Phospholipid esters from herring roe promotes SPM biosynthesis in human monocyte-derived macrophages and a keratinocyte/fibroblast co-culture with implications for the treatment of psoriasis

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Abstract

Immune-mediated inflammatory disease (IMID) is a major public health issue which can affect a number of organs and tissues with a profound impact on quality of life and often present with comorbidities. Psoriasis is a chronic IMID affecting the skin which presents with both local and systemic inflammation as part of its pathophysiology. An oil rich in polar lipids extracted from the roe sacks of herring has been shown to have immunomodulatory functions and to improve the clinical symptoms and impact inflammatory cytokine pathways in psoriasis in a clinical trial. The lipidic nature of herring roe oil and its high content of marine polyunsaturated fatty acids could suggest involvement of lipid mediator pathways for the observed alleviation of psoriatic inflammation. Of particular interest is the super-family of lipid mediators termed specialized pro-resolving mediators (SPMs), due to their known involvement in the resolution of inflammation and return to homeostasis. We have therefore explored the influence of herring roe oil and phospholipid esters from herring roe on lipid mediator and SPM biosynthesis in IFN-γ and LPS-stimulated human monocyte-derived macrophages and an IL-17A-stimulated skin cell co-culture with keratinocytes and fibroblasts. Lipid mediators including SPMs were quantified from resulting cell supernatants using a validated LC-MS/MS protocol. In these experiments we observed broad SPM biosynthesis with dominant upregulation of RvE2 and RvE3 in both cell systems and upregulation of DHA-derived SPMs such as RvD2 and PDX. Observations of PCTR2 in the macrophage cell supernatants also indicate activation of reparative pathways upon treatment with herring roe oil. In conclusion, we observed a promotion of SPM biosynthesis associated with a shift towards a protective and possibly

reparative macrophage phenotype as well as promotion of biosynthesis of pro-resolving lipid mediators in a skin cell co-culture, thus demonstrating a possible mechanism for resolution of inflammation in the skin niche using herring roe oil.

Introduction

Inflammation is a physiological process that has been described by scholars for centuries, even millennia, where inflammation was famously described two thousand years ago by Aulus Cornelius Celsus (25 BC – 50 AD) by the four cardinal signs of redness, swelling, heat, and pain (1–3). With the progress of science, including growing access to modern powerful analytical instruments, an enormous amount of work has since then been put into expanding the knowledge of initiation and perpetuation of inflammation by describing the actions of cytokines and chemokines, as well as the pro-inflammatory eicosanoids (e.g. prostaglandins and leukotrienes) and their importance in driving inflammatory processes (4–6). And it has not been until the most recent decades that scientists have been able to elucidate the active processes such as the lipid mediator class switch for containing inflammation and restoring tissue homeostasis (4,7). Where before it was thought that resolution of inflammation was a passive process that took place through gradual dilution of chemoattractants, it has now been shown that these are active processes regulated through the expression of a super-family of lipid mediators termed specialized pro-resolving mediators (SPMs) (2,5,6,8). SPMs are potent and transient small molecule signaling agents that are endogenously biosynthesized from polyunsaturated fatty acids (PUFAs) and that, as implied by their name, promote the shift towards resolution of inflammation (4,6). The biosynthesis of SPMs from their parent fatty acids (FAs) is regulated by cell type and phenotype, enzyme expression, and substrate availability (2,9–11). In regulating this vital response, SPMs bind to and activate G protein-coupled receptors (GPCRs) (6,12,13) that result in, amongst other, the reduction of polymorphonuclear leukocyte (PMN) infiltration (6), promotion of efferocytosis/phagocytosis (6), and promotion of tissue regeneration (14,15).

The lack of, or incomplete, resolution of inflammation perpetuates what should have been appropriate and self-limiting inflammatory processes and has been linked to chronic inflammation and multiple disease states (16). A group of chronic inflammatory disorders with unknown etiology that share common inflammatory pathways has been collectively termed as immune-mediated inflammatory diseases (IMIDs) (17). IMIDs are a major public health issue with a global incidence of over 67 million cases (18). IMIDs can affect a number of organs and tissues, including the skin, and can have a profound impact on quality of life, often present with comorbidities (19–21). In 2019, 6.84% of new IMID cases were psoriasis (18), a chronic inflammatory skin disease which presents with both local and systemic inflammation as part of its pathophysiology. Hippocrates (460 – 377 BC) described the symptoms of psoriasis as *psora* (itch), and *lopoi (*scaling) and recommended treatment with tar followed by sun exposure (22). It is now known that albeit IMIDs display various symptoms depending on the organ they are affecting, IMIDs share an underlying pathogenesis and, indeed, many of the same drugs are therefore used to treat several of these diseases (23,24). Insights in the molecular basis of inflammatory disease has led to therapies targeting specific IMID-associated immune and inflammatory pathways, with recent focus on developing drugs targeting the cytokines IL-17 and IL-23 (25,26). However, due to their intricate involvement in cessation of inflammation responses, imbalances in lipid-mediated regulation of resolution/inflammation due to dysregulated lipid mediator and SPM biosynthesis and signaling have also been proposed to play a role in the pathophysiology of IMIDs (27–29), and it has been shown that intervention with PUFAs (30) or

81 SPMs (31,32) may hold promise in correcting these imbalances. Lack of immunoresolution due to
82 dysregulated SPM biosynthesis may arise both from dysregulated enzyme expression as a
83 consequence of the disease state (33) or substrate competition from pro-inflammatory precursors
84 released from membrane phospholipids (PLs) (34).

85 Marine PUFA oils have traditionally been considered to have anti-inflammatory properties and have
86 been reported to have potential in treatment of selected IMIDs (35,36). Furthermore, supplementation
87 with marine oils has also been reported to promote SPM and SPM precursor biosynthesis (37–39),
88 where biosynthesis and downstream SPM profiles changes according to different marine oils (39).
89 The difference in conversion between oils implies that the pharmacology may be impacted by other
90 parameters, such as lipid composition and oil source, rather than just the PUFA content/ratios. One
91 marine oil of interest in this regard is herring (*Clupea harengus*) roe oil (HRO), where the nature of
92 the raw material used for extraction causes HRO to have a high content of membrane phospholipid
93 esters enriched with marine PUFAs (PEHeRo = phospholipid esters from herring roe). The benefits
94 of marine PLs have been described previously in literature as having anti-inflammatory effects (40–
95 42) and reports also imply that marine PLs could be superior to other marine lipid forms with regards
96 to bioavailability and active uptake across the blood-brain barrier (43–47), neuroprotective properties
97 (48), and ability to reduce lesion progress in atherosclerosis (49).

98 The long-chain PUFAs (LC-PUFAs) provided by PEHeRo in HRO are primarily docosahexaenoic
99 acid (DHA) and eicosapentaenoic acid (EPA) in a ratio of approximately 3:1. As reported for other
100 marine PLs, the fatty acids in PEHeRo have also been shown to be readily bioavailable and
101 supplementation has shown reduction of plasma and RBC PC n-6/n-3 ratios (50), as well as a higher
102 acute uptake of DHA and EPA when compared to a triacylglycerol source in a head to head study
103 (51). The bioavailability of lipids and their corresponding fatty acids upon administration is relevant
104 for lipid mediator biosynthesis, as LC-PUFA supplementation has been shown to give increased
105 blood and plasma levels of lipid mediators (52–55). Being able to increase blood levels of lipid
106 mediators through supplementation coincides with observations of positive effects on inflammation
107 through direct supplementation of lipid mediators (33,56,57).

108 In addition to the general reports on benefits of marine PLs pertaining to inflammation, HRO
109 specifically has been shown to display effect on peripheral inflammation (58) and has been shown to
110 dampen psoriasis *in vivo* in a clinical trial (59–61). The dampening effect of HRO on psoriasis has
111 been shown in human trials where oral daily treatment with HRO gave statistically significant
112 improvement of mean Psoriasis Area Severity Index (PASI) score (59,60). *Post hoc* analyses of
113 blood samples from the study by Tveit *et al.* (59) further supports the beneficial effects of HRO for
114 dampening of psoriasis by showing that HRO had a beneficial effect on immune cell activation and
115 influenced the immune cell profile by, amongst others, reducing CCL2 over time and leading to an
116 increase in IFN-γR1 (61). The *in vitro* experiments performed by Mildenberger *et al.* (62) further
117 corroborates the potential benefit of HRO in the treatment of psoriasis by showing that HRO limits
118 secretion of IL-23 in macrophages in a dose-dependent manner and limits secretion of IL-17 from
119 CD^{4+} T-cells. Thus, showing that HRO positively impacts immune cells and skin cells impacted by
120 psoriasis in multiple ways.

121 To understand how HRO exerts anti-inflammatory actions, we have studied its impact on lipid
122 mediator biosynthesis in several cell systems. We have studied the effects of HRO on SPM
123 biosynthesis in primary human monocyte-derived-macrophages (MDMs), as these cells have a role in
124 all inflammatory processes and are known to readily express SPMs as part of their signaling in
125 inflammation regulation. Macrophages are also known to change their phenotype towards more anti-

126 inflammatory phenotypes upon stimulation with lipid mediators (63), which is followed by a shift in
127 lipid mediator biosynthesis. In addition, the effects of HRO on SPM biosynthesis in a skin cell model
128 consisting of a co-culture of IL-17A-treated keratinocytes and fibroblasts, which mimics psoriasis,
129 are studied (64). Current knowledge about SPM biosynthesis in skin cells is limited. Therefore, the
130 study aims to elucidate the role of these cells in SPM and lipid mediator biosynthesis during
131 resolution of inflammation. Studying both the immune cells and skin cells simultaneously may give
132 an image of the cell-cell interplay between these cell types locally in the skin niche. Lastly, we have
133 also mapped the lipid mediator content of HRO and isolated PEHeRo in order to have a greater
134 understanding of which mediators are presented to the cells exogenously compared to the output after
135 treatment, and if any of the identified lipid mediators has described roles in specific interleukin
136 pathways relevant to the pathogenesis of inflammatory diseases including psoriasis.

137

138 **Materials and methods**

139 **Preparation of HRO and PEHeRo**

140 Immature herring roe (IHR) was dried under reduced pressure (until H_2O <20% w/w), then contacted
141 with EtOH/H_2O to extract the lipids. Centrifugation of the resulting suspension yielded a clarified
142 lipid extract which then was concentrated under reduced pressure, desalinated by crystallization in
143 EtOH (abs.), and filtered to give a desalinated phospholipid concentrate. The final HRO was prepared
144 by standardization and viscosity modification through addition of a reconstituted triglyceride oil and
145 evaporation under reduced pressure.

146 IHR was contacted with EtOH/H_2O to extract lipids before the resulting extract was clarified by
147 centrifugation and dried under reduced pressure. The dried crude extract was then desalinated by
148 crystallization in EtOH (abs.) and filtration. PEHeRo (>90% PLs w/w dry matter, 85% DM) was
149 purified from the desalinated extract using normal-phase flash column chromatography (EtOH/H_2O
150 88:12, 200 nm).

151 Phospholipid analyses were performed using quantitative ^{31}P NMR (Spectral Service AG) and oil
152 fatty acid analyses were performed using GC-FID according to Ph. Eur. 2.4.29 with modifications to
153 accommodate for PL-rich oils (Mylnefield Lipid Analysis).

154 **HRO and PEHeRo emulsions**

155 HRO and PEHeRo were prepared as 5% emulsions in dH_2O by stirring at 800 rpm under inert
156 atmosphere (N_2 flushing) at 37˚C for 1 hour. Emulsions were then pasteurized under inert
157 atmosphere by sonication at 70˚C for 2 min in an ultrasonic water bath (Branson 2200, 40 kHz) or by
158 an ultrasonic probe. Emulsions were stored at 4˚C for maximum 4 days.

159 **Macrophage cell culture**

160 MDMs from each donor were treated in 1.5 mL per well in a 6-well plate (3 wells per condition) with
161 indicated final percentages (w/w) of HRO or PEHeRo, by dilution of the prepared emulsions in
162 phenol- and serum-free CTS™ AIM-V™ Medium (Gibco, A3830801) supplemented with Pen-Strep.
163 MDMs were either pretreated with HRO for 16 h, followed by change of medium with addition of 1
164 µg/mL lipopolysaccharide (LPS, Sigma-Aldrich, L2630) and 50 ng/mL IFN-γ (Miltenyi Biotec, 130-
165 096-481) for further 24 h or co-treated with either HRO or PEHeRo and 1 ng/mL LPS for 24 h.

166 Supernatants were collected, pooled per condition, centrifuged and frozen at -80°C until shipping on
167 dry ice for analysis. For additional information on MDM isolations refer to Mildenberger *et al.* (62).

168 **Keratinocyte and fibroblast co-culture**

169 HaCaT keratinocytes (CLS Cell line service GmbH, cat no 300493) were co-cultured with normal
170 human primary dermal fibroblasts (ATCC PCS-201-012) in Dulbecco´s modified Eagle´s medium
171 (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL
172 streptomycin and 250 µg/mL fungizone (all purchased from Thermo Fisher Scientific) in T25 tissue
173 culture flasks (Nunc™ EasYFlask™, VWR) and stimulated with and without 100 ng/mL IL-17A
174 (Sigma-Aldrich, cat.no SRP0675) and 5 µg/mL HRO for 96 h, before media were collected and
175 stored at -80 ºC for further fatty acid and lipid mediator analysis. The co-culture was established by
176 first seeding fibroblasts at $1x10^3$ cells per cm^2 and pre-cultured in the media described above for 2-
177 days, before adding 4,3 $x10^3$ keratinocytes per cm^2 and co-culturing additional 5 days before
178 initiating stimulation with HRO with and without IL-17A for 96 h as described above.

179 **Fatty acid composition of cells**

180 Total lipids were extracted from the cells according to the protocol by Folch *et al*. (65). The $CHCl_3$
181 phase was dried under nitrogen gas, and thereafter the samples were methylated over night with
182 benzene, 2% H_2SO_4 in MeOH, and dimethoxypropanate at 50 °C. The methyl esters of the fatty acids
183 were separated using GC (Hewlett Packard 6890) with flame-ionizing detector (FID) using a BPX70
184 column (60 m, 0.25 mm i.d., 0.25 µm film) with a temperature program starting at 50 °C, and
185 increasing at a rate of 4 °C /min until 170 °C, then raised to 0.5 °C/min until 200°C, and finally to 10
186 °C/min until 240 °C. The peaks were integrated using GC ChemStation software (Agilent
187 Technologies, Rev. B.01.01 SR1, 2001-2005). The fatty acids were identified by an external standard
188 (GLC 463, Nu-Chek Prep), and the concentration of the individual fatty acids was calculated based
189 on an internal standard (C23:0, Larodan). For analysis of fatty acid composition in lipid classes, the
190 $CHCl_3$ lipid extracts were first evaporated under nitrogen gas and re-dissolved in hexane. The lipid
191 classes (phospholipids, triacylglycerides and free fatty acids) were separated by thin layer
192 chromatography using a mixture of petroleum ether, Et_2O and AcOH (113:20:2, by volume) as the
193 mobile phase. The lipids were visualized by spraying the TLC plates with 0.2% (w/v) 2′,7′-
194 dichlorofluorescein in MeOH, and then identified by comparing with known standards (Sigma
195 Chemical Co.) under UV light. The spots corresponding to the different fractions were scraped off
196 into glass tubes, trans-methylated following the aforementioned procedure and the fatty acid
197 composition determined by GC-FID.

198 **Lipid mediator analyses**

199 Lipid mediator analysis was performed using methodologies and approaches recently published by
200 Dooley *et al.* (66).

201 Snap frozen media was placed in 2 volumes of MeOH containing deuterated internal standards (IS)
202 (d_4-LTB_4, d_5-MaR1, d_5-MaR2, d_4-PGE_2, d_5-LXA_4, d_5-RvD_3, d_5-RvD2, d_4-RvE1, d_5-17R-RvD1, d_5-
203 LTE_4, d_5-LTD_4 and d_5-LTC_4), representing the chromatographic regions of interest, were added to
204 facilitate lipid mediator identification and quantification. This was placed at -20 ºC for at least 45
205 minutes then centrifuged and supernatants were subjected to solid-phase extraction (SPE) using the
206 ExtraHera system (Biotage) and Isolute C18 500 mg columns (Biotage).

207 Oils samples were placed in 2 volumes of MeOH containing 2.5 ng of deuterated IS (d_4-LTB$_4$, d_5-
208 MaR1, d_5-MaR2, d_4-PGE$_2$, d_5-LXA$_4$, d_5-RvD$_3$, d_5-RvD2, d_4-RvE1, d_5-17R-RvD1, d_5-LTE$_4$, d_5-LTD$_4$
209 and d_5-LTC$_4$) and incubated in 2 volumes of KOH (1N) for 45 minutes at 45 ºC. At the end of the
210 incubation the samples were immediately acidified and subjected to SPE using the ExtraHera system
211 (Biotage) and Isolute C18 500 mg columns (Biotage). Oils analysed for unesterified lipid mediators
212 were not saponified prior to being subjected to SPE.

213 Methyl formate and MeOH fractions from C18 SPE were collected, brought to dryness, and
214 suspended in phase (MeOH/H_2O, 1:1. v/v) for injection on a Shimadzu LC-20AD HPLC and a
215 Shimadzu SIL-20AC autoinjector, paired with a QTrap 6500+ (Sciex). Analysis of mediators isolated
216 in the methyl formate fraction was conducted as follows: an Agilent Poroshell 120 EC-C18 column
217 (100 mm × 4.6 mm × 2.7 µm) was kept at 50 °C and mediators eluted using a mobile phase consisting
218 of MeOH/H_2O/AcOH of 20:80:0.01 (v/v/v) that was ramped to 50:50:0.01 (v/v/v) over 0.5 min and
219 then to 80:20:0.01 (v/v/v) from 2 min to 11 min, maintained till 14.5 min and then rapidly ramped to
220 98:2:0.01 (v/v/v) for the next 0.1 min. This was subsequently maintained at 98:2:0.01 (v/v/v) for
221 5.4 min, and the flow rate was maintained at 0.5 mL/min. In the analysis of mediators isolated in the
222 MeOH fraction, the initial mobile phase was MeOH/H_2O/AcOH of 20:80:0.5 (v/v/v) which was
223 ramped to 55:45:0.5 (v/v/v) over 0.2 min and then to 70:30:0.5 (v/v/v) over 5 min and then ramped to
224 80:20:0.5 (v/v/v) for the next 2 min. The mobile phase was maintained for 3 min and ramped to
225 98:2:0.5 (v/v/v) for 2 min. QTrap 6500+ was operated using a multiple reaction monitoring (MRM)
226 method. Each lipid mediator was identified using the following criteria: (1) matching retention time
227 to synthetic or authentic standards (±0.05 min), (2) a signal-to-noise ratio ≥ 5 for a primary transition,
228 and (3) a signal-to-noise ratio of ≥3 for a secondary transition. Data was analyzed using validated
229 protocols (66) and Sciex OS v3.0. Chromatograms were reviewed using the AutoPeak algorithm,
230 using 'low' smoothing setting and signal to noise ratios were calculated using the relative noise
231 algorithm (66). External calibration curves were used to quantify identified mediators. Where
232 available calibration curves were obtained for each mediator using synthetic compound mixtures that
233 gave linear calibration curves with R^2 values of 0.98–0.99. These calibration curves were then used
234 to calculate the abundance of each mediator. Where synthetic standards were not available for the
235 construction of calibration curves, calibration curves for mediators with similar physical properties
236 (e.g. carbon chain length, number of double bonds, number of hydroxyl groups and similar elution
237 time) were used.

238 **Statistical analysis**

239 Statistical significances were determined using; the Mann-Whitney U test (one-tailed/two-tailed as
240 appropriate) for comparison of two groups, one-factor ANOVA and one-way/two-way Dunnett's
241 multiples comparison test or Tukey's HSD for comparison of >2 groups. Statistical significance
242 criteria for difference between groups was $P < 0.05$. All results are presented as mean values ± the
243 standard error of the mean (SEM). PLS-DA analyses were performed using MetaboAnalyst v6.0
244 (67), all other statistical analyses were performed using Microsoft Excel for Microsoft 365 MSO.

245

246 **Results**

247 **Oil composition and lipid mediator content in HRO and PEHeRo**

248 In order to understand the effects of phospholipid ester rich oils on cellular systems, it is important to
249 understand the composition of the oils both with regards to fatty acid profile and lipid class

250 distribution, but also with regards to lipid mediators potentially present in the oil. We therefore
251 started off with characterizing the composition of the oils used.

252 Herring roe oil (HRO) is a lipid extract from herring roe that has been standardized with regards to
253 content of *phospholipid esters from herring roe* (PEHeRo) using a marine triacylglyceride (TAG) oil.
254 The phospholipid esters in PEHeRo are predominantly phosphatidylcholine (>80%) that are enriched
255 with LC-PUFAs. The fatty acid profiles in both HRO and PEHeRo display a high content of DHA
256 with a DHA/EPA ratio of approximately 3:1, where DHA and EPA make up 50-60% of the total fatty
257 acid profile in area %. The HRO and PEHeRo investigated in this work had a phospholipid content of
258 34% and 91% (per anhydrous substance) and 425 mgTAG/g and 318 mgTAG/g DHA+EPA,
259 respectively. Lastly, HRO and PEHeRo were found to contain 6% area and 1% area of omega-3
260 docosapentaenoic acid (n-3 DPA), respectively.

261 After establishing the macroscopic lipid composition, the content of lipid mediators in HRO and
262 PEHeRo was determined, as it is possible that the lipid mediator levels in the oils could influence the
263 behavior and SPM biosynthesis in the cell systems. The oils were screened for SPMs and
264 monohydroxylated fatty acids (including SPM precursors) originating from DHA, n-3 DPA, EPA,
265 and arachidonic acid (AA). The qualitative lipid mediator profiles of the two oils were,
266 unsurprisingly, found to be similar, with the predominant SPMs originating mostly from the DHA
267 metabolome in form of RvD5, RvD6, PDX, 17R-PD1, MaR1, and MaR2 (Fig. 1A). The most similar
268 relative abundance between HRO and PEHeRo was found for 18-HEPE, RvD5, MaR2, and RvD5 $_{n\text{-}3\ DPA}$
269 where the groups rank sums indicated a $P > 0.1$ (two-way) as denoted with # in (Fig 1 A-C).
270 Notable differences between the oils included a relative lower abundance of PDX and higher relative
271 abundance of MaR1 and RvE4 in PEHeRo compared to HRO. Furthermore, there were also notable
272 contributions from SPMs originating from the n-3 DPA (RvT4 and RvD5 $_{n\text{-}3\ DPA}$) and EPA (RvE4)
273 metabolomes, whereas lipid mediators from the AA metabolome, as expected, only made up 1-2% of
274 the total SPM content in the oils.

275 The key difference between HRO and PEHeRo pertaining to SPM content, was the >10 times higher
276 concentration of overall SPMs in the less refined HRO compared to the concentrated phospholipid oil
277 PEHeRo. The same was also observed for the monohydroxylated fatty acid levels in the two oils.

278 Furthermore, the monohydroxylated fatty acid (HDHAs, HEPEs, HDPAs, and HETEs) content was
279 found to be surprisingly high in HEPEs given the overall fatty acid composition, with 5-HEPE and
280 18-HEPE being the two major contributors (Fig. 1B and Fig. 1C). The HEPE/HDHA ratio was found
281 to be 1.8 and 1.4 for HRO and PEHeRo respectively (Fig. 1B), where a significant portion (>50%) of
282 total HEPEs and HDHAs were found to be present as metabolically available free fatty acids not
283 bound in lipids. The sum of HEPEs and HDHAs made up >95% of the total amount of
284 monohydroxylated fatty acids in the two oils. Lastly, we found higher levels of unesterified EPA in
285 both HRO and PEHeRo compared to DHA free fatty acid.

286 **Supplemented HRO is taken up by macrophages and keratinocytes/fibroblasts**

287 The uptake of fatty acids from HRO was analyzed by GC-FID, showing a significant increase in
288 DHA, n-3 DPA, and EPA in total lipids in the MDMs (Fig. 2A and supplementary table S1). In the
289 direct keratinocyte/fibroblast co-culture, it was further shown that the changes introduced by HRO
290 could be observed in the phospholipid fractions of the cells with similarly significantly increased
291 EPA and DHA and decreased AA (Fig. 2B and supplementary table S2).

292 **HRO and PEHeRo promote SPM biosynthesis in monocyte-derived macrophages**

293 The macrophage experiments conducted by Mildenberger *et al.* demonstrated reduction in cytokine
294 secretion, and we therefore wanted to study SPM biosynthesis in the macrophages treated with HRO
295 in order to better understand how this oil and its phospholipids (PEHeRo) elicits their anti-
296 inflammatory and possibly proresolving activities (62).

297 We therefore quantified the SPMs present in the supernatants from likewise stimulated MDMs,
298 which showed a significant upregulation of SPM concentrations when macrophages were pre-treated
299 with HRO prior to stimulation with LPS+IFN-γ (Fig. 3A-D and supplementary table S3). The
300 biosynthesis was found to be significantly upregulated for SPMs from the DHA, EPA, and AA
301 metabolomes, including a non-significant trend for upregulation of n-3 DPA SPMs. The EPA SPM
302 RvE3 was found in highest concentration in the treated samples. The overall SPM profiles of the
303 treated macrophages showed activation of both ALOX5 and ALOX15 pathways.

304 Partial least squares discriminant analysis (PLS-DA) of the SPMs in the cell supernatants from the
305 treated and untreated macrophages (including a fresh media control sample) as shown in Fig 2C also
306 displayed a difference between the groups. This shift in SPM concentrations indicated by the
307 separation of the clusters in green (2, untreated) and blue (3, treated with HRO) in the PLS-DA
308 scores plot. The chart shown in Fig. 3D shows the variable importance in projection (VIP) scores of
309 individual SPMs that contribute to the separation of the clusters in the PLS-DA with a VIP score >1
310 identifying those mediators that contribute to the observed separation to the greatest extent. VIP
311 scores >1 were observed for 4 SPMs from the DHA metabolome (RvD1, RvD4, PD1, and PDX) and
312 2 SPMs from the n-3 DPA metabolome ($RvD5_{n\text{-}3\ DPA}$ and $PD1_{n\text{-}3\ DPA}$).

313 Considering the promotion of SPM biosynthesis, we observed in the macrophages when pretreated
314 with HRO, we wanted to further explore this and see if we were able to optimize the experiment
315 setup with regards to measured concentrations of SPMs in the supernatant after
316 treatment/stimulation. Also, considering that we this far have only looked at the complete HRO, we
317 also wanted to evaluate if there were any differences between HRO and a purified sample of PEHeRo
318 in terms of ability to promote SPM biosynthesis in primary immune cells. So, we tested two
319 concentrations of HRO (0.05% and 0.25%) and one concentration of PEHeRo (0.25%) in an
320 experiment where the macrophages were co-stimulated with the oils and 1.0 ng/mL LPS for 24 hours
321 before SPM analysis of the cell supernatants, similarly to the conditions employed by Sobrino *et al.*
322 (39).

323 Similarly to the initial experiment, we also observed a significant upregulation of SPM biosynthesis
324 in the macrophages treated with HRO and PEHeRo (Fig. 4A-D and supplementary table S4). The
325 results also showed, as expected, generally higher concentrations of SPMs in the cell supernatants,
326 thus offering a potential for greater understanding of the pathways activated upon treatment with
327 HRO and PEHeRo.

328 All three treatments conditions (0.05% HRO, 0.25% HRO, and 0.25% PEHeRo) gave significant
329 upregulation of the sum of SPMs from the DHA and EPA metabolomes (Fig. 4A-B) when compared
330 to media stimulated with LPS (control). We also observed a nonsignificant trend for upregulation of
331 SPMs from the DPA metabolome and, in contrast to the first experiment, no upregulation of AA
332 SPMs. Similarly to the first experiments, the most strongly expressed SPM was the EPA-derived
333 SPM RvE3.When comparing the two treatments concentrations of HRO (0.05% and 0.25%), we
334 observed a nonsignificant dose-dependent trend where the higher treatment concentration gave higher
335 mean values than the lower concentration. The macrophage SPM biosynthetic response to the

336 treatment did not reflect the 5-times increase in treatment concentration and could indicate saturation
337 of the system with regards to accessible fatty acids for SPM biosynthesis.

338 Looking at individual SPMs through the PLS-DA analysis from the experiments, we also see here
339 that PEHeRo elicits a different response from the treated macrophages compared to the two
340 concentrations of HRO. This is well visualized through the clear separation of the cluster for
341 PEHeRo and the two HRO clusters in Fig. 4C. The partial overlap of the two different doses of HRO
342 is in alignment with the observation made for the abundance of SPMs from the different
343 metabolomes, where a nonsignificant trend for dose-dependency was observed. The VIP scores
344 shown in Fig. 4D indicate which individual SPMs are drivers for the separation of the clusters
345 similarly to Fig. 3C. The VIP scoring shows also here that RvD1 is the biggest driver for separation
346 of the clusters, followed by RvE3 which was found to be the most abundant SPM present in both
347 experiments. Furthermore, we see that individual SPMs from all four metabolomes (DHA, n-3 DPA,
348 EPA, and AA) contribute to the separation of the different clusters. Despite not observing significant
349 upregulation of total n-3 DPA SPMs between the groups, the individual n-3 DPA SPMs with a VIP-
350 score >1 were found to be significantly upregulated: RvD1 $_{\text{n-3 DPA}}$ for HRO and PEHeRo at 0.25%,
351 RvT2 for PEHeRo at 0.25%, and RvT1 for both concentrations of HRO, thus corroborating their
352 significance in the separation of the clusters in the PLS-DA analysis.

353 Interestingly, the VIP scoring in Fig. 4D also shows the cysteinyl DHA-derived SPM PCTR2 (PCTR
354 = protectin conjugates in tissue regeneration) from the DHA metabolome to be a significant
355 contributor to the separation of the different clusters, thus indicating activation of reparative
356 pathways and not only protective SPM pathways.

357 **HRO promotes SPM biosynthesis in IL-17A stimulated fibroblast/keratinocyte co-culture**

358 To study the potential effects of HRO on the IL-23/IL-17 cytokine axis in psoriasis, a co-culture of
359 keratinocytes and fibroblasts that mimics psoriasis upon stimulation with IL-17A was developed.
360 Mildenberger *et al.* (62) showed that HRO dampens psoriatic marker S100A7 (psoriasine) and the
361 IL-17 response gene NFKBIZ in this co-culture. Considering the dampening effects observed in these
362 co-culture experiments, we wanted to see if the SPM biosynthesis was upregulated from the co-
363 culture in this *in vitro* psoriasis-mimicking cell model.

364 Quantification of SPMs and monohydroxylated fatty acids in the co-culture cell media showed
365 significant upregulation of SPMs from the DHA and EPA metabolomes, as well as significant
366 upregulation of monohydroxylated DHA, EPA, and n-3 DPA (Fig. 5A-D and supplementary table
367 S5). It was encouraging to see a significant promotion of SPM biosynthesis taking place despite only
368 treating the cells with 1/100 (0.0005%) of the amount of HRO compared to the 0.05% HRO used in
369 the macrophages and reporting on 1/3 of the amount of media (3 mL vs 1 mL). The amounts
370 measured in the media were of course affected by the decreased concentration of HRO, and the
371 overall quantities are therefore lower compared to what was observed in the macrophage
372 experiments. It should be noted that a parallel co-culture experiment (n=6) showed similar results
373 albeit with somewhat more moderate levels of lipid mediators. This experiment did not show
374 significant upregulation of sum DHA-derived SPMs (only PD1 in the HRO group). Nor was a
375 significant increase in the sum of HDPAs observed, only a significant increase of 17-HDPA was
376 observed compared to the stimulated control (see supplementary table S6 for more information).

377 The DHA SPMs that contributed to the significant upregulation of sum of DHA SPMs (Fig. 5A) in
378 both the groups treated with HRO included RvD1, RvD2, RvD3, RvD5, RvD6, and PD1, of which
379 RvD5 and RvD6 were significant in HRO+IL-17A compared to the stimulated control. The

380 contributors to the overall activation of the DHA SPM metabolome coincide with observations made
381 in the macrophage experiments where RvD1 and PD1 had VIP scores >1 when HRO was used to
382 pretreat macrophages prior to stimulation with LPS+IFN-γ and RvD1 and RvD2 had VIP scores >1
383 when macrophages were costimulated with HRO/PEHeRo + LPS. Significant upregulation of
384 monohydroxylated fatty acids (HDHAs and HDPAs) also could indicate that the HRO indeed
385 promotes activation of the metabolic pathways for SPM biosynthesis from the DHA and DPA
386 metabolomes. The HDHA profile measured in the media supports the observations made of the
387 SPMs (Fig. 5C), as the main monohydroxylated fatty acid found was 17-HDHA where the 17S-
388 HDHA isomer is a precursor for the D-series resolvins formed through ALOX15. 17-HDHA was
389 significantly favored over 14-HDHA where the 14S isomer is a pathway marker for the biosynthesis
390 of MaR1 and MaR2 through ALOX12. The apparent preference for 17-HDHA thus correspond with
391 the observed levels of MaR1 and MaR2 where neither of which were measured above the LLOQ.
392 The same also being the case for the experiments with the macrophages where no consistent or
393 significant measurements of MaR1 and MaR2 were made. It should be noted that this was more
394 pronounced for the HDPAs, as only 17-HDPA was measured in the media samples.

395 Furthermore, SPMs from the EPA metabolome were also significantly increased in both treatment
396 groups compared to the stimulated control (Fig. 5B). This was primarily driven by RvE3 and to a
397 lesser extent RvE2 (approximately 1:40 RvE2/RvE3), both of which were also separately
398 significantly upregulated compared to the stimulated control. RvE2 and RvE3 being the main EPA
399 SPMs measured is not surprising when looking at the HEPE profile of both treatment groups (Fig.
400 5D), where 18-HEPE was found in the, by far, highest concentration in the media. The 18R isomer of
401 18-HEPE is a SPM precursor biosynthesized via CYP450 and GPX4 and is the main intermediate
402 before a one-step conversion via ALOX15 into RvE3 or the conversion to RvE2 via ALOX5. The
403 abundance of 15-HEPE was far lower than the abundance of 18-HEPE, which may explain why
404 RvE4 was not found above the LLOQ as 15S-HEPE is the main precursor for RvE4.

405 Including a group of "healthy" cells only treated with HRO shows, that there may be an impact to the
406 lipid mediator biosynthesis when the cells are stimulated with IL-17A. The greatest impact of IL-17A
407 stimulation was observed for the EPA metabolome, with a significant reduction of 18-HEPE, sum
408 HEPEs, and EPA SPMs when comparing the HRO group with the HRO+IL-17A group.
409 Interestingly, only RvE3 was significantly reduced, whereas only a nonsignificant trend was seen for
410 RvE2. An impact of IL-17A stimulation was also observed for SPMs in the DHA metabolome, albeit
411 to a lesser extent. Here we observed that both RvD2 and PD1 were significantly upregulated in the
412 HRO group, but not in the HRO+IL-17A group. The negating effect of IL-17A on lipid mediator
413 biosynthesis was only observed in one out of two data sets, as can be seen from Table S6 in the
414 supplementary data.

415

416 **Discussion**

417 An interesting finding from the macrophage experiments in the present study was the comparison of
418 total SPMs across different metabolomes. Where notably, treatment with PEHeRo significantly
419 increased the biosynthesis of SPMs from the DHA metabolome compared to HRO. In addition to the
420 significant difference between PEHeRo and HRO in the DHA metabolome, a similar observation was
421 made for SPMs from the EPA metabolome albeit as a nonsignificant trend. The observed increase in
422 SPM biosynthesis in macrophages treated with PEHeRo cannot be explained by availability of
423 metabolically available free fatty acid precursors or availability of mono-hydroxylated fatty acids,

424 including lipid mediator precursors, as PEHeRo was shown to contain far less of these compounds
425 compared to the HRO used in these experiments. Thus, any normalization based on availability of
426 free EPA/DHA, bound EPA/DHA, or monohydroxylated fatty acids (18-HEPE and 17-HDHA) only
427 increases the difference observed when comparing HRO and PEHeRo based on overall
428 concentration. This indicates that the more purified PEHeRo more efficiently promotes upregulation
429 of biosynthesis and that the oils' ability to upregulate these pathways depends on other factors such
430 as matrix complexity or lipid class composition and not just the precursor availability. For instance,
431 phospholipids have been indicated to give increased *in vivo* bioavailability of PUFAs compared to
432 corresponding triacylglycerides (51,68), and their lysoforms may also themselves act as signaling
433 molecules (69). The observations correspond with existing data that indicates that different oils elicit
434 different ability to promote conversion of PUFAs and SPM precursors into SPMs (39). Pinpointing
435 the impacting factors for these observations needs further studies to be verified.

436 Consistent dominant upregulation of the EPA-derived SPMs RvE2 and RvE3 was observed in all
437 experiments, with RvE3 being the most abundant of the two. The higher relative concentration of
438 RvE3 compared to other SPMs can possibly be attributed to RvE3 being available in one enzymatic
439 transformation from 18R-HEPE by ALOX15, which was likely abundant in both HRO and PEHeRo
440 from the measured levels of 18-HEPE (achiral analysis). RvE2 is also biosynthesized from 18R-
441 HEPE but through two steps by ALOX5 and a dehydrogenase. The availability of 18-HEPE in the
442 oils does not fully explain the strong upregulation of RvE3, as more RvE3 was found in cell media
443 when the macrophages were stimulated with PEHeRo which contained >10x less 18-HEPE than
444 HRO. The consistent upregulation of RvE2 and RvE3 across both the macrophages and the skin cell
445 co-culture experiments is particularly interesting given that RvE2 has been reported to be a regulator
446 of neutrophil infiltration in addition to enhancing phagocytosis (70), andmore importantly, RvE3 has
447 been reported to attenuate inflammation via the IL-23/IL-17A-axis (71) which is highly relevant for
448 psoriatic inflammation.

449 The most recurring DHA-derived SPM was found to be RvD2, which was observed consistently in
450 the macrophage experiments and for the HRO treated group in the keratinocyte/fibroblast co-culture
451 experiments. RvD2 takes part in regulating inflammation by increasing phagocytosis and reducing
452 neutrophil infiltration (72), promoting macrophage polarization (73), and reducing expression of
453 proinflammatory cytokines (72). The protectin PDX has been reported to be implicated in regulation
454 of psoriasis, as it has been found to be elevated in psoriasis lesional skin (74), and was measured in
455 all the macrophage experiments. By being upregulated in the immune cells treated with HRO and
456 PEHeRo and involved in regulation of inflammation in psoriasis, PDX could be a promising
457 biomarker for future studies in psoriasis using marine phospholipids.

458 Showing that we were able to upregulate SPM biosynthesis in the keratinocyte/fibroblast co-culture
459 was intriguing, considering that there, to our knowledge, are few studies detailing the ability of lipid
460 supplementation to regulate SPM production in these cells. The monohydroxylated fatty acid profiles
461 could imply that a key pathway for synthesis of lipid mediators from DHA and DPA goes through
462 17-HDHA, as there were found significantly higher concentrations of 17-HDHA compared to 14-
463 HDHA. This was even more pronounced for n-3 DPA, where only 17-HDPA was found in the media
464 upon analysis. Also, it was interesting to see activation of the DPA metabolome considering the
465 much lower content of this PUFA in HRO, thus implicating the importance of this pathway in
466 regulation of inflammation. The apparent preference for the 17-hydroxylated DHA and n-3 DPA are
467 aligned with literature showing 17-HDHA biosynthesis through ALOX15 in normal human
468 keratinocytes when treated with DHA (75). Morin *et al.* have shown that supplementation of a
469 psoriatic skin cell model with pure EPA also led to increased epidermal production of 17-HDHA,

470 besides PGE3 and 12-HEPE, but not in the presence of polarized T-cells (76). Future experiments in
471 the keratinocyte/fibroblast co-cultures should include chiral analyses of the monohydroxylated fatty
472 acids in order to further elucidate which enzymes that are involved. Our findings furthermore
473 indicates that the SPM biosynthesis from DHA and DPA is maintained in an inflamed state, as the
474 precursor levels were found to be very similar between the HRO and the HRO + IL-17A groups.
475 However, where ALOX15 seems to be important for conversion of DHA and DPA, this does not
476 seem to be the case for EPA. Biosynthesis via 18-HEPE seems to be the main pathway for EPA
477 derived lipid mediators with 15-HEPE levels being comparatively lower. This could be due to
478 substrate vs enzyme availability in the system, but the finding is also consistent with the SPMs found
479 in both the skin cells and the macrophages, where the downstream product of 15S-HEPE RvE4 was
480 not measured in any of the experiments.

481 The observed biosynthesis of the cysteinyl SPM PCTR2 in the macrophages may also indicate a shift
482 towards a reparative phenotype as well as a protective one, as the PCTRs have been reported to be
483 biosynthesized by macrophages and to be involved in macrophage phenotype regulation and tissue
484 regeneration (14,77,78). The PCTRs are biosynthesized similarly to PD1 through ALOX15
485 conversion of 17S-HpDHA into 16S,17S-epoxy-DHA, but with a last step through a
486 glutamyltransferase instead of an epoxide hydrolase to give PCTR1 instead of PD1. PCTR1 is then
487 hydrolyzed to PCTR2 which may be taken through a final hydrolysis to PCTR3. The linear nature of
488 their biosynthesis implies that PCTR1 also was produced by these cells prior to being hydrolyzed to
489 PCTR2, which is interesting given that PCTR1 has been shown to stimulate keratinocyte migration
490 and facilitate host defense (14).

491 In addition to promoting lipid mediator biosynthesis, we also observed that HRO significantly
492 increased the levels of EPA/DHA in the total fatty acids in the MDMs and significantly increased the
493 levels of EPA/DHA and reduced the levels of AA in the PL-fraction of the fibroblast/keratinocyte co-
494 culture. Allowing for potential replacement of AA in cell membranes could positively impact
495 psoriasis, as overexpressed PLA2 enzymes liberating AA for conversion into pro-inflammatory lipid
496 mediators is a driver for psoriatic inflammation (79).

497 The results presented in this work also shows that both HRO and PEHeRo contain SPMs, including:
498 1) PDX that has been shown to ameliorate inflammation and promote macrophage polarization in
499 experimental models of infection (80). 2) MaR1 that has been reported to display immunoresolving
500 effects in models of skin inflammation through regulation of IL-23 (81), and 3) RvE4 that increases
501 efferocytosis of apoptotic cells (82). The impact of these existing SPMs in the oils was not elucidated
502 in this work, but considering their reported effects, they may provide anti-inflammatory effects and
503 could potentially impact SPM biosynthesis. The effect of exogenous administration of lipid
504 mediators has been shown in humans, where supplementation has been shown to increase blood lipid
505 mediator levels (83). However, as stated above, the treatment of MDMs indicated that the existing
506 lipid mediator levels in HRO and PEHeRo were not the primary drivers for biosynthesis, as the
507 PEHeRo contained far less SPMs and monohydroxylated fatty acids than HRO but resulted in
508 significantly increased levels of DHA-derived SPMs in the cell media.

509 The results shown in this work indicates relevance for understanding more of the underlying
510 mechanisms that allows for regulation of cell processes involved in the pathophysiology of psoriasis
511 through lipid mediators and SPMs. The key limitation to the data, however, is this being *in vitro*
512 experiments, where all fatty acids and lipid mediators are made very accessible in concentrations that
513 may not reflect realistic *in vivo* concentrations. Thus, the effects of directly metabolically available

514 PUFAs and, particularly, lipid mediators present in the oils may therefore be over-emphasized. It is
515 therefore important to take this into further *in vivo* trials for confirming the relevance of the findings.

516 The data on skin cells mimicking psoriasis indicates a possible mechanism of resolution of
517 inflammation in the local skin niche which could provide a treatment modality for psoriasis, and it is
518 plausible that these mechanisms also could contribute to resolution of inflammation in other organs
519 and IMIDs. The findings will also contribute to the broader understanding of the pharmacological
520 factors that are in play when administering exogenous sources of SPMs and SPM precursors into
521 biological systems.

522 In summary, we observed that HRO promotes broad SPM and lipid mediator biosynthesis in
523 macrophages and keratinocytes/fibroblasts, with a subset of SPMs that are consistently upregulated
524 throughout the stimulation/treatment conditions and cell types. Thus, indicating that these pathways
525 may carry significance for signaling and inflammation resolution across several cell types. When
526 treating macrophages with HRO and PEHeRo we found that the macrophages exhibited a dose-
527 dependent trend for biosynthesis of SPMs associated with resolution of inflammation and a shift
528 towards a more protective and possibly reparative phenotype. Given the human interventions and
529 preclinical data that are available pertaining to the use of HRO in the management of psoriasis, the
530 presented results suggest as a model of inflammatory disease for studies of local cellular resolution of
531 inflammation processes.

532

533 **Conflict of Interest**

534 TRB, MB, and RG are employed by Arctic Bioscience AS.

535 The authors declare that all experiments performed were partially funded by Arctic Bioscience AS.

536 JD is an inventor on patents related to the composition of matter and/or use of pro-resolving
537 mediators some of which are licensed by Brigham and Women's Hospital or Queen Mary University
538 of London for clinical development.

539

540 **Author Contributions**

541 TRB performed all statistical tests aside from the PLS-DA and the fatty acid uptake experiments,
542 evaluated the data, and wrote the original draft of the paper. JD, MD and AS performed all lipid
543 mediator/SPM analyses on both oils and cell media and performed the PLS-DA analyses. TKØ
544 performed fatty acid uptake experiments including statistical analysis of these experiments and was
545 part of performing all co-culture experiments. JM performed all macrophage experiments. MB
546 provided HRO and PEHeRo. MP and NS performed the co-culture experiments. RG contributed to
547 the writing of the paper. RG, TRB, TKØ, JM, and MP also contributed to describing the scope of the
548 project.

549 All authors contributed to review and finalization of the final article for publication.

550

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792

793

794 **Figure legends**

795 **Fig. 1** A) Relative concentrations of select SPMs in HRO and PEHeRo (mean of 3 replicates ±SEM)
796 as a percentage of total SPMs found in the oils. B) Relative concentrations of monohydroxylated
797 fatty acids in HRO and PEHeRo (mean of 3 replicates ±SEM) as a percentage of the total amount of
798 monohydroxylated fatty acids found in the oils. C) Relative distribution of monohydroxylated fatty
799 acids in HRO and PEHeRo (mean of 3 replicates ±SEM) as a percentage of the sum
800 monohydroxylated fatty caids from DHA, EPA, and n-3 DPA. SPMs were identified and quantified
801 using LC-MS/MS. # $P > 0.1$ (Mann-Whitney U test, two-tailed) and indicating similarity between
802 HRO and PEHeRo for the analyte, remaining parameters were $P < 0.1$.

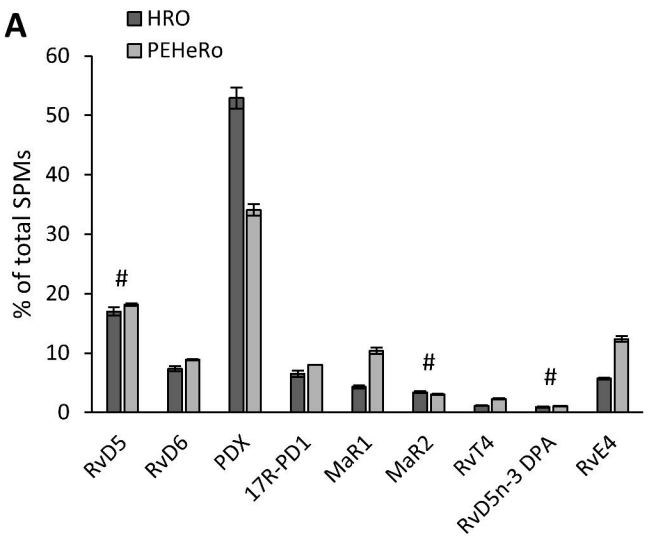
803 **Fig. 2** A) Relative concentrations from GC-FID of fatty acids as % of total fatty acids in MDMs
804 (mean of 3 replicates ±SEM) when treated with HRO versus control. B) Relative concentrations from
805 GC-FID of fatty acids as % of total fatty acids in the phospholipid fraction of the
806 keratinocyte/fibroblast co-culture (mean of 9 replicates ±SEM) when treated with HRO versus
807 control. *$P < 0.05$ (One-factor ANOVA with Tukey's HSD).

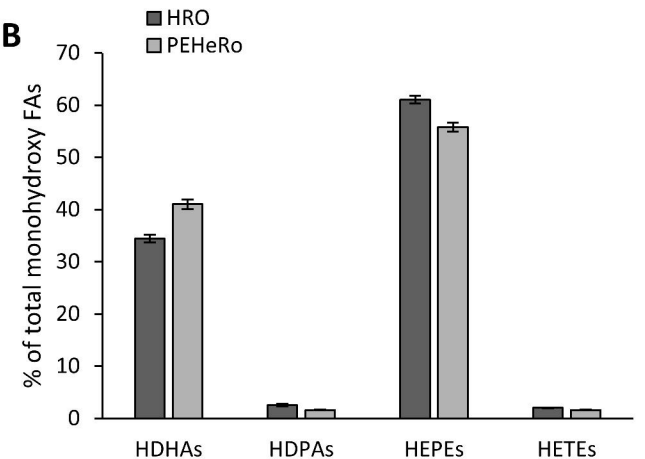
808 **Fig. 3** A) SPM levels (mean of 4 donors/experiments ± SEM) in supernatants from macrophages
809 incubated with HRO prior to stimulation with LPS+IFN-γ versus only stimulated with LPS+IFN-γ
810 (control). B) Partial least squares discriminant analysis (PLS-DA) of supernatants from macrophages
811 incubated with HRO prior to stimulation with LPS+IFN-γ versus only stimulated with LPS+IFN-γ
812 (control). Results are compared to lipid mediator concentrations identified in fresh media. C)
813 Variable importance in projection (VIP) scores of SPMs that contribute to the difference between the
814 tested conditions. D) Figure showing the treatment conditions and the highlighted measured
815 outcomes. SPMs were identified and quantified using LC-MS/MS. *$P < 0.05$ (Mann-Whitney U test,
816 two-tailed).

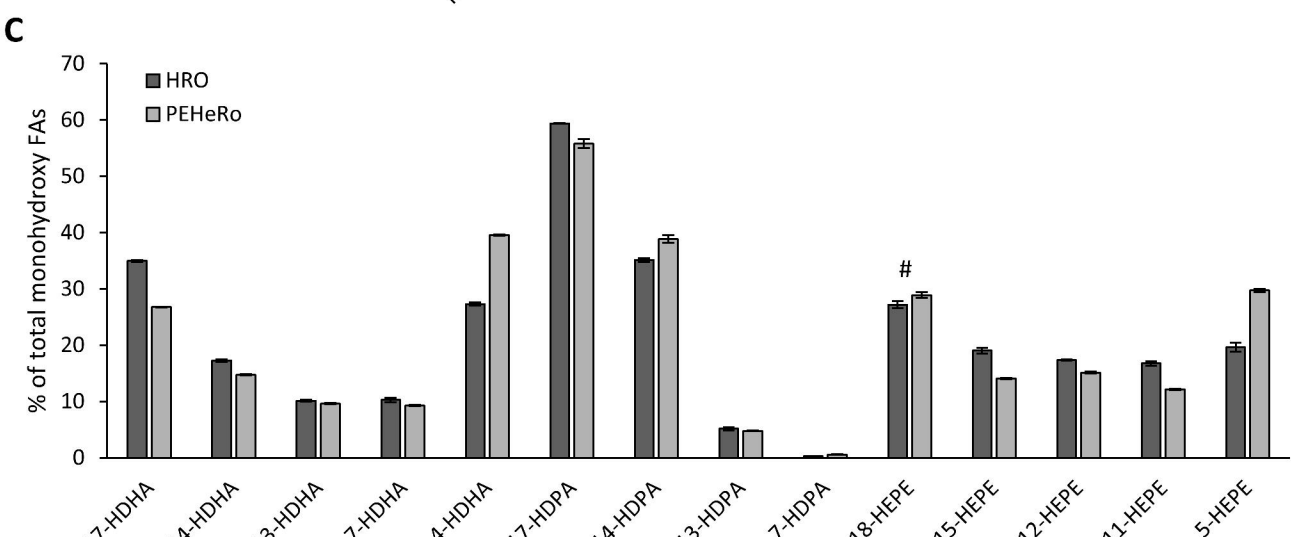
817 **Fig. 4** SPM levels (mean of 4 donors/experiments ± SEM) from DHA (A) and EPA (B) metabolomes
818 in macrophage cell supernatants (pg/3 mL) when incubated with HRO+LPS, PEHeRo+LPS, LPS
819 (control), or untreated. C) Partial least squares discriminant analysis (PLS-DA) of supernatants from
820 macrophages incubated with HRO+LPS, PEHeRo+LPS, LPS (control), or untreated. D) Variable
821 importance in projection (VIP) scores of SPMs that contribute to the difference between the tested
822 conditions. SPMs were identified and quantified using LC-MS/MS. *$P < 0.05$ and **$P < 0.01$ (One-
823 factor ANOVA with one-way Dunnett's multiple comparisons test for comparison to control and
824 Tukey's HSD for comparison between treatment groups).

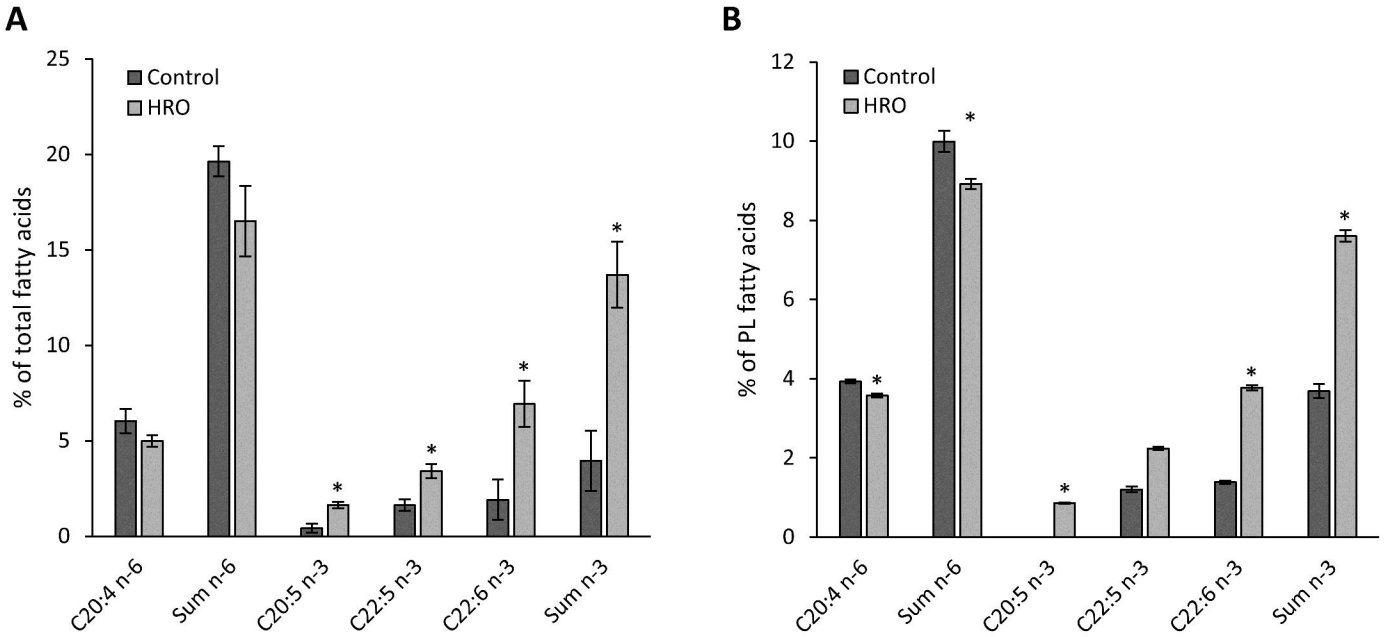
825 **Fig. 5** SPM and SPM precursor levels (mean of 3 biological replicates ± SEM) in
826 fibroblast/keratinocyte co-culture cell supernatants (pg/mL) incubated with IL-17A (control), HRO,
827 HRO+IL-17A, or untreated. SPMs were identified and quantified using LC-MS/MS. A) SPMs and
828 monohydroxylated fatty acids from DHA and monohydroxylated fatty acids from DPA. B) SPMs and
829 monohydroxylated fatty acids from EPA. C) Monohydroxylated fatty acids from DHA. D)
830 Monohydroxylated fatty acids from EPA. *$P < 0.05$ and **$P < 0.01$ (One-factor ANOVA with one-
831 way Dunnett's multiple comparisons test for comparison to control and Tukey's HSD for comparison
832 between treatment groups and between different monohydroxylated fatty acids).

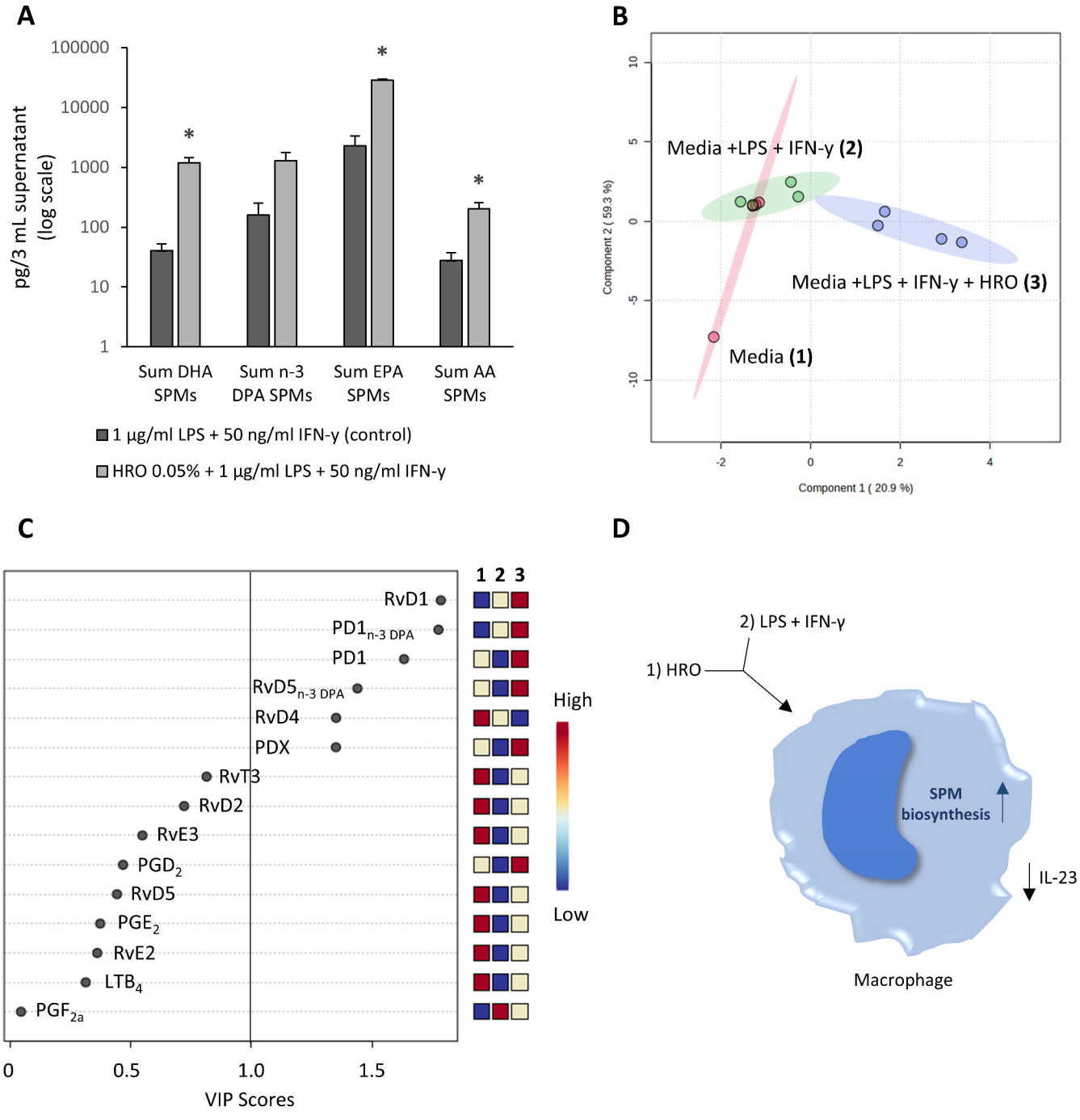
833

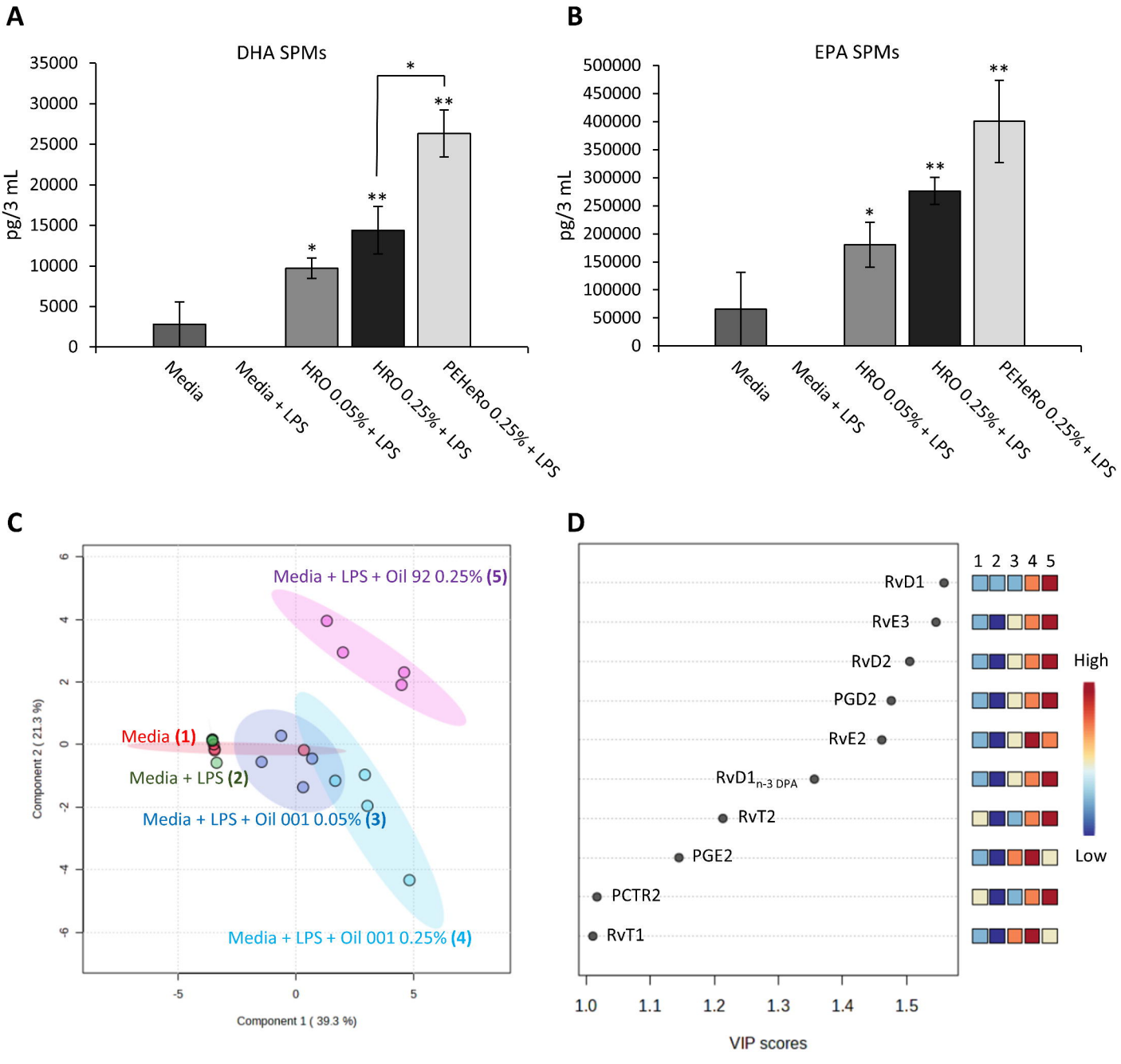

A
HRO
PEHeRo
% of total SPMs
60
50
40
30
20
10
0
#
#
#
RvD5
RvD6
PDX
17R-PD1
MaR1
MaR2
RvT4
RvD5n-3 DPA
RvE4

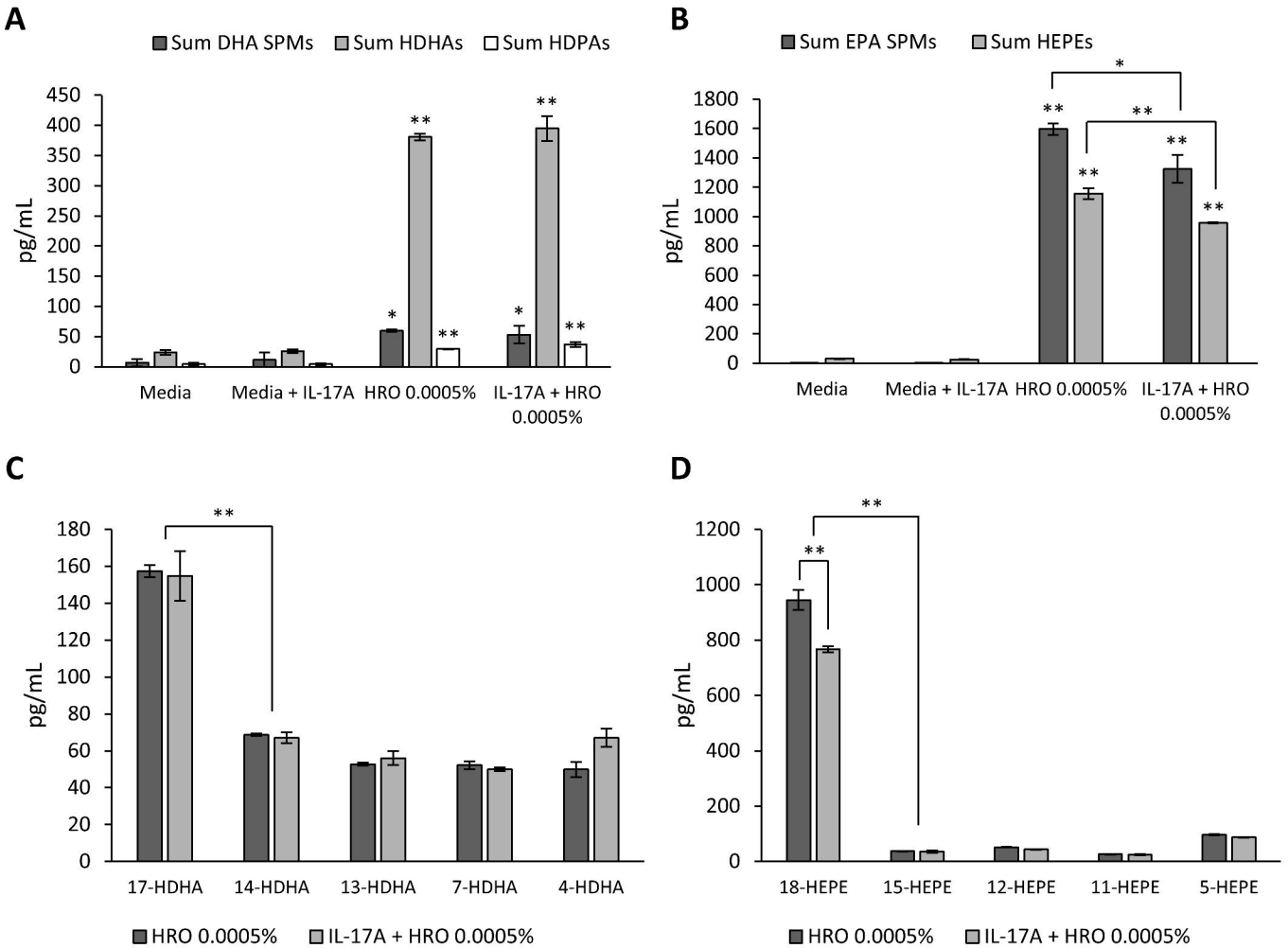

B
HRO
PEHeRo
% of total monohydroxy FAs
70
60
50
40
30
20
10
0
HDHAs
HDPAs
HEPEs
HETEs


C
HRO
PEHeRo
% of total monohydroxy FAs
70
60
50
40
30
20
10
0
#
17-HDHA
14-HDHA
13-HDHA
7-HDHA
4-HDHA
17-HDPA
14-HDPA
13-HDPA
7-HDPA
18-HEPE
15-HEPE
12-HEPE
11-HEPE
5-HEPE


A
Control
HRO
% of total fatty acids
25
20
15
10
5
0
*
C20:4 n-6
Sum n-6
C20:5 n-3
C22:5 n-3
C22:6 n-3
Sum n-3
B
Control
HRO
% of PL fatty acids
12
10
8
6
4
2
0
*
C20:4 n-6
Sum n-6
C20:5 n-3
C22:5 n-3
C22:6 n-3
Sum n-3


A
pg/3 mL supernatant (log scale)
100000
10000
1000
100
10
1
*
*
*
Sum DHA SPMs
Sum n-3 DPA SPMs
Sum EPA SPMs
Sum AA SPMs
1 µg/ml LPS + 50 ng/ml IFN-γ (control)
HRO 0.05% + 1 µg/ml LPS + 50 ng/ml IFN-γ
B
Media +LPS + IFN-γ (2)
Media +LPS + IFN-γ + HRO (3)
Media (1)
Component 2 (59.3 %)
Component 1 (20.9 %)
C
1 2 3
RvD1
PD1n-3 DPA
PD1
RvD5n-3 DPA
RvD4
PDX
RvT3
RvD2
RvE3
PGD2
RvD5
PGE2
RvE2
LTB4
PGF2a
High
Low
VIP Scores
D
1) HRO
2) LPS + IFN-γ
SPM biosynthesis
IL-23
Macrophage


A
DHA SPMs
pg/3 mL
35000
30000
25000
20000
15000
10000
5000
0
*
**
*
**
Media
Media + LPS
HRO 0.05% + LPS
HRO 0.25% + LPS
PEHeRo 0.25% + LPS
B
EPA SPMs
pg/3 mL
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
*
**
**
Media
Media + LPS
HRO 0.05% + LPS
HRO 0.25% + LPS
PEHeRo 0.25% + LPS
C
Media + LPS + Oil 92 0.25% (5)
Media (1)
Media + LPS (2)
Media + LPS + Oil 001 0.05% (3)
Media + LPS + Oil 001 0.25% (4)
Component 2 (21.3 %)
Component 1 (39.3 %)
D
RvD1
RvE3
RvD2
PGD2
RvE2
RvD1n-3 DPA
RvT2
PGE2
PCTR2
RvT1
1 2 3 4 5
High
Low
VIP scores


A
Sum DHA SPMs
Sum HDHAs
Sum HDPAs
pg/mL
450
400
350
300
250
200
150
100
50
0
Media
Media + IL-17A
HRO 0.0005%
IL-17A + HRO 0.0005%
B
Sum EPA SPMs
Sum HEPEs
pg/mL
1800
1600
1400
1200
1000
800
600
400
200
0
Media
Media + IL-17A
HRO 0.0005%
IL-17A + HRO 0.0005%
C
pg/mL
180
160
140
120
100
80
60
40
20
0
17-HDHA
14-HDHA
13-HDHA
7-HDHA
4-HDHA
HRO 0.0005%
IL-17A + HRO 0.0005%
D
pg/mL
1200
1000
800
600
400
200
0
18-HEPE
15-HEPE
12-HEPE
11-HEPE
5-HEPE
HRO 0.0005%
IL-17A + HRO 0.0005%