Pro-Resolving Mediator Profiles And 5-Lipoxygenase Activity In Cerebrospinal Fluid Correlate with Disease Severity and Outcome in Adults with Tuberculous Meningitis

Romain A. Colas[1*], Le Thanh Hoang Nhat[2*], Nguyen Thuy Thuong Thuong[2], Esteban Alberto Gomez Cifuentes[1], Lucy Ly[1], Hai Hoang Thanh[2], Nguyen Hoan Phu[2], Nguyen Thi Hoang Mai[2], Guy E. Thwaites[2,3,$] and Jesmond Dalli[1,4,$,+].

[1]William Harvey Research Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London, EC1M 6BQ UK.

[2]Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam

[3]Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, UK

[4] Centre for Inflammation and Therapeutic Innovation, Queen Mary University of London, London, UK.

* Equal first authorship

[$] Equal senior authorship

[+]Corresponding author: Dr Jesmond Dalli Ph.D, William Harvey Research Institute, John Vane Science Centre, Charterhouse Square, London. EC1M 6BQ. E-mail: j.dalli@qmul.ac.uk, Tel: +44 (0) 207 882 8263

Short Title: Relationships between SPM levels and outcomes in Tuberculous meningitis

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Abstract

Tuberculous meningitis (TBM) is the most lethal form of tuberculosis infection, characterized by a dysregulated immune response that frequently leads to neurological injury and death despite the best available treatment. The mechanisms driving the inflammatory response in TBM are not well understood. To gain insights into these mechanisms we used a lipid mediator profiling approach to investigate the regulation of a novel group of host protective mediators, termed specialized pro-resolving mediators (SPM), in the cerebrospinal fluid (CSF) of adults with TBM enrolled into a randomised placebo-controlled trial of adjunctive aspirin treatment. We found distinct lipid mediator profiles with increasing disease severity, changes that were linked with an upregulation of inflammatory eicosanoids in patients with severe TBM and a decrease in the production of a number of 5-lipoxygenase (ALOX5)-derived SPM. CSF pro-resolving mediator concentrations were also associated with 80-day survival. In survivors, we found a significant increase in pro-resolving mediator concentrations, including the ALOX5-derived resolvin (Rv)T2, RvT4 and 15-epi-Lipoxin (LX)B_4, compared to those who died. Aspirin administration increased the ratio of pro-resolving to pro-inflammatory mediators decreasing the concentrations of the prothrombic mediator TxA_2, changes that were linked with early reductions in brain infarcts and deaths. Together, these findings identify a CSF SPM signature that is associated with disease severity and 80-day mortality in TBM. Furthermore, the therapeutic manipulation of the ratio between pro-resolving mediators and pro-inflammatory/thrombogenic mediators in the CSF, by aspirin for example, offers a novel treatment strategy to reduce the morbidity and mortality caused by TBM.

Authors Summary

Infections of the brain and the meninges by *Mycobacterium tuberculosis* (*M. tb*) lead to severe inflammation and are associated with poor outcomes. The mechanisms leading to this disease remain poorly defined. Herein, we investigated how *M. tb* infection regulates the concentrations of specialized pro-resolving mediators that are central in controlling the body's ability to clear infections. In these investigations, we found that disease survival was linked with increased concentrations of a number of these protective molecules including resolvins and lipoxins. Treatment of *M. tb*-infected patients with aspirin decreased the production of the immunosuppressive and thrombogenic mediator thromboxane A_2 improving the balance between protective and inflammatory molecules. Of note, these changes were linked with reduced disease severity and improved survival. Therefore, the present findings suggest a previously unappreciated role for pro-resolving mediators in TBM pathogenesis.

Introduction

Mycobacterium tuberculosis (*M. tb*) is responsible for more deaths globally than any other infectious disease. When it infects the brain and meninges to cause tuberculous meningitis (TBM), which represents 1-5% of all forms of tuberculosis, it either kills or severely disables around a half of all sufferers despite the best available treatment (1). The pathogenesis of TBM is not well understood, but poor outcomes have been linked to dysregulated intracerebral inflammation (2). Current therapeutic approaches are aimed at killing *M. tb* infecting the brain or controlling the inflammatory response (3, 4). To date, the latter has primarily involved adjunctive corticosteroid therapy and has been associated with increased survival, although how corticosteroids modulate intra-cerebral inflammation to influence outcomes remains uncertain (5).

It is now well established that under ideal conditions the body activates evolutionarily conserved programs to terminate inflammation and promote the repair and regeneration of damaged tissues (6). At the helm of these programs is a recently uncovered genus of mediators produced *via* the stereoselective conversion of essential fatty acids and termed specialized pro-resolving mediators (SPM). These mediators include the arachidonic acid (AA)-derived lipoxins and the n-3 docosapentaenoic acid (DPA)-derived resolvins, protectins and maresins (6). Recent studies demonstrate that SPM via the activation of cognate receptors regulate the phagocytosis and killing of bacteria during infections. They counter regulate the production of pro-inflammatory mediators including cytokines and eicosanoids, and control both leukocyte trafficking and phenotype (7-11). These autacoids also mediate the protective actions of several widely used therapeutics, including atorvastatin, pravastatin and aspirin (7, 12, 13).

We recently tested the hypothesis that the addition of aspirin to standard TBM treatment (anti-tuberculosis drugs and corticosteroids) may further improve outcomes by inhibiting thromboxane A_2 (TxA_2) and preventing brain infarcts (a common life-

threatening complication of TBM), and by enhancing the resolution of intra-cerebral inflammation through the increased expression of SPMs (14). The trial found that in patients with microbiologically confirmed TBM, aspirin was associated with reduced brain infarcts and/or death in the first 60 days of treatment.

Little is known about the regulation of lipid mediators, and in particular SPM, in the cerebrospinal fluid (CSF) during TBM. Thus, in the present studies we investigated whether CSF lipid mediator concentrations were altered with increasing disease severity. We found that concentrations of a number of SPM families were reduced with increasing disease severity. This was linked with an upregulation of inflammation-initiating eicosanoids, including prostaglandins and cystenyl leukotrienes. Pre-treatment CSF lipid mediator concentrations were also found to be predictive of outcome with distinct lipid mediator profiles obtained in those patients that were alive at the end of the study versus those that died during the study. Finally, we also found that in aspirin-treated patients there was a dose-dependent alteration of the CSF lipid mediator profiles improving the balance between pro-resolving and pro-inflammatory/pro-thrombogenic mediators.

Results

Increased disease severity is associated with a reduction in CSF SPM concentrations

In order to determine whether there was a relationship between CSF lipid mediator concentrations and disease severity in TBM we investigated lipid mediator profiles of patients recruited to the Aspirin TBM study (NCT02237365). Here CSF was collected from enrolled patients with known or suspected TBM just prior to the start of treatment in a randomised comparison of aspirin versus placebo as an adjunct to dexamethasone administration for the first 60 days of TBM treatment (14). Disease severity was assessed using the Medical Research Council grade (15) that uses Glasgow coma score and focal neurological deficits to categorise disease severity as mild (grade 1), moderate (grade 2), or severe (grade 3; See Supplemental Table 1 for patient information). Using targeted liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based profiling of CSF we identified mediators from all four major bioactive metabolomes, including the AA-derived lipoxin (LX)s, leukotriene (LT)s and prostaglandin (PG)s and the n-3 DPA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)-derived resolvin (Rv)s (Supplemental Table 2). In order to gain insights into the relationship between CSF lipid mediator concentrations and disease severity we first grouped the mediators by biological function assessing the differences between pro-resolving mediators (lipoxins, resolvins, protectins and maresins) and pro-inflammatory mediators (leukotrienes, prostaglandins and thromboxane) in patients with moderate or severe disease (MRC grades 2 and 3) when compared with those with mild disease (MRC grade 1)(16)(Figure 1A, Supplemental Table 2). Results of this analysis demonstrated that with increasing disease severity there was a decrease in overall pro-resolving lipid mediator concentrations in the CSF of patients with TBM (Figure 1A). This relationship was coupled with a trend towards an increase in the concentrations of pro-inflammatory mediators in those with more severe disease (Figure 1B). Assessment of the

resolution index, that is the ratio between the concentrations of pro-resolving mediators and pro-inflammatory eicosanoids, (16) also demonstrate a reduction in the resolution index, indicative of increased inflammation, in patients with the more severe disease (Figure 1C).

In order to gain further insights into the mediator pathways that were differentially regulated between these patient groups we next interrogated the biosynthetic pathways for each of the essential fatty acid metabolomes. This demonstrated that in patients with an MRC score of 2 and 3, when compared with patients with an MRC score of 1, there was a significant reduction in two pro-resolving mediator families, the n-3 docosapentaenoic acid-derived resolvins ($RvD_{n-3\ DPA}$) and the arachidonic acid-derived lipoxins (LX). This was coupled with a significant increase in the pro-inflammatory arachidonic acid-derived prostaglandins (PG) and leukotrienes (LT; Figure 1D).

We next employed Partial Least Squares Discriminant Analysis (PSL-DA), a regression model that identifies variables that contribute to the separation of experimental groups, to investigate the relationship between lipid mediator profiles (i.e. the concentrations of individual mediators identified in the CSF) and disease severity. This analysis demonstrated that the individual lipid mediator concentrations were markedly different between patients with MRC grades 1, 2 and 3 as demonstrated by the distinct clustering of patients from the different disease severity groups (Figure 2A). Assessment of the variable importance in projection (VIP) scores, which identify the contribution of each mediator in the observed separation between each of the groups, identified 24 mediators that had a VIP score greater than 1, showing that they were differentially regulated depending on disease severity (Figure 2A). Amongst the mediators found to be differentially regulated between these three disease groups were several ALOX5-derived mediators that are involved in coordinating the host response to clear bacterial infections, including RvT4, RvD1 and RvE1 (Figure 2B)(7, 17, 18).

Having identified that there was a differential regulation of lipid mediator profiles with increasing disease severity we next assessed the association between distinct lipid mediators and disease severity. There was a significant negative correlation between a select group of pro-resolving mediators, 15-epi-LXB_4, $RvD2_{n-3\ DPA}$, 22-OH-PD1, MaR1 and 15-epi-LXA_4, and increasing disease severity (Figure 2C and Supplemental Table 2). In addition, we also observed increased concentrations of LTE_4, the terminal product in the cysteinyl leukotriene biosynthetic metabolome and that was recently found to be also bioactive (19), with increasing disease severity, although the correlation was not statistically significant after correction for multiple testing (Figure 2C, Supplemental Table 2).

To further validate the potential utility of these mediators as biomarkers of disease severity we conducted multiple regression analysis, using Least Absolute Shrinkage and Selection Operator (LASSO) regression analysis. This demonstrated that decreased concentrations of identified pro-resolving mediators, primarily 15-epi-LXB_4, LXB_4, PGE_2, 22-OH-PD1, were associated with increased disease severity (Supplementary Table 3).

Given the role that immune cells play in the biosynthesis of lipid mediators (7, 9), we next investigated whether disease severity influenced CSF leukocyte numbers. Here we found that cell numbers were reduced in those with more severe disease, from 416±53 cells/mm^3 in patients with an MRC score of 1, to 239±27 cells/mm^3 and 164±39 cells/mm^3 in patients with an MRC score of 2 and 3 respectively, although these changes were not statistically significant ($p>0.05$). We next assessed whether there was an association between CSF leukocyte counts and the concentrations for each of the identified LM families. With adjustment for multiple testing, no significant correlations were found between these parameters (data not shown), suggesting that the observed difference may be due to a differential activation of the leukocyte population.

Pre-treatment SPM and eicosanoid concentrations in CSF correlate with mortality

Having found SPM concentrations were associated with TBM disease severity we investigated whether CSF lipid mediator concentrations correlated with mortality (See Supplemental Table 4 for patient information). We first assessed the CSF concentrations of SPM and pro-inflammatory eicosanoids, finding that SPM concentrations were significantly reduced in patients that died during the study, while pro-inflammatory mediator concentrations tended to be higher in those who survived, although this did not reach statistical significance (Figure 3A,B and Supplemental Table 5). Of note, comparison of the resolution index in survivors and those who died demonstrated a significantly higher CSF resolution index in survivors (Figure 3C), suggesting that disruption of pro-resolving mediator production and a concomitant increase in pro-inflammatory eicosanoid production are linked with outcome from TBM.

We next conducted lipid mediator biosynthetic pathway analysis to evaluate which pathways are contributing to the observed differences in lipid mediator concentrations. This demonstrated that there was a downregulation in the expression of 13-series resolvins (RvT), $RvD_{n\text{-}3\ DPA}$, and LX with an upregulation in the pro-inflammatory LT in non-survivors when compared with survivors (Figure 3D).

Orthogonal PLS-DA (OPLS-DA) analysis of CSF lipid mediator profiles provided further support for the differences in lipid mediator concentrations between those who died and those who survived, with CSF lipid mediator concentrations from each of these patient groups giving two distinct clusters (Figure 4A). This separation between the two groups was linked with a differential regulation of 18 lipid mediators, which gave a VIP score greater than 1 and included MaR2, RvT2 and 15-epi-LXB_4. Of note, RvT2 and 15-epi-LXB_4 activate the innate immune response to clear bacterial infections and counter-regulate the production of pro-inflammatory mediators (6, 7). Statistical assessment of mediators found to be differentially

regulated demonstrated that the concentrations of MaR2, RvT2 and 15-epi-LXB_4 were significantly lower in non-survivors when compared with survivors (Supplemental Table 5 and Figure 4B). Out of these mediators 15-epi-LXB_4 was found to be significantly reduced after multiple correction. This lipid mediator was also a strong predictor of mortality in TBM as demonstrated by LASSO regression analysis (Supplemental Table 6).

Aspirin administration upregulates CSF concentrations of select pro-resolving mediators during TBM

We recently reported that treatment with aspirin, dexamethasone and anti-tuberculosis drugs was associated with reduction in new brain infarcts and deaths within 60 days in patients with microbiologically confirmed TBM (14). Therefore, we investigated the impact of aspirin on CSF lipid mediator pathways in this patient sub-population. We compared lipid mediator profiles obtained after 30 days of aspirin (1000mg/day or 81mg/day) or placebo (Supplementary Table 7). Assessment of overall lipid mediators by function demonstrated a decrease in the concentrations of proinflammatory eicosanoids and an overall increase in the resolution index of patients given 1000mg of aspirin when compared with those receiving placebo (Figure 5A-C). However, these changes did not reach statistical significance. Pathway analysis of lipid mediator families identified in the CSF of these patients demonstrated a trend towards the upregulation of a number of pro-resolving families that included the $RvD_{n\text{-}3\ DPA}$ and $MaR_{n\text{-}3\ DPA}$ in patients receiving 1000mg and 81mg aspirin respectively, although these changes did not reach statistical significance (n= 28 for patients in 81mg aspirin group, n = 27 patients in 1000mg aspirin group and n= 34 patients in placebo group).

To further evaluate the regulation of CSF lipid mediator concentrations by aspirin we conducted OPLS-DA. This analysis demonstrated that while day 30 CSF lipid mediator profiles between the 81mg and placebo groups were not markedly

different, lipid mediator profiles from patients given 1000mg aspirin gave distinct clusters to that from patients given placebo (Figure 5D,E). This separation between the two patient groups was linked with a differential regulation of 18 mediators from all the four bioactive metabolomes, including RvT4 and TxB_2, the inactive breakdown product of the prothrombotic TxA_2 (20)(Figure 5E). Statistical assessment of their concentrations in CSF demonstrated that after 30 days TxB_2 was reduced with both 81 mg and 1000mg of aspirin, reaching statistical significance in those given 1000mg after correcting for multiple testing (Supplemental Table 8,9).

Discussion

In the present study we investigated the regulation of CSF lipid mediator profiles before and during the treatment of adults with TBM. We found that pre-treatment disease severity was associated with the concentrations of both inflammatory and pro-resolving mediators, with more severe disease linked to lower SPM concentrations and increased concentrations of immunosuppressive, vasoconstrictive and nociceptive eicosanoids. Pre-treatment SPM concentrations were also associated with 80-day mortality, with survivors having higher concentrations of several SPM families, including the ALOX5-derived Rvs and LXs, compared to those who died. Aspirin co-administration with dexamethasone was observed to increase the CSF resolution index after 30 days of treatment, and decrease TxB_2 concentrations.

Recent decades have seen significant advances in our ability to manage patients with TBM, however, morbidity and mortality remain high (1). This is at least part due to our limited understanding of the underlying mechanisms that perpetuate inflammation within the CNS leading to disability and ultimately death despite the best available treatment. In the present studies we investigated the relationship between disease severity and local mediator biosynthesis. Results from these analysis demonstrate that even prior to the initiation of treatment patients that died during the course of the study presented with a profound dysregulation in both protective and inflammatory mediator pathways.

Lipid mediator biosynthesis is a tightly coordinated process where essential fatty acids are sequentially oxygenated, primarily by lipoxygenase and cyclooxygenase enzymes, to produce stereochemically defined and structurally unique products (6, 21). Regulation of these biosynthetic enzymes occurs at both a transcriptional/translational level as well as via post-translational modifications. Recent studies demonstrate that nitric oxide synthase promotes the S-nitrosylation of COX-2 increasing its catalytic activity and upregulating the production of protective

292 mediators including PGI_2 (22) and RvT (7). On the other hand, the calcium-
293 calmodulin-dependent protein kinase II - p38 mediated phosphorylation of ALOX5
294 leads to a switch in the product profile of the enzyme from SPM production to the
295 formation of leukotrienes (23). The observation that with increasing disease severity
296 there is a downregulation of ALOX5-derived SPM and a concomitant upregulation in
297 cysteinyl leukotriene production suggest that as the disease progresses there is an
298 increase in ALOX5 phosphorylation that leads to an alteration in the product profile of
299 the enzyme.

300 Leukocytes play an important role in the biosynthesis of lipid mediators, with
301 their product profile reflecting their activation status (23-25). Different macrophage
302 subsets, for example, display distinct lipid mediator profiles with monocyte-derived-
303 macrophages skewed towards a classic phenotype expressing higher amounts of
304 pro-inflammatory eicosanoids, whereas cells with an alternatively activated
305 phenotype display higher concentrations of pro-resolving mediators (23-25). This
306 shift is also linked with a differential expression of both lipid mediator biosynthetic
307 enzymes as well as the phosphorylation status of ALOX5 (23, 25, 26). In the present
308 study we found a decrease in the number of leukocytes in the CSF. However there
309 was no correlation between leukocyte numbers and lipid mediator concentrations
310 suggesting that regulation of enzyme activity, possibly reflecting a shift in leukocyte
311 phenotype or population, is responsible for the altered lipid mediator concentrations.

312 Studies conducted by Vane and colleagues demonstrate that aspirin inhibits
313 the production of prostaglandins and thromboxane, a mechanism that is dependent
314 on the acetylation of COX-1 and COX-2 (27). Later investigations by Serhan and
315 colleagues found that acetylation of COX-2 also led to a switch in the catalytic activity
316 of the enzyme, from the production of PGG_2 to the formation of epimeric forms of
317 resolvins, lipoxins and protectins (28, 29). In the preset study we did not observe
318 significant increases in these epimeric forms of the SPM in the CSF of TBM patients
319 treated with aspirin. This may be because the present study was not adequately

powered for this analysis or because at the interval tested, i.e. 30 days post initiation of treatment, the biosynthetic pathways leading to the formation of these molecules were downregulated. Future studies powered to interrogate this question will need to establish whether daily regulation of high dose aspirin downregulates these pathways in the CSF. Of note, we found high dose aspirin administration reduced TxB_2 concentrations and was linked with improved outcomes.

In summary, the present findings uncover novel mechanisms in the pathophysiology of TBM that may have relevance to all forms of tuberculosis. Disease severity is associated with an alteration in lipid mediator expression and a dysregulation in the production of several SPM pathways; a mechanism that is also linked with a poor prognosis. This dysregulation was at least in part restored by aspirin, lending support to the hypothesis that TBM arises from a failure of the host to engage resolution mechanisms and offering novel treatment strategies.

Materials and Methods

Clinical study:

The patients in the current study were enrolled in a clinical trial of adjunctive aspirin, the design and conduct of which have been previously described (14). Briefly, we conducted a parallel group, double blind, randomised, placebo-controlled trial in HIV-uninfected adults with TBM to assess the safety and efficacy of either 81mg or 1000mg aspirin daily for the first 60 days of treatment with standard anti-tuberculosis drugs and dexamethasone. The trial enrolled in-patients at the Hospital for Tropical Diseases, a 550-bed tertiary referral hospital in Ho Chi Minh City, Vietnam, and was approved by the Oxford Tropical Research Ethics Committee and the Institutional Review Board of the Hospital for Tropical Diseases and the Ethical Committee of the Ministry of Health, Vietnam. Adults (≥ 18 years old) with suspected TBM (at least 5 days of meningitis symptoms, nuchal rigidity, and CSF abnormalities) and a negative HIV test were eligible to enter the trial. Written informed consent to participate in the study was obtained from all participants or from their relatives if the participant could not provide consent due to incapacity.

Lumbar puncture was performed before the start of treatment and on days 30 and 60 as per normal clinical care with CSF archived at -80^{o}C until later testing. Clinical progress and neurological and drug-related adverse events were assessed daily until discharge from hospital and monthly thereafter until 8 months, when a final clinical assessment was made.

Targeted lipid mediator profiling. All samples were extracted using solid-phase extraction columns as in (30). Prior to sample extraction, deuterated internal standards, representing each region in the chromatographic analysis (500 pg each) were added to facilitate quantification in 4V of cold methanol. Samples were kept at -20°C for a minimum of 45 min to allow protein precipitation. Supernatants were subjected to solid phase extraction, methyl formate and methanol fractions were

collected, brought to dryness and suspended in phase (methanol/water, 1:1, vol/vol) for injection on a Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector, paired with a QTrap 6500 plus (Sciex). For identification and quantitation of products eluted in the methyl formate, an Agilent Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 µm) was kept at 50°C and mediators were eluted using a mobile phase consisting of methanol-water-acetic acid of 20:80:0.01 (vol/vol/vol) that was ramped to 50:50:0.01 (vol/vol/vol) over 0.5 min and then to 80:20:0.01 (vol/vol/vol) from 2 min to 11 min, maintained till 14.5 min and then rapidly ramped to 98:2:0.01 (vol/vol/vol) for the next 0.1 min. This was subsequently maintained at 98:2:0.01 (vol/vol/vol) for 5.4 min, and the flow rate was maintained at 0.5 ml/min. QTrap 6500+ was operated in negative ionization mode using a multiple reaction monitoring method as in (30).

In the analysis of peptide-lipid conjugated mediators eluted in the methanol fraction, an Agilent Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 µm) was kept at 50°C and mediators were eluted using a mobile phase consisting of methanol-water-acetic acid at 55:45:0.1 (vol:vol:vol) over 5 min, that was ramped to 80:20:0.1 (vol:vol:vol) for 2 min, maintained at 80:20:0.1 (vol:vol:vol) for the next 3 min and ramped to 98:2:0.1 (vol:vol:vol) over 3 min. This was kept at 98:2:0.1 (vol:vol:vol) for 3 min. A flow rate of 0.65 ml/min was used throughout the experiment. QTrap 6500+ was operated in positive ionization mode using scheduled multiple reaction monitoring (MRM) coupled with information-dependent acquisition and enhanced product ion scan (30).

Each LM was identified using established criteria including matching retention time to synthetic and authentic materials and at least 6 diagnostic ions (30). Calibration curves were obtained for each using synthetic compound mixtures at 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 pg that gave linear calibration curves with an r^2 values of 0.98–0.99.

Statistical analysis.

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390 We performed all statistical analyses and data derivation and prepared the
391 manuscript in R (31), Prism 8 and Microsoft Excel. Results are expressed as mean
392 and 95% confidence interval or interquartile range as indicated in the figures and
393 tables. Summary tables of the baseline characteristics of the study population with
394 respect to disease severity, mortality outcome and treatment allocation as median
395 (IQR) for continuous data and n (%) for categorical data were provided in the
396 supplemental document.
397 To assess the overall balance between pro-inflammatory mediators and pro-
398 resolving mediators in the CSF we combined the concentrations of pro-resolving
399 mediators, combining the concentrations of the DHA-derived RvD (RvD1, RvD2,
400 RvD3, RvD4, RvD5, RvD6, 17R-RvD1 and 17R-RvD3) PD (PD1, 10S,17S-diHDHA,
401 17R-PD1 and 22-OH-PD1) PCTRs (PCTR1, PCTR2 and PCTR3) and MaR (MaR1,
402 7S, 14S-diHDHA, MaR2, 4S, 14S-diHDHA and 22-OH-MaR1) MCTRs (MCTR1,
403 MCTR2 and MCTR3), the n-3 DPA derived RvT (RvT1, RvT2, RvT3 and RvT4),
404 $RvD_{n\text{-}3\ DPA}$ ($RvD1_{n\text{-}3\ DPA}$, $RvD2_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$), $PD_{n\text{-}3\ DPA}$ ($PD1_{n\text{-}3\ DPA}$ and 10S,
405 17S-diHDPA) and $MaR_{n\text{-}3\ DPA}$ ($MaR1_{n\text{-}3\ DPA}$ and 7S, 14S-diHDPA), the EPA-derived
406 RvE (RvE1, RvE2 and RvE3) and the AA-derived LX (LXA_4, LXB_4, 5S, 15S-diHETE,
407 15R-LXA_4 and 15R-LXB_4). Separately we combined the concentrations of pro-
408 inflammatory eicosanoids: AA-derived LT (LTB_4, 5S, 12S-diHETE, 12-epi-LTB_4, 6-
409 trans, 12-epi-LTB_4 and 20-OH-LTB_4), cysLT (LTC_4, LTD_4 and LTE_4), PG (PGD_2,
410 PGE_2 and $PGF_{2\alpha}$) and Tx (TxB_2). The resolution index was obtained by dividing the
411 overall concentration of pro-resolving mediators by the overall concentrations of pro-
412 inflammatory eicosanoids. Investigations into the *flux down each of the mediator*
413 *families* was conducted by determining the fold change in the concentration of the
414 different mediator families indicated above, between the control groups (ie MRC1 or
415 survivors) and test groups (ie MRC2 + MRC3 or non-survivors respectively).
416

417 Statistical differences between the concentrations (expressed as the log2-fold
418 change) of the mediators in each analysis group was determined using Hochberg
419 Multiple Testing Correction or Benjamini-Hochberg Multiple Testing Correction as
420 indicated. Lipid mediator networks were constructed using Cytoscape 3.7.1. and the
421 pathways that were statistically up or down regulated were denoted using red and
422 blue lines respectively.
423 Investigators were not blinded to group allocation or outcome assessment. The
424 criterion for statistical significance was $p \leq 0.05$. OPLS-DA and PLS-DA (32) were
425 performed using SIMCA 14.1 software (Umetrics, Umea, Sweden) following mean
426 centering and unit variance scaling of LM levels. PLS-DA is based on a linear
427 multivariate model that identifies variables that contribute to class separation of
428 observations (MRC scores, survivors/non-survivors; Placebo/aspirin groups) on the
429 basis of their variables (LM levels). During classification, observations were projected
430 onto their respective class model. The score plot illustrates the systematic clusters
431 among the observations (closer plots presenting higher similarity in the data matrix).
432 Loading plot interpretation identified the variables with the best discriminatory power
433 (Variable Importance in Projection greater than 1) that were associated with the
434 distinct intervals and contributed to the tight clusters observed in the Score plot.
435 Comparisons of lipid mediators by disease severity (MRC grade) or mortality
436 outcome were based on the Spearman-test for the test for trend of ordinary groups
437 for non-normal continuous outcomes. The test were implemented in R package
438 “Comparegroup”. In addition, to identify which lipid mediators are the strong predictor
439 for disease severity and mortality outcomes in TBM, we performed a Lasso
440 regression model in which the disease severity or mortality as the outcome, and all
441 the lipid mediators as the covariates. The lipid mediators which were likely chosen by
442 the model demonstrated as the strong predictors of the disease severity or mortality
443 in TBM.
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445 The reduction of lipid mediator was computed as the difference between the two
446 timepoints from baseline and day 30. The reduction was compared between
447 treatment arms based on a simple linear regression. In this model, the lipid mediator
448 reduction is the outcome and treatment arm is the main covariate. To increase the
449 power for the analysis, the model was also adjusted for the baseline lipid mediator.
450 For all the comparison in this study, we performed multiplicity adjustments of p-
451 values for all comparisons based on Hochberg Multiple Testing Correction, as
452 implemented in R package “stats”.
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Author Contributions

THP, NTHM recruited and cared for the patients; LTHN and HHT helped to analyse the data; NTTT helped design the study and coordinated the laboratory assessments in Vietnam; GT, RAC, LL, EAGC and JD carried out experiments and analysed data; All authors contributed to manuscript preparation; GT and JD conceived overall research plan.

Declaration of Interests

The authors declare no competing interests.

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Figure Legends:

Figure 1: Reduced pre-treatment CSF SPM concentrations are is associated with increased disease severity in TBM. CSF were collected from patients with TBM before the start of treatment and lipid mediators were extracted, identified and quantified using lipid mediator profiling. (A) Sum of pro-resolving mediators (DHA-derived RvD, PD, PCTR, MaR, MCTR; n-3 DPA-derived RvT; $RvD_{n\text{-}3\ DPA}$, $PD_{n\text{-}3\ DPA}$, $MaR_{n\text{-}3\ DPA}$; EPA-derived RvE; AA-derived LX) (B) Sum of pro-inflammatory eicosanoids (AA-derived LT, PG, Tx). (C) Resolution index, the ratio between the sum of pro-resolving mediators and the sum of pro-inflammatory eicosanoids. Statistical differences were determined using Spearmans correlation. Results are mean ± 95% C.I.. n = 44 for MRC grade 1; n = 47 for MRC grade 2 and n = 12 for MRC grade 3. (D) Pathway interaction analysis down each of the lipid mediator families. Figure depicts the fold change, expressed as log_2-fold change, in lipid mediator concentrations between patients with an MRC grade of 1 (MRC1) and those with MRC grade of 2 (MRC2) and MRC grade of 3 (MRC3). Scales represent fold increase or decrease for each mediator family. Mediator families coloured in red or blue represent those families that were found to be significant regulated. Statistical significance between mediator concentrations in patients with MRC1 and those with MRC2 and MRC3 grades was determined using Benjamini-Hochberg multiple testing correction. Red lines depict pathways that are upregulated, in patients with an MRC grade of 2 and 3 when compared with MRC1 patients. Blue lines depict pathways that are downregulated in patients with an MRC grade of 2 and 3 when compared with MRC1 patients. Pentagons depict the distinct essential fatty acids, squares the lipid mediator biosynthetic enzymes and circles the distinct lipid mediator families. DHA = docosahexaenoic acid; n-3 DPA = n-3 docosapentaenoic acid; EPA = eicosapentaenoic acid; AA = arachidonic acid; ALOX = lipoxygenase; COX = cyclooxygenase; CYP450 = Cytochrome P450; LTC_4S = leukotriene C_4 synthase; GSTM = Glutathione S-transferase Mu; LTA_4H = Leukotriene A_4 hydrolase; PGS =

598 Prostaglandin synthase; TBXAS1 = thromboxane-A synthase; Rv = resolvins; PD =
599 Protectins; MaR = Maresins; LX = Lipoxins; LT-Leukotrienes; PG = prostaglandins;
600 Tx = Thromboxane.
601
602 **Figure 2: TBM disease severity is linked with a switch in the ALOX5 product**
603 **profile.** CSF were collected from patients with TBM before the start of treatment and
604 lipid mediators were extracted, identified and quantified using lipid mediator profiling.
605 An interaction network was constructed depicting the number of mediators in each of
606 the lipid mediator families that were significantly downregulated (blue) or upregulated
607 (red) in CSF from patients with an MRC grade of 2 and 3 when compared with
608 patients that had an MRC grade of 1. (B) Box-plot of lipid measurement vs. MRC
609 grade of 6 lipid mediators which are significantly associated with disease severity. n
610 = 44 for MRC grade 1; n = 47 for MRC grade 2 and n = 12 for MRC grade 3
611
612
613 **Figure 3: Pre-treatment CSF resolution status is associated with poor outcome**
614 **from TBM.** CSF was collected before the start of treatment and lipid mediators
615 identified and quantified using lipid mediator profiling. Sum of pro-resolving
616 mediators; (B) Sum of pro-inflammatory eicosanoids; (C) Resolution index. Statistical
617 analysis was conducted using one-way ANOVA with Dunnet post-hoc test. Results
618 are mean ± 95% C.I. n = 95 survivors and 8 non-survivors. (D) Interaction networks
619 were constructed to comparing the pre-treatment CSF lipid mediator profiles from
620 patients that died during the 80-day trial period to those that survived. Scales
621 represent fold increase or decrease for each mediator family. Mediator families
622 coloured in red or blue represent those families that were found to be significant
623 regulated. Statistical significance was determined using Benjamini-Hochberg multiple
624 testing correction. Results are representative of n = 8 patients. Red lines depict
625 pathways that are upregulated in non-survivors. Blue lines depict pathways that are

626 downregulated in non-survivors. Pentagons depict the distinct essential fatty acids,
627 squares the lipid mediator biosynthetic enzymes and circles the distinct lipid mediator
628 families.
629
630
631 **Figure 4: Death from TBM is associated with a downregulation of CSF ALOX5-**
632 **derived SPM and an upregulation of ALOX5 derived LTE_4.**
633 (A) CSF lipid mediator profiles obtained from patients that died during the 80-day
634 duration of the study (non-survivors) were compared with those that were alive at the
635 end of the study (survivors) using OPLS-DA (left panel) Score plot. (right panel)
636 loading plot. (B) Box-plot of lipid measurement vs. 80-day mortality outcome of 6 lipid
637 mediators which are significant associated with mortality. n = 95 survivors and 8 non-
638 survivors
639
640 **Figure 5. Aspirin 1000mg/day increases CSF resolution status and reduces**
641 **CSF TxB_2 concentrations after 30 days of treatment.** CSF fluids were collected
642 30 days after administration of 81mg, 1000mg aspirin per day or placebo. Lipid
643 mediators were extracted, identified, quantified using lipid mediator profiling. (A)
644 cumulative pro-resolving mediator concentrations (B) cumulative pro-inflammatory
645 eicosanoid concentrations (C) resolution index. (D,E) OPLS-DA of lipid mediator
646 profiles from patients given (D) 81mg (E) 1000mg aspirin per day in comparison to
647 those receiving placebo. (*Left panel*) score plot; (*right panel*) loading plot. Mediators
648 with a VIP score > 1 are identified in red (placebo) green (1000mg aspirin) or blue
649 (81mg aspirin) circles that denote the association with the placebo and aspirin group.
650 Results for A-C are mean ± 95% C.I. n= 28 for patients in 81mg aspirin group and n
651 = 27 patients in 1000mg aspirin group and n= 34 patients in placebo group.
652
653

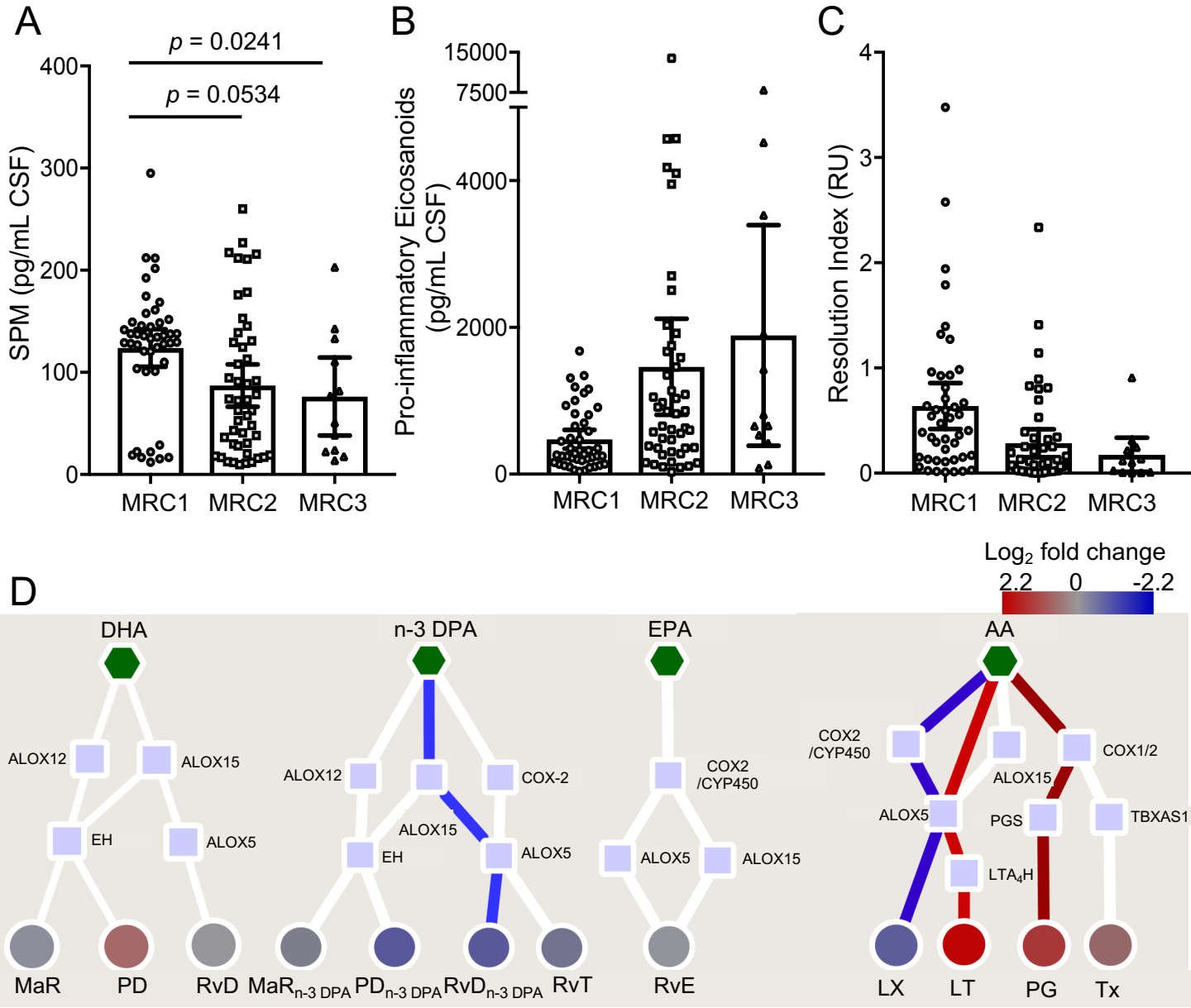
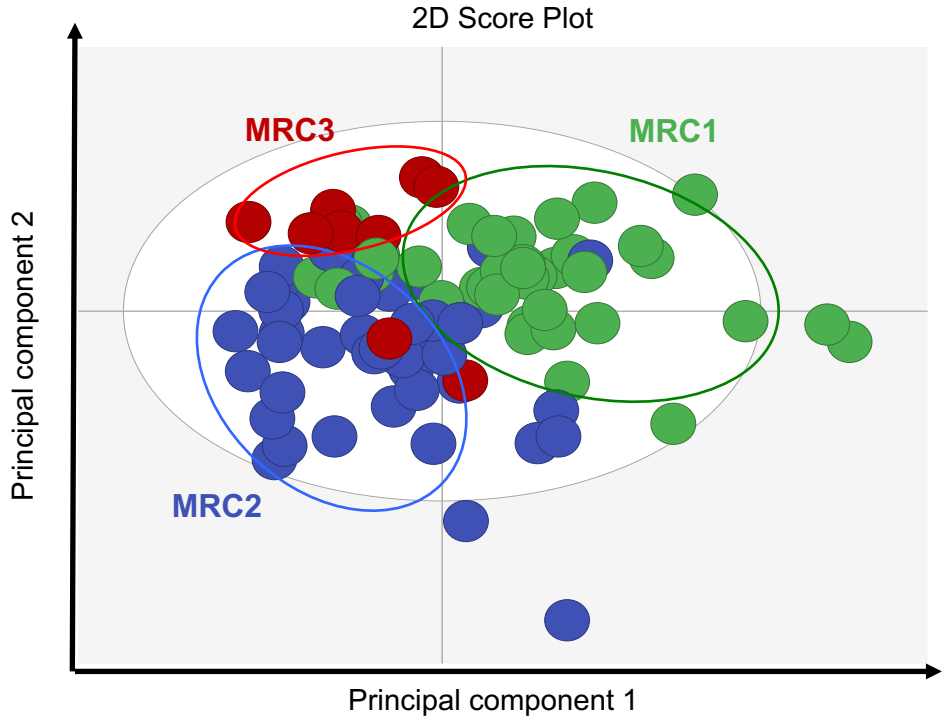
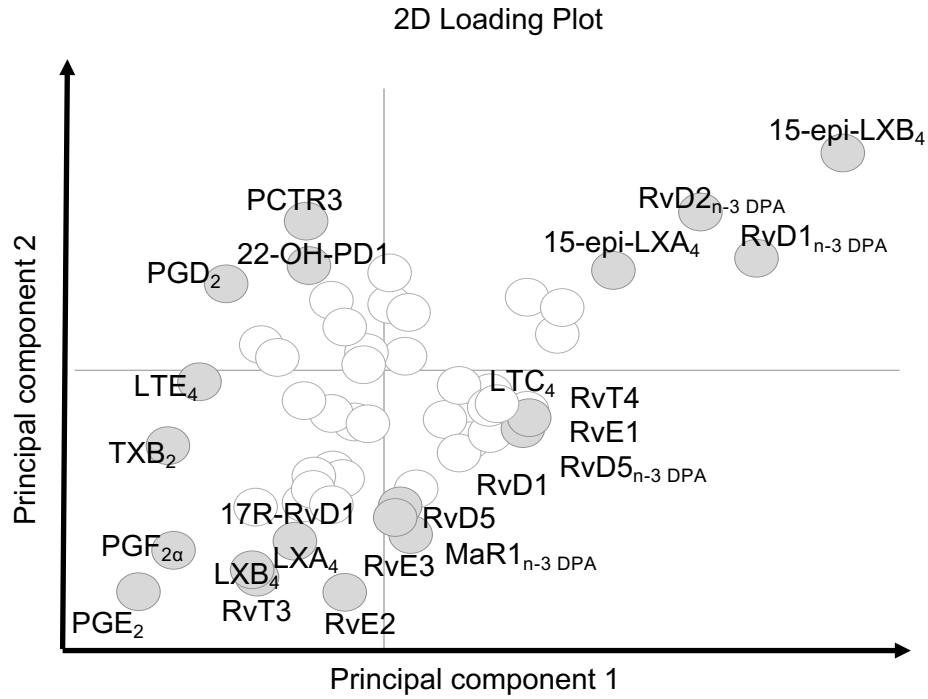

A
p = 0.0241
p = 0.0534
SPM (pg/mL CSF)
400
300
200
100
0
MRC1
MRC2
MRC3
B
Pro-inflammatory Eicosanoids (pg/mL CSF)
15000
7500
4000
2000
0
MRC1
MRC2
MRC3
C
Resolution Index (RU)
4
3
2
1
0
MRC1
MRC2
MRC3
D
Log2 fold change
2.2
0
-2.2
DHA
ALOX12
ALOX15
EH
ALOX5
MaR
PD
RvD
n-3 DPA
ALOX12
COX-2
ALOX15
EH
ALOX5
MaRn-3 DPA
PDn-3 DPA
RvDn-3 DPA
RvT
EPA
COX2 /CYP450
ALOX5
ALOX15
RvE
AA
COX2 /CYP450
ALOX15
COX1/2
ALOX5
PGS
TBXAS1
LTA4H
LX
LT
PG
Tx

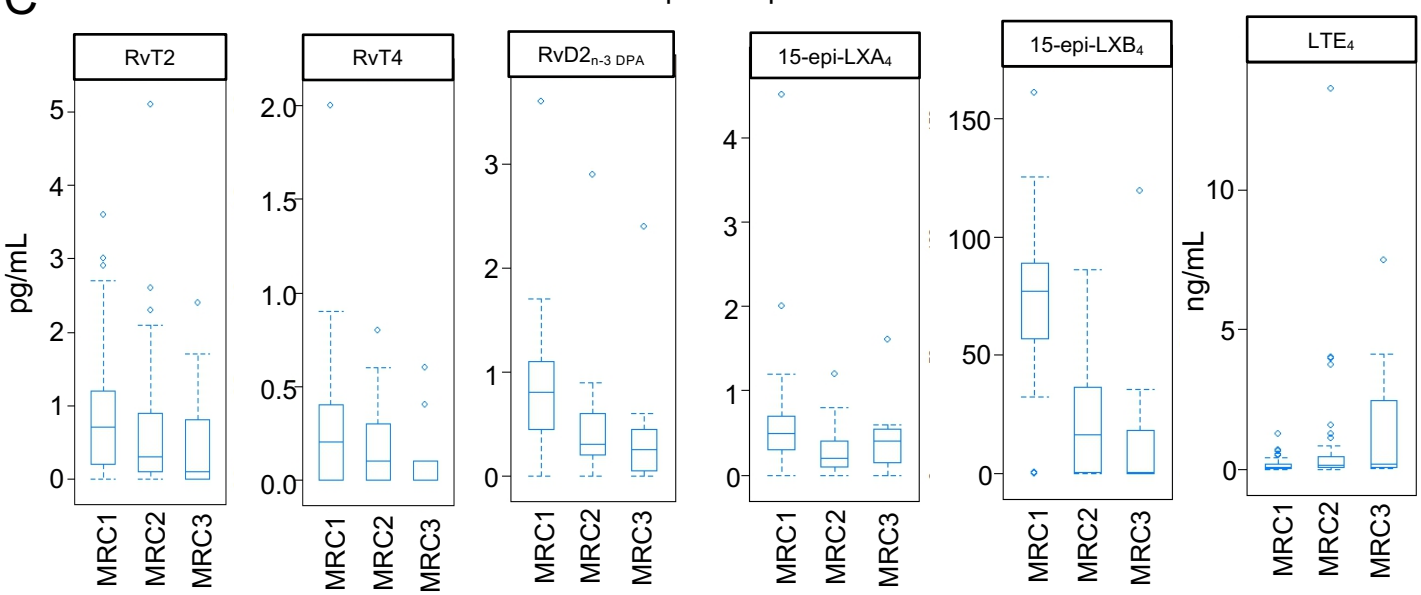
Figure 2

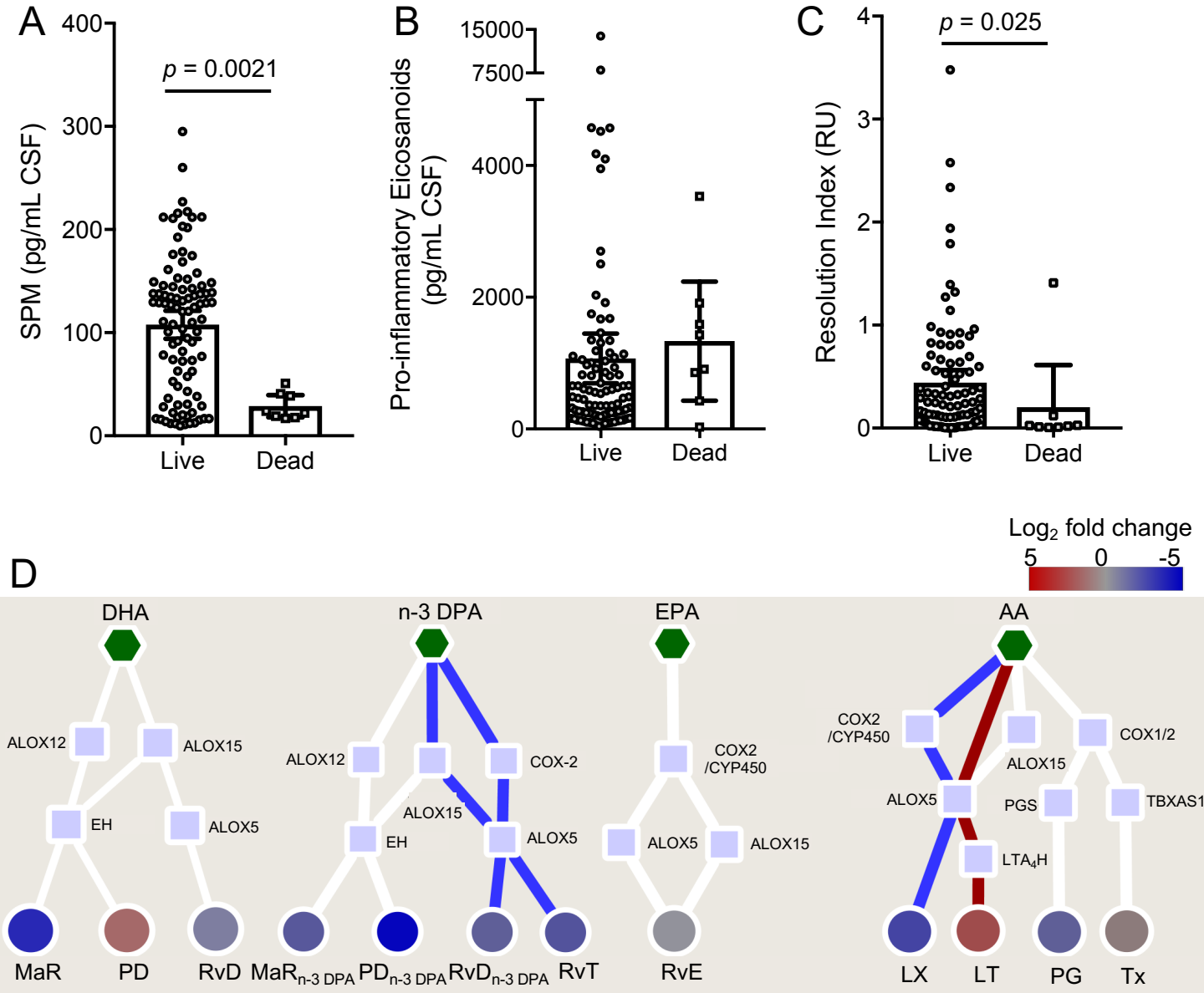
A


2D Score Plot
MRC3
MRC1
MRC2
Principal component 2
Principal component 1


2D Loading Plot
15-epi-LXB4
PCTR3
RvD2n-3 DPA
15-epi-LXA4
RvD1n-3 DPA
22-OH-PD1
PGD2
LTE4
LTC4
RvT4
RvE1
TXB2
RvD5n-3 DPA
RvD1
17R-RvD1
RvD5
PGF2α
LXA4
RvE3
MaR1n-3 DPA
LXB4
RvT3
PGE2
RvE2
Principal component 2
Principal component 1

C


RvT2
RvT4
RvD2n-3 DPA
15-epi-LXA4
15-epi-LXB4
LTE4
pg/mL
ng/mL
MRC1
MRC2
MRC3


A
SPM (pg/mL CSF)
400
300
200
100
0
p = 0.0021
Live
Dead
B
Pro-inflammatory Eicosanoids (pg/mL CSF)
15000
7500
4000
2000
0
Live
Dead
C
Resolution Index (RU)
4
3
2
1
0
p = 0.025
Live
Dead
D
Log2 fold change
5
0
-5
DHA
ALOX12
ALOX15
EH
ALOX5
MaR
PD
RvD
n-3 DPA
ALOX12
COX-2
ALOX15
EH
ALOX5
MaRn-3 DPA
PDn-3 DPA
RvDn-3 DPA
RvT
EPA
COX2 /CYP450
ALOX5
ALOX15
RvE
AA
COX2 /CYP450
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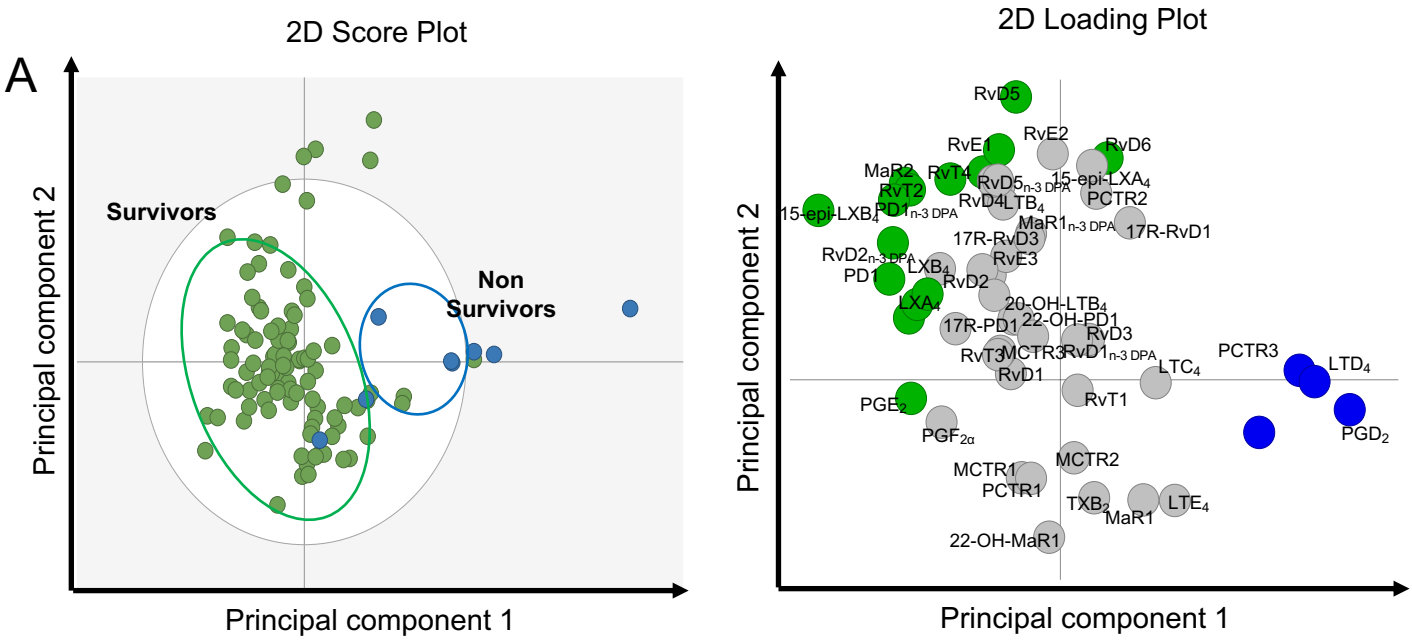
TBXAS1
LTA4H
LX
LT
PG
Tx

Figure 4


A
2D Score Plot
Survivors
Non Survivors
Principal component 2
Principal component 1
2D Loading Plot
RvD5
RvE2
RvE1
RvD6
MaR2
RvT4
RvD5n-3 DPA
15-epi-LXA4
RvT2
PCTR2
15-epi-LXB4
PD1n-3 DPA
RvD4
LTB4
MaR1n-3 DPA
17R-RvD1
17R-RvD3
RvD2n-3 DPA
RvE3
PD1
LXB4
RvD2
LXA4
20-OH-LTB4
17R-PD1
22-OH-PD1
RvD3
RvT3
MCTR3
RvD1n-3 DPA
PCTR3
LTD4
LTC4
RvD1
RvT1
PGE2
PGD2
PGF2α
MCTR2
MCTR1
PCTR1
TXB2
LTE4
MaR1
22-OH-MaR1
Principal component 2
Principal component 1

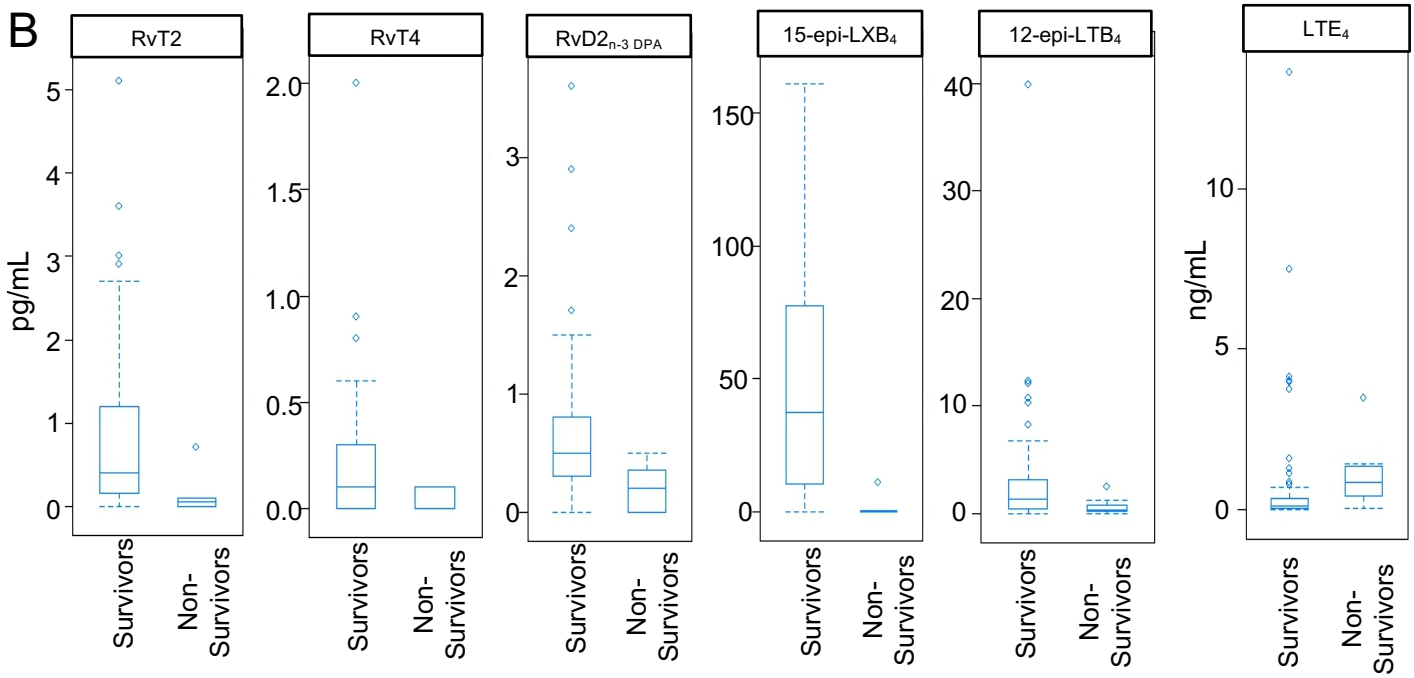
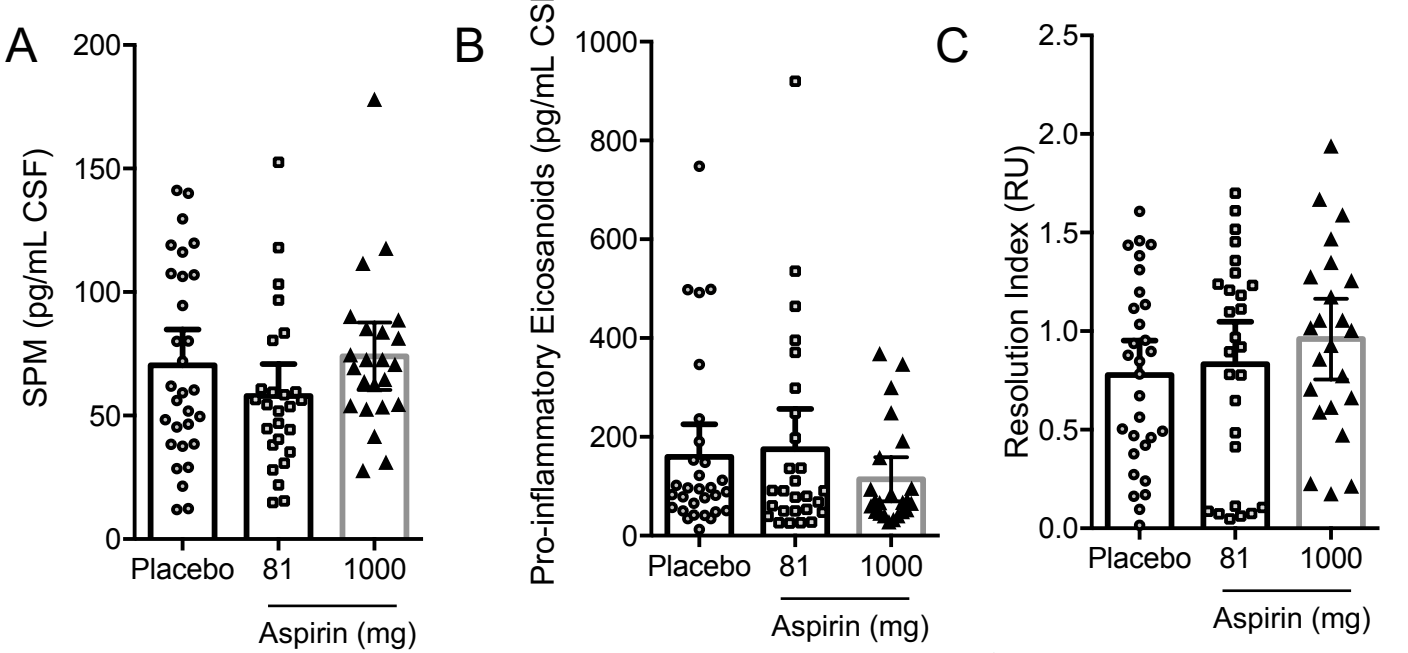
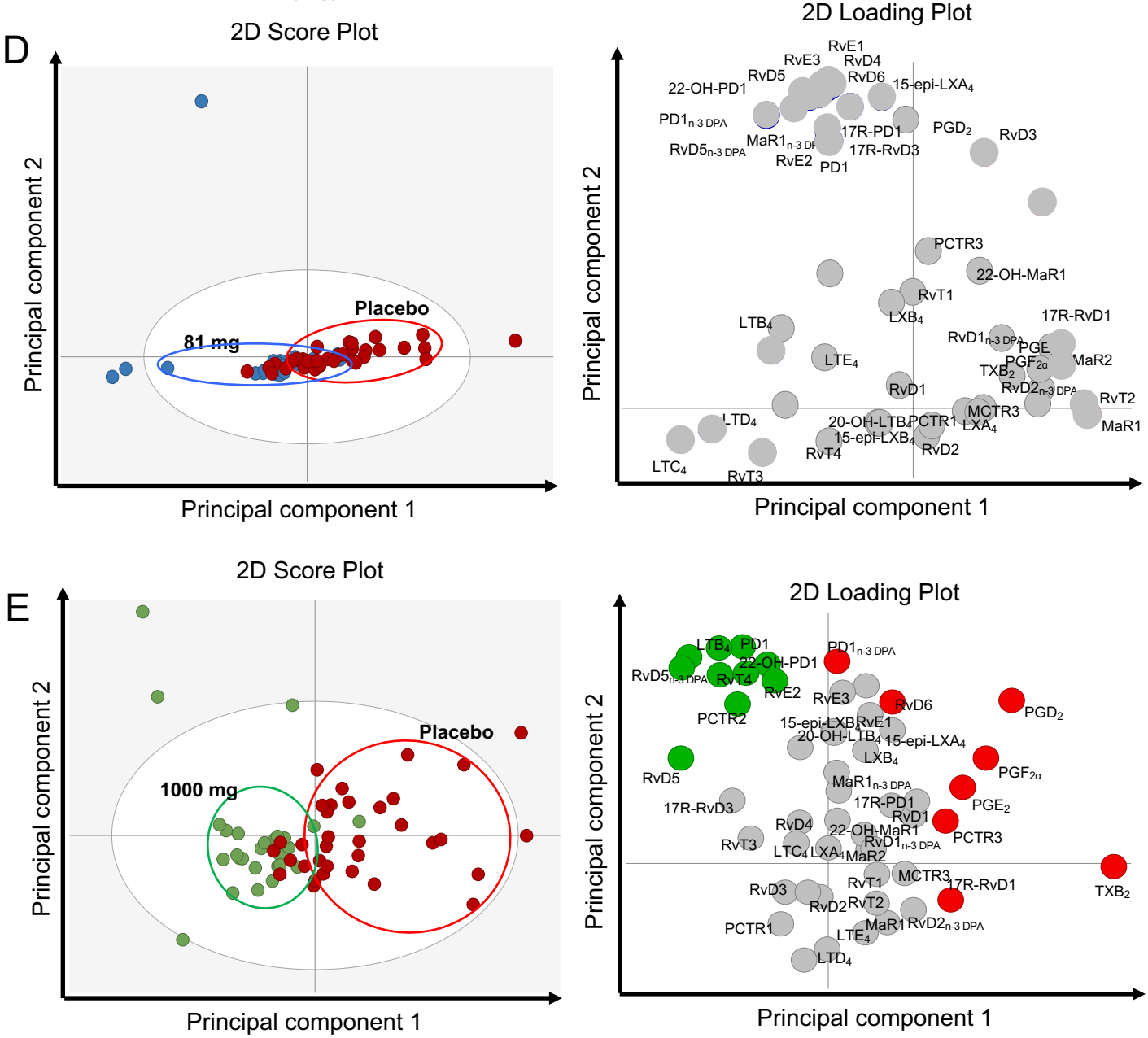

B
RvT2
RvT4
RvD2n-3 DPA
15-epi-LXB4
12-epi-LTB4
LTE4
pg/mL
ng/mL
Survivors
Non-Survivors

Figure 5


A
SPM (pg/mL CSF)
200
150
100
50
0
Placebo
81
1000
Aspirin (mg)
B
Pro-inflammatory Eicosanoids (pg/mL CSF)
1000
800
600
400
200
0
Placebo
81
1000
Aspirin (mg)
C
Resolution Index (RU)
2.5
2.0
1.5
1.0
0.5
0.0
Placebo
81
1000
Aspirin (mg)


D
2D Score Plot
Principal component 2
Principal component 1
Placebo
81 mg
2D Loading Plot
Principal component 2
Principal component 1
RvE1
RvE3
RvD4
RvD5
RvD6
22-OH-PD1
15-epi-LXA4
PD1n-3 DPA
17R-PD1
PGD2
RvD3
RvD5n-3 DPA
MaR1n-3 DPA
17R-RvD3
RvE2
PD1
PCTR3
22-OH-MaR1
RvT1
LXB4
LTB4
17R-RvD1
RvD1n-3 DPA
PGE2
LTE4
PGF2α
MaR2
TXB2
RvD1
RvD2n-3 DPA
RvT2
LTD4
MCTR3
20-OH-LTB4
PCTR1
LXA4
MaR1
15-epi-LXB4
RvT4
RvD2
LTC4
RvT3
E
2D Score Plot
Principal component 2
Principal component 1
Placebo
1000 mg
2D Loading Plot
Principal component 2
Principal component 1
LTB4
PD1
PD1n-3 DPA
22-OH-PD1
RvD5n-3 DPA
RvT4
RvE2
RvE3
RvD6
PGD2
PCTR2
15-epi-LXB4
RvE1
20-OH-LTB4
15-epi-LXA4
LXB4
RvD5
PGF2α
MaR1n-3 DPA
17R-RvD3
17R-PD1
PGE2
RvD1
RvD4
22-OH-MaR1
PCTR3
RvT3
LTC4
LXA4
RvD1n-3 DPA
MaR2
MCTR3
RvT1
17R-RvD1
TXB2
RvD3
RvD2
RvT2
PCTR1
MaR1
RvD2n-3 DPA
LTE4
LTD4