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Pro-resolving fatty acids and oxylipids in osteoarthritis and rheumatoid arthritis

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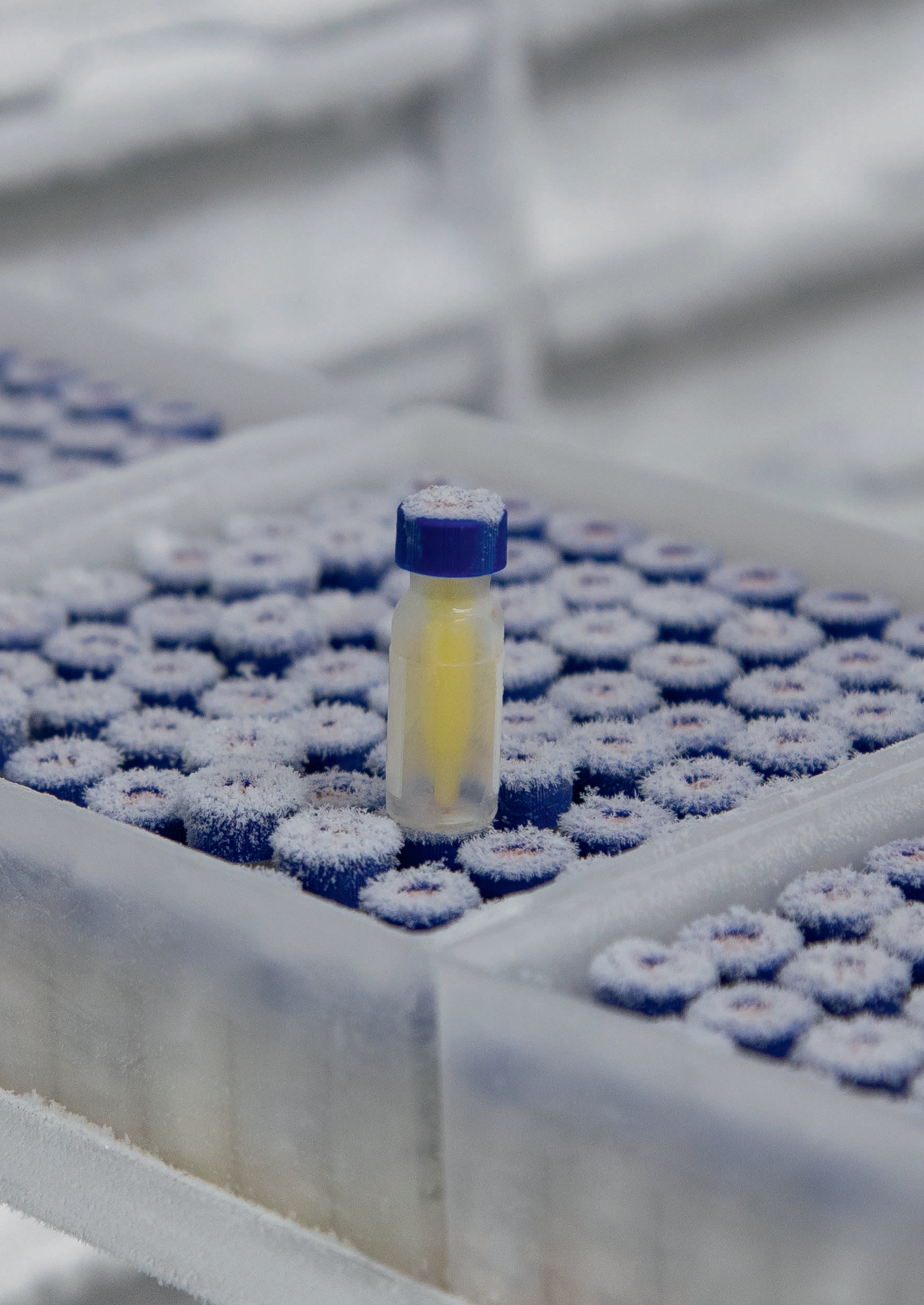

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Effects of anticoagulants and storage conditions on clinical oxylipid levels in human plasma

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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.

Keywords: LC-MS/MS, Lipidomics, Storage, Oxylipids, Stability, Plasma

Introduction

Clinical metabolomics and lipidomics stand at the basis of 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 used extensively 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 has 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 more than 25

years of research that *ex vivo* radical reactions and peroxidation of PUFAs are of big 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 the 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 (K_2EDTA, from here on referred to as EDTA) and sodium heparin (from here on referred to as heparin). 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.

Materials and Methods

Chemicals and materials

K_2EDTA (EDTA) and sodium heparin vacutainers were from BD (Mississauga, ON, Canada)). LC-MS grade methanol (MeOH), glacial acetic acid *pro analysi* (*p.a.),* and LC-MS grade water, were from Sigma Aldrich (Schnelldorf, Germany). Ethanol *p.a.* was from Merck (Darmstadt, Germany). ACN and were from Sigma Aldrich BHT. All substances used as standards were from Cayman Chemicals (Ann Arbor, MI, USA), except 17-hydroxydocosatetraenoic acid (17-HDoTE) which was made in-house *(see Production of 17-HDoTE below).* Sample tubes (1.5 mL) were from Eppendorf (Hamburg, Germany). Autosampler vials, caps and inserts were from Agilent Technologies (Waldbronn, Germany). The internal standard (IS) solution used for oxylipid analysis consisted of LTB_4-d4, 15(*S*)-HETE-d8, PGE_2-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.

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 LUMC. Two EDTA and 2 heparin tubes of blood from each volunteer were collected. The blood was spun at 2100 ×g 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 µg/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. After treatment, argon was blown over the samples before storing the aliquots at 4 different storage temperatures, for different amounts of time, see figure 1, resulting in 134 samples.

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

Targeted lipidomics

Targeted lipidomics analysis of the plasma was carried 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 N_2 before reconstituting with 36 µL MeOH, vortexing and sonicating and adding 54 µL H_2O, 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 C_{18} 50 × 2.1 mm, 1.7 *µ*m, 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 *µ*L and the flow rate 400 *µ*L/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.

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.

Data analysis

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

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 Figures 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 temperature and refrigerated; 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 Figure 1.

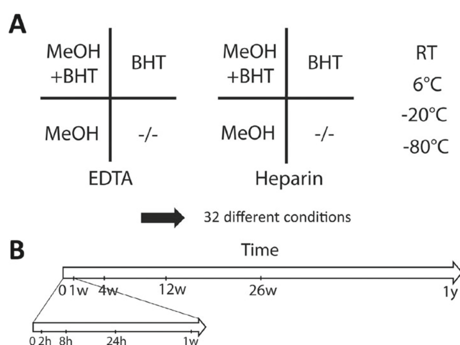

Figure 1. Study design. Blood was collected into both EDTA and heparin tubes and plasma was immediately obtained by centrifugation. **A:** All EDTA and heparin samples were pooled and aliquoted. The aliquots were then split in four groups, MeOH was either added or not, and BHT either added or not (for detailed information see *Materials and Methods*) giving 8 different conditions. These aliquots were then stored at 4 different storage temperatures, resulting in 32 different storage conditions. Abbreviations: BHT butylated hydroxytoluene**,** RT room temperature **B:** In addition to a baseline measurement, samples from the different storage conditions in **A** were analyzed at different time points, spanning from two hours after preparation of the plasma for short-term storage at room temperature and 6°C to long-term storage for one year in -20°C and -80°C freezers.

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 Figure 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 Figure 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 (Figure 2B and C), except for 5-HETE (ratio 0.3), and 12-HETE (ratio 0.1). Moreover, three other oxylipids, TXB_2 (ratio 0.2) and LTB_4 and LTE_4 (ratio 0.0) were much higher/only present in the heparin samples compared to the EDTA samples (Figure 2D).

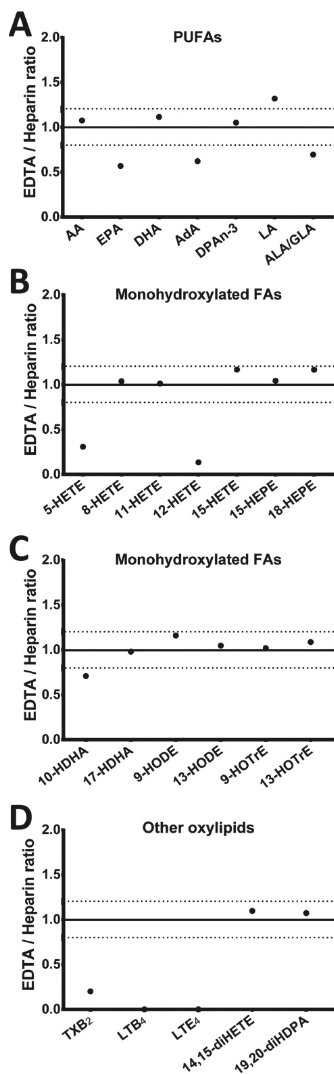

Figure 2. Comparison of lipid analytes in EDTA and heparin plasma at baseline. Heparin and EDTA were added to separate aliquots. The indicated ratios result from dividing the concentration of each analyte in the EDTA sample with the one in the heparin sample. For each analyte the ratio of the average (of technical duplicates) corrected area (to internal standard) from EDTA plasma to the heparin plasma samples is depicted for **A:** PUFAs; **B:** monohydroxylated FA derived from AA and EPA or from **C:** DHA, LA and ALA; and **D:** other oxylipids**.** The dotted line shows the range 0.8-1.2. Abbreviations: AA arachidonic acid, EPA eicosapentaenoic acid, DHA docosahexaenoic acid, AdA adrenic acid, DPA_{n-3} omega-3 docosapentaenoic acid, LA linoleic acid, ALA alpha-linolenic acid, GLA gamma-linolenic acid, HETE hydroxyeicosatetraenoic acid, HEPE hydroxyeicosapentaenoic acid, HDHA hydroxydocosahexaenoic acid, HODE hydroxyoctadecadienoic acid, HOTrE hydroxyoctadecatrienoic acid, TXB_2 thromboxane B2, LTB_4 leukotriene B4, diHETE dihydroxyeicosatetraenoic acid, and diHDPA dihydroxydocosapentaenoic acid.

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 Figure S1). The new measurements confirmed our initial results, indicating that LTB_4, TXB_2, 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.

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 eight hours at room temperature and after two, eight, or 24 hours at 6 °C. In addition, levels were established after both 24 hours and one week at -20 °C (see Supplementary Figures 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 Figures 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.

Short-term storage at room temperature

After two hours, no clear differences were observed for any of the PUFAs in any storage condition (Fig.3 and Supplementary Figures S2-S8). However, an increase in levels could be observed for some PUFAs after eight hours 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 Figures S2-S8).

Similarly, little 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 (Figure 3 and Supplementary Figures

S9-S22). However, a significant increase compared to baseline could be observed after eight hours for several monohydroxylated FAs and this increase was prevented by MeOH precipitation for both anticoagulants (Figure 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 (Figure 3A and Supplementary Figure S12).

For the CYP450 products 14,15-diHETE and 19,20-diHDPA, no clear increase compared to baseline was observed over a period of eight hours, as well as no difference between the storage conditions (Figure 3 and Supplementary Figures S23 and S24). For TXB_2, a significant increase was only observed in the EDTA plasma without MeOH precipitation, especially after 8 h of storage (Figure 3A and Supplementary Figure 25). Neither a change over time nor differences between storage conditions were observed for LTB_4, while for LTE_4 a clear increase was observed in heparin plasma samples without MeOH (Figure 3B and Supplementary Figures S26 and S27).

Short-term storage at 6 °C

Samples kept at 6 °C were worked-up after two, eight, and 24 hours. An increase in PUFA levels was observed already after eight hours for EDTA plasma and after 24 hours 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 Figure 4 and Supplementary Figures 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 LTB_4 (see Figure 4 and Supplementary Figures 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 (Figure 4A and Suppl. Fig S9). LTE_4 levels in heparin plasma also increased over time and the accumulation was prevented by MeOH addition (Figure 4B and Supplementary Figure S26).

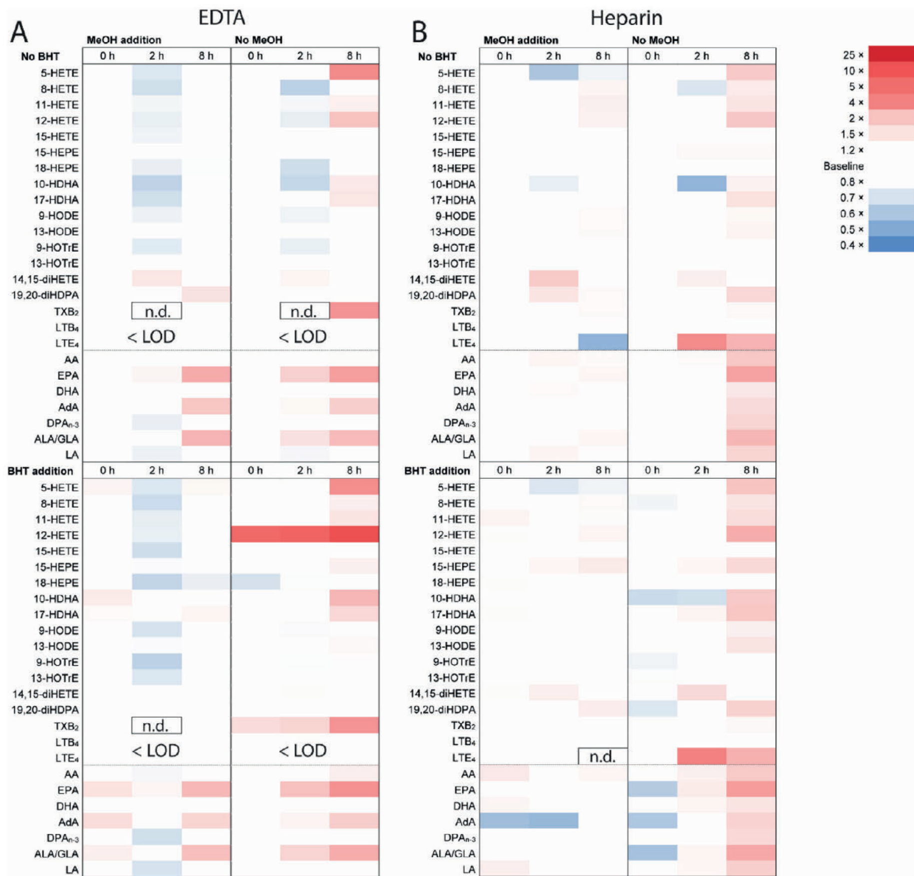

Figure 3. Short-term storage at room temperature. Heatmap showing the fold increase of the corrected area to baseline over time. **A**: EDTA plasma and **B**: heparin plasma. LTB_4 and LTE_4 were not detected in EDTA samples. Abbreviations: n.d. not determined, LOD limit of detection.

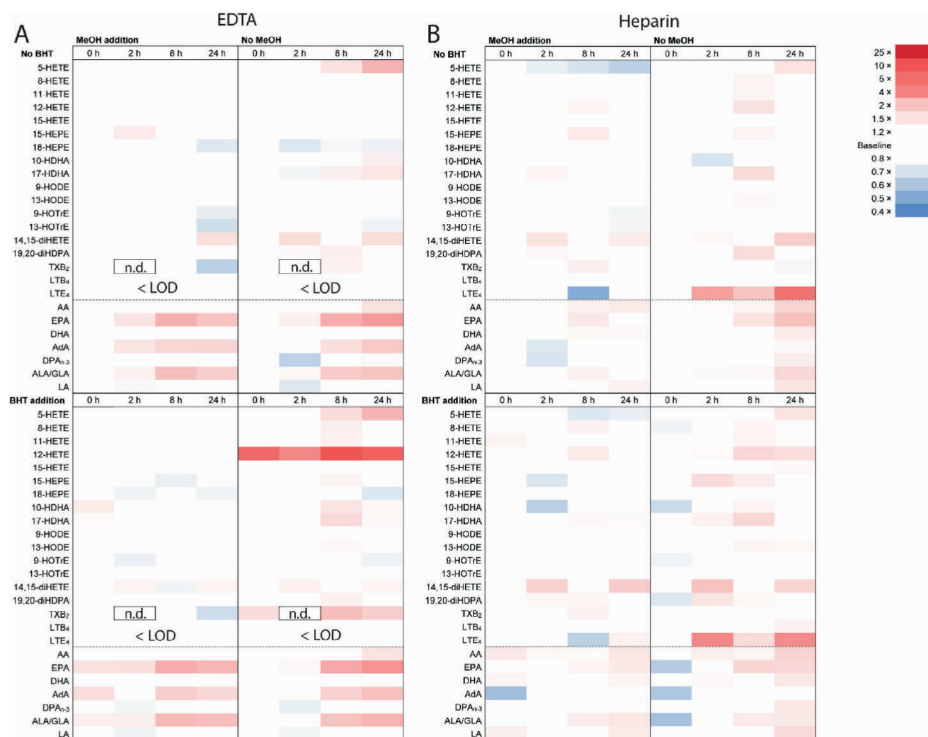

Figure 4. Short-term storage at 6 °C. Heatmap showing the fold increase of the corrected area to baseline over time. **A**: EDTA plasma and **B**: heparin plasma. LTB_4 and LTE_4 were not detected in EDTA samples. Abbreviations: n.d. not determined, LOD limit of detection.

Short-term storage at -20 °C

Samples kept short-term at -20 °C were worked-up after 24 hours 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 hours in the EDTA plasma. MeOH precipitation and BHT addition had little effect. (Figure 5 and Supplementary Figures 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 (Figure 5 and Supplementary Figures S9-S27). A significant increase in 12-HETE and TXB_2 levels was observed in EDTA plasma and this was prevented by addition of MeOH (Figure 5A and Supplementary Figures S12 and S25. Note the possible outlier in Supplementary Figure S12, see *Discussion*). Similar to samples kept at higher temperatures, LTE_4 levels in heparin plasma had increased after 24 hour storage and addition of MeOH had little effect on this change (Figure 5B and Supplementary Figure S26).

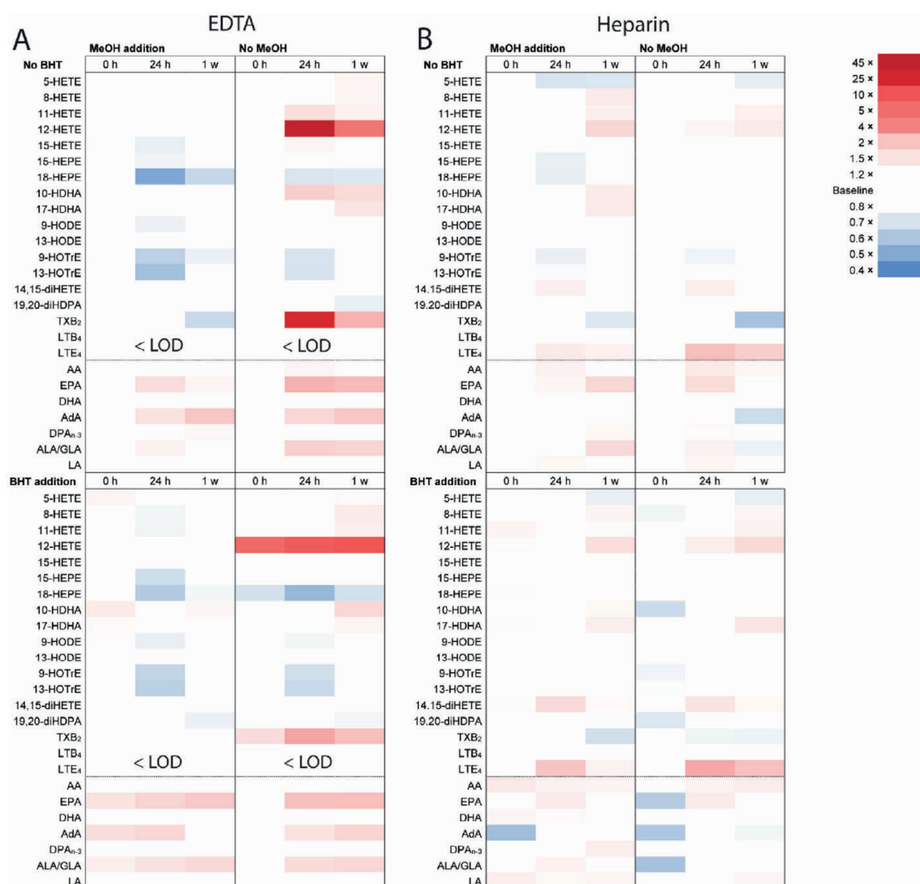

Figure 5. Short-term storage at -20 °C. A heatmap showing the fold increase of the corrected area to baseline over time. **A**: EDTA plasma and **B**: heparin plasma. LTB_4 and LTE_4 were not detected in EDTA samples. Abbreviations: LOD limit of detection. 12-HETE levels without additives in EDTA plasma, possible outlier, see Supplementary Fig S12.

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 Figures S28-S34). Compared to baseline, only EPA, AdA, and ALA/GLA levels increased over one year for all conditions (Figure 6 and Supplementary Figures 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 (Figure 6 and Supplementary Figures S35-S53) and these changes were slowed down by MeOH addition. The increase in 12-HETE and TXB_2 levels in EDTA plasma and LTE_4 in heparin plasma were the most pronounced and

were diminished by the addition of MeOH prior to storage (Figure 6 and Supplementary Figures S38, S51, and S52).

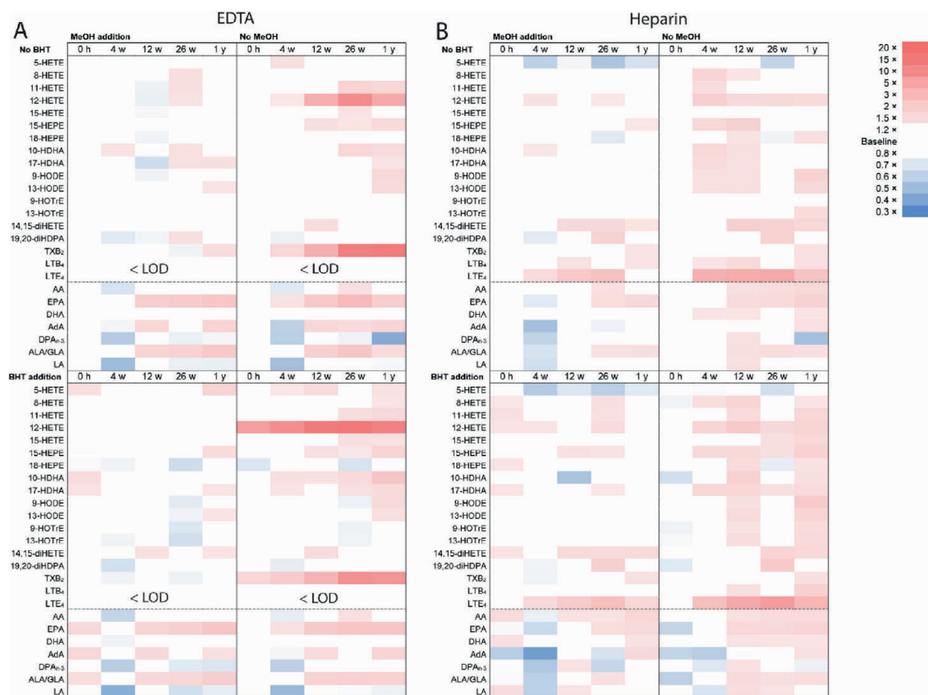

Figure 6. Long-term storage at -80 °C. Heatmap showing the fold increase of average corrected area to baseline over time. **A**: EDTA plasma and **B**: heparin plasma. LTB_4 and LTE_4 were not detected in EDTA samples. Abbreviations: LOD limit of detection.

Long term storage at -20 °C

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

Without MeOH, levels of monohydroxylated FAs were significantly increased by week 4 in EDTA plasma and by week 26 in heparin plasma (Figure 7 and Supplementary Figures S35-S48) and this was diminished by MeOH additon. 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 HOTrEs (Figure 7 and Supplementary Figures 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. (Figure 7 and Supplementary Figures S49 and S50). Levels of TXB_2 were significantly increased only in EDTA plasma without MeOH by week 4 (Figure 7 and Supplementary Figure S51), while LTB_4 and LTE_4 levels were significantly increased in heparin plasma without MeOH by week 26 and week 4 respectively (Figure 7B and Supplementary Figures S52 and S53). As there was no LTB_4 detected at baseline in EDTA plasma, and the figure depicts ratio to basal levels, its fold-increase is not presented in Figure 7A. The addition of BHT did not have a significant effect on lipid levels.

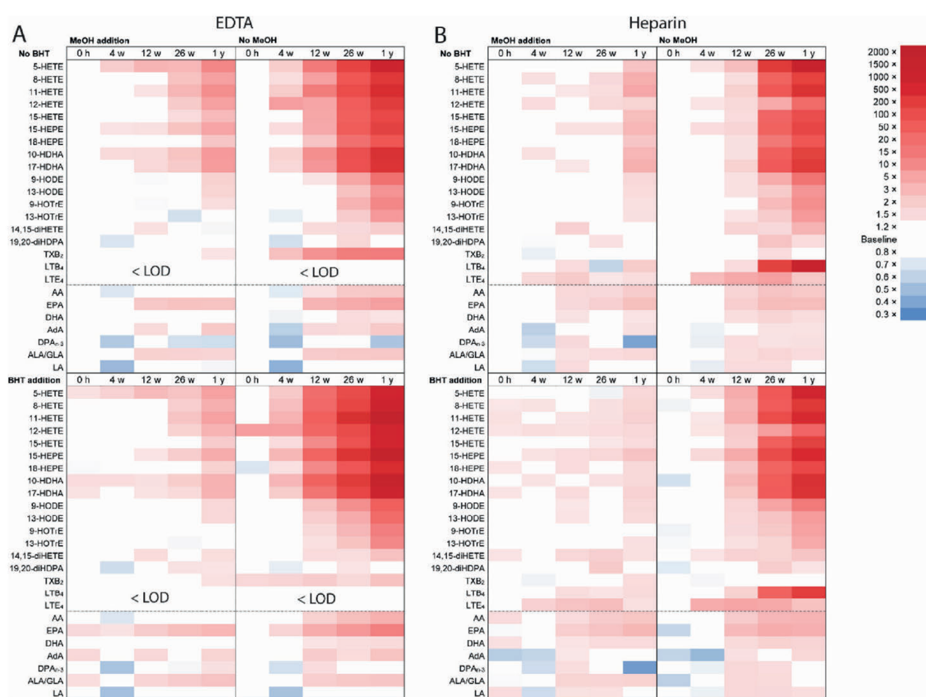

Figure 7. Long-term storage at -20 °C. Heatmap showing the fold increase of corrected area to baseline over time. **A:** EDTA plasma and **B:** heparin plasma. Note the increase in LTB_4 from week 12 is most likely not LTB_4 but the isomer 5*S*,12*S*-diHETE, for more detail see chapter *Appearances of new oxylipids over time*. Abbreviations: LOD limit of detection.

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 LTA_4, 6-trans-LTB_4 and 6-trans-12-epi-LTB_4, became detectable after 12 weeks in plasma from both anticoagulants (Supplementary Figures S54 and S55). A small increase in the LTB_4 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 5*S*,12*S*-diHETE that is not distinguishable from LTB_4 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. Figure S53) and the relative retention time (RRT) had clearly shifted towards the 5*S*,12*S*-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 Figure S56). In addition, detectable levels of 7*S*,17*S*-diHDPA ($RvD5_{n\text{-}3\ DPA}$,), 5*S*,15*S*-diHETE, and 8*S*,15*S*-diHETE, were present when samples had been stored at -20°C without MeOH precipitation for 26 weeks (Supplementary Figures S57-S59).

Surprisingly, we also observed the appearance of features that had the characteristics of bioactive lipid mediators (LMs) such as MaR1, PD1, and PDX (10*S*,17*S*-diHDHA), starting at 26 weeks (Supplementary Figures 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 (Figure 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 lipid mediators 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.

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 (Figures 8A and 9A) and on the chiral platform (Figures 8B and 9B) (see *Materials and Methods*). The signal referring to PDX falls within a window of no more than 0.5% RRT deviation on both chromatographic platforms, when compared to standard material (Figure 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.

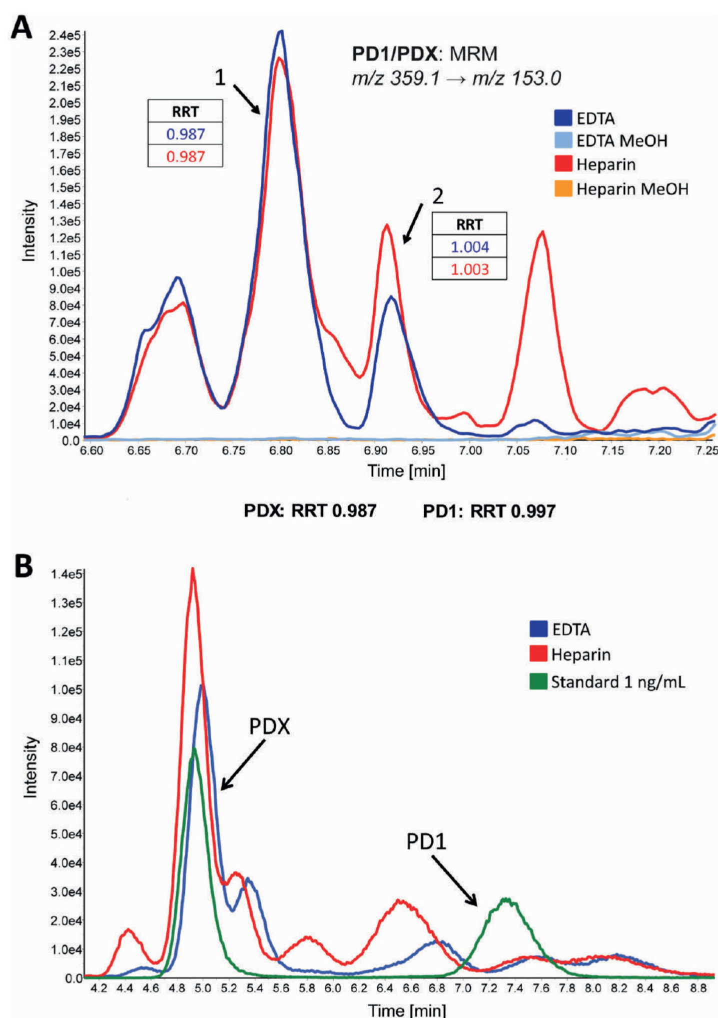

Figure 8. Identification of PD1/PDX (10*S*,17*S*-diHDHA) in samples stored at -20 °C for 74 weeks. **A**: MRM trace of PD1/PDX after separation on RPLC. RRT relative to IS LTB_4-d4 of standard material and unidentified peaks **1** and **2**. **B**: MRM trace of PD1/PDX after separation on chiral column. 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 the interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

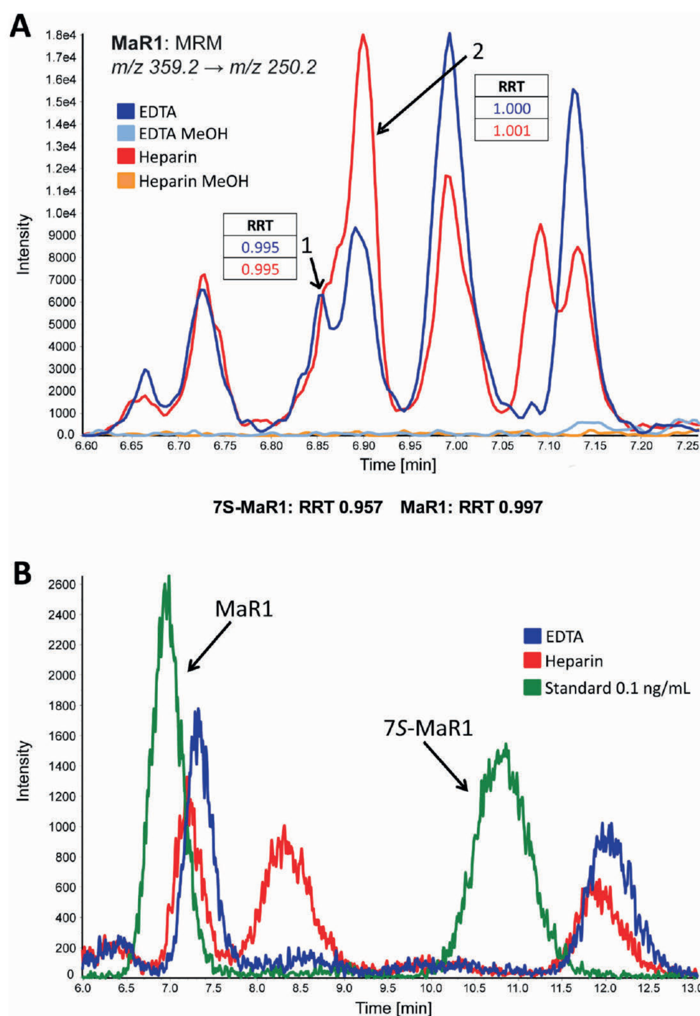

Figure 9. Identification of MaR1 in samples stored at -20 °C for 74 weeks. **A**: MRM trace of MaR1 after separation on RPLC. RRT relative to IS LTB_4-d4 of standard material and unidentified peaks **1** and **2** **B**: MRM trace of MaR1 after separation on chiral column. RRT on column to IS LTB_4-d4 of standard material. 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 0.1 ng/mL each MaR1 and 7*S*-MaR1. (For the interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Chiral analysis of HETEs

In addition to the chiral analysis of lipid mediators, 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 15*S*-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 (Figure 6) and showed a high *S/R* ratio. As expected from the results in figure 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.

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 suggests 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 TXB_2 and 12-HETE, and the 5-LOX products 5-HETE, LTB_4 and LTE_4 proved highly affected. These lipids were all higher in heparin than in EDTA plasma. Higher concentration of 12-HETE and TxB_2 (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 TXB_2 and LTE_4. The increase in levels is likely due to (residual) enzymatic 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 Figures S38 and S51). This could indicate the slow activation of 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 Figure 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 oxylipin 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 5*S*-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 chapters *Appearances of new oxylipids over time* and *Chiral analysis of lipid mediators*, Figure 8, Figure 9, and Supplementary Figures 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); 5*S*,12*S*-diHETE (17), 6-trans-LTB_4 and 6-trans-12-epi-LTB_4 (24,25) via 5-H(p)ETE, and $RvD5_{n\text{-}3\ DPA}$ via 17-$HpDPA_{n\text{-}3}$ (26,27) *in vivo*. In contrast, 5*S*,15*S*-diHETE can be made *in vivo* via 5-LOX (28,29) and/or via 15-LOX and 8*S*,15*S*-diHETE via 15-LOX(34) from 8*S*-H(p)ETE. For PDX a double oxygenation catalyzed by 15-LOX might be an alternative pathway as shown for soybean 15-LOXby Chen et al (30).

However, as is apparent in Figures 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 (35). 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 TXB_2 in EDTA plasma and LTE_4 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 two hours at room temperature or refrigerated for eight hours without significant changes on levels, except for LTE_4 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.

Author contribution

HB and HSJ carried out the experiments and were responsible for data analysis. AIF 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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Supplementary material

Analyte name	Average baseline concentration in EDTA plasma, n=2 [ng/mL]	Average baseline concentration in heparin plasma, n=2 [ng/mL]
5-HETE	0.042	0.166
8-HETE	0.040	0.039
11-HETE	0.017*	0.017
12-HETE	0.072	0.595
15-HETE	0.082	0.068
15-HEPE	0.012*	0.011*
18-HEPE	0.041	0.034
10-HDHA	0.008*	0.014*
17-HDHA	0.049	0.051
14,15-diHETE	0.095	0.087
19,20-diHDPA	0.473	0.442
TXB2	0.032, n=1	0.138
LTB4	n.d	0.020
LTD4	n.d	0.027, n=1
LTE4	n.d	0.012
AA	843+	785+
EPA	49.8	87.3
DHA	598+	536+
AdA	33.3	53.3
DPAn-3	223	213
ALA/GLA	624+	903+
LA	4754+	3607+

Supplementary Table 1. Calculated concentrations of oxylipids and PUFAs at baseline. Pooled plasma directly precipitated with MeOH after aliquotting. Average of the concentration of two technical replicates for each anticoagulant unless otherwise specified.

* Concentration below lowest standard

+ Concentration above highest standard

Abbreviations: AA arachidonic acid, EPA eicosapentaenoic acid, DHA docosahexaenoic acid, AdA adrenic acid, DPAn-3 omega-3 docosapentaenoic acid, LA linoleic acid, ALA alpha-linolenic acid, GLA gamma-linolenic acid, HETE hydroxyeicosatetraenoic acid, HEPE hydroxyeicosapentaenoic acid, HDHA hydroxydocosahexaenoics acid, TXB2 thromoboxane B2, LTB4 leukotriene B4, diHETE dihydroxyeicosatetraenoic acid, and diHDPA dihydroxydocosapentaenoic acid

Supplementary figures S1-S64 can be found in the online version of this article.