

Clinical Chorioamnionitis at Term: The Amniotic Fluid Fatty acyl Lipidome

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Abbreviations:

AF: amniotic fluid, COX: cyclooxygenase, DAMP: damage associated molecular patterns, EpDPE: epoxy docosapentaenoic acid, EpETE: epoxy eicosatetraenoic acid, EpETrE: epoxy eicosatrienoic acid, FDR: false discovery rate, HDoHE: hydroxy docosahexaenoic acid, HEDE:, hydroxy eicosadienoic acid, HEPE: hydroxy eicosapentaenoic acid, HMGB: high mobility gene box-1, HOTrE: hydroxy octadecatrienoic acid, LTB₄: leukotriene B₄, MIAC: microbial invasion of the amniotic cavity, MRM: multiple reaction monitoring, PG: prostaglandin, SI: sterile inflammation, TCC: clinical chorioamnionitis at term, TLB: spontaneous labor at term

Abstract

Clinical chorioamnionitis at term (TCC) is the most common obstetrical affliction diagnosed in labor and delivery units worldwide, and is associated with a substantial increase in maternal and neonatal morbidity and mortality. This obstetrical complication is a heterogeneous condition, as only half of patients have detectable microorganisms in the amniotic cavity. Since bioactive lipids play a key role in the initiation and resolution of an inflammatory response, we aimed to characterize the amniotic fluid lipidome in patients with TCC. We studied the amniotic fluid of patients in the following groups: 1) spontaneous labor at term without clinical chorioamnionitis (TLB); and 2) spontaneous labor at term with clinical chorioamnionitis (TCC). The TCC group was subdivided into: a) those with microbial invasion of the amniotic cavity (TCC-MIAC); and b) those without microbial invasion of the amniotic cavity (TCC-noMIAC). The amniotic fluid concentration of pro-inflammatory lipid mediators did not differ between patients in TLB and TCC. In contrast, lipids with anti-inflammatory/pro-resolution properties was significantly lower in all patients with TCC than in TLB. These results suggest that while pro-inflammatory lipid mediators are involved in infection-driven intra-amniotic inflammation, a relative deficiency of anti-inflammatory/pro-resolution lipid mediator biosynthesis is a characteristic of TCC.

Keywords:

Lipoxygenase, Eicosanoids, Inflammation, omega-3 fatty acids, lipidomics, intra-amniotic inflammation, infection, parturition, epoxy fatty acids, epoxygenase

Introduction

Intra-amniotic inflammation in both preterm labor patients with intact membranes and in pre-labor rupture of membranes is often subclinical in nature (1-3). However, this process can lead to a systemic maternal inflammatory response that is known as clinical chorioamnionitis (4). Clinical chorioamnionitis is the most common infection-related condition diagnosed in labor and delivery units around the world. It is most frequent in young primiparous women and is estimated to have affected nearly 38,000 live births in the United States alone in 2014 (5, 6). In addition to intense maternal inflammatory response, intra-amniotic infection elicits an acute histologic chorioamnionitis (7) and frequently, a fetal inflammatory response, which is manifested as funisitis or chorionic vasculitis (8-11). Clinical chorioamnionitis and puerperal endomyometritis are the leading causes of infection related complications in pregnant women. In addition to the maternal morbidity associated with clinical chorioamnionitis and puerperal infections, neonates born to mothers with clinical chorioamnionitis at term are at increased risk for long-term complications, notably cerebral palsy (11-13).

The features of clinical chorioamnionitis include maternal fever, maternal or fetal tachycardia, leukocytosis, uterine tenderness, and foul-smelling amniotic fluid (14-16). We recently reported that clinical chorioamnionitis at term is a heterogeneous condition, as only 54% of patients have intra-amniotic infection (also called microbial-associated intra-amniotic inflammation), 24% have intra-amniotic inflammation without detectable microorganisms (sterile intra-amniotic inflammation), and 22% have no intra-amniotic inflammation (6). While the precise causes of sterile intra-amniotic inflammation are unknown, studies have shown that damage-associated molecular patterns (DAMPs), such as high mobility gene box-1 (HMGB1), can elicit a sterile inflammatory response (17) and are elevated in the amniotic fluid of patients with clinical chorioamnionitis at term (18), and that this alarmin can induce labor (19). There is an urgent need for tests allowing the differential diagnosis of the three subgroups of patients with clinical chorioamnionitis, as these patients require different treatment. For example, patients with microbial-associated intra-amniotic inflammation should be managed with antibiotics, whereas the

administration of these agents is unnecessary for those without intra-amniotic inflammation/infection. Identification of biomarkers to delineate clinical chorioamnionitis in the presence and absence of intra-amniotic infection would significantly advance our understanding of the disease as well as meet our diagnostic needs.

Bioactive lipids derived from the metabolism of polyunsaturated fatty acids (PUFA) such as arachidonic acid act as mediators in the inflammatory response to injury caused by both microorganisms (bacterial or viral) and cellular damage (sterile) (20-23). These lipids also ensure resolution of inflammation (21, 22). Prostaglandins play an important role in parturition at term (24-26) and it is possible that an increase in amniotic fluid inflammatory lipids such as prostaglandin E₂ (PGE₂), leukotriene B₄ (LTB₄), etc., in clinical chorioamnionitis can be observed. In fact, amniotic fluid concentrations of LTB₄ (as well as other 5-lipoxygenase derived metabolites of arachidonic acid) are significantly higher in patients with clinical chorioamnionitis and microbial-associated intra-amniotic inflammation than in those with sterile intra-amniotic inflammation (27). Recent evidence suggests that, in the normal course of events, a typical inflammatory response is followed by the synthesis of pro-resolution mediators such as lipoxins, resolvins, and protectins from PUFA to inhibit further leukocyte infiltration (20, 21). Biosynthesis of these resolution mediators is facilitated by the same polymorphonuclear leukocytes that generate inflammatory lipid mediators, following an acute inflammatory response (28-30). Such lipid mediator class switching is crucial to limit inflammation to a homeostatic function and prevent a chronic inflammatory response (28). As an acute inflammatory condition, clinical chorioamnionitis at term is expected to elicit an amniotic fluid lipid mediator profile distinct from that of spontaneous labor at term. Therefore, the bioactive lipid profiles of amniotic fluid in clinical chorioamnionitis at term may reflect an imbalance between pro- and anti-inflammatory lipid mediators in favor of the pro-inflammatory state, when compared to spontaneous labor.

To test this hypothesis, the amniotic fluid fatty acyl lipidome of patients at term in spontaneous labor with or without clinical chorioamnionitis was analyzed, utilizing an unbiased liquid

chromatography–mass spectrometric (LC-MS) approach. Results of this study demonstrate that the concentrations of inflammatory lipid mediators such as PGE₂, known to participate in the initiation of labor, are similar, while a significantly lower concentration of anti-inflammatory lipid mediators was observed in patients with clinical chorioamnionitis, especially those without detectable microbial invasion in the amniotic cavity.

Materials and Methods:

Study design and population:

A retrospective cross-sectional study was conducted by searching the clinical database and Bank of Biological samples of Wayne State University, Detroit Medical Center and the Perinatology Research Branch (NICHD/NIH). Women with singleton pregnancies who had amniotic fluid samples obtained by transabdominal amniocentesis were included. The details of patient groups are described elsewhere (27).

All patients provided written, informed consent before the collection of amniotic fluid samples. The collection and utilization of the samples were approved by the Human Investigation Committee of the Sotero del Rio Hospital, a major affiliate of the Catholic University of Santiago, Chile, and the Institutional Review Board (IRB), of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD/NIH/DHHS). Many of these samples have been used in previous studies of the biology of cytokines and inflammatory mediators (4, 6, 16, 31-33).

Clinical definitions:

Spontaneous term labor was defined as the presence of regular uterine contractions with a frequency of at least one every 10 minutes and cervical changes after 37 weeks of gestation. Clinical chorioamnionitis was diagnosed by the presence of maternal fever (temperature $>37.8^{\circ}\text{C}$) accompanied by two or more of the following criteria: 1) maternal tachycardia (heart rate >100 beats/min); 2) uterine tenderness; 3) foul-smelling odor of the amniotic fluid; 4) fetal tachycardia (heart rate >160 beats/min); and 5) maternal leukocytosis (leukocyte count $>15,000$ cells/ mm^3) (16).

Intra-amniotic inflammation was diagnosed when the amniotic fluid interleukin (IL)-6 concentrations were ≥ 2.6 ng/mL as determined by enzyme-linked immunosorbent assay (ELISA) (34). Microbial invasion of the amniotic cavity (MIAC) was defined according to the results of amniotic fluid culture and/or PCR coupled with electrospray ionization mass spectrometry (PCR/ESI-MS; Ibis® Technology - Athogen, Carlsbad, CA). Based on the results of amniotic fluid cultures and/or PCR/ESI-MS, patients were classified as: 1) having MIAC; or 2) those without MIAC.

Sample collection:

Amniotic fluid samples were obtained by transabdominal amniocentesis performed for evaluation of the microbial and inflammatory status of the amniotic cavity in patients presenting with clinical chorioamnionitis at term, whereas patients approaching term underwent an amniocentesis for assessment of fetal lung maturity. This information was used by obstetricians and neonatologists in the management of mothers and neonates in terms of treatment with antibiotics. Women at term in labor consisted of those who were admitted for suspected preterm labor because of uncertain dates and had an amniocentesis for the assessment of fetal lung maturity. The criteria for considering whether these patients were at term in labor were derived retrospectively, and were: 1) spontaneous labor; 2) delivery within 24 hours of amniocentesis; 3) analysis of amniotic fluid consistent with fetal lung maturity; 4) birth weight >2500 grams; 5) absence of respiratory distress syndrome or other complications of prematurity; and 6) physical examination of the newborn by a pediatrician that was consistent with a term neonate. Samples of amniotic fluid were transported to the laboratory in a sterile capped syringe and cultured for aerobic/anaerobic bacteria and genital Mycoplasmas. White blood cell (WBC) count (35), glucose concentration (36) and Gram stain (37) were also performed shortly after collection, as previously described. The results of these tests were used for clinical management. Results of IL-6 concentrations in amniotic fluid were used only for research purposes. Amniotic fluid not required for clinical assessment was centrifuged at 1300 x g for 10 min at 4°C and the supernatant was stored at -70°C.

Detection of Microorganisms with Molecular Methods:

In addition to standard cultivation techniques, the amniotic fluid of patients with clinical chorioamnionitis was analyzed using broad-range real-time polymerase chain reaction with electrospray ionization mass spectrometry (PCR/ESI-MS) (Ibis®Technology - Athogen, Carlsbad, CA, USA), as previously described (34). Briefly, DNA was extracted from 300µL of amniotic fluid using a method that combines bead-beating cell lysis with a magnetic-bead-based extraction method (38, 39). The extracted

DNA was amplified by the previously described broad bacteria and candida (BAC) detection assay, according to the manufacturer's instructions. PCR/ESI-MS can identify 3400 bacteria and 40 *Candida* spp., which are represented in the platform's signature database (40).

After PCR amplification, 30 μ L aliquots of each PCR product were desalted and analyzed via electrospray ionization mass spectrometry (ESI-MS). The presence of microorganisms was determined by signal processing and triangulation analysis of all base composition signatures obtained from each sample and compared to a database. The sensitivity (lower limit of detection - LOD) of the assay for the detection of bacteria in blood is, on average, 100 colony-forming unit (CFU)/mL (95% CI, 6–600 CFU/mL). A comparison of detection limits between blood and amniotic fluid shows that the assays have comparable detection limits (100 CFU/mL).

Determination of IL-6 concentrations in amniotic fluid:

Concentrations of IL-6 in amniotic fluid were determined by sensitive and specific enzyme immunoassays obtained from R&D Systems (Minneapolis, MN). The initial assay validation was performed in our laboratory prior to the conduction of this study. The concentrations were determined by interpolation from the standard curves. The inter- and intra-assay coefficients of variation for IL-6 were 8.7% and 4.6%, respectively. The sensitivity of the assay for IL-6 was 0.09 pg/mL.

Sample preparation and LC-MS analysis:

Fatty acyl lipids from amniotic fluid samples were extracted and analyzed by LC-MS, as described earlier (26). However, the LC-MS analysis included Multiple Reaction Monitoring (MRM) of metabolites of linoleic, eicosapentaenoic, and docosahexaenoic acids in addition to arachidonic acid. A complete list of the analyzed PUFA metabolites, the internal standards used, mass spectrometric conditions, as well as the detection limits of individual lipid mediators were presented in Supplementary Data, Table S1. Under standardized conditions, the detection limits of most eicosanoids are ca. 2 pg on the column and the limit of quantitation was 5 pg at a signal/noise ratio of 3. Since the sample volume

used was 200 μ L, this translates to an assay sensitivity of 0.03 nM for an average molecular mass of 330 of the detected eicosanoids.

Statistical Analysis:

For any detectable lipid analyte in a subject group, a zero value observed in any sample was replaced with half the average detection limit of the LC-MS method used for the eicosanoids (i.e. 0.015 nM). This ensures that information from all samples was used in the statistical analysis and that the fold change between groups was finite for each analyte. A Wilcoxon test, which does not rely on any distributional assumptions about the data, was used for all pair-wise group comparisons. The significance of p value from the Wilcoxon test is independent on the choice of the threshold concentration used to replace the values below the quantitation limits of the assay.

A parametric alternative to the Wilcoxon test was also applied by using a t-test for analytes with concentrations above the quantitation limits of the assay in all samples, or using censored regression otherwise. In the presence of a perfect separation between groups, the maximum likelihood estimation involved in censored regression could not be applied, and hence a t-test was used instead.

To account for multiple testing, the p value obtained for all analytes in a given group comparison was adjusted to control the False Discovery Rate (FDR) (41). A threshold of 10% on the FDR was used to infer significance.

All analyses were performed under the R statistical language and environment, version 3.0 (www.r-project.org), using the *censReg* R package for the censored regression analysis (42).

Results

Clinical characteristics of the study population:

The demographic and clinical characteristics of patients are described in Table 1. The patient groups include women at term gestation with spontaneous labor (TLB, $n=35$) and those with clinical chorioamnionitis at term (TCC, $n=24$). Patients with clinical chorioamnionitis at term were further subdivided into: 1) those with MIAC (TCC-MIAC, $n=12$); and 2) those without MIAC (TCC-noMIAC, $n=12$). IL-6 concentrations in amniotic fluid were greater in the TCC-MIAC group than in the TCC-noMIAC group, however, the statistical significance was marginal ($p = 0.07$). The TLB group had, yet, lower IL-6 concentrations than the TCC-noMIAC group.

LC-MS analysis of amniotic fluid fatty acyl lipidome:

The LC-MS method included MRM transitions to encompass stable metabolites of linoleic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid produced by cyclooxygenase, lipoxygenase, and the epoxygenase pathways of PUFA metabolism (details of the metabolites analyzed and the standard abbreviations are provided in Supplementary data - Table S1). Each detected fatty acyl lipid was positively identified by a combination of matching High Performance Liquid Chromatography (HPLC) retention time with the authentic standard and the unique MRM transition. Coefficient of variation between different LC-MS runs of samples was $<5\%$. Of the total 144 individual PUFA metabolites monitored by the LC-MS method, 51 metabolites were detectable in more than 50% of the samples in any group (Supplementary data - Table S2). Only the lipid mediators that showed significant differences between TLB and TCC-noMIAC with $p < 0.005$ were included in the figures.

Prostaglandins:

Concentrations of prostaglandins and their metabolites such as PGE_2 , $\text{PGF}_2\alpha$, bicyclo PGE_2 , etc., derived primarily from ω -6 PUFA in the cyclooxygenase pathway were comparable in the amniotic fluid of patients with spontaneous labor at term (TLB) and those with clinical chorioamnionitis at term with

MIAC (TCC-MIAC) and without MIAC (TCC-noMIAC) (Table 2). These similarities were also evident when the median concentrations of primary prostaglandins (PGE_2 and $\text{PGF}_2\alpha$) and their corresponding downstream metabolites (15-keto PGE_2 , PGA_2 , 13,14-dihydro-15-keto PGE_2 (measured as bicyclo PGE_2) from PGE_2 and 15-keto $\text{PGF}_2\alpha$, 13,14-dihydro-15-keto $\text{PGF}_2\alpha$ from $\text{PGF}_2\alpha$) were summed to assess their aggregate biosynthesis (Supplementary Data, Table S3). Despite higher concentrations in TCC-MIAC and lower concentrations in TCC-noMIAC groups compared to the TLB group, there was no statistically significant difference in the median concentrations of the cyclooxygenase-derived eicosanoids between any of the patient groups. The exception was 13,14-dihydro-15-keto $\text{PGF}_2\alpha$, which was higher in TCC-MIAC than in the TLB group (Table 2).

Lipoxygenase pathway metabolites:

Hydroxy fatty acids derived from both ω -6 and ω -3 PUFA in the lipoxygenase pathway were detectable in all three patient groups (Table 3). There were no significant differences between spontaneous labor at term (TLB) and clinical chorioamnionitis with detectable microorganisms in the amniotic cavity (TCC-MIAC) with respect to the median concentrations of the hydroxy fatty acids from either ω -6 or ω -3 PUFA, except for 12-HETE, 15-HETE, and 11-HEPE, which are higher in TCC-MIAC than in the TLB group (Table 3). On the other hand, virtually all hydroxy fatty acids derived from ω -3 PUFA were significantly lower in patients with clinical chorioamnionitis without detectable microorganisms in the amniotic cavity (TCC-noMIAC) than in those with TLB (Table 3, Figures 1 and 2). While 5 of 9 hydroxy fatty acids from ω -6 PUFA (Table 3) were also lower in patients with TCC-noMIAC than in those with TLB, the difference in concentration was more pronounced in ω -3 PUFA-derived hydroxy fatty acids (HOTrEs, HEPEs, and HDoHEs, 5 fold lower in TCC-noMIAC than TLB) than ω -6 PUFA-derived hydroxy fatty acids (HOTrE- γ , HEDE, and HETEs, 2 fold lower in TCC-noMIAC than TLB) (Supplementary Data, Table S2).

Epoxygenase pathway metabolites:

The concentration of epoxy fatty acids derived from the metabolism of PUFA in the epoxygenase pathway, especially from ω -3 PUFA (EpETEs and EpDPEs), were significantly lower in patients with clinical chorioamnionitis at term, regardless of the status of MIAC (TCC-noMIAC and TCC-MIAC) than in spontaneous labor at term (TLB) ($p < 10^{-7}$ to 10^{-3}) (Table 4, Figures 3 and 4). Alternatively, the epoxy fatty acids derived from the epoxygenase metabolism of ω -6 PUFA (EpOMEs and EpETrEs) were only lower in patients with clinical chorioamnionitis without MIAC (TCC-noMIAC, $p < 10^{-7}$ to 10^{-4}), but not in those with TCC-MIAC, ($p = 0.1$ to 0.97) than in those with TLB (Table 4, Figure 3). Vicinyl dihydroxy PUFA resulting from the hydrolysis of these epoxy fatty acids were also included in the analysis (Supplementary Data, Table S1) but were not detectable in any of these samples (data not shown).

Discussion

Principal findings of the study: 1) The amniotic fluid concentrations of anti-inflammatory/pro-resolution lipid mediators are significantly lower in women with clinical chorioamnionitis at term without detectable microorganisms in the amniotic cavity than in those with spontaneous labor at term; and 2) there were no significant differences in the amniotic fluid concentrations of prostaglandins and other known inflammatory lipid mediators between spontaneous labor and women in labor at term with clinical chorioamnionitis.

Amniotic fluid prostaglandin concentrations are similar between patients with spontaneous labor at term regardless of the presence or absence of intra-amniotic inflammation:

Spontaneous parturition at term is akin to inflammation by virtue of leukocyte infiltration in gestational tissues (43, 44) as well as elevated concentrations of inflammatory cytokines such as IL-1 α , IL-1 β (45), IL-6 (44, 46), IL-8 (47), tumor necrosis factor- α (TNF- α) (48), and monocyte chemoattractant protein-1 (MCP-1) (49). Induction of cyclooxygenase-2 (COX-2) expression in placental membranes in response to elevated cytokines also results in enhanced biosynthesis of prostaglandins such as PGE₂ and PGF₂ α in amniotic fluid, even in the absence of intra-amniotic infection and precedes the onset of labor at term (24, 25, 50-52). Our recent mass spectrometry-based lipidomic analysis of human amniotic fluid at term confirmed significantly higher concentrations of prostaglandins in spontaneous labor at term than in term without labor (26). In addition, we have identified several other arachidonic acid derived lipid mediators of epoxigenase and lipoxygenase pathways.

The median IL-6 concentrations in amniotic fluid of patients with clinical chorioamnionitis at term are significantly higher than in those with spontaneous labor at term (TLB) (Table 1). However, the difference in the concentrations of IL-6 are not significantly different between the two sub-groups of clinical chorioamnionitis ($p = 0.07$). Regardless of the presence of microorganisms in the amniotic cavity in clinical chorioamnionitis (TCC-noMIAC and TCC-MIAC), there was no statistically significant

increase in the inflammatory eicosanoids such as PGE₂ or other arachidonic acid derived lipid mediators over spontaneous labor at term (TLB) (Table 2). In fact, the concentrations of prostaglandins (both PGE₂ and PGF₂α as well as their downstream metabolites [Supplementary data, Table S3]) were lower in clinical chorioamnionitis compared to TLB, albeit, statistically not significant. LTB₄, the chemotactic leukotriene, and other 5-lipoxygenase pathway derived metabolites are virtually undetectable in patients with clinical chorioamnionitis without MIAC and intra-amniotic inflammation [sterile intra-amniotic inflammation (TCC-SI)] or in those with TLB, except in patients with clinical chorioamnionitis with intra-amniotic infection (TCC-IAI-MIAC) (27). Therefore, sterile intra-amniotic inflammation associated with clinical chorioamnionitis is not due to a higher concentration of prostaglandins and other inflammatory lipid mediators that are normally increased in amniotic fluid during spontaneous labor (and play an important role in the physiological process of parturition). Thus, despite higher amniotic fluid concentrations of the inflammatory cytokine IL-6, in clinical chorioamnionitis compared to TLB, there is no corresponding increase in prostaglandins. These data strongly suggest a dissociation in the normally coordinated expression of inflammatory cytokines and COX-2 mediated prostaglandin biosynthesis, at least, in the context of clinical chorioamnionitis with or without intra-amniotic infection.

Anti-inflammatory and precursors of specialized pro-resolution lipid mediators are lower in the amniotic fluid of patients with clinical chorioamnionitis at term:

Our recent eicosanomic analysis of human amniotic fluid in labor at term demonstrated the presence of substantial concentrations of epoxygenase pathway metabolites of arachidonic acid (median concentration of EpETrEs: 39 – 191 nM) (26). An expanded fatty acyl lipidomic analysis to encompass metabolites of other essential PUFA such as linoleic acid, eicosapentaenoic acid, and docosahexaenoic acid, along with arachidonic acid, revealed the presence of epoxides of these PUFA as well, in human amniotic fluid in spontaneous labor at term (Table 4 and Supplementary Data, Table S2). However, the concentration of epoxygenase pathway metabolites of PUFA, especially those derived from ω-3 PUFA,

were significantly lower in the amniotic fluid of patients with clinical chorioamnionitis, regardless of the presence/absence of bacteria in the amniotic fluid, than in those with spontaneous labor at term (TLB) (Table 4, Figures 3 and 4). As can be expected, not all epoxy PUFA concentrations were lower (in relation to prostaglandins) to the same extent (Table 4, and Supplementary Data, Table S3). While 8(9)-EpETE is undetectable in both clinical chorioamnionitis groups and 17(18)-EpETE was not detectable in TCC-no MIAC, the rest of the epoxy PUFA were lower to varying degrees (Table 4). The differences in the concentrations (as well as the differences in reduced concentrations in clinical chorioamnionitis) of the epoxy PUFA may represent different, yet unknown, physiological effects in clinical chorioamnionitis. This lower concentration of the epoxy PUFA is not due to their metabolism by epoxide hydrolase, since little, if any, of the corresponding dihydroxy PUFA were detectable in amniotic fluid of any of these patients. Although an effective efflux of the epoxide hydrolysis products from the amniotic cavity is a possibility, their complete absence in amniotic fluid, despite high concentrations of the PUFA epoxides, suggests minimal, if any, epoxide hydrolase activity in the amniotic cavity. Further studies on the expression and enzymatic activity of epoxide hydrolases in placental membranes are needed to confirm these results.

Concentrations of lipoxygenase pathway metabolites of ω -3 PUFA (α -linolenic, eicosapentaenoic, and docosahexaenoic acids) (Figures 1 and 2) were also significantly lower in patients with clinical chorioamnionitis at term without bacteria in the amniotic cavity (TCC-noMIAC) than in patients with spontaneous labor at term (TLB) or those with clinical chorioamnionitis at term with intra-amniotic infection (TCC-MIAC) (Table 3). Any differences in concentration of hydroxy PUFA between TLB and TCC-MIAC were not significant. Thus, while the cyclooxygenase pathway metabolism essentially remains unchanged in labor at term regardless of clinical chorioamnionitis, epoxigenase and lipoxygenase pathway metabolism, especially that of ω -3 PUFA, is significantly lower only in TCC-noMIAC.

Metabolites of ω -3 PUFA are generally considered anti-inflammatory by virtue of their partial agonist/antagonist properties against the eicosanoid receptors (53-55). Metabolism of ω -6 PUFA, especially arachidonic acid, is also inhibited by their ω -3 analogs (56, 57). In addition to the reduced agonist activity of ω -3 PUFA derived prostaglandins, resolution of inflammation by lipoxygenase metabolites of ω -3 PUFA as an active process is now firmly established as a new paradigm by the identification and biological activity of lipoxins, resolvins, and protectins (collectively termed as Specialized Pro-resolution Mediators, SPM) (21, 58, 59). While SPMs such as Resolvins D1 and E1, Protectin D1, Lipoxins A4, B4, and A5 were included in the analysis, they were not detected in these samples of human amniotic fluid. However, the detected hydroxy fatty acids of ω -3 PUFA such as HEPes and HDoHEs are known precursors of SPMs and several additional pro-resolution mediators that were reported in recent years (60, 61). Significantly lower concentrations or absence of these SPM precursors likely signify a failure of resolution of inflammation that characterizes clinical chorioamnionitis in the absence of microbial invasion of the amniotic cavity (TCC-noMIAC).

Although little is known about the role of epoxy PUFA in reproductive biology, studies in cardiovascular and renal physiology showed that epoxy PUFA are anti-inflammatory by inhibition of cytokine-mediated activation of NF- κ B as well as their analgesic, antihypertensive, and antifibrotic activities (62-64). It is likely that the anti-inflammatory activities of epoxy PUFA provide a balance to the pro-inflammatory properties of prostaglandins (e.g. PGE₂) in human amniotic fluid during spontaneous labor at term as a normal physiological response. Thus, the ratio of prostaglandins to the epoxy PUFA in spontaneous labor at term (TLB) likely signifies a homeostatic response in a normal physiological event. Any significant change in this ratio in favor of prostaglandins, either by an increase in prostaglandins or by a decrease in epoxy PUFA, could result in an inflammatory condition. Indeed, the ratios of both PGE₂ and PGF₂ α (as well as the sums of the metabolites of each of these prostaglandins) tilted 50-100% in favor of prostaglandins, primarily due to decreased concentrations of epoxy PUFA (Supplementary Data, Table S3). This suggests an intriguing possibility that a loss of balance between pro- and anti-

inflammatory lipid mediators in clinical chorioamnionitis at term, represented by cyclooxygenase and epoxygenase metabolites, respectively, manifests in sterile intra-amniotic inflammation. This inflammatory response is further exacerbated by a failure of pro-resolution mediator biosynthesis as suggested by lower concentrations of hydroxy ω -3 PUFA, the precursors of SPMs (*vide supra*). Furthermore, it can be surmised that physiological responses such as fever and tachycardia that are mediated by prostaglandins, are held in abeyance during spontaneous labor at term by the antipyretic, analgesic, and anti-inflammatory actions of the epoxygenase and lipoxygenase derived lipid mediators. This represents a novel concept that clinical chorioamnionitis, in the absence of microbial invasion of the amniotic cavity, is a result of failed endogenous anti-inflammatory lipid mediator biosynthesis in an ordered and latent 'inflammatory' physiological process, i.e. parturition.

In conclusion, the amniotic fluid fatty acyl lipidomic profile of clinical chorioamnionitis at term is distinctly different from that of spontaneous labor. Clinical chorioamnionitis, either driven by intra-amniotic infection or idiopathic, is characterized by significantly lower concentrations of anti-inflammatory as well as pro-resolution lipid mediators in human amniotic fluid. Spontaneous parturition at term is characterized by increased expression of PUFA metabolizing enzymes such as COX-2 (65, 66), elevated cytokine expression (44, 45, 48, 49, 67), leukocyte chemotaxis (44), and is generally considered a physiological inflammatory response. Recent unbiased, myometrial transcriptomic analysis further confirmed the overexpression of inflammatory genes in spontaneous labor at term (68, 69). Despite the heightened inflammatory molecular signatures during spontaneous parturition at term, return to homeostasis is normally uneventful, except in clinical chorioamnionitis. While intra-amniotic infection (microbial-associated intra-amniotic inflammation) can be understood as a pathological event, data from the lipidomic analysis presented in this report strongly suggest that clinical chorioamnionitis at term in the absence of MIAC (as well as that resulting from intra-amniotic infection, at least, in part) is a result of 'unchecked inflammation' of a physiological process due to insufficient anti-inflammatory and pro-resolution lipid mediator response to return the system to homeostasis. Further studies to elucidate the mechanisms of this anti-inflammatory and pro-resolution insufficiency could identify valuable biomarkers

of impending clinical chorioamnionitis and/or pharmacological targets to alleviate both short- and long-term complications associated with clinical chorioamnionitis to the neonate.

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Footnotes:

^aThis article is a continuation of our biochemical characterization of Clinical Chorioamnionitis at term and represents eighth in the series.

^bThe content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Table 1: Clinical and obstetrical characteristics of women in spontaneous labor at term and with clinical chorioamnionitis at term

	TLB (n=35)	TCC- noMIAC (n=12)	TCC-MIAC (n=12)	<i>p</i> (TCC-noMIAC vs TCC-MIAC)
Maternal age (years)	23 (20-29)	21.5 (19.5-23.5)	19 (17.8-25.3)	0.4
GA at amniocentesis and delivery (weeks)	39 (38-40.2)	39.6 (38.9-40.0)	40.5 (39.9-40.8)	0.04
IL-6 (ng/ml)	1.08* (0.76- 1.71)	3.2 (2.0-12.0)	13.9 (5.0-22.0)	0.07
Birth weight (grams)	3250 (3100- 3730)	3500 (2978-3725)	3710 (3565-3795)	0.09

TLB: spontaneous labor at term. TCC: clinical chorioamnionitis at term. TCC-MIAC: clinical chorioamnionitis at term with microbial invasion of the amniotic cavity. TCC-noMIAC: clinical chorioamnionitis at term without microbial invasion of the amniotic cavity. GA: Gestational age. Values are expressed as median (interquartile range). *p*: Wilcoxon Rank Sum test

Table 2: Cyclooxygenase pathway derived lipid mediators from polyunsaturated fatty acids in human amniotic fluid in spontaneous labor at term with or without clinical chorioamnionitis.

Lipid	TLB	TCC-no MIAC	TCC-MIAC	<i>p</i> (TLB vs TCC-no MIAC)	<i>p</i> (TLB vs TCC-MIAC)	<i>p</i> TCC-no MIAC vs TCC-MIAC)
PGE ₂	65.2 (28.5-157.4) [34]	37.5 (20.9-105.5) [11]	94.4 (64.3-128.7) [12]	0.19	0.67	0.11
Bicyclo PGE ₂	173.0 (101.2-264.0) [35]	123.6 (39.4-206.6) [11]	185.1 (109.1-334.3) [12]	0.21	0.34	0.13
PGA ₂	56.3 (39.7-85.6) [34]	44.6 (22.1-73.0) [11]	52.5 (32.4-101.1) [12]	0.33	0.59	0.24
19-hydroxy PGE ₂	147.7 (88.4-243.3) [35]	132.7 (95.6-157.7) [12]	171.5 (82.0-299.4) [12]	0.48	0.78	0.44
PGE ₃	18.0 (12.8-27.1) [34]	13.0 (8.12-20.2) [11]	20.7 (18.0-31.2) [11]	0.24	0.37	0.13
Bicyclo PGE ₁	12.4 (5.54-16.3) [27]	3.29 (0.02-12.4) [6]	12.7 (5.26-19.1) [9]	0.07	0.83	0.15
19-hydroxy PGE ₁	33.7 (21.7-60.3) [32]	30.9 (17.7-49.1) [12]	37.5 (21.0-66.2) [12]	0.53	0.85	0.29
PGJ ₂	25.9 (12.1-33.2) [29]	24.9 (5.20-27.7) [9]	26.8 (14.3-45.6) [10]	0.46	0.58	0.37
PGF ₂ α	10.2 (0.02-24.6) [23]	3.86 (0.02-14.8) [6]	22.5 (0.02-43.6) [8]	0.41	0.30	0.22

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Lipid	TLB	TCC-no MIAC	TCC-MIAC	<i>p</i> (TLB vs TCC-no MIAC)	<i>p</i> (TLB vs TCC-MIAC)	<i>p</i> (TCC-no MIAC vs TCC-MIAC)
15-keto PGF₂α	0.02 (0.02-13.2) [13]	0.02 (0.02-0.02) [2]	0.02 (0.02-16.1) [5]	0.21	0.69	0.21
13,14-dihydro-15-keto- PGF₂α	10.8 (0.02-20.3) [24]	15.2 (9.15-22.0) [10]	18.5 (17.2-28.3) [12]	0.26	<i>0.01</i>	0.17

All concentrations are in nM. Values in parentheses are interquartile ranges of analyte concentrations in nM and those in brackets are number of samples with detectable levels of the corresponding lipid mediators. All *p* values are derived from Wilcoxon tests. *p* Values <0.05 were italicized. TLB: spontaneous labor at term. TCC: clinical chorioamnionitis at term. TCC-MIAC: clinical chorioamnionitis at term with microbial invasion of the amniotic cavity. TCC-noMIAC: clinical chorioamnionitis at term without microbial invasion of the amniotic cavity. MIAC: microbial invasion of the amniotic cavity. PG: prostaglandin

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Table 3: Lipoxygenase pathway derived lipid mediators from ω -3 and ω -6 polyunsaturated fatty acids in human amniotic fluid at term in spontaneous labor with or without clinical chorioamnionitis.

Lipid	TLB	TCC-noMIAC	TCC-MIAC	<i>P</i> (TLB vs TCC- noMIAC)	<i>P</i> (TLB vs TCC- MIAC)	<i>P</i> (TCC-noMIAC vs TCC-MIAC)
9(S)-HOTrE (ω-3)	3.63 (2.72-4.92) [32]	0.82 (0.02-2.57) [6]	2.48 (0.02-4.99) [8]	5.32×10^{-4}	0.18	0.22
13(S)-HOTrE (ω-3)	5.95 (2.98-8.51) [34]	2.47 (1.38-3.88) [9]	4.07 (2.29-9.25) [12]	2.28×10^{-3}	0.75	0.13
5-HEPE (ω-3)	0.02 (0.02-1.89) [16]	0.02 (0.02-0.02) [1]	0.02 (0.02-1.44) [5]	0.02	0.56	0.06
11-HEPE (ω-3)	2.01 (1.66-2.24) [30]	2.67 (1.99-3.45) [12]	2.50 (2.17-3.02) [12]	0.02	0.01	0.67
12-HEPE (ω-3)	10.9 (8.74-18.1) [35]	4.11 (3.37-5.34) [11]	9.53 (6.48-13.9) [12]	1.99×10^{-6}	0.25	2.32×10^{-3}
15-HEPE (ω-3)	1.50 (0.02-2.58) [23]	0.02 (0.02-0.17) [3]	2.18 (0.02-2.95) [8]	0.01	0.73	0.02
4-HDoHE (ω-3)	3.66 (1.79-6.65) [32]	0.81 (0.02-2.51) [6]	4.44 (2.56-5.70) [11]	4.69×10^{-3}	0.81	0.01
7-HDoHE (ω-3)	3.37 (2.40-5.85) [33]	0.02 (0.02-1.13) [4]	1.93 (1.00-3.32) [10]	1.35×10^{-5}	0.05	0.01
10-HDoHE (ω-3)	2.69 (1.51-4.06) [29]	0.02 (0.02-1.58) [4]	2.92 (2.37-4.18) [10]	8.07×10^{-4}	0.49	1.09×10^{-3}

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Lipid	TLB	TCC-noMIAC	TCC-MIAC	P (TLB vs TCC- noMIAC)	P (TLB vs TCC- MIAC)	P (TCC-noMIAC vs TCC-MIAC)
11-HDoHE (ω -3)	9.70 (7.26-13.3) [35]	2.98 (2.64-6.36) [11]	11.0 (6.31-17.0) [12]	3.18×10^{-5}	0.97	1.11×10^{-3}
13-HDoHE (ω -3)	2.15 (1.24-3.46) [30]	0.02 (0.02-0.93) [4]	1.79 (0.79-3.45) [9]	1.14×10^{-4}	0.46	0.01
14-HDoHE (ω -3)	19.2 (11.8-24.0) [35]	6.81 (5.17-11.4) [12]	14.9 (10.4-31.2) [12]	4.93×10^{-4}	0.80	0.02
17-HDoHE (ω -3)	3.29 (1.67-5.79) [30]	1.27 (0.02-2.51) [7]	5.06 (2.72-8.04) [11]	0.01	0.47	0.01
20-HDoHE (ω -3)	1.61 (1.14-4.47) [27]	0.02 (0.02-0.27) [3]	1.82 (1.06-3.82) [10]	3.35×10^{-4}	0.92	1.77×10^{-3}
13(S)-HOTrE (γ) (ω -6)	10.6 (7.50-17.7) [35]	5.57 (2.73-8.28) [12]	7.88 (4.29-10.4) [12]	1.03×10^{-3}	0.07	0.27
8-HETrE (ω -6)	16.8 (11.4-28.9) [35]	7.00 (5.67-11.5) [12]	17.1 (12.7-31.1) [12]	1.55×10^{-4}	0.76	0.01
8-HETE (ω -6)	7.43 (4.66-11.9) [34]	3.25 (2.62-4.46) [11]	11.6 (6.27-16.6) [12]	5.3×10^{-4}	0.09	7.17×10^{-5}
11-HETE (ω -6)	19.2 (11.7-29.5) [35]	10.9 (4.64-16.5) [12]	28.9 (15.1-39.9) [12]	0.009	0.25	0.005
12-HETE (ω -6)	144.6 (108.7-207.1) [35]	100.7 (69.2-137.8) [12]	252.8 (163.7-373.4) [12]	0.02	0.005	2.74×10^{-4}
15-HETE (ω -6)	21.8 (15.5-30.4) [35]	14.7 (6.51-22.7) [12]	54.6 (24.7-83.8) [12]	0.09	0.03	0.003

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All concentrations are in nM. Values in parentheses are interquartile ranges of analyte concentrations in nM and those in brackets are number of samples with detectable levels of the corresponding lipid mediators. All *p* values are derived from Wilcoxon tests. *p* Values <0.05 were italicized. TLB: spontaneous labor at term. TCC: clinical chorioamnionitis at term. TCC-MIAC: clinical chorioamnionitis at term with microbial invasion of the amniotic cavity. TCC-noMIAC: clinical chorioamnionitis at term without microbial invasion of the amniotic cavity. MIAC: microbial invasion of the amniotic cavity.

Table 5: Epoxygenase pathway derived lipid mediators from ω -6 and ω -3 polyunsaturated fatty acids in human amniotic fluid in spontaneous labor at term with or without clinical chorioamnionitis.

Lipid	TLB	TCC-noMIAC	TCC-MIAC	<i>p</i> (TLB vs TCC- noMIAC)	<i>p</i> (TLB vs TCC- MIAC)	<i>p</i> (TCC-noMIAC vs TCC-MIAC)
9(10)-EpOME	217.2 (172.2-384.9) [35]	113.0 (76.2-166.1) [12]	239.8 (157.4-370.2) [12]	1.20×10^{-4}	0.97	0.03
12(13)-EpOME	150.2 (106.4-212.7) [35]	59.9 (51.5-107.3) [12]	116.7 (79.5-212.7) [12]	1.06×10^{-4}	0.28	0.16
8(9)-EpETrE	45.7 (28.9-59.9) [35]	14.4 (10.2-21.5) [12]	33.2 (25.2-39.7) [12]	4.97×10^{-7}	0.21	0.02
11(12)-EpETrE	200.2 (166.1-307.6) [35]	67.4 (52.1-113.1) [12]	177.8 (129.3-210.0) [12]	6.77×10^{-6}	0.24	0.01
14(15)-EpETrE	109.6 (83.7-162.9) [35]	45.1 (23.2-67.3) [12]	80.7 (71.8-115.6) [12]	2.86×10^{-6}	0.10	0.02
8(9)-EpETE (ω-3)	2.94 (1.83-3.86) [33]	0.02 (0.02-0.02) [1]	0.02 (0.02-0.02) [2]	1.26×10^{-6}	5.33×10^{-6}	NA
11(12)-EpETE (ω-3)	14.6 (11.1-19.4) [35]	1.94 (1.42-2.96) [10]	3.45 (2.79-6.00) [11]	6.87×10^{-7}	7.70×10^{-8}	0.07
17(18)-EpETE (ω-3)	5.94 (3.92-8.80) [35]	0.02 (0.02-0.93) [5]	1.62 (0.51-2.02) [9]	5.81×10^{-7}	7.55×10^{-6}	0.04
16(17)-EpDPE (ω-3)	9.62 (7.39-14.5) [35]	3.00 (2.32-3.84) [11]	5.21 (2.76-7.58) [12]	6.01×10^{-6}	3.55×10^{-3}	0.05

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Lipid	TLB	TCC-noMIAC	TCC-MIAC	<i>p</i> (TLB vs TCC- noMIAC)	<i>p</i> (TLB vs TCC- MIAC)	<i>p</i> (TCC-noMIAC vs TCC-MIAC)
19(20)-EpDPE (ω-3)	13.4 (8.63-18.9) [35]	3.02 (2.17-4.12) [12]	6.46 (3.54-6.89) [12]	<i>1.06x10⁻⁶</i>	<i>1.55x10⁻⁴</i>	<i>0.02</i>

All concentrations are in nM. Values in parentheses are interquartile ranges of analyte concentrations in nM and those in brackets are number of samples with detectable levels of the corresponding lipid mediators. All p values are derived from Wilcoxon tests. p Values <0.05 were italicized. TLB: spontaneous labor at term. TCC: clinical chorioamnionitis at term. TCC-MIAC: clinical chorioamnionitis at term with microbial invasion of the amniotic cavity. TCC-noMIAC: clinical chorioamnionitis at term without microbial invasion of the amniotic cavity. MIAC: microbial invasion of the amniotic cavity.

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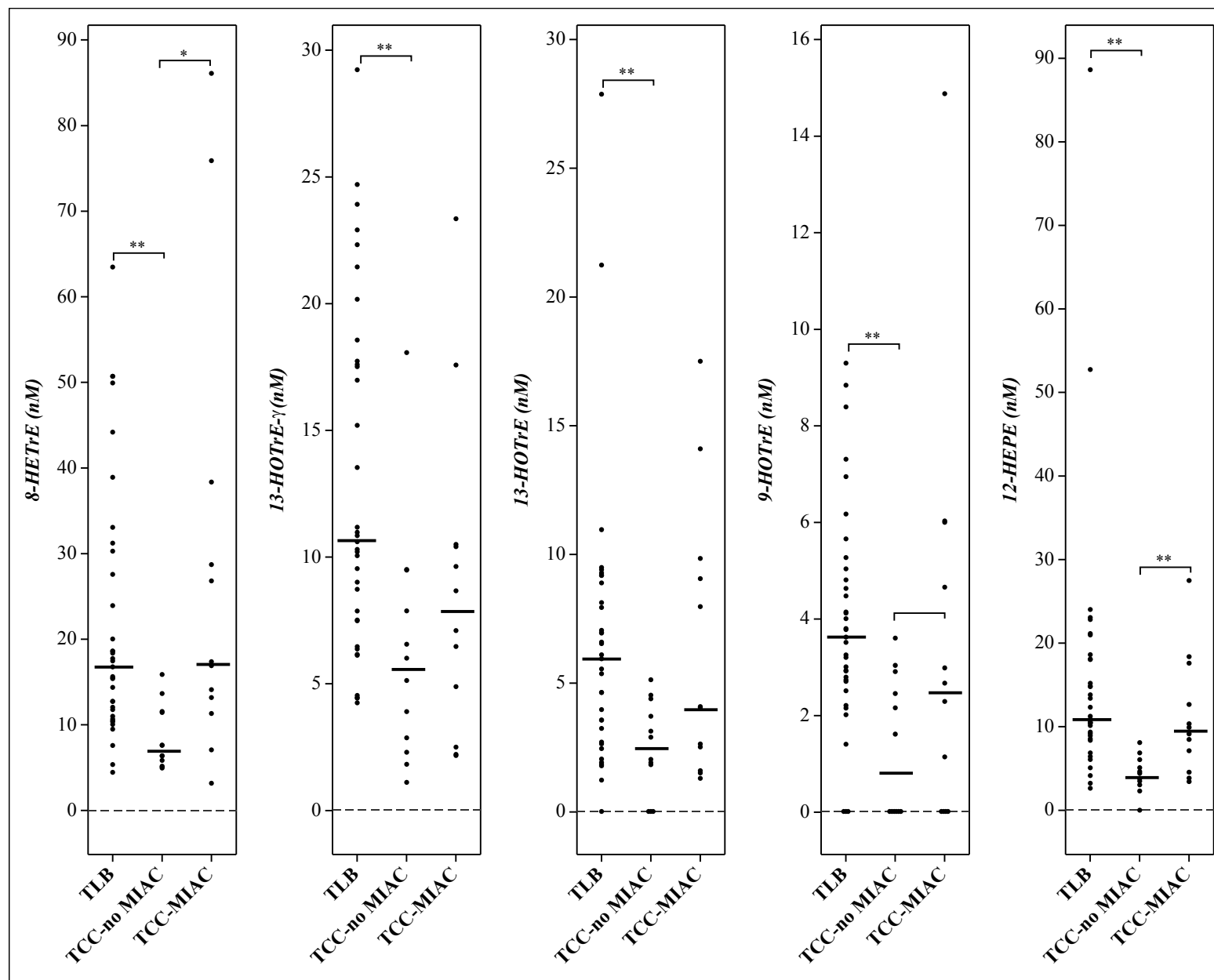


Figure 1: Lipoxigenase pathway metabolites of ω -6 PUFA, dihomo- γ -linolenic (8-HETrE), γ -linolenic (13-HOTrE- γ), and ω -3 PUFA, α -linolenic (9-HOTrE and 13-HOTrE), eicosapentaenoic (12-HEPE) acids detected in amniotic fluid from women in spontaneous labor at term (TLB), compared to those with clinical chorioamnionitis at term without (TCC-noMIAC), or with microbial invasion of the amniotic cavity (TCC-MIAC). Cross bars: Median concentrations. * $p < 0.05$. ** $p < 0.005$.

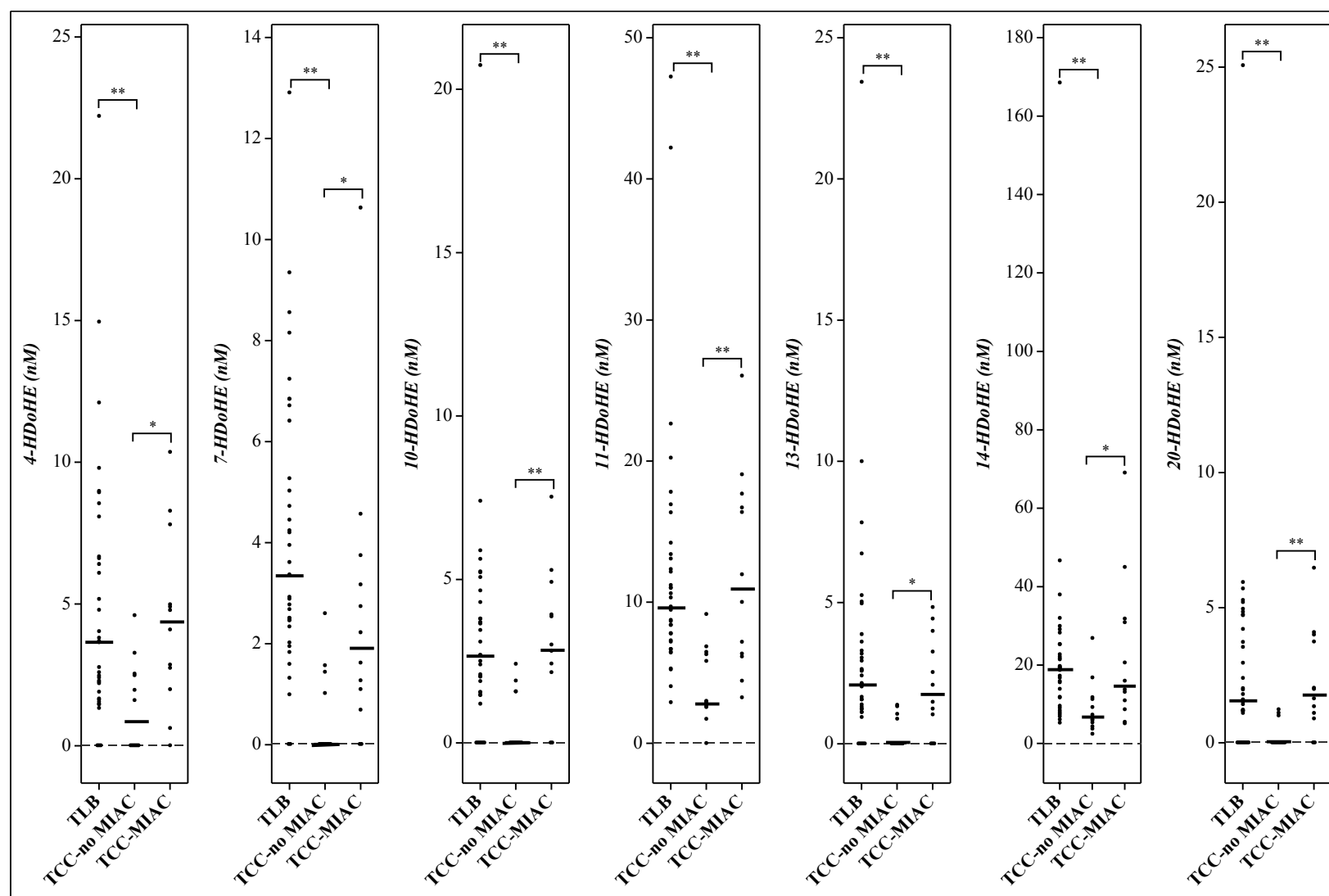


Figure 2: Lipoxigenase pathway metabolites of docosahexaenoic acid (ω -3 PUFA) that exhibited significant difference between patient groups. Patient group abbreviations and other details are the same as in Figure 1.

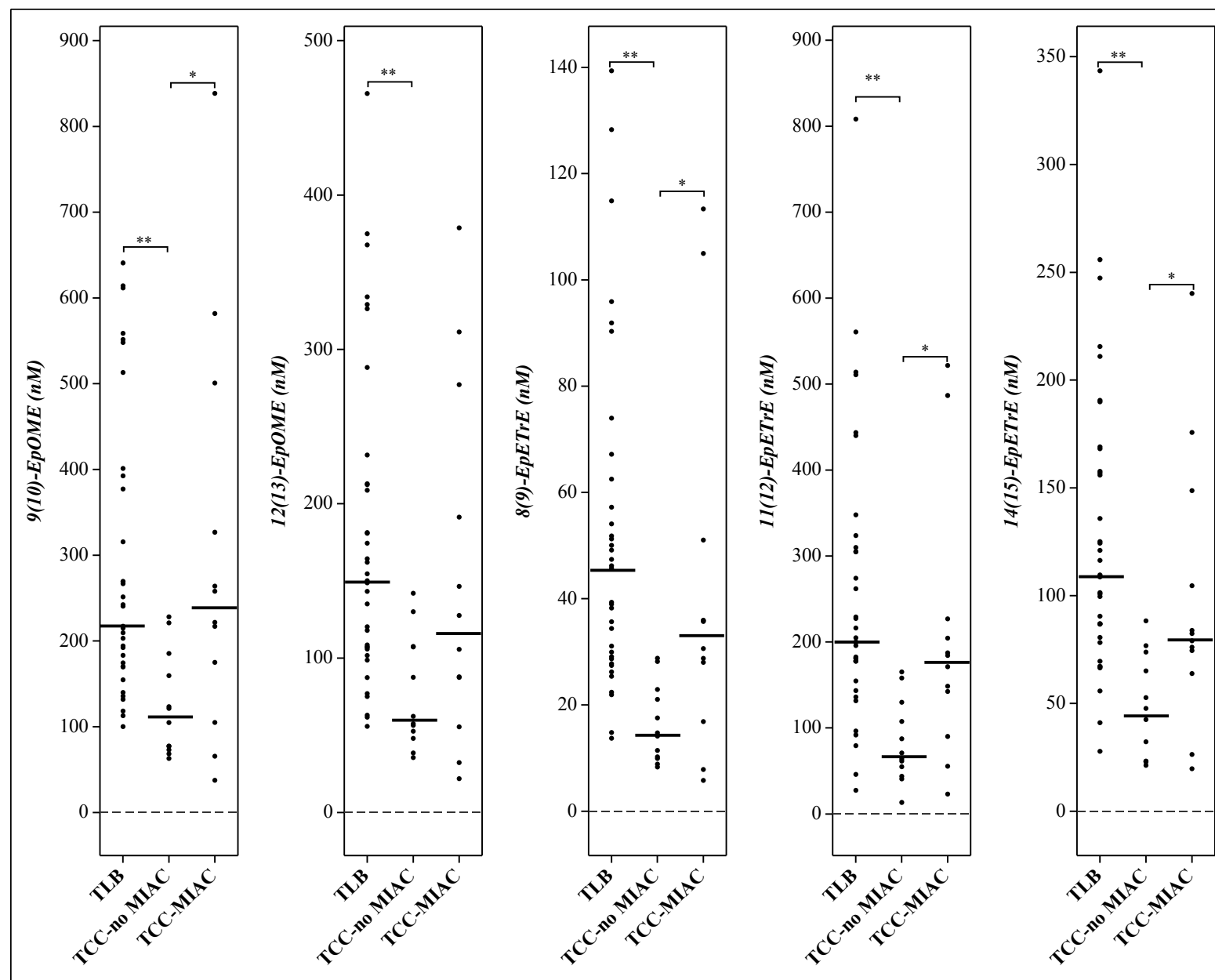


Figure 3: Epoxygenase pathway metabolites of linoleic and arachidonic acids (ω -6 PUFA) that exhibited significant difference between patient groups. Patient group abbreviations and other details are the same as in Figure 1.

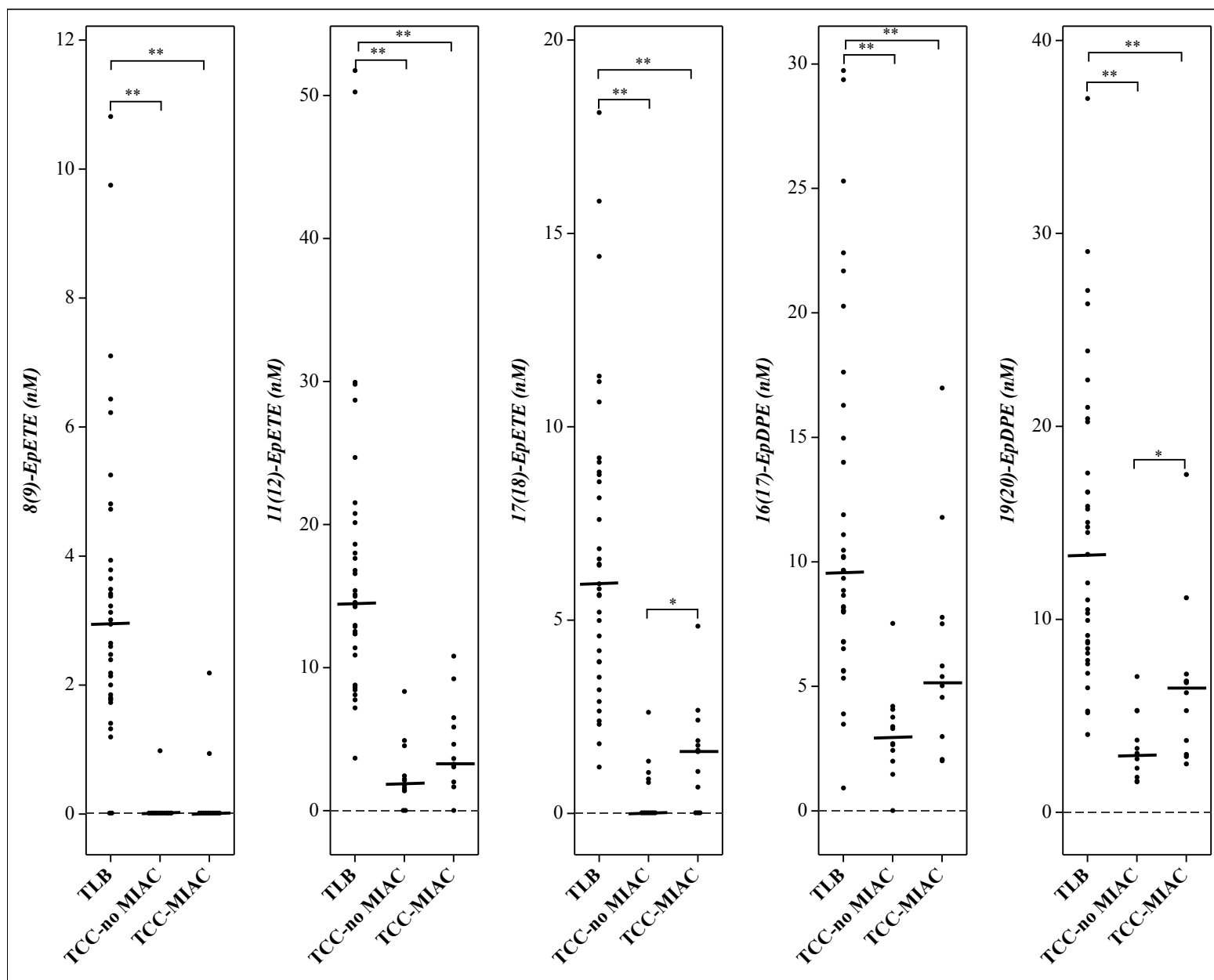


Figure 4: Epoxygenase pathway metabolites of eicosapentaenoic and docosahexaenoic acids (ω -3 PUFA) that exhibited significant difference between patient groups. Patient group abbreviations and other details are the same as in Figure 1.