Impaired production and diurnal regulation of vascular $RvD_{n\text{-}3\ DPA}$ increases systemic inflammation and cardiovascular disease

Romain A. Colas*[1], Patricia R. Souza*[1], Mary E. Walker[1], Maudrian Burton[2], Zbigniew Zasłona[3], Annie M. Curtis[4], Raquel M. Marques[1],and Jesmond Dalli[1]

* share first authorship

[1]William Harvey Research Institute, Barts and the London School of Medicine, Queen Mary University of London, Charterhouse Square London, United Kingdom. EC1M 6BQ
[2]William Harvey Research Institute, NIHR Cardiovascular Biomedical Research Unit at Barts, Queen Mary University of London, Charterhouse Square London, United Kingdom. EC1M 6BQ
[3]School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin, Ireland
[4]Molecular and Cellular Therapeutics Department, Royal College of Surgeons in Ireland, Dublin, Ireland

Contact: Dr Jesmond Dalli, 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

Online Information
Online Methods:
Materials.
Liquid chromatography (LC)-grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA); Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 µm) was obtained from Agilent (Cheshire, UK); C18 SPE columns were from Biotage (Uppsala, SE); synthetic standards for LC-tandem mass spectrometry (MS-MS) quantitation and deuterated (d) internal standards (d_8-5S-HETE, d_5-RvD2, d_5-LXA_4, d_4-PGE_2, and d_4-LTB_4) were purchased from Cambridge Bioscience (Cambridge, UK) or provided by Charles N. Serhan (Harvard Medical School, Boston, Massachusetts, USA; supported by NIH-funded P01GM095467); Dulbecco's Phosphate-Buffered Saline (DPBS, without calcium and magnesium, Sigma), PAF C-16 (Cambridge Bioscience); Whole Blood Lysing Reagent Kit (Beckman Coulter, Inc); VioBlue-anti-CD41 (Miltenyi Biotec, clone REA386), PE-Cy5-anti-CD62P (BioLegend, clone AK4), Brilliant Violet 711-anti-CD11b (BioLegend, clone ICRF44), APC-Cy7-anti-CD16 (Abcam, clone 3G8), Alexa Fluor 647-anti-CD14 (BioLegend, clone HCD14). Anti-mouse CD11b-PE-Texas Red, CD62P-Brilliant Violet 650™, CD115-Brilliant Violet 711™, and CD41-Brilliant Violet 510™ PerCP/Cy5.5-anti-CD63 and FITC-ant-CD42b (Biolegend). Alexa Fluor 647 rabbit anti-human-15-LOX, Dylight 405 rabbit anti-human-5-LOX (Bioss Antibodies); Mifepristone and carvedilol (Tocris). The authors declare that all supporting data are available within the article and its online supplementary files.

Methods
Healthy volunteers blood collection: Venous Peripheral blood was collected at indicated intervals in sodium citrate (3.2%) from fasting volunteers that declared not taking NSAIDS for at least 14 days, caffeine and alcohol for at least 24h and fatty fish for 48h. Blood was collected via sequel bleeds from the same volunteers on the same day. Food was provided after the 12:00 h blood-draw to all volunteers. Volunteers gave written consent in accordance with a Queen Mary Research Ethics Committee (QMREC 2014:61) and the Helsinki declaration. Blood was then taken for flow cytometry and lipid mediator profiling analysis.

CVD patients blood collection: Fasting patients were screened and those that met the inclusion/ exclusion criteria were consented for blood to be obtained between 8:00 to 9:00 h, 12:00 h and between 16:00 to 18:00 h in accordance with East of England- Cambridge Central Research Ethics Committee and the Joint Research Management Office (JRMO), Queen Mary University of London. The inclusion criteria were i) severe coronary artery disease requiring treatment; ii) hospital admission for percutaneous coronary intervention (PCI); iii) >24hour post PCI; iv) able to provide informed consent; v) >18 years and vi) at least 2 of the following risk factors: hypertension, high cholesterol, smoker, diabetes, known ischemic heart disease
The exclusion criteria were: i) sustained ventricular tachycardia and/or ventricular fibrillation or appropriate ICD valve disease requiring intervention; ii) contra-indications to PCI; iii) women who are pregnant; iv) <18 years and v) enrolled in other studies. These blood samples were processed within 60 minutes of collection for *lipid mediator profiling* and *whole blood stimulations* as detailed in the sections below.

Targeted lipid mediator profiling. Plasma was obtained from peripheral blood of healthy volunteers and patients following centrifugation at 1500 x *g* for 10 min at room temperature. Descending aortas were weighed, placed in ice-cold methanol and homogenized using a glass dounce. All samples for LC-MS-MS-based profiling were extracted using solid-phase extraction columns as in[1, 2]. 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 fraction 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 5500 or QTrap 6500 plus (Sciex). An Agilent Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 µm) was kept at 50°C and mediators 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 5500 or QTrap 6500 plus were operated using a multiple reaction monitoring method as in[1, 2]. Each LM was identified using established criteria including matching retention time to synthetic and authentic materials and at least 6 diagnostic ions[1, 2]. 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.

Profiling of Acetylcholine, Norepinephrine and Adenosine. Plasma was placed in ice cold MeOH containing deuterated (d_9)-choline and kept at -20 ^{o}C for 45 min to allow for protein precipitation. Samples were then centrifuged for 10 minutes at 4000 x *g*. Supernatant were collected and evaporated under a gentle stream of nitrogen gas using a TurboVap LV (Biotage) at 37°C until dryness. Products were then suspended in MeOH profiled using an LC/MS-MS system. A Qtrap 5500 (AB Sciex) equipped with a Shimadzu SIL-20AC autoinjector and LC-20AD binary pump (Shimadzu Corp.) was used with an Agilent Eclipse Plus C18 column (100x4.6mmx1.8µm). The mobile phase consisted of methanol/water/acetic acid, 80:20:0.01 (vol:vol:vol) for 2.5 min that was ramped to 98:2:0.01 (vol:vol:vol) over 0.2 min and maintained for 1.3 min. The flow rate was maintained at 0.5ml/min. To monitor and quantify the levels of acetylcholine and norepinephrine, the Qtrap 5500 was operated in positive mode and a multiple reaction monitoring (MRM) method was developed with signature ion fragments (m/z) for each molecule monitoring the parent ion (Q1) and a daughter ion (Q3). The MRM transition employed for Acetylcholine was146>87, for norepinephrine was 170>152 and for adenosine 268>136.

Preparation of RvD1$_{n\text{-}3\ DPA}$ and RvD2$_{n\text{-}3\ DPA}$. RvD1$_{n\text{-}3\ DPA}$ and RvD2$_{n\text{-}3\ DPA}$ were prepared and isolated as in[1]. n-3 DPA (10 µM) was incubated with 100 U/ml isolated soybean-LOX (Borate buffer, 4°C, pH 9.2). 17S-HpDPA was isolated using UV-RP-HPLC (Infinity 1260; Agilent Technologies). 17S-HpDPA (10 µg) was then incubated with human neutrophils (80x10^6 cells/ml; PBS$^{+/+}$) and calcium ionophore (5 µM, 37°C). After 45 min the reaction was quenched using 2 volumes ice-cold methanol, reduced using sodium borohydrate, and products extracted using C18 SPE. RvD1$_{n\text{-}3\ DPA}$ and RvD2 $_{n\text{-}3\ DPA}$ were isolated using RP-HPLC (Infinity 1260; Agilent Technologies). Here, an Agilent Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 µm) was kept at 50°C and LM isolated with a mobile phase consisting of methanol-water-acetic acid of 60:40:0.01 (vol/vol/vol) maintained for 2 minutes, then ramped to 80:20:0.01 (vol/vol/vol) from 2 min to 16 min and to 98:2:0.01 (vol/vol/vol) over 3 minutes. This was maintained for 2 min. Flow rate was kept at 0.5 mL/min.

Human whole blood incubations. In select experiments, whole blood was incubated with RvD1$_{n\text{-}3\ DPA}$, RvD2$_{n\text{-}3\ DPA}$, RvD5$_{n\text{-}3\ DPA}$ (0.1, 1, 10 nM) or vehicle (PBS) for 15 min (37^{o}C). Blood was then incubated with PAF (100nM) for 30 min (37^{o}C).

After stimulation, samples were washed twice with PBS for 12 min at 800 x *g*. Samples were stained for flow cytometry as described below.
In select experiments, venous blood from healthy volunteers was collected and incubated with acetylcholine (ACh) at 0.1 μM for 45 min (37^{o}C). Plasma was then separated by centrifugation at 1,500 x *g* for 10 min for LM profiling.
In select incubations, human peripheral blood was incubated with an anti-human anti-human PSGL1 (2μg/ml), or Isotype control for 30 min at RT. This was then incubated with either vehicle, PAF (100nM) or FMLP (1μM) for 45 min at 37^{o}C. Incubations were stopped and platelet leukocyte aggregates were assessed using flow cytometry whereas $RvD_{n\text{-}3\ DPA}$ concentrations were determined using LM profiling.

Flow chamber: Whole blood was incubated with or 1 unit of ADA or 10nM of ACh for 20 min. The using an automated syringe pump (Harvard Apparatus) connected to small-diameter tubing (1.6 mm inner diameter) and chamber slides (15μ-Slide VI$^{0.4}$, Ibidi) the blood was perfused at a sheer rate of 0.1 or 0.3 pascals for 15 min and plasma was collected as detailed above.

PRP incubations: Peripheral blood from healthy volunteers was collected in acidified-citrate-dextrose. Blood was centrifuged at 500 x *g* for 20 min. PRP was collected and cells incubated with $RvD5_{n\text{-}3\ DPA}$ or vehicle (0.01% EtOH + PBS) for 15 min at 37^{o}C. Cells were then incubated with PAF (100nM) or Vehicle (0.01% EtOH) for 30 min at 37^{o}C. Cells were then washed with PBS and cellular activation was assessed using flow cytometry as detailed below.

Mouse Experiments: *ApoE$^{-/-}$ mice***.** Experiments strictly adhered to UK Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986) and Laboratory Animal Science Association (LASA) Guidelines (Guiding Principles on Good Practice for Animal Welfare and Ethical Review Bodies, 3rd Edition, 2015). Apo E$^{-/-}$ mice were a kind gift of Prof. Fulvio D'Acquisto (Queen Mary University of London). Mice (male and female) were fed a western diet for 6 weeks from 4 weeks of age and kept of a 12h light dark cycle. At 8 weeks of age mice were given $RvD5_{n\text{-}3\ DPA}$ (100ng/mouse; i.v.) or vehicle on alternate days for a 2-week period. Mice were culled, aortic arches were collected and stained using oil-red O as in[3]. Staining intensity was determined using ImageJ and expressed as relative units per mm^2. The descending aorta was collected, placed in ice-cold methanol and lipid mediators identified and quantified as described above.
WT mice: All mice were kept on a 12h light dark cycle, with lights turned on at 7:00 h and lights turned off at 19:00h. In select experiments, mice were administered mifepristone (Mife; 20mg/Kg; i.v.), carvedilol (Cav; 10mg/Kg; i.v) or vehicle at zeitgeber time (ZT) 8 (i.e., 8 hr after light onset; 3 pm), blood was collected at 19:00 H (ZT 12) and 10:00 h (ZT 3) and plasma $RvD_{n\text{-}3\ DPA}$ concentrations were determined using LM profiling as detailed above. In all experiments animals were given numerical identifiers and randomly assigned to the experimental groups with the experimenter being blinded to group assignments.

Bmal-1$^{Mye\text{-}/\text{-}}$ mice: BMAL1-LysM$^{-/-}$ mice were bred under specific pathogen–free conditions at the Trinity Biomedical Science Institute animal facility. All experiments were approved by the Trinity College Dublin Animal Research Ethics Committee and the Health Products Regulatory Authority (HPRA). Blood was collected from mice at 19:00 h (ZT12) and 10:00 h (ZT3) and $RvD_{n\text{-}3\ DPA}$ concentrations determined using lipid mediator profiling.

Flow Cytometry. Whole blood was incubated with lineage-specific markers for 45 min (4^{o}C, in DPBS containing 0.02% BSA). The following anti-human antibodies were used: VioBlue-anti-CD41, PE-Cy5-anti-CD62P, Brilliant Violet 711-anti-CD11b,

APC-Cy7-anti-CD16, Alexa Fluor 647-anti-CD14. After staining, red blood cells were lysed using Whole Blood Lysing Reagent Kit, according to the manufacturer's instructions. Data was collected using BD LSR Fortessa and analysis was conducted using FlowJo (Tree Star Inc., V10).
In separate experiments blood was collected from Apo $E^{-/-}$ mice using heparin-lined syringes *via* cardiac puncture. Cells were incubated with Fc-blocking IgG and anti-mouse CD11b-PE-Texas Red, CD62P-Brilliant Violet 650™, CD115-Brilliant Violet 711™, and CD41-Brilliant Violet 510™ (Biolegend) for 45 minutes on ice. Red blood cells were lysed and fixed using Whole Blood Lysing Reagent Kit. Staining was then evaluated using LSR Fortessa cell analyser (BD Biosciences) and analysed using FlowJo software (Tree Star Inc., V10).

For the analysis of the biosynthetic enzymes, whole blood was incubated with lineage-specific markers for 30 min (4°C, in DPBS containing 0.02% BSA). The following anti-human antibodies were used: Brilliant Violet 786-anti-CD14, APC-Cy7-anti-CD16, PerCP-Cy5.5-anti-CD4. After staining, red blood cells were lysed using Whole Blood Lysing Reagent Kit, according to the manufacturer's instructions. Samples were washed twice with PBS for 12 min at 800 x *g,* and incubated with Fc block for 20 min at RT (dilution 1:2, in Permeabilization buffer). Next followed the intracellular staining for 30 min (RT, in Permeabilization buffer). The following anti-human antibodies were used: Alexa Fluor 647-anti-15-LOX, Dylight 405-anti-5-LOX. Staining was then evaluated using LSR Fortessa cell analyser (BD Biosciences) and analysed using FlowJo software (Tree Star Inc., V10).

In select experiments platelet adhesion molecule expression was assessed. Here platelets were incubated with fluorescently labelled mouse anti-human VioBlue-anti-CD41, PE-Cy5-anti-CD62P, PerCP/Cy5.5-anti-CD63 and FITC-ant-CD42b for 30 min at 4°C. Cells were then washed and fluorescence staining evaluated using LSRFortessa cell analyser (BD Biosciences) and analysed using FlowJo software (Tree Star Inc., V10).

ImageStream. Whole blood was incubated with lineage-specific markers for 45 min (4°C, in DPBS containing 0.02% BSA). The following anti-human antibodies were used: eFluor450-anti-CD41, PE-Cy5-anti-CD62P, APC-Cy7-anti-CD16, FITC-anti-CD14. After staining, red blood cells were lysed using Whole Blood Lysing Reagent Kit, according to the manufacturer's instructions. Staining was then assessed using ImageStream X MK2 and analysis was performed using IDEAS® (Image Data Exploration and Analysis Software, Version 6.0). To identify the number of monocytes and neutrophils interacting with platelets we measured the number of cells expressing CD41. To determine the number of platelets tethered to each leukocyte we used the spot count feature in the IDEAS® software that measures pixel intensity in the CD41 channel after background subtraction and assesses whether this connects to a particular spot on the image (i.e. a platelets) as in[4].

Statistical analysis. Results are expressed as mean ± s.e.m. We assumed normality and equal distribution of variance between the different groups analyzed. Sample sizes for each experiment were determined on the variability observed in preliminary experiments. Differences between groups were assessed using one-sample t test (normalized data), Mann Whitney test (2 groups), Wilcoxon Signed Rank Test or Friedman's test followed by Dunn's multiple comparisons test (multiple groups). Investigators were not blinded to group allocation or outcome assessment. The criterion for statistical significance was $p \leq 0.05$. Sample sizes for each experiment were determined on the variability observed in prior experiments[2] and preliminary experiments. Partial least squares-discrimination analysis (PLS-DA) and principal component analysis (PCA)[5] were performed using SIMCA 14.1 software

(Umetrics, Umea, Sweden) following mean centering and unit variance scaling of LM levels. PLS-DA is based on a linear multivariate model that identifies variables that contribute to class separation of observations (Blister exudates) on the basis of their variables (LM levels). During classification, observations were projected onto their respective class model. The score plot illustrates the systematic clusters among the observations (closer plots presenting higher similarity in the data matrix). Loading plot interpretation identified the variables with the best discriminatory power (Variable Importance in Projection greater than 1) that were associated with the distinct intervals and contributed to the tight clusters observed in the Score plot. The present study is exploratory and primarily mechanistic in nature.

References

1. Dalli J, Colas RA, Serhan CN. Novel n-3 immunoresolvents: Structures and actions. *Sci Rep*. 2013;3:1940
2. Rathod KS, Kapil V, Velmurugan S, Khambata RS, Siddique U, Khan S, Van Eijl S, Gee LC, Bansal J, Pitrola K, Shaw C, D'Acquisto F, Colas RA, Marelli-Berg F, Dalli J, Ahluwalia A. Accelerated resolution of inflammation underlies sex differences in inflammatory responses in humans. *J Clin Invest*. 2017;127:169-182
3. Khambata RS, Ghosh SM, Rathod KS, Thevathasan T, Filomena F, Xiao Q, Ahluwalia A. Antiinflammatory actions of inorganic nitrate stabilize the atherosclerotic plaque. *Proc Natl Acad Sci U S A*. 2017;114:E550-E559
4. Singh MV, Davidson DC, Jackson JW, Singh VB, Silva J, Ramirez SH, Maggirwar SB. Characterization of platelet-monocyte complexes in hiv-1-infected individuals: Possible role in hiv-associated neuroinflammation. *J Immunol*. 2014;192:4674-4684
5. Janes KA, Yaffe MB. Data-driven modelling of signal-transduction networks. *Nat Rev Mol Cell Biol*. 2006;7:820-828

Online Tables
Online Table I. Healthy volunteers demographics

Sex	Age (years)	Weight (Kg)	BMI (Kg/m^2)
3M/4F	34 ± 4.1	65.6 ± 11.3	23.2 ± 3.0

Online Table II: Diurnal lipid mediator profiles in healthy volunteer peripheral blood

			Healthy volunteer plasma Lipid mediators concentration (pg/mL)				
DHA bioactive metabolome	**Q1**	**Q3**	**18:00**	**7:00**	**9:00**	**12:00**	**15:00**
RvD1	375	141	0.8 ± 0.4	0.8 ± 0.3	1.4 ± 0.4	0.8 ± 0.3	0.8 ± 0.2
RvD2	375	141	0.9 ± 0.4	0.9 ± 0.2	0.6 ± 0.3	0.3 ± 0.2	0.3 ± 0.2
RvD3	375	147	0.1 ± 0.0	0.6 ± 0.4	0.4 ± 0.2	0.2 ± 0.1	0.1 ± 0.0
RvD4	375	101	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	1.3 ± 0.8	0.8 ± 0.6
RvD5	359	199	0.6 ± 0.2	0.3 ± 0.2	0.3 ± 0.1	0.5 ± 0.2	0.5 ± 0.1
RvD6	359	101	0.6 ± 0.2	0.4 ± 0.1	0.7 ± 0.4	0.7 ± 0.2	0.8 ± 0.3
17R-RvD1	375	141	0.2 ± 0.1	0.4 ± 0.2	0.3 ± 0.2	0.4 ± 0.2	0.4 ± 0.1
17R-RvD3	375	147	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
PD1	359	153	0.6 ± 0.2	0.7 ± 0.4	0.8 ± 0.3	1.0 ± 0.4	1.0 ± 0.6
17R-PD1	359	153	0.6 ± 0.3	0.4 ± 0.3	0.4 ± 0.2	0.3 ± 0.2	0.3 ± 0.2
10S,17S-diHDHA	359	153	0.6 ± 0.2	1.4 ± 0.4	1.1 ± 0.6	0.7 ± 0.4	0.7 ± 0.2
22-OH-PD1	375	153	0.8 ± 0.4	1.3 ± 0.7	1.1 ± 0.5	1.4 ± 0.7	2.7 ± 2.1
MaR1	359	221	1.0 ± 0.3	0.6 ± 0.2	1.2 ± 0.2	1.1 ± 0.4	1.0 ± 0.4
7S,14S-diHDHA	359	221	1.1 ± 0.4	1.0 ± 0.3	0.6 ± 0.4	1.0 ± 0.3	0.8 ± 0.4
4S,14S-diHDHA	359	101	11.3 ± 8.5	8.5 ± 5.7	7.8 ± 4.8	9.1 ± 5.6	8.1 ± 5.5
n-3 DPA bioactive metabolome							
$RvD1_{n\text{-}3\ DPA}$	377	143	1.9 ± 0.4	10.9 ± 4.2*	7.5 ± 2.3*	6.0 ± 1.8	1.6 ± 0.3
$RvD2_{n\text{-}3\ DPA}$	377	261	2.3 ± 1.3	1.8 ± 1.0	2.5 ± 1.1	1.7 ± 1.2	1.1 ± 0.7
$RvD5_{n\text{-}3\ DPA}$	361	263	2.6 ± 1.2	2.6 ± 1.2	4.5 ± 0.3*	2.3 ± 1.2	3.2 ± 2.2
$PD1_{n\text{-}3\ DPA}$	361	183	1.1 ± 0.3	2.3 ± 0.5	1.6 ± 0.3	1.6 ± 0.3	1.1 ± 0.3
$MaR1_{n\text{-}3\ DPA}$	361	249	1.7 ± 0.7	3.5 ± 1.9	3.2 ± 1.4	0.9 ± 1.0	1.3 ± 0.6
RvT1	377	193	0.1 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.4 ± 0.2	-
RvT2	377	143	0.3 ± 0.2	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.3	0.3 ± 0.2
RvT3	377	255	-	-	-	-	-
RvT4	359	193	2.0 ± 0.8	2.6 ± 1.4	3.0 ± 1.3	1.4 ± 0.6	1.4 ± 0.6
EPA bioactive metabolome							
RvE1	349	195	3.9 ± 1.5	3.7 ± 1.5	4.5 ± 1.5	4.9 ± 1.9	4.9 ± 2.0
RvE2	333	199	2.3 ± 0.6	1.9 ± 0.7	2.5 ± 0.8	2.3 ± 0.8	2.7 ± 1.1
RvE3	333	201	1.2 ± 0.3	1.4 ± 0.5	1.6 ± 0.4	1.4 ± 0.6	1.3 ± 0.6
AA bioactive metabolome							
LXA_4	351	217	0.3 ± 0.1	0.8 ± 0.4	0.7 ± 0.3	0.6 ± 0.2	0.6 ± 0.2
LXB_4	351	221	0.9 ± 0.4	0.8 ± 0.3	0.2 ± 0.2	0.5 ± 0.2	0.6 ± 0.2
5S,15S-diHETE	335	235	11.7 ± 3.6	9.2 ± 3.5	19.0 ± 11.2	8.9 ± 2.4	8.5 ± 3.0
15epi-LXA_4	351	217	7.0 ± 3.1	8.3 ± 5.5	4.2 ± 1.2	4.6 ± 1.2	4.2 ± 1.1
15epi-LXB_4	351	221	1.7 ± 0.9	1.0 ± 0.2	1.5 ± 0.4	0.5 ± 0.2	2.3 ± 1.0
LTB_4	335	195	2.0 ± 0.5	2.5 ± 0.7	2.6 ± 1.2	1.8 ± 0.6	1.9 ± 0.5
5S,12S-diHETE	335	195	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
20-OH-LTB_4	351	195	0.1 ± 0.0	0.3 ± 0.2	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
PGD_2	351	189	5.8 ± 1.7	7.5 ± 1.5	4.7 ± 0.4	6.6 ± 1.8	7.6 ± 1.6
PGE_2	351	189	5.9 ± 0.8	8.7 ± 3.6	4.4 ± 1.2	5.3 ± 1.2	6.5 ± 1.5
$PGF_{2\alpha}$	353	193	6.1 ± 1.5	11.8 ± 2.5	7.0 ± 1.3	6.3 ± 0.9	8.9 ± 0.7
TxB_2	369	169	233.6 ± 149.7	364.5 ± 176.9	92.3 ± 34.2	183.6 ± 100.0	415.8 ± 305.8

Peripheral blood was collected from healthy volunteers at the indicated intervals. Plasma was placed in ice-cold methanol and lipid mediators (LM) were assessed using LM-profiling (see methods for details). Q1, M-H (parent ion) and Q3, diagnostic ion in the MS-MS (daughter ion). Results are mean ± s.e.m. and expressed as pg/mL. n = 7 volunteers per interval. The detection limit was ~ 0.1 pg. -, Below limits of detection * $p<0.05$ vs 18:00h values using paired Mann–Whitney test.

Online Table III. ACh regulation of n-3 DPA metabolome in human whole blood

			Lipid mediators concentration (pg/mL)	
n-3 DPA bioactive metabolome	**Q1**	**Q3**	**Vehicle**	**ACh 0.1 µM**
$RvD1_{n\text{-}3\ DPA}$	377	143	3.4 ± 1.8	4.9 ± 2.8
$RvD2_{n\text{-}3\ DPA}$	377	261	2.8 ± 1.2	7.4 ± 2.6*
$RvD5_{n\text{-}3\ DPA}$	361	201	0.8 ± 0.7	1.5 ± 0.6
$PD1_{n\text{-}3\ DPA}$	361	155	0.4 ± 0.2	0.5 ± 0.1
$MaR1_{n\text{-}3\ DPA}$	361	223	0.3 ± 0.2	0.9 ± 0.3*
RvT1	377	211	0.3 ± 0.3	2.0 ± 1.3
RvT2	377	197	1.2 ± 0.8	1.6 ± 0.9
RvT3	377	255	1.2 ± 0.9	1.4 ± 0.9
RvT4	359	211	0.5 ± 0.3	1.0 ± 0.4

Peripheral blood from healthy volunteers was collected and incubated with ACh (0.1 µM; 45 min; 37^{o}C). Incubations were quenched with ice-cold methanol and n-3 DPA-derived LM identified and quantified using LM-profiling (see methods for details). Q1, M-H (parent ion) and Q3, diagnostic ion in the MS-MS (daughter ion). Results are expressed as pg/mL, mean ± s.e.m, n = 9 donors per group. * $p < 0.05$ vs Vehicle group using paired Mann–Whitney test.

Online Table IV: Peripheral blood SPM substrate and precursor concentrations.

			Lipid mediators precursors concentration (pg/mL)	
n-3 DPA bioactive metabolome	**Q1**	**Q3**	**Vehicle**	**ACh 0.1 µM**
17HDHA	343	245	101.9 ± 26.7	103.6 ± 25.9
14HDHA	343	205	205.8 ± 47.3	229.2 ± 41.2
7HDHA	343	141	9.1 ± 3.3	8.6 ± 3.7
4HDHA	343	101	20.4 ± 5.47	19.9 ± 5.1
DHA	327	283	23117.7 ± 7852.2	28104.0 ± 10103.4
18HEPE	317	259	33.8 ± 11.3	36.2 ± 12.2
15HEPE	317	219	35.3 ± 13.6	31.3 ± 8.7
12HEPE	317	179	382.6 ± 116.1	402.4 ± 106.0
5HEPE	317	115	45.5 ± 11.7	42.7 ± 11.3
EPA	301	257	5371.5 ± 514.6	5867.2 ± 713.2
15HETE	319	219	287.6 ± 88.1	336.4 ± 95.2
12HETE	319	179	4751.4 ± 1271.4	5148.0 ± 1153.2
5HETE	319	115	61.2 ± 5.5	69.9 ± 13.5
AA	303	259	20584.4 ± 4222.3	24205.0 ± 4783.4*
17-HDPA	345	247	64.4 ± 14.9	58.9 ± 13.2
14-HDPA	345	207	112.6 ± 25.5	137.2 ± 25.8*
13-HDPA	345	193	1.4 ± 0.4	1.9 ± 0.6
7-HDPA	345	143	56.4 ± 19.3	44.9 ± 10.7
DPA	327	283	3703.3 ± 703.0	3873.1 ± 706.3

Peripheral blood from healthy volunteers was collected and incubated with acetylcholine (ACh) at 0.1 µM for 45 min. Plasma was isolated, placed in ice cold methanol containing deuterium labelled internal standards and SPM precursors together with their pathway markers were extracted, identified and quantified using lipid mediator (see methods for details). Q1, M-H (parent ion) and Q3, diagnostic ion in the MS-MS (daughter ion). Results are expressed as pg/mL. Mean ± SEM of n = 9 per condition. * $p < 0.05$ vs Vehicle group using paired Mann–Whitney test.

Online Table V: SPM concentrations in peripheral blood incubations with cortisol.

			Lipid mediators concentration (pg/mL)		
n-3 DPA bioactive metabolome	**Q1**	**Q3**	**Vehicle**	**Cortisol 1 µM**	**Cortisol 10 µM**
$RvD1_{n\text{-}3\ DPA}$	377	143	1.4 ± 1.1	1.9 ± 1.2	1.7 ± 1.5
$RvD2_{n\text{-}3\ DPA}$	377	261	1.8 ± 1.3	1.0 ± 0.9	1.2 ± 0.8
$RvD5_{n\text{-}3\ DPA}$	361	201	1.9 ± 0.3	1.1 ± 0.6	1.7 ± 0.3
$PD1_{n\text{-}3\ DPA}$	361	155	0.9 ± 0.2	1.5 ± 1.1	2.5 ± 1.5
$MaR1_{n\text{-}3\ DPA}$	361	223	0.9 ± 0.5	0.3 ± 0.2	0.2 ± 0.2
RvT1	377	211	0.7 ± 0.3	0.5 ± 0.2	0.6 ± 0.2
RvT2	377	197	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0
RvT3	377	255	3.9 ± 2.5	1.6 ± 0.5	2.2 ± 1.5
RvT4	359	211	2.1 ± 1.3	2.7 ± 1.5	3.0 ± 1.8
17-HDPA	345	247	73.3 ± 20.8	74.9 ± 18.2	71.5 ± 17.5
14-HDPA	345	207	282.0 ± 39.0	286.2 ± 91.0	241.0 ± 40.9
13-HDPA	345	193	3.3 ± 0.7	3.2 ± 0.7	3.0 ± 0.6
7-HDPA	345	143	239.7 ± 95.6	268.9 ± 83.5	257.8 ± 109.6
DPA	327	283	21213.7 ± 7408.2	24568.2 ± 6300.4	28823.5 ± 8019.0

Peripheral blood from healthy volunteers was collected and incubated with cortisol (1 -10 µM) or vehicle for 45 min. Plasma was obtained, placed in ice cold methanol containing deuterium labelled internal standards and lipid mediators were extracted, identified and quantified using lipid mediator profiling (see methods for details). Q1, M-H (parent ion) and Q3, diagnostic ion in the MS-MS (daughter ion). Results are expressed as pg/mL. Mean ± SEM of n = 5 per condition.

Online Table VI: CVD – demographics and clinical data

Participants	9
Age (years)	65.2 ± 8.6
Sex	7 Male, 2 Female
CRP mg/L	35.4 ± 42.2
IL-6 pg/mL	2.5 ± 1.0
TNF-α pg/mL	108.2 ± 74.9
Creatine µmol/L	119.1 ±90.5
LDL mmol/L	3.0 ± 0.2
HDL mmol/L	0.5 ± 0.1
Type II Diabetes	3
Hypertension	9
Current Smoking	0
Obese *n*	4
Previous AMI	1
Previous PCI	4
LVEF ≤50%	4
Aspirin (*n*)	9
Statins (*n*)	Atorvastatin (4), Simvastatin (3) and Rosuvastatin (1)
Other medications (*n)*	Allopurinol (1), Amitriptyline (2), Amlodipine (2), Apixaban (1), Bisoprolol (6), Candesartan (1), Citalopram (1), Clopidogrel (2), Codeine (1), Cyanacobalamin (1), Dorzolamide (1), Doxazosin (2), Enoxaparin (1), Fentanyl (1), Finasteride (1), Flucloxacillin (1), Fluoxetine (2), Furosemide (2), Isosorbide mononitrate (1), Lansoprazole (4), Lantus Insulin (1), Lisinopril (1), Metformin (1), Nicorandil (1), NoroRapid Insulin (1), Omeprazole (3), Paracetamol (1), Phyllocontine (1), Priadel (1), Ramipril (5), Salbutamol (1), Salmeterol (1), Sertraline (1), Setagliptin (1), Tamoxifen (1), Tamsulosin (2) Temazepam (1), Thiamine (1), Tildiem (1), Timolol (1), Tioropium bromide (1), Warfarin (1), Xalatan (1).

Online Table VII. Peripheral blood LM profiles in patients with CVD

			Plasma from CVD patients Lipid mediators concentration (pg/mL)		
DHA bioactive metabolome	**Q1**	**Q3**	**PM**	**AM**	**Midday**
RvD1	375	141	1.0 ± 0.5	0.6 ± 0.5	0.5 ± 0.2
RvD2	375	141	0.3 ± 0.1	0.2 ± 0.2	0.2 ± 0.1*
RvD3	375	147	0.2 ± 0.1	0.4 ± 0.2	0.1 ± 0.1
RvD4	375	101	1.8 ± 0.5	2.5 ± 1.2	2.3 ± 1.6
RvD5	359	199	1.8 ± 0.4	2.1 ± 0.9	0.7 ± 1.3
RvD6	359	101	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.2
17R-RvD1	375	141	0.5 ± 0.3	0.5 ± 0.2	1.2 ± 0.9
17R-RvD3	375	147	0.3 ± 0.3	0.3 ± 0.2	1.2 ± 0.8
PD1	359	153	0.3 ± 0.1	0.8 ± 0.2*	0.5 ± 0.5
17R-PD1	359	153	0.4 ± 0.3		0.2 ± 0.1
10S,17S-diHDHA	359	153	0.3 ± 0.2	0.1 ± 0.1	
22-OH-PD1	375	153	2.4 ± 2.3	0.1 ± 0.1	0.4 ± 0.3
MaR1	359	221	0.8 ± 0.4	0.9 ± 0.5	1.0 ± 0.7
7S,14S-diHDHA	359	221	0.2 ± 0.2	0.8 ± 0.5	-
4S,14S-diHDHA	359	101	0.6 ± 0.3	0.4 ± 0.2	0.1 ± 0.1*
n-3 DPA bioactive metabolome					
$RvD1_{n\text{-}3\ DPA}$	377	143	1.1 ± 0.2	1.4 ± 0.4	0.5 ± 0.3*
$RvD2_{n\text{-}3\ DPA}$	377	261	1.0 ± 0.4	1.7 ± 0.6	0.5 ± 0.2
$RvD5_{n\text{-}3\ DPA}$	361	263	0.9 ± 0.3	0.9 ± 0.4	1.0 ± 0.6
$PD1_{n\text{-}3\ DPA}$	361	183	0.3 ± 0.1	0.2 ± 0.1	0.7 ± 0.2
$MaR1_{n\text{-}3\ DPA}$	361	249	0.3 ± 0.2	-	0.6 ± 0.7
RvT1	377	193	0.1 ± 0.1	0.3 ± 0.2	-
RvT2	377	143	-	0.3 ± 0.2	-
RvT3	377	255	0.2 ± 0.1	0.9 ± 0.3	0.2 ± 0.2
RvT4	359	193	0.4 ± 0.2	0.3 ± 0.1	-
EPA bioactive metabolome					
RvE1	349	195	2.5 ± 2.2	1.8 ± 1.2	0.4 ± 0.2
RvE2	333	199	0.5 ± 0.4	0.2 ± 0.1	0.1 ± 0.0
RvE3	333	201	1.5 ± 0.9	2.1 ± 0.8	1.7 ± 1.9
AA bioactive metabolome					
LXA_4	351	217	-	0.1 ± 0.1	0.4 ± 0.3
LXB_4	351	221	1.1 ± 0.4	1.1 ± 0.6	0.5 ± 0.4*
5S,15S-diHETE	335	235	18.4 ± 5.6	15.8 ± 7.9	8.1 ± 3.0
15epi-LXA_4	351	217	4.6 ± 2.6	1.6 ± 0.6	2.3 ± 1.6
15epi-LXB_4	351	221	13.5 ± 5.3	20.5 ± 7.6	5.5 ± 2.6
LTB_4	335	195	2.2 ± 0.4	2.1 ± 0.6	1.1 ± 0.6
5S,12S-diHETE	335	195	0.3 ± 0.1	0.8 ± 0.5	0.2 ± 0.2
20-OH-LTB_4	351	195	0.4 ± 0.2	0.2 ± 0.1	0.1 ± 0.1
PGD_2	351	189	5.7 ± 2.5	2.6 ± 0.6	5.5 ± 2.4
PGE_2	351	189	13.3 ± 3.6	9.6 ± 2.4*	20.3 ± 9.2
$PGF_{2\alpha}$	353	193	9.1 ± 4.0	7.4 ± 1.9	14.1 ± 13.7
TxB_2	369	169	36.2 ± 23.9	26.6 ± 20.0	86.1 ± 93.5

Peripheral blood from CVD patients was collected at 9:00 h (AM) 12:00 h (Midday) and between 16:00-18:00 h (PM). Plasma was placed in ice-cold methanol containing internal standards. Lipid mediators (LM) were extracted, identified and quantified using LM-profiling (see methods for details). Q1, M-H (parent ion) and Q3, diagnostic ion in the MS-MS (daughter ion). Results are mean ± s.e.m. and

expressed as pg/mL. n = 9 patients at each interval. The detection limit was ~ 0.1 pg. -, Below limits of detection * $p<0.05$ vs PM values using paired Mann–Whitney test.

Online Table VIII: $RvD5_{n\text{-}3\ DPA}$ administration upregulated SPM and reduced pro-inflammatory eicosanoids in aortic tissues from $ApoE^{-/-}$.

DHA Bioactive Metabolome	Q1	Q3	$ApoE^{-/-}$ WD (pg/10mg aorta)	$ApoE^{-/-}$ WD + $RvD5_{n\text{-}3\ DPA}$ (pg/10mg aorta)
RvD1	375	233	3.7 ± 1.1	1.7 ± 0.9*
RvD2	375	215	0.7 ± 0.7	0.4 ± 0.4
RvD3	375	147	5.7 ± 3.9	2.4 ± 0.6
RvD4	375	225	0.2 ± 0.1	-
RvD5	359	199	0.3 ± 0.1	0.4 ± 0.2
RvD6	359	159	1.0 ± 0.8	1.5 ± 1.1
17R-RvD1	375	215	0.6 ± 0.1	1.6 ± 0.9
17R-RvD3	375	147	1.2 ± 0.7	1.3 ± 0.3
PD1	359	153	2.1 ± 0.7	2.6 ± 0.7
10S,17S-diHDHA	359	153	32.2 ± 6.0	66.0 ± 35.4
17R-PD1	359	137	0.8 ± 0.4	0.3 ± 0.2
22-OH-PD1	375	153	7.2 ± 1.7	6.0 ± 1.0
MaR1	359	177	17.7 ± 2.3	35.4 ± 22.7*
7S,14S-diHDHA	359	177	0.3 ± 0.3	1.8 ± 0.3*
4,14-diHDHA	359	159	3.5 ± 2.1	4.9 ± 2.0
n-3 DPA Bioactive Metabolome				
RvT1	377	211	-	-
RvT2	377	255	-	-
RvT3	377	173	-	-
RvT4	361	193	-	-
$RvD1_{n\text{-}3\ DPA}$	377	215	0.1 ± 0.1	0.1 ± 0.1
$RvD2_{n\text{-}3\ DPA}$	377	261	-	0.1 ± 0.1
$RvD5_{n\text{-}3\ DPA}$	361	263	0.1 ± 0.1	0.1 ± 0.1
$PD1_{n\text{-}3\ DPA}$	361	183	0.1 ± 0.1	0.1 ± 0.1
$MaR1_{n\text{-}3\ DPA}$	361	223	-	-
EPA Bioactive Metabolome				
RvE1	349	161	1.8 ± 0.6	1.8 ± 0.5
RvE2	333	159	1.2 ± 1.3	0.1 ± 0.1
RvE3	333	201	0.3 ± 0.2	0.7 ± 0.1
AA Bioactive Metabolome				
LXA_4	351	115	0.4 ± 0.2	0.3 ± 0.0
LXB_4	351	221	1.3 ± 0.8	0.6 ± 0.4
5S,15S-diHETE	335	235	35.6 ± 24.0	51.9 ± 15.3
15-epi-LXA_4	351	115	6.2 ± 2.2	9.6 ± 1.9*
15-epi-LXB_4	351	221	4.6 ± 4.3	1.4 ± 0.5
LTB_4	335	195	1.4 ± 0.3	1.7 ± 0.3
5S,12S-diHETE	335	195	1.3 ± 0.4	0.6 ± 0.2
20-OH-LTB_4	351	195	-	-
PGE_2	351	189	24.1 ± 2.2	20.5 ± 4.5*
PGD_2	351	189	18.5 ± 2.9	14.9 ± 3.5
PGF_{2a}	353	193	10.8 ± 0.7	10.0 ± 1.7
TxB_2	369	169	46.9 ± 6.5	34.5 ± 4.1*

ApoE$^{-/-}$ mice were fed a Western diet (WD) for 6 weeks. On week 4 mice were administered vehicle or 100ng/mouse RvD5$_{n\text{-}3\ DPA}$ (via i.v. injection) on alternative days. Descending aortas were harvested and placed in ice-cold methanol containing internal standards. Lipid mediators (LM) were extracted, identified and quantified using LM-profiling (see methods for details). Q1, M-H (parent ion) and Q3, diagnostic ion in the MS-MS (daughter ion). Results are mean ± s.e.m. and expressed as pg/10mg tissue. n = 4 mice per group. * $p < 0.05$ vs Vehicle mice using Mann–Whitney test.

Online Figures

Online Figure I. Diurnal regulation of LM-SPM healthy volunteers. Peripheral blood was collected from healthy volunteers at the indicated intervals and plasma placed in ice-cold methanol containing deuterium labeled internal standards. Lipid mediators (LM) were extracted, identified and quantified using LM profiling (see methods for details). (A) Representative MRM for identified LM, (B) MS-MS spectra used for the identification of $RvD5_{n\text{-}3\ DPA}$. Results are representative of n = 7 healthy volunteers. Concentration of (C) DHA metabolome, (D) EPA metabolome, (E) PG and (F) LTB_4 metabolome. Results are mean ± s.e.m, expressed as pg/mL. n = 7 volunteers per interval. (G) Blood was collected from WT (*left panel*) and BMAL1-$LysM^{-/-}$ (*right panel*) mice at zeitgeber time (ZT) 3 (10 am) and 19:00 (ZT12) and $RvD_{n\text{-}3\ DPA}$ were identified and quantified using lipid mediator profiling. Results are mean ± s.e.m, n = 4-5 mice per group.

Online Figure II: Morning $RvD_{n\text{-}3\ DPA}$ concentrations negatively correlate with peripheral blood neutrophil activation. Peripheral blood was collected from healthy volunteers at the indicated intervals, LM concentrations determined using LM profiling (see methods for details) and neutrophil and platelet activation determined using fluorescently labelled antibodies and flow cytometry. (A) Neutrophil CD41 expression. Results are mean ± s.e.m, n = 7 volunteers per interval and expressed as percentage of 18:00 h antigen expression. Results in the grey panel are re-plotted from the white portion to aid in visualization of rhythmicity. (B, C) Correlation between changes in neutrophil (B) CD11b and (C) CD62P (9:00 to 18:00) expression and 9:00 $RvD_{n\text{-}3\ DPA}$ concentrations. Results are representative of n=8 volunteers. Dashed line represents 95% confidence interval.

Online Figure III. Acetylcholine up-regulates n-3 DPA SPM in peripheral blood from healthy volunteers. (A) Blood was collected from healthy volunteers and acetylcholine levels determined using LC/MS-MS. Results are mean ± s.e.m, n = 7 healthy donors per condition and expressed as ng/mL. *, $p \leq 0.05$ vs 18h concentrations using Wilcoxon Signed Rank Test. Results in the grey panel are re-plotted from the white portion to aid in visualization of rhythmicity (B-D) Blood was collected from healthy volunteers and incubated with acetylcholine (ACh; 0.1 μM; 45 min; 37°C). Incubations were quenched with ice-cold methanol and n-3 DPA-derived LM identified and quantified using LM-profiling (see methods for details). (B) Representative MRM for the identified n-3 DPA SPM (B,C) MS-MS spectra used for the identification of (C) $RvD2_{n\text{-}3\ DPA}$. Results are representative of n = 9 healthy donors. (D) Plasma $RvD_{n\text{-}3\ DPA}$ concentrations. Results are mean ± s.e.m, n = 9 healthy donors per condition and expressed as pg/mL. **, $p \leq 0.01$ vs vehicle incubations (Veh) using paired Mann–Whitney test. (E) Peripheral blood was incubated in with ACh (10nM) or Veh (PBS) then perfused at 0.1 Pa for 20 min at 37°C. n = 6 healthy volunteers. Plasma was collected and $RvD_{n\text{-}3\ DPA}$ concentrations ascertained using LM profiling. *, $p \leq 0.05$ vs vehicle incubations (Veh) using paired Mann–Whitney test.

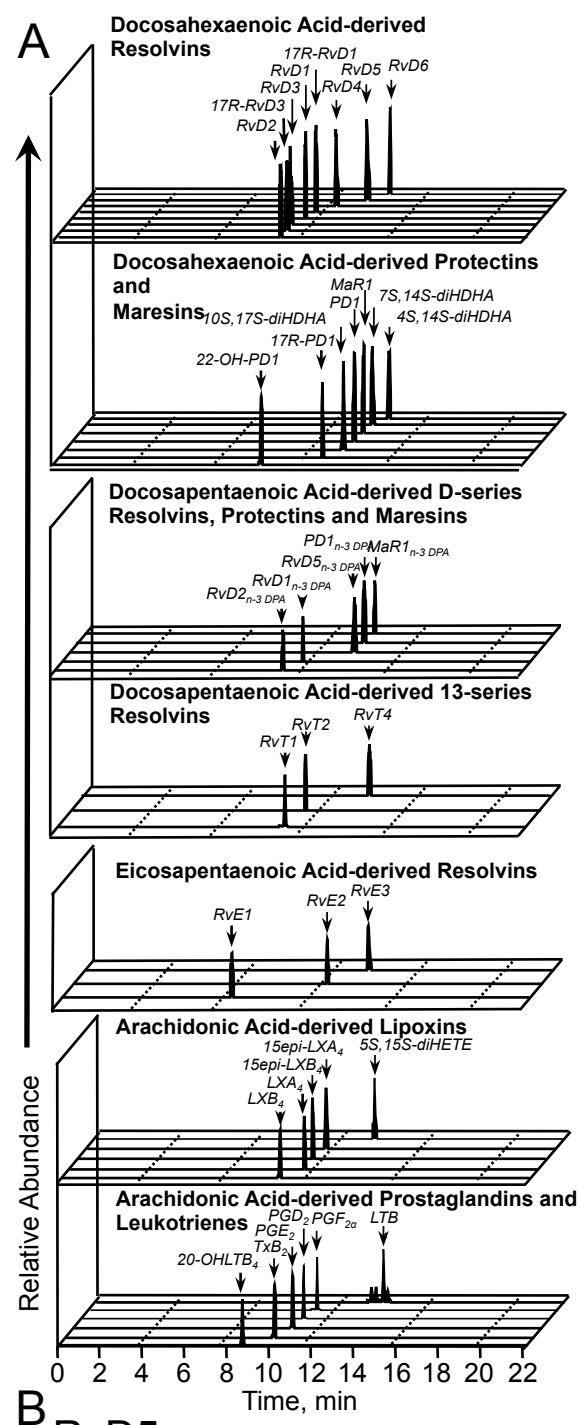
Online Figure IV. (A,B) Mice were administered mifepristone (Mife; 20mg/Kg; i.v.), carvedilol (Cav; 10mg/Kg; i.v) or vehicle at zeitgeber time (ZT) 8 (15:00 h), blood was collected at ZT 12 and ZT 3 and plasma $RvD_{n\text{-}3\ DPA}$ concentrations ascertained using LM profiling. Results are mean ± s.e.m. n = 4 mice per group. *, $p \leq 0.05$ vs ZT3 values #, $p \leq 0.05$ vs ZT12 +Veh values using Mann–Whitney test. (C) Human peripheral blood was incubated with Isotype antibody or an anti-human-PSGL1 antibody (30 min at RT) then with 100nM PAF (left panel), 1μM fMLP (right panel) or vehicle (PBS+0.01% EtOH) for 45 min at 37°C. Plasma was then collected and lipid mediators identified and quantified using LM profiling. Results are mean±sem n=4-5 donors per group. *, $p \leq 0.05$ vs Isotype incubations using Mann–Whitney test.

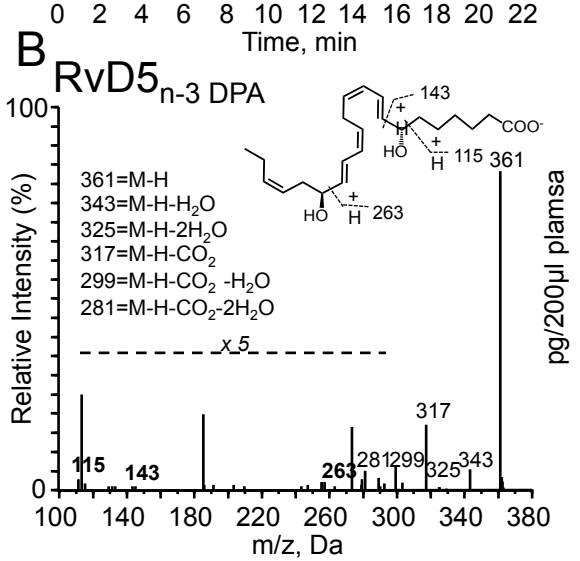
Online Figure V: Dysregulated diurnal regulation of $RvD_{n\text{-}3\ DPA}$ biosynthetic enzymes in peripheral blood leukocytes from CVD patients. Peripheral blood from healthy volunteers (HV) and patients diagnosed with cardiovascular disease (CVD) was collected at 9:00 h (AM), 12:00 h (Midday) and 16:00 h (PM). Leukocyte subsets and biosynthetic enzymes were identified by using fluorescently labeled antibodies and flow cytometry. Expression of 15-LOX and 5-LOX in (A) leukocytes (B) monocytes (C) and neutrophils. Results are mean ± s.e.m. and expressed as mean fluorescence intensity units (MFI); n = 3 HV, n = 4 CVD patients. *, $p \leq 0.05$ **$p<0.01$ ***$p<0.001$ compared to HV determined using Unpaired t-test, and 1-way ANOVA with Tukey multiple comparisons test for comparison between AM, Midday and PM groups.

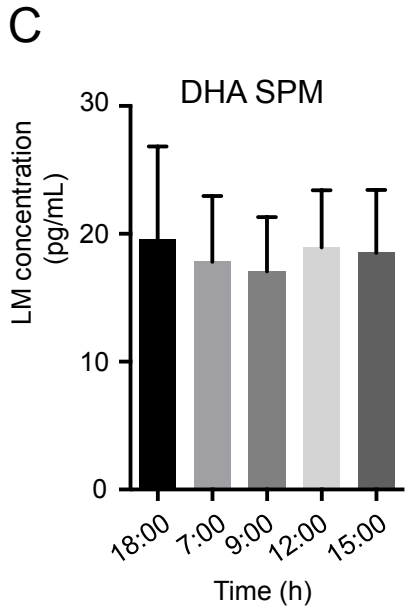
Online Figure VI. Dysregulated $RvD_{n\text{-}3\ DPA}$ pathway and ACh in CVD Patients. Peripheral blood was collected from patients with CVD and healthy volunteers (HV) at 9:00h (AM), 12:00 (midday) and 16:00-18:00 h (PM). Plasma was collected and placed in ice-cold methanol containing deuterium labelled internal standards. LM extracted, identified and quantified using LM profiling (see methods for details). (A) 7-HDPA, (B) 17-HDPA and (C) n-3 DPA concentrations. Results are mean ± s.e.m, expressed as pg/mL. For A-C n = 7 HV per interval,14 CVD patients for AM, PM and 5 for midday interval. * $p \leq 0.05$ and ** $p \leq 0.01$ compared to respective HV $RvD_{n\text{-}3\ DPA}$ concentrations using Mann–Whitney test. (D) Plasma ACh concentrations. n = 9 patients. ** $p \leq 0.01$ and compared to PM values using paired Mann–Whitney test.

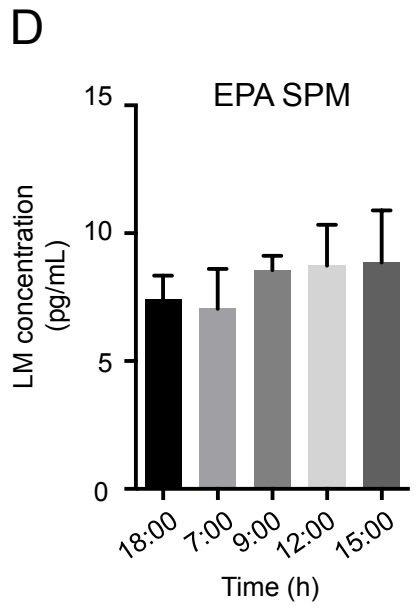
Online Figure VII. $RvD2_{n\text{-}3\ DPA}$ and $RvD5_{n\text{-}3\ DPA}$ counter-regulate PAF induced platelet and leukocyte activation in peripheral blood from CVD patients. Whole blood was incubated with $RvD2_{n\text{-}3\ DPA}$ or $RvD5_{n\text{-}3\ DPA}$ (0.1nM, 1nM or 10nM) or vehicle (PBS containing 0.01% EtOH) for 15min then with PAF (100ng/ml) for 30min (37^{o}C). Expression of CD62P on (A) neutrophils (B) monocytes and CD11b on (C) neutrophils and (D) monocytes was investigated using flow cytometry and fluorescently labeled antibodies. (E-F) Flow cytometric assessment of platelet (E) CD62P and (F) CD63 expression. Results are mean ± s.em. and expressed as percentage of PAF incubated cells. n = 9 patients per group. * $p<0.05$ *vs* PAF group using Wilcoxon Signed Rank Test.

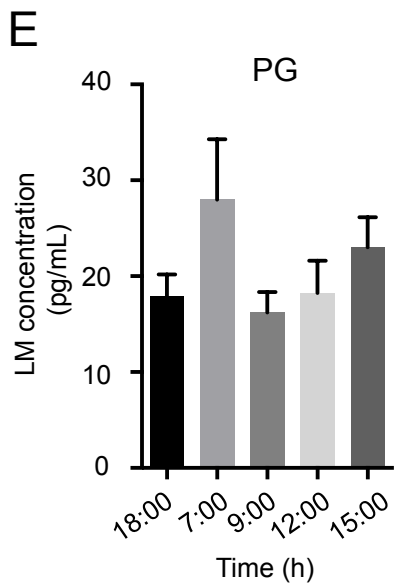
Online Figure VIII: $RvD5_{n\text{-}3\ DPA}$ reduces systemic platelet and leukocyte activation as well as vascular inflammation in $ApoE^{-/-}$ mice. $ApoE^{-/-}$ mice were fed a western diet for 6 weeks and given $RvD5_{n\text{-}3\ DPA}$ (100ng/mouse; i.v.) on alternate days for 2 weeks. Blood was obtained and (A) monocyte and (B) neutrophil expression of CD41, CD62P and CD11b were determined using flow cytometry. (C) Descending aortas were harvested, lipid mediators were extracted, identified and quantified using LC/MS-MS-based lipid mediator profiling. PLS-DA 2-dimensional score plot of the distinct LM-SPM profiles identified in mouse aortic tissues *(Top panel)* and corresponding 2-dimensional loading plot. Grey ellipse in the score plots denotes 95% confidence regions. Green and blue circles represent LM with a variable in importance score ≥ 1 *(Bottom panel).* (D) Aortic arches were stained using Oil red-O and staining intensity was determined using ImageJ. Results are mean ± s.e.m of 4 mice per group. *$p<0.05$ and ** $P<0.01$ vs Vehicle treated mice using Mann-Whitney Test.

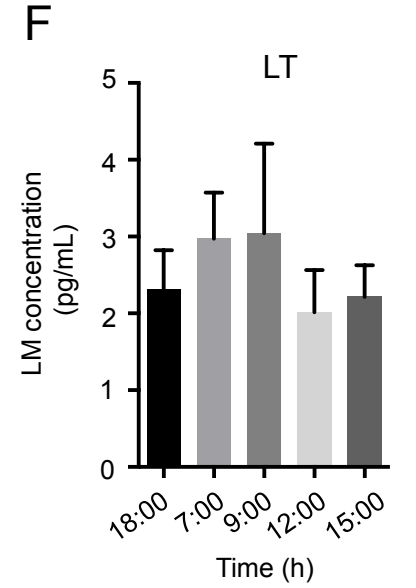

A
Docosahexaenoic Acid-derived Resolvins
17R-RvD1
RvD1
RvD5
RvD6
RvD3
RvD4
17R-RvD3
RvD2
Docosahexaenoic Acid-derived Protectins and Maresins
MaR1
PD1
7S,14S-diHDHA
10S,17S-diHDHA
4S,14S-diHDHA
17R-PD1
22-OH-PD1
Docosapentaenoic Acid-derived D-series Resolvins, Protectins and Maresins
PD1n-3 DPA
MaR1n-3 DPA
RvD5n-3 DPA
RvD1n-3 DPA
RvD2n-3 DPA
Docosapentaenoic Acid-derived 13-series Resolvins
RvT2
RvT4
RvT1
Eicosapentaenoic Acid-derived Resolvins
RvE1
RvE2
RvE3
Arachidonic Acid-derived Lipoxins
15epi-LXA4
5S,15S-diHETE
15epi-LXB4
LXA4
LXB4
Arachidonic Acid-derived Prostaglandins and Leukotrienes
PGD2
PGF2α
PGE2
LTB
TxB2
20-OHLTB4
Relative Abundance
0 2 4 6 8 10 12 14 16 18 20 22
Time, min

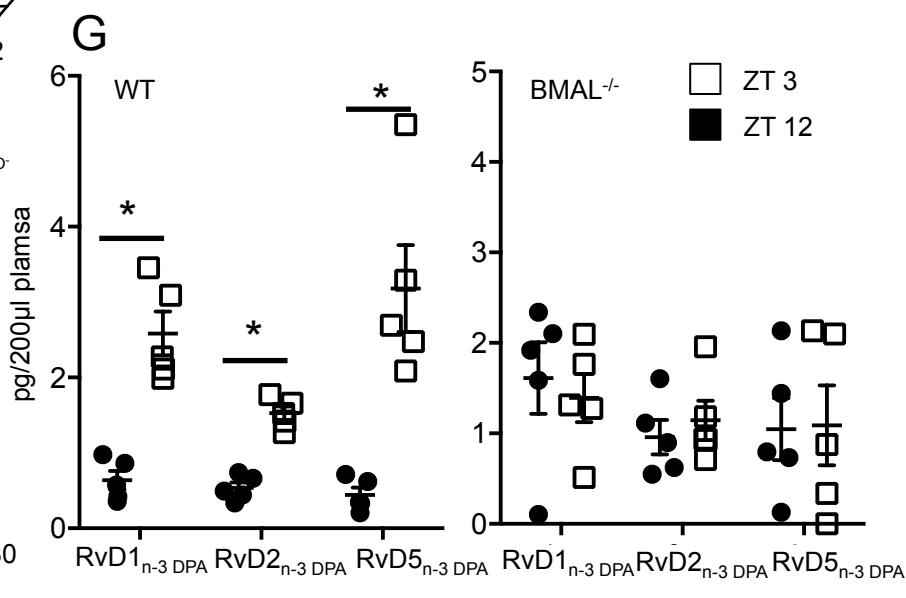

B
RvD5n-3 DPA
143
COO-
HO
H
115
361
263
361=M-H
343=M-H-H2O
325=M-H-2H2O
317=M-H-CO2
299=M-H-CO2 -H2O
281=M-H-CO2-2H2O
x 5
317
115
143
263
281
299
325
343
Relative Intensity (%)
100 140 180 220 260 300 340 380
m/z, Da


C
DHA SPM
LM concentration (pg/mL)
0 10 20 30
18:00 7:00 9:00 12:00 15:00
Time (h)

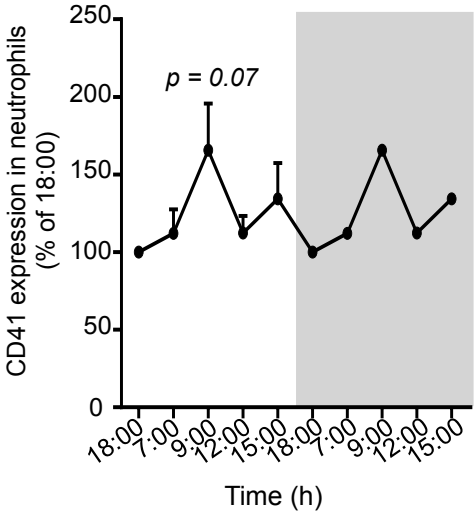

D
EPA SPM
LM concentration (pg/mL)
0 5 10 15
18:00 7:00 9:00 12:00 15:00
Time (h)


E
PG
LM concentration (pg/mL)
0 10 20 30 40
18:00 7:00 9:00 12:00 15:00
Time (h)

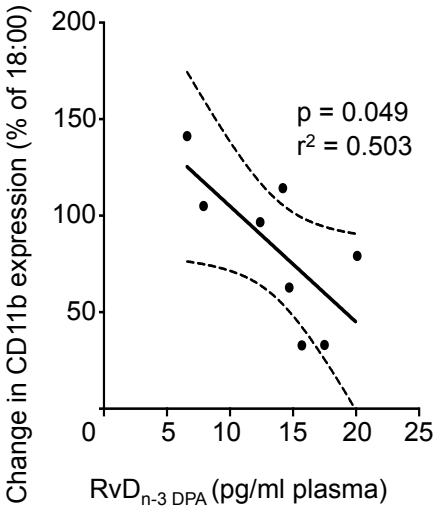

F
LT
LM concentration (pg/mL)
0 1 2 3 4 5
18:00 7:00 9:00 12:00 15:00
Time (h)


G
WT
BMAL-/-
ZT 3
ZT 12
pg/200µl plamsa
*
RvD1n-3 DPA
RvD2n-3 DPA
RvD5n-3 DPA

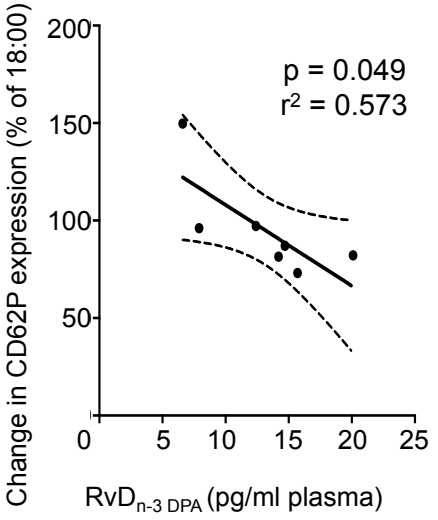
A

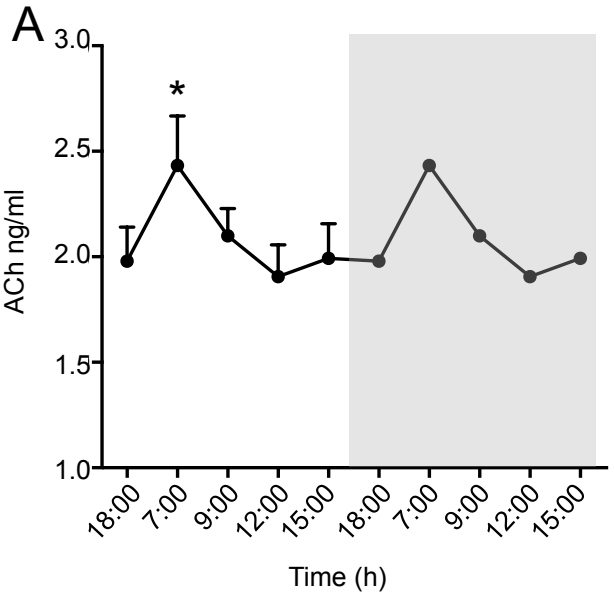

p = 0.07
CD41 expression in neutrophils (% of 18:00)
250
200
150
100
50
0
18:00
7:00
9:00
12:00
15:00
18:00
7:00
9:00
12:00
15:00
Time (h)

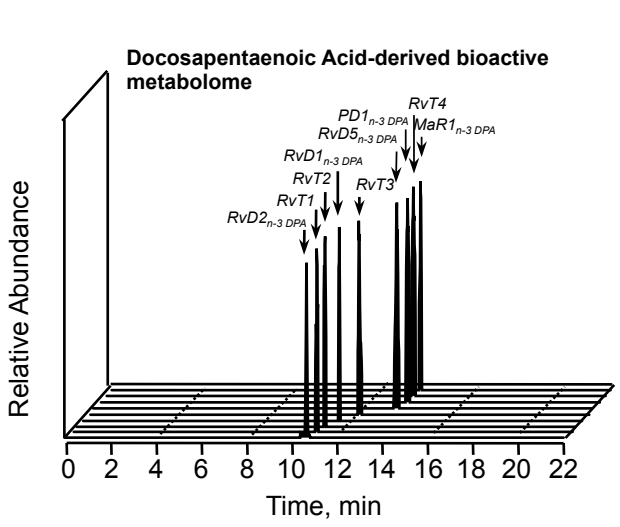
B

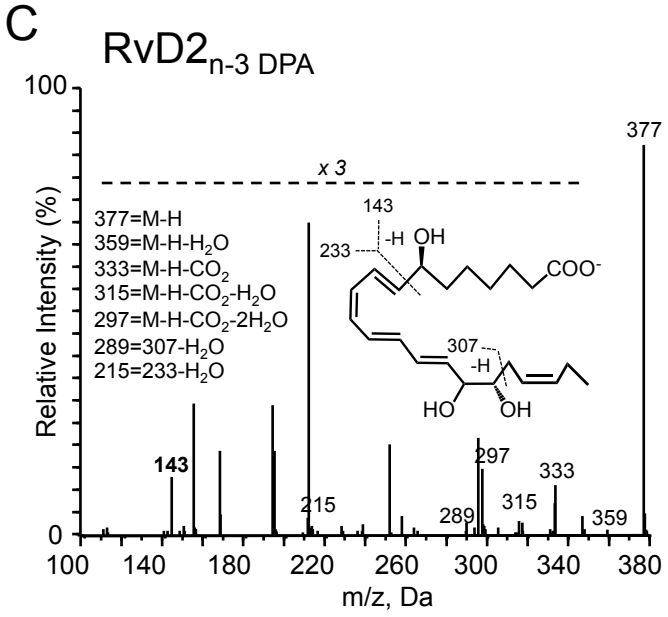

p = 0.049
r2 = 0.503
Change in CD11b expression (% of 18:00)
200
150
100
50
0
5
10
15
20
25
RvDn-3 DPA (pg/ml plasma)

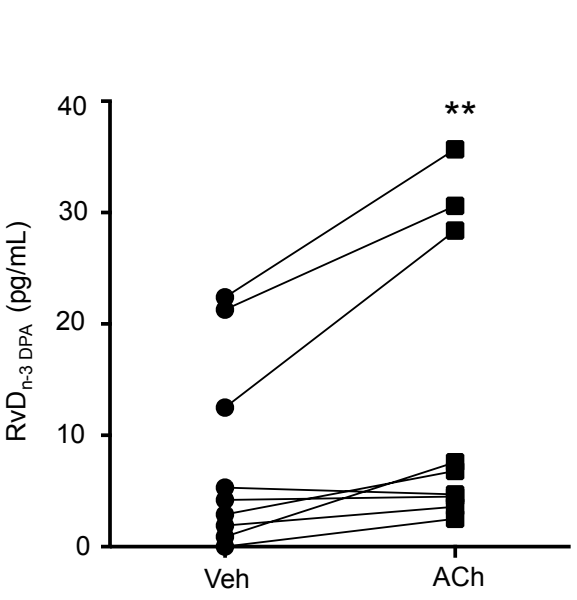
C

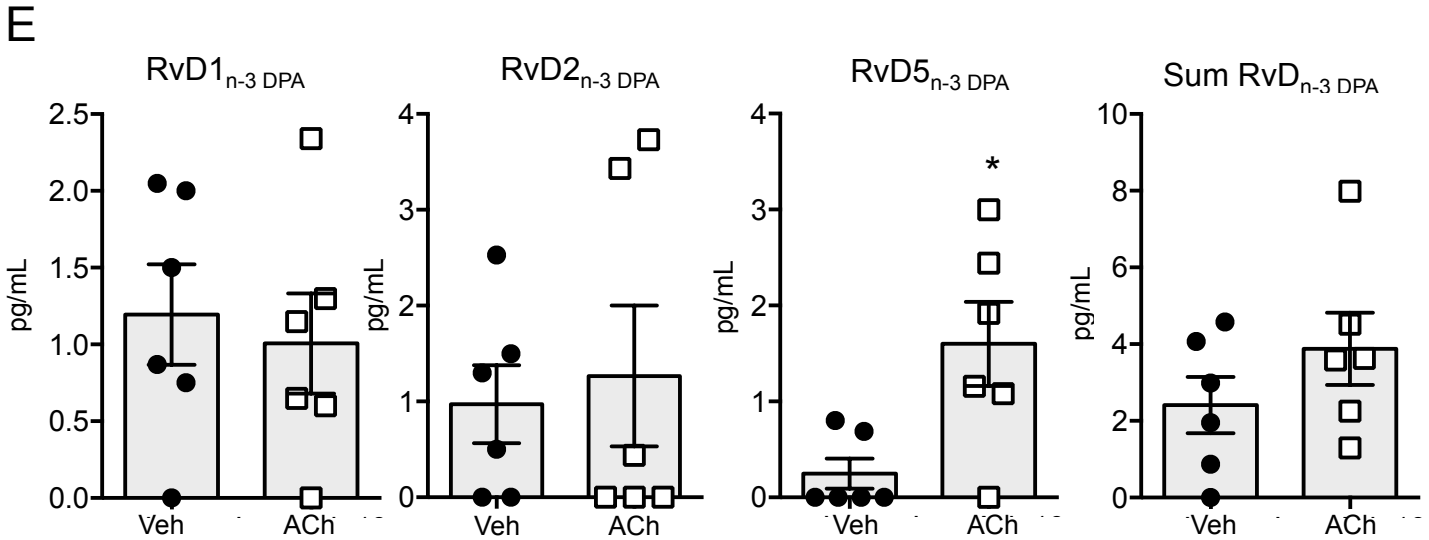

p = 0.049
r2 = 0.573
Change in CD62P expression (% of 18:00)
200
150
100
50
0
5
10
15
20
25
RvDn-3 DPA (pg/ml plasma)

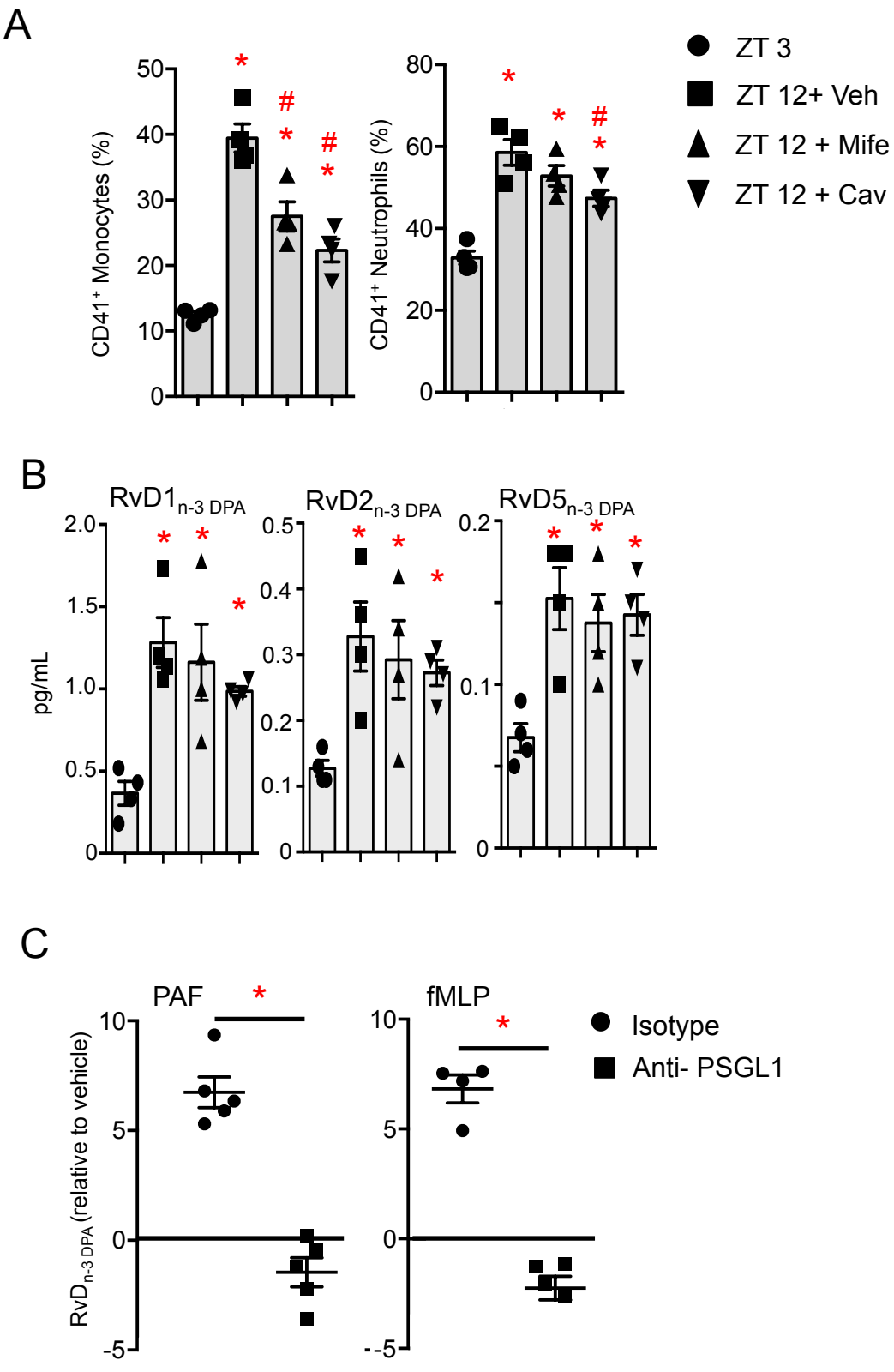

A
ACh ng/ml
3.0
2.5
2.0
1.5
1.0
*
18:00
7:00
9:00
12:00
15:00
18:00
7:00
9:00
12:00
15:00
Time (h)

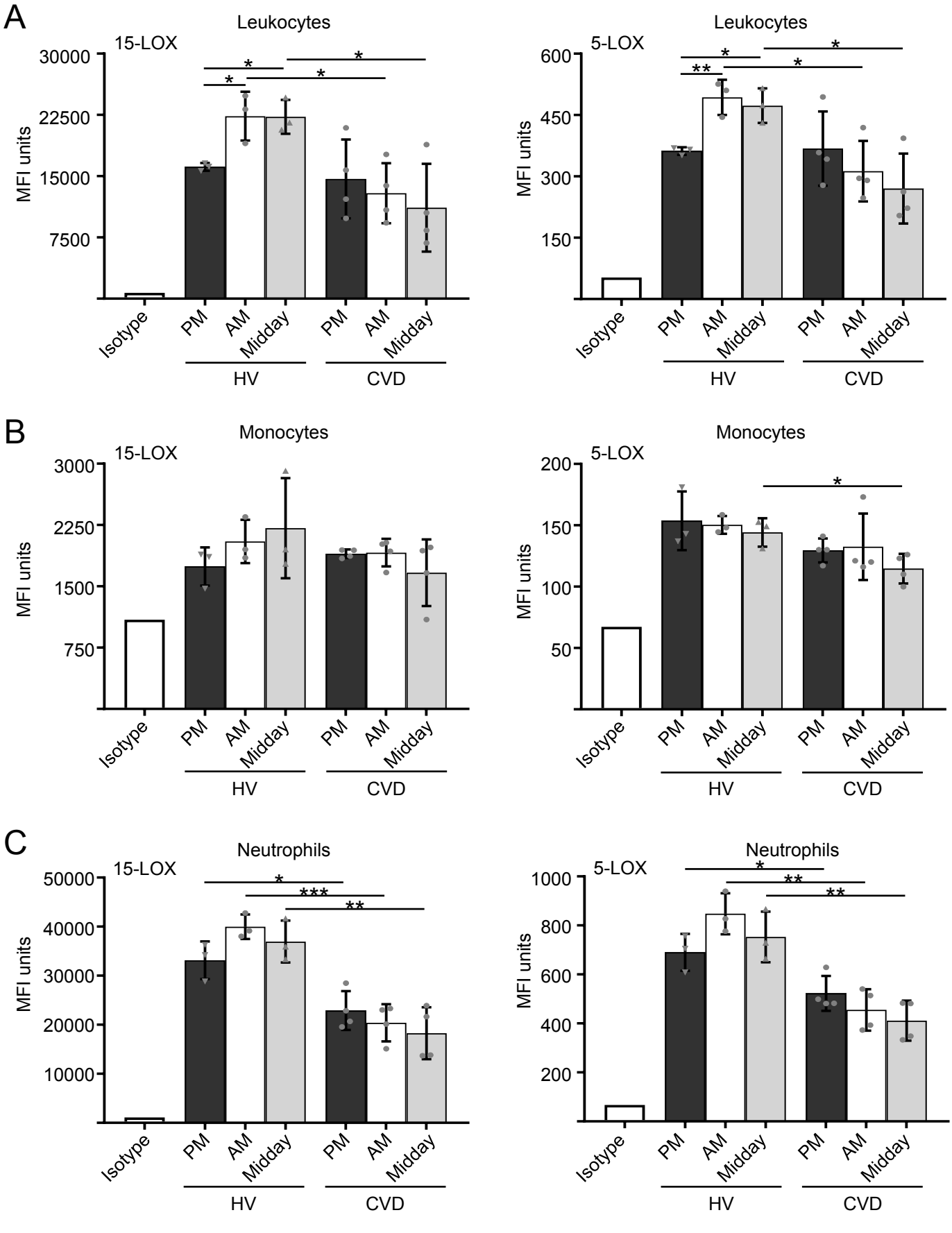

B
Docosapentaenoic Acid-derived bioactive metabolome
RvT4
PD1n-3 DPA
MaR1n-3 DPA
RvD5n-3 DPA
RvD1n-3 DPA
RvT2
RvT3
RvT1
RvD2n-3 DPA
Relative Abundance
0 2 4 6 8 10 12 14 16 18 20 22
Time, min

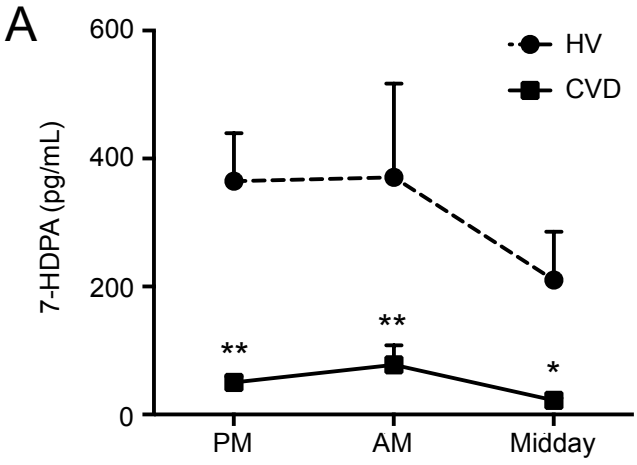

C
RvD2n-3 DPA
100
Relative Intensity (%)
0
x 3
377
377=M-H
359=M-H-H2O
333=M-H-CO2
315=M-H-CO2-H2O
297=M-H-CO2-2H2O
289=307-H2O
215=233-H2O
143
-H
OH
233
COO-
307
-H
HO
OH
143
215
289
297
315
333
359
100 140 180 220 260 300 340 380
m/z, Da

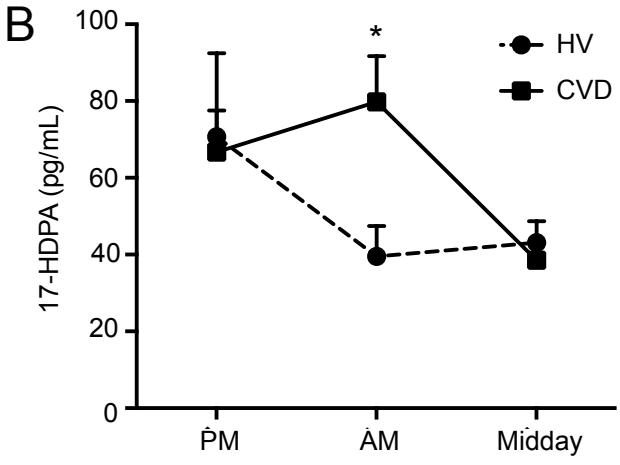

D
RvDn-3 DPA (pg/mL)
40
30
20
10
0
**
Veh
ACh

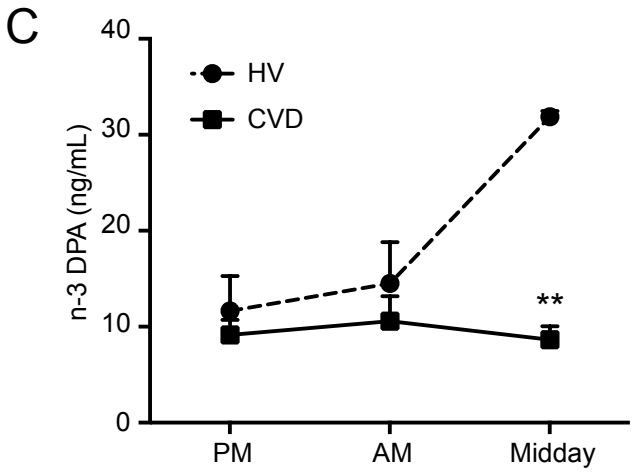

E
RvD1n-3 DPA
RvD2n-3 DPA
RvD5n-3 DPA
Sum RvDn-3 DPA
pg/mL
Veh
ACh
*

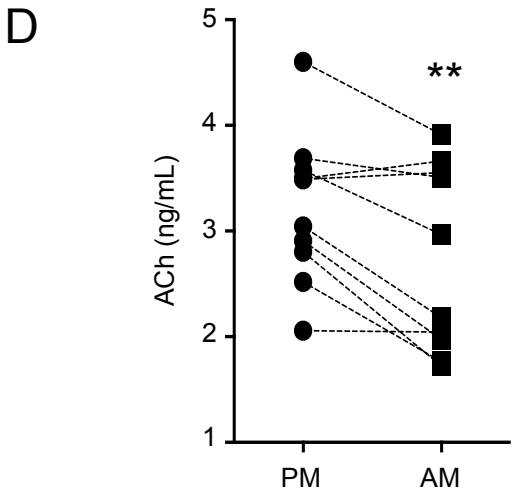

A
CD41+ Monocytes (%)
CD41+ Neutrophils (%)
ZT 3
ZT 12+ Veh
ZT 12 + Mife
ZT 12 + Cav
B
RvD1n-3 DPA
RvD2n-3 DPA
RvD5n-3 DPA
pg/mL
C
PAF
fMLP
RvDn-3 DPA (relative to vehicle)
Isotype
Anti- PSGL1

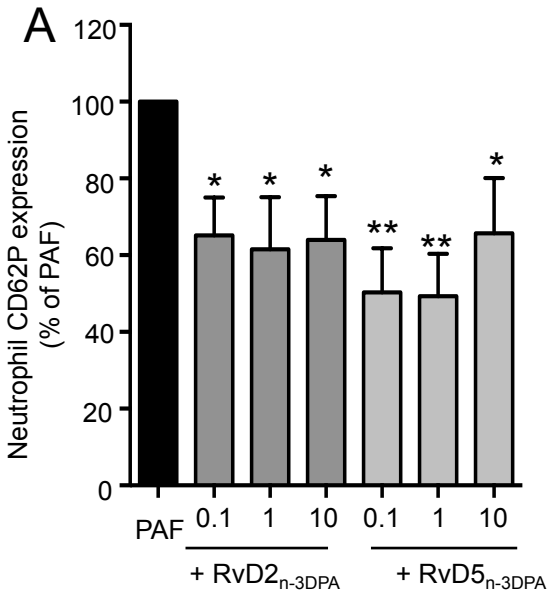

A
Leukocytes
15-LOX
MFI units
30000
22500
15000
7500
Isotype
PM
AM
Midday
PM
AM
Midday
HV
CVD
Leukocytes
5-LOX
600
450
300
150
B
Monocytes
15-LOX
3000
2250
1500
750
Monocytes
5-LOX
200
150
100
50
C
Neutrophils
15-LOX
50000
40000
30000
20000
10000
Neutrophils
5-LOX
1000
800
600
400
200

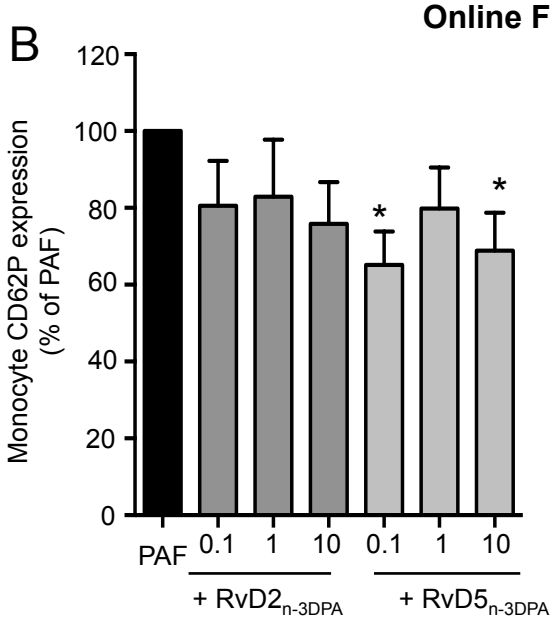

A
600
400
200
0
7-HDPA (pg/mL)
HV
CVD
**
**
*
PM
AM
Midday

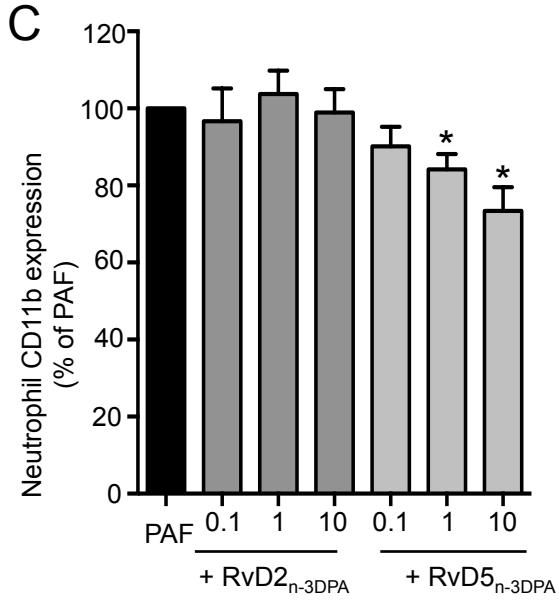

B
100
80
60
40
20
0
17-HDPA (pg/mL)
HV
CVD
*
PM
AM
Midday

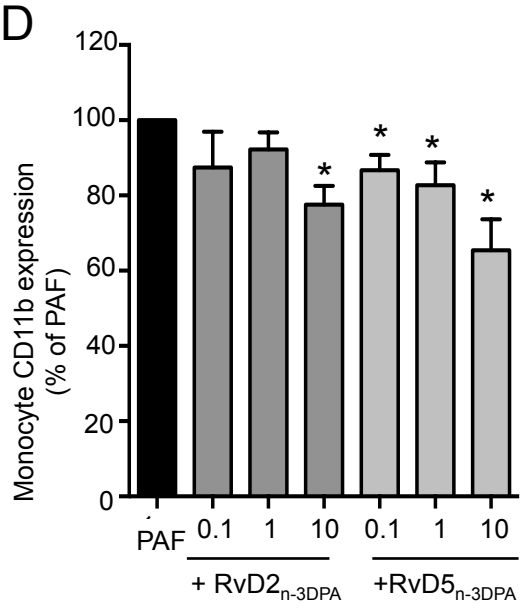

C
40
30
20
10
0
n-3 DPA (ng/mL)
HV
CVD
**
PM
AM
Midday

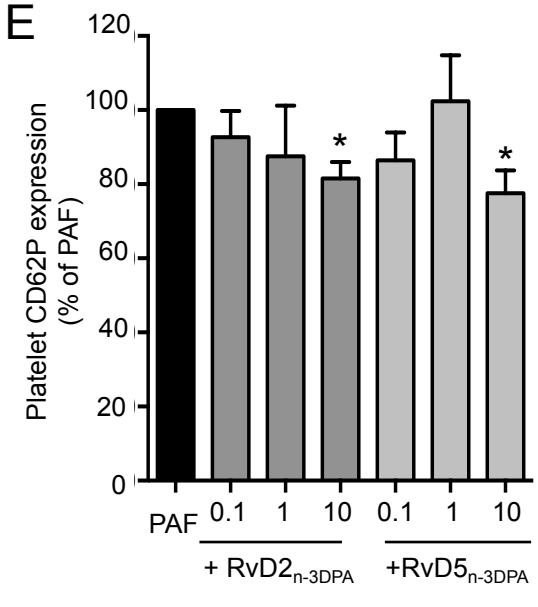

D
5
4
3
2
1
ACh (ng/mL)
**
PM
AM

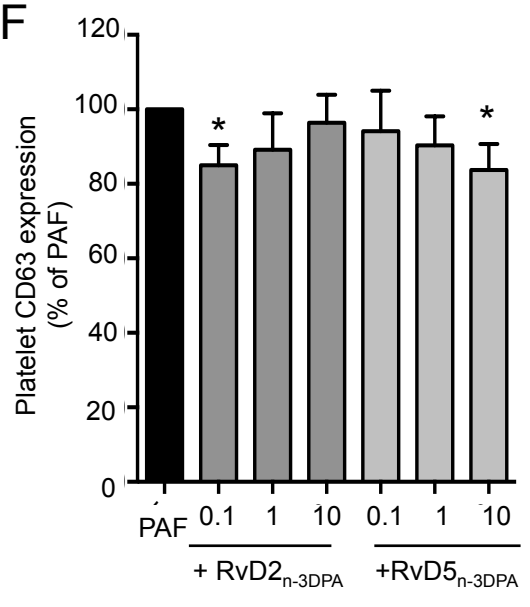

A
Neutrophil CD62P expression (% of PAF)
120
100
80
60
40
20
0
*
*
*
**
**
*
PAF
0.1
1
10
0.1
1
10
+ RvD2n-3DPA
+ RvD5n-3DPA

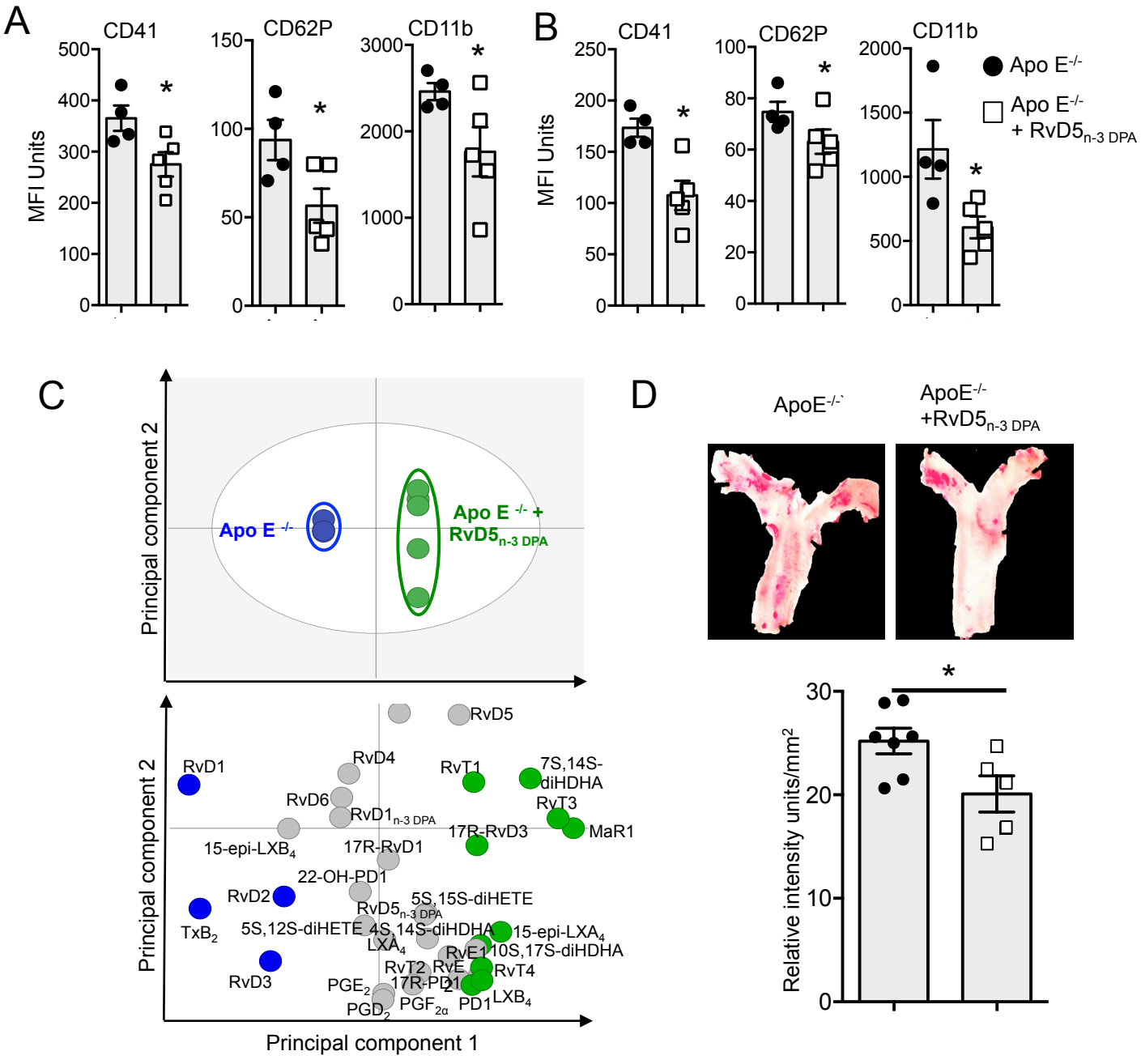

B
Monocyte CD62P expression (% of PAF)
120
100
80
60
40
20
0
*
*
PAF
0.1
1
10
0.1
1
10
+ RvD2n-3DPA
+ RvD5n-3DPA


C
Neutrophil CD11b expression (% of PAF)
120
100
80
60
40
20
0
*
*
PAF
0.1
1
10
0.1
1
10
+ RvD2n-3DPA
+ RvD5n-3DPA


D
Monocyte CD11b expression (% of PAF)
120
100
80
60
40
20
0
*
*
*
PAF
0.1
1
10
0.1
1
10
+ RvD2n-3DPA
+RvD5n-3DPA


E
Platelet CD62P expression (% of PAF)
120
100
80
60
40
20
0
*
*
PAF
0.1
1
10
0.1
1
10
+ RvD2n-3DPA
+RvD5n-3DPA


F
Platelet CD63 expression (% of PAF)
120
100
80
60
40
20
0
*
*
PAF
0.1
1
10
0.1
1
10
+ RvD2n-3DPA
+RvD5n-3DPA


A
CD41
CD62P
CD11b
MFI Units
B
Apo E-/-
Apo E-/- + RvD5n-3 DPA
C
Principal component 2
Apo E -/-
Apo E -/- + RvD5n-3 DPA
RvD5
RvD1
RvD4
RvT1
7S,14S-diHDHA
RvD6
RvD1n-3 DPA
RvT3
17R-RvD3
MaR1
15-epi-LXB4
17R-RvD1
22-OH-PD1
RvD2
5S,15S-diHETE
RvD5n-3 DPA
TxB2
5S,12S-diHETE
4S,14S-diHDHA
15-epi-LXA4
LXA4
RvE1
10S,17S-diHDHA
RvT2
RvE2
RvT4
RvD3
PGE2
17R-PD1
PGF2α
PD1
LXB4
PGD2
Principal component 1
D
ApoE-/-
ApoE-/- +RvD5n-3 DPA
Relative intensity units/mm2