig. 1.

3.1. Effect of anticoagulants at baseline

Baseline levels of oxylipids and PUFAs (see concentrations in Supplementary Table 1) in both EDTA and heparin plasma directly precipitated with MeOH were determined first. The ratio of EDTA/heparin (see Fig. 2) was determined for all analytes detected at baseline. This can also be referred to as a nominal accuracy determination with the values obtained for heparin set to 1.0 (100%). As can be observed in Fig. 2A, most of the ratios for PUFAs were between 0.6 and 1.3. Similarly, most ratios for the monohydroxylated fatty acids derived from AA, EPA, DHA, LA and ALA were between 0.7 and 1.2 (Fig. 2B and C), except for 5-HETE (ratio 0.3), and 12-HETE (ratio 0.1). Moreover, three other oxylipids, TXB₂ (ratio 0.2) and LTB₄ and LTE₄ (ratio 0.0) were much higher/only present in the heparin samples compared to the EDTA samples (Fig. 2D).

To confirm that certain eicosanoids and leukotrienes are indeed only detectable in heparin plasma, we repeated this analysis with blood from four (two female, two male) additional volunteers > 1.5 years after the initial measurements (see Supplementary Fig. S1). The new measurements confirmed our initial results, indicating that LTB₄, TXB₂, 5-HETE and 12-HETE measurements are particularly sensitive to the anti-coagulant used for samples collection. Our results clearly show that the choice of anticoagulant can significantly affect the reported concentrations of some oxylipids.

3.2. Short- and long-term storage

Next, we investigated the effect of short- and long-term storage
under different conditions.

After blood collection and plasma preparation, samples are often kept at room temperature or refrigerated for a limited amount of time. To assess for how long samples can be kept at room temperature or refrigerated without changes in measured analyte concentrations, we established oxylipid and PUFA levels after two or 8 h at room temperature and after two, eight, or 24 h at 6 °C. In addition, levels were established after both 24 h and one week at −20 °C (see Supplementary Figs. S2–S27).

Moreover, since large cohorts are needed in clinical research, samples are sometimes collected over a long period of time and measured at the study endpoint. To assess the effect of long term storage, samples were kept at −20 °C or −80 °C for four weeks, 12 weeks, 26 weeks, or one year before oxylipid and PUFA measurement (see Supplementary Figs. S28–S61). The effects of BHT, a known commonly-used anti-oxidant, and of immediate MeOH precipitation on the analyte measurements were assessed. The samples without addition of BHT precipitated with MeOH at baseline were used as reference.

### 3.3. Short-term storage at room temperature

After 2 h, no clear differences were observed for any of the PUFAs in any storage condition (Fig. 3 and Supplementary Figs. S2–S8). However, an increase in levels could be observed for some PUFAs after 8 h compared to baseline. This effect was independent of MeOH.
precipitation and of the presence of BHT in the EDTA samples (Fig. 3A). In the heparin plasma samples, this increase could be prevented by MeOH precipitation, but was independent of BHT-addition (Fig. 3B and Supplementary Figs. S2–S8).

Similarly, few differences were observed for monohydroxylated FAs between the different sample treatment conditions (for both anticoagulants) except for 12-HETE in EDTA plasma w/ BHT after a two-hour storage period (Fig. 3 and Supplementary Figs. S9–S22). However, a significant increase compared to baseline could be observed after 8 h for several monohydroxylated FAs and this increase was prevented by MeOH precipitation for both anticoagulants (Fig. 3). Interestingly, in the case of 12-HETE the addition of BHT significantly affected the measured signal in the unprecipitated samples particularly in EDTA plasma (Fig. 3A and Supplementary Fig. S12).

For the CYP450 products 14,15-diHETE and 19,20-diHDPA, no clear increase compared to baseline was observed over a period of 8 h, as well as no difference between the storage conditions (Fig. 3 and Supplementary Figs. S23 and S24). For TXB₂, a significant increase was only observed in the EDTA plasma without MeOH precipitation, especially after 8 h of storage (Fig. 3A and Supplementary Fig. 25). Neither a change over time nor differences between storage conditions were observed for LTE₄, while for LTE₄ a clear increase was observed in heparin plasma samples without MeOH (Fig. 3B and Supplementary Figs. S26 and S27).

3.4. Short-term storage at 6°C

Samples kept at 6°C were worked-up after two, eight, and 24 h. An increase in PUFA levels was observed already after 8 h for EDTA plasma and after 24 h for heparin plasma. This increase was prevented by MeOH precipitation only in the heparin plasma samples. Addition of BHT did not have a significant effect on PUFA levels (see Fig. 4 and Supplementary Figs. S3, S5, and S7).

Except for 12-HETE in EDTA plasma w/ BHT, no significant differences were observed between different storage conditions within the 24 hours storage period for most of the monohydroxylated FAs, the two CYP450 products, and LTE₄ (see Fig. 4 and Supplementary Figs. S5–S27). Similar to samples stored at room temperature, the measurement of 12-HETE seemed to be affected by BHT addition in the unprecipitated EDTA plasma samples. Moreover, 5-HETE showed accumulation over time in EDTA plasma but not in heparin plasma, and this increase was prevented by MeOH addition (Fig. 4A and Suppl. Fig. S9). LTE₄ levels in heparin plasma also increased over time and the accumulation was prevented by MeOH addition (Fig. 4B and Supplementary Fig. S26).

3.5. Short-term storage at −20°C

Samples kept short-term at −20°C were worked-up after 24 h and...
one week. In contrast to room temperature and 6 °C conditions, PUFA levels were only marginally affected in heparin plasma after 1 week, while they were already increased after 24 h in the EDTA plasma. MeOH precipitation and BHT addition had little effect. (Fig. 5 and Supplementary Figs. S2–S8). Up to 1 week of storage at −20 °C resulted in small changes for most monohydroxylated FAs and CYP450 products in EDTA plasma and was prevented by addition of MeOH, while few changes were observed in heparin plasma (Fig. 5 and Supplementary Figs. S9–S27). A significant increase in 12-HETE and TXB₂ levels was observed in EDTA plasma and this was prevented by addition of MeOH (Fig. 5A and Supplementary Figs. S12 and S25). Note the possible outlier in Supplementary Fig. S12, see Discussion). Similar to samples kept at higher temperatures, LTE₄ levels in heparin plasma had increased after 24 h storage and addition of MeOH had little effect on this change (Fig. 5B and Supplementary Fig. S26).

3.6. Long-term storage at −80 °C

For PUFAs, only minor differences could be seen between storage conditions during long term storage at −80 °C (Supplementary Figs. S28–S34). Compared to baseline, only EPA, AdA, and ALA/GLA levels increased over one year for all conditions (Fig. 6 and Supplementary Figs. S29, S31, and S33). The addition of MeOH had no effect in the EDTA samples, but seemed to slow down the increase of these PUFA levels when plasma was collected in heparin. For most of the oxylipids, small changes were observed over the time span of one year (Fig. 6 and Supplementary Figs. S35–S53) and these changes were slowed down by MeOH addition. The increase in 12-HETE and TXB₂ levels in EDTA plasma and LTE₄ in heparin plasma were the most pronounced and were diminished by the addition of MeOH prior to storage (Fig. 6 and Supplementary Figs. S38, S51, and S52).
3.7. Long term storage at −20°C

During a one-year storage period, most PUFAs remained stable (Fig. 7 and Supplementary Fig. S34). Only for EPA, a significant increase was observed after 12 weeks in EDTA plasma and this was diminished by addition of MeOH (Supplementary Fig. S29).

Without MeOH, levels of monohydroxylated FAs were significantly increased by week 4 in EDTA plasma and by week 26 in heparin plasma (Fig. 7 and Supplementary Figs. S35–S48) and this was diminished by MeOH addition. One year after plasma preparation a clear increase compared to basal levels could be observed for all monohydroxylated FAs even in the samples with MeOH, with the exception of the HODEs and HOTEs (Fig. 7 and Supplementary Figs. S35–S48).

The levels of 14,15-diHETE and 19,20-diHDPA without MeOH addition (both anticoagulants) increased less than that of monohydroxylated FAs and the increase was diminished by MeOH addition. (Fig. 7 and Supplementary Figs. S49 and S50). Levels of TXB2 were significantly increased only in EDTA plasma without MeOH by week 4 (Fig. 7 and Supplementary Fig. S51), while LTB4 and LTE4 levels were significantly increased in heparin plasma without MeOH by week 26 and week 4 respectively (Fig. 7B and Supplementary Figs. S52 and S53). As there was no LTB4 detected at baseline in EDTA plasma, and the figure depicts ratio to basal levels, its fold-increase is not presented in Fig. 7A. The addition of BHT did not have a significant effect on lipid levels.

3.8. Appearances of new oxylipids over time

In addition to an increase in signal intensity for lipids already detectable at baseline, we also observed the appearance of some lipid species during storage. This was mostly the case in samples stored long-term without MeOH precipitation at −20°C.

The non-enzymatic products of LTA4, 6-trans-LTB4 and 6-trans-12-epi-LTB4, became detectable after 12 weeks in plasma from both anticoagulants (Supplementary Figs. S54 and S55). A small increase in the LTB4 area ratio in samples without MeOH compared to samples with MeOH, was observed in heparin plasma after 12 weeks, which could be a result of additional 5S,12S-diHETE that is not distinguishable from LTB4 due to the very small differences in retention time, as previously identified by our lab [17,18]. After 26 weeks these levels in both EDTA and heparin plasma had increased significantly (Suppl. Fig. S53) and the relative retention time (RRT) had clearly shifted towards the 5S,12S-diHETE value (data not shown). The hydroxylated product of AdA (17-HDoTE) was first detectable after four weeks of storage at −20°C in plasma from both anticoagulants, and it increased further over time (Supplementary Fig. S56). In addition, detectable levels of 7S,17S-diHDPA (RvD5n-3 DPA,), 5S,15S-diHETE, and 8S,15S-diHETE, were present when samples had been stored at −20°C without MeOH precipitation for 26 weeks (Supplementary Figs. S57–S59).

Surprisingly, we also observed the appearance of features that had the characteristics of bioactive LMs such as MaR1, PD1, and PDX.
(10S,17S-diHDDHA), starting at 26 weeks (Supplementary Figs. S60 and S61). The obtained chromatographic signals closely matched the relative retention times of purified standard material (within the limit of 0.5% deviation), as well as showing the characteristic fragmentation (Figs. 8 and 9). As this finding is highly relevant for the field of bioactive lipid research we further characterized these products. As it has been described that LM may be accompanied by multiple isomers that share their fragmentation pattern in MS/MS data acquisition, we used a chiral column to obtain orthogonal chromatographic separation. The results of the chiral analysis are discussed in the following section.

3.9. Chiral analysis of lipid mediators

Samples that had been kept at −20 °C for 74 weeks with or without MeOH were analyzed on both our standard RP C-18 oxylipid platform (Figs. 8A and 9A) and on the chiral platform (Figs. 8B and 9B) (see Materials and methods). The signal referring to PDX falls within a window of no > 0.5% RRT deviation on both chromatographic platforms, when compared to standard material (Fig. 8). Therefore, we concluded that the signal appearing during long term storage refers to PDX. For both PD1 and MaR1, chiral analysis revealed that the signals obtained at correct RRT (C-18), presenting genuine MS/MS spectra for both components, do not correspond to the correct analytes for which bioactivity has been assigned.

3.10. Chiral analysis of HETEs

In addition to the chiral analysis of LM, we also investigated the S and R enantiomers of monohydroxylated FAs over time starting at 12 weeks. Therefore, we analyzed 5-HETE, 12-HETE, and 15-HETE on the chiral platform in samples with or without MeOH stored at −20 °C or −80 °C for up to 74 weeks. Peak areas of S isomers and R isomers were corrected to 15S-HETE-d8 and S/R ratios calculated.

As can be seen in the Supplementary material S62–S64 the levels of 5-HETE and 15-HETE at −80 °C remained stable over the 74 weeks storage time. Particularly 12-HETE levels increased during storage at −80 °C in EDTA plasma (Fig. 6) and showed a high S/R ratio. As expected from the results in Fig. 7, much stronger effects were observed for samples store at −20 °C. Particularly for 12-HETE a severe change in the S/R ratio occurred over time in the heparin samples. This was also visible for 15-HETE, albeit to a lower extent. In contrast, the S/R ratio increased for 5-HETE and this was prevented by the addition of MeOH. For further details please refer to the Supplementary material.

4. Discussion

In this study, we assessed the effect of anti-coagulants, storage temperature and length as well as sample treatment on the levels of PUFAs and PUFA-derived oxylipids in plasma. Our data indicate that 1) EDTA and heparin differently affected the levels of specific lipids both...
at baseline and after storage, 2) less changes in lipid levels were observed upon storage at lower temperatures and 3) MeOH precipitation prevented changes in lipid levels at all temperatures, while BHT had little effect. Moreover, chiral analyses of mono-hydroxylated lipids suggest that some enzymes displayed a residual activity even at −20°C and this could be prevented by MeOH treatment before storage.

With respect to the employed anticoagulant, particularly platelet-derived oxylipids such as TXB₂ and 12-HETE, and the 5-LOX products 5-HETE, LTB₄ and LTE₄ were highly affected. These lipids were all higher in heparin than in EDTA plasma. Higher concentration of 12-HETE and TXB₂ (platelet-derived oxylipids) could be explained by heparin activating residual platelets in the platelet-poor plasma used in this study [19], possibly due to re-suspension of the platelets after the centrifugation step. Similarly, higher concentration of 5-HETE and the leukotrienes in heparin plasma could be due to residual platelets and possible re-suspension of leukocytes after the centrifugation [20–22] as well as to reduced AA release in EDTA plasma leading to lower residual 5-LOX AA products. In any case, it underlines the importance of careful handling of samples after centrifugation, and special caution should be applied to the interpretation of data obtained for these metabolites, upon long-term storage. Moreover, one must be cautious when comparing results from studies that have used different anticoagulants for plasma collection.

We have also tested the effect of storage temperature for different periods of time on the levels of different lipid classes. For all lipid classes, the levels were stable for longer periods of time at lower storage temperatures. The most affected lipids were the PUFAs EPA, AdA and ALA/GLA, and the oxylipids 5-HETE and 12-HETE, and TXB₂ and LTE₄. Interestingly, DHA accumulated to a somewhat lower extent than EPA. To this point we can only speculate about this discrepancy, however, it is conceivable that DHA is increasingly metabolized when compared to EPA. The overall increase in levels is likely due to (residual) enzymatic}

![Fig. 8. Identification of PD1/PDX (10S,17S-diHDHA) in samples stored at −20 °C for 74 weeks. A: MRM trace of PD1/PDX after separation on RPLC. RRT relative to IS LTB₄-d₄ of standard material and unidentified peaks 1 and 2. B: MRM trace of PD1/PDX after separation on chiral column. Dark blue, EDTA plasma without MeOH. Light blue EDTA plasma stored with MeOH, red heparin plasma stored without MeOH, orange heparin plasma stored with MeOH, green standard with 1 ng/mL each PD1 and PDX. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image-url)
activity and/or non-enzymatic oxidation. In the case of PUFAs, most increase was observed in the EDTA samples and the increase was marginally affected by MeOH addition, indicating that this is predominantly a non-enzymatic process. In contrast, the increase in oxylipids was significantly reduced by addition of MeOH prior to storage, indicating the involvement of enzymatic processes in the observed changes in levels at all temperatures.

To further investigate this, we have performed chiral analysis of the main 5-, 12- and 15-LOX products derived from AA: 5-HETE, 12-HETE and 15-HETE after long-term storage at −20°C and −80°C, as these are the most relevant storage conditions for clinical studies.

The least changes were observed in (oxy)lipid levels after storage at −80°C. Consequently, the effect of MeOH addition prior to storage at −80°C is minor, but still detectable, especially in 12-HETE and TXB$_2$ levels in EDTA plasma at −80°C. The levels of these compounds after one year of storage were similar to the baseline heparin levels (Supplementary Figs. S38 and S51). This could indicate the slow activation of residual platelets over time in EDTA plasma at −80°C, supported also by a higher S/R-ratio of 12-HETE in EDTA plasma without additives compared to MeOH addition at 74 weeks at −80°C (Supplementary Fig. 63). At −20°C, the S/R-ratio in EDTA plasma is relatively constant over time, suggesting that non-enzymatic processes have a higher contribution at this temperature. In heparin, the S/R ratio at −20°C approaches 1 over time, albeit slower in samples with MeOH, indicating the contribution of both enzymatic and non-enzymatic, non-stereospecific pathways to the synthesis of 12-HETE.

At −20°C, most of the oxylipid levels were increased over time, starting with 4 weeks of storage.

Chiral analysis for 5-HETE in heparin shows the increase of $S$ over $R$ for 5-HETE at −20°C, while for 15-HETE, $S$ and $R$ increase similarly over time and the $S/R$ ratio decreases slightly towards 1 at 74 weeks. This indicates that the 5-HETE increase at −20°C is mostly due to enzymatic synthesis of 5S-HETE while 15-HETE is likely made in a non-enzymatic, non-stereospecific way. In EDTA, no clear differences were observed in $S/R$ ratio over time, indicating the predominant contribution of a non-enzymatic, non-stereospecific production of both 5- and 15-HETE.

The appearance of some other features over time at −20°C (see
Sections 3.8. Appearances of new oxylipids over time and 3.9. Chiral analysis of lipid mediators, Fig. 8, Fig. 9, and Supplementary Figs. S53–S61) could then also be explained by the activity of 5-LOX (among other proteins), as this enzyme can be involved in the synthesis of PDX via 17-HpDHA [23]; 55,12S-diHETE [17], 6-trans-LTB4, and 6-trans-12-epi-LTB4, [24,25] via 5-HpETE, and RvDS3,3_dpa, Via 17-HpDPA_m3 [26,27] in vivo. In contrast, 55,15S-diHETE can be made in vivo via 5-LOX [28,29] and/or via 15-LOX and 85,15S-diHETE via 15-LOX(2) from 85-Hp(ET)E. For PDX a double oxygenation catalyzed by 15-LOX might be an alternative pathway as shown for soybean 15-LOX by Chen et al. [30].

However, as is apparent in Figs. 8 and 9, multiple isomers have formed during long-term storage at ~20 °C, so to assign the formation of these oxylipids to enzyme activity rather than non-enzymatic, non-stereospecific radical reactions would require a more detailed investigation.

Interestingly, BHT addition in general had very little effect on analyte levels, even in the EDTA samples, in which the contribution of non-enzymatic, non-stereospecific reactions seems to be predominant. However, it strongly affected the 12-HETE levels in EDTA at baseline, which could be explained by a possible interference with the measurement method, activation of platelets [31–33] or contamination of the baseline EDTA samples with erythrocytes and/or platelets. It is conceivable that the combination of BHT in particular with EDTA results in activation of residual platelets in plasma samples. As described by Ruzzene et al. [31], BHT can activate phospholipase C via protein kinase C, while EDTA has been described to cause irreversible damage to platelets [34]. The combination of these two processes could lead to an increased production of 12-HETE and TXB2, in particular when proteins have not been precipitated with MeOH.

To prevent the protein-catalyzed oxidation process in samples where proteins have not been precipitated with MeOH, we added a 10-fold increased amount of BHT. However, our data indicate that even these high concentrations of BHT were not able to significantly limit the production of oxylipids. Therefore, we deem BHT addition to plasma samples an unnecessary effort in oxylipid analysis that, in combination with EDTA, might even be counterproductive in the analysis of 12-HETE and TXB2.

To conclude, oxylipid datasets from plasma prepared with different anticoagulants cannot be directly compared, especially for platelet-derived metabolites and leukotrienes. For long-term studies, samples should be stored at ~80 °C, but MeOH addition to plasma prior to storage can maintain sample integrity at least for 12 (EDTA) to 26 (heparin) weeks at ~20 °C. For some analytes, like 12-HETE and TXB2 in EDTA plasma and LTE4 in heparin plasma, addition of MeOH prior to storage at ~80 °C appears necessary to prevent ex vivo accumulation over time. In addition, for measuring of EPA, AdA, and ALA/GLA, addition of MeOH to heparin plasma kept at ~80 °C is recommended.

For short-term, samples can be kept for 2 h at room temperature or refrigerated for 8 h without significant changes on levels, except for LTE4 in heparin and EPA, AdA and ALA/GLA in EDTA. For oxylipids, MeOH addition prolongs the time at which a sample can be kept at these temperatures, but does not affect the increase of EPA, AdA and ALA/GLA in EDTA plasma. These PUFA levels also increase at ~20 °C in EDTA plasma (slightly less with MeOH than without), so for PUFA analysis heparin should be the preferred choice.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbbalip.2018.10.003.

Author contribution

HB and HSJ carried out the experiments and were responsible for data analysis. AIP and MG designed and supervised the study. All authors contributed to the critical assessment of the data and manuscript preparation.

Additional information

The authors declare no competing interests.

The study was approved by the Medical Ethical Committee of the Leiden University Medical Center. All methods were performed in accordance with the relevant guidelines and regulations outlined by the Medical Ethical Committee of the Leiden University Medical Center.

Transparency document

The Transparency document associated with this article can be found, in online version.

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