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Identification and Profiling of Specialized Pro-Resolving Mediators in Human Tears by Lipid Mediator Metabolomics

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Abstract

Specialized pro-resolving mediators (SPM), e.g. Resolvin D1, Protectin D1, Lipoxin A₄, and Resolvin E1 have each shown to be active in ocular models reducing inflammation. In general, SPMs have specific agonist functions that stimulate resolution of infection and inflammation in animal disease models. The presence and quantity of SPM in human emotional tears is of interest. Here, utilizing a targeted LC-MS-MS metabololipidomics based approach we document the identification of pro-inflammatory (Prostaglandins and Leukotriene B₄) and pro-resolving lipid mediators (D-series Resolvins, Protectin D1, and Lipoxin A₄) in human emotional tears from 12 healthy individuals. SPMs from the Maresin family (Maresin 1 and Maresin 2) were not present in these samples. Principal Component Analysis (PCA) revealed gender differences in the production of specific mediators within these tear samples as the SPMs were essentially absent in these female donors. These results indicate that specific SPM signatures are present in human emotional tears at concentrations known to be bioactive. Moreover, they will help to further appreciate the mechanisms of production and action of SPMs in the eye, as well as their physiologic roles in human ocular disease resolution.

Keywords

Lipid Mediators; Omega-3 fatty acids; inflammation; resolution; resolvins; protectins

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1. Introduction

The resolvins and the other specialized pro-resolving mediators (SPM) protectins and maresins were initially uncovered in the resolution phase of acute inflammatory responses as molecules that carry unique bioactions to resolve inflammation. These include: a) limiting neutrophilic infiltration in tissues; b) enhancing macrophage uptake of apoptotic cells and microbial clearance; and c) stimulating tissue regeneration [reviewed in ref. 1]. The SPM include four families of mediators with potent actions: lipoxins, resolvins, protectins and maresins. Each family and their members are structurally distinct and display stereospecific actions and biosynthetic mechanisms [1]. The lipoxins are biosynthesized from arachidonic acid. The resolvins are biosynthesized from the omega-3 (n-3) essential fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [1] and are produced via two main pathways from these substrates to give E-series resolvins from EPA (RvE1, RvE2 and RvE3) and DHA-derived D-series resolvins (e.g., resolvin D1, resolvin D2, resolvin D3, resolvin D4, resolvin D5 and resolvin D6), with each member possessing unique structures and potent functions in vivo in animal models of disease and with isolated human cells [1].

In addition to SPM production during resolution of inflammation, increasing evidence indicates that SPM are also produced in human tissues rich in omega-3 essential fatty acids [2]. For example, these tissues, to name a few, include human blood [3, 4], lymph nodes [4], human brain [5], human placenta [6], breast milk [7, 8] and human kidney [2]. The SPM are clearly endogenous mediators that, given their presence in human organs and tissues, may have additional physiologic roles in their tissue of origin in addition to their functions in activating endogenous resolution circuits in inflammation.

The lipoxin A₄ receptor is present in human corneal epithelial cells [9], where lipoxin A₄ was found to display potent protective actions in murine eye wound repair [10] and in autoimmune dry eye disease [11, 12]. The n-3-PUFA-derived SPM such as resolvin D1, resolvin E1 and neuroprotectin D1 are also produced in murine models of eye disease and are increased with supplementation of EPA and DHA in the diet [13]. Both E-series resolvins and D-series resolvins have potent actions in the anterior as well as posterior ocular function [14–17], suggesting that the local production of SPM may control specific ocular functions. Along these lines, resolvin D1 reduces uveitis in rat eye [17], lipoxin A₄ stimulates corneal wound healing [18, 19], resolvin E1 reduces ocular inflammation [20] and corneal inflammation [16] and regulates retinal neovascularization [13], and resolvin D1 and lipoxin A₄ regulate pain [21] and histamine-stimulated conjunctival goblet cell mucin secretion [11, 22]. Together, these findings provide evidence for a potential role of endogenous SPM in ocular function.

Tear fluid can serve as a means to identify and monitor novel biomarkers in ocular and systemic disease that can also be used in personalized medicine and for prevention [23]. The omega-3 (DHA and EPA) and omega-6 (arachidonic acid) fatty acid precursors are present in human tears and have been shown to correlate with clinical measures of dry eye systems in humans [24]. Recently, we have operationalized the identification and profiling of lipid mediators from arachidonic acid – prostaglandins, leukotrienes and lipoxins [25] – as well as n-3-derived SPM, resolvins, protectins and maresins in human tissues employing rigorous

LC-MS-MS-based profiling and matching (> six diagnostic ions) with authentic standards [4, 26]. Because human tears contain many autacoids [27], in the present report we profiled LM and SPM and identified resolvins and SPM at bioactive concentrations in human tears.

2. Materials and Methods

Human tears, acquired through an induction of an emotional response and immediately frozen at -20°C , were purchased from Lee Biosolutions (Maryland Heights, MO USA). These samples were analyzed in accordance with Partners Human Research Committee Protocol 1999P001279. All were de-identified; 6 samples from male donors and 6 samples from females. Prior to solid phase extraction (vide infra), all tear samples were stored at -80°C . All purchased samples were recorded as having a fixed initial volume of 100 μL . Lipid mediator metabololipidomics was carried out; first, the human emotional tear samples (100 μL each) were thawed on an ice-water bath. Prior to sample extraction, 5 deuterium labeled internal standards were added to each sample: d8-5-HETE, d5-RvD2, d5-LXA₄, d4-LTB₄, d4-PGE₂ (500 pg each; Cayman Chemical, Ann Arbor, MI) along with two volumes of ice-cold LC-MS-MS grade methanol (EMD Millipore, MA USA). These labeled standards were added for quantification and recovery of endogenous tear lipid mediators. Samples were then covered on ice for approximately 45 minutes to allow for protein precipitation, followed by centrifugation (1,000g, 10 min, 4°C). Supernatants were collected from each sample, and solid phase extraction (SPE) was carried out according to optimized and reported methods as in [4]. Briefly, the samples were expeditiously acidified to an apparent pH 3.5, loaded onto Isolute C18 SPE 500 mg cartridges (Biotage, Charlotte, NC USA), and rapidly neutralized using double-distilled H₂O, followed by a 6 mL hexane wash step. Methyl formate fractions were concentrated in a gentle stream of nitrogen gas (N₂) contained within an automated evaporator (TurboVap LV, Biotage). Products eluted within the methyl formate fraction from solid phase extractions were then rapidly suspended in a 50:50 mixture of 50 μL Methanol/Water prior to injection for LC-MS-MS.

2.1 LM-Metabololipidomics: LC-MS-MS Acquisition Parameters

The resulting samples were then taken and injected into the liquid chromatography-tandem mass spectrometry system. This system consisted of a Qtrap 5500 (Sciex) equipped with a Shimadzu LC-20AD HPLC (Tokyo, Japan). The column was a Poroshell 120 EC-18 column (100 mm $\times$ 4.6 mm $\times$ 2.7 μm ; Agilent Technologies, Santa Clara, CA, USA), jacketed in a column oven regulated at 50°C . The mobile phases used were A) LC-MS-MS grade water (Fisher Chemical, NJ, USA) in a ratio of 99.99% H₂O: 0.01% acetic acid, and B) LC-MS-MS grade methanol (Fisher Chemical, NJ, USA) with the same ratio 99.99% MeOH: 0.01% acetic acid. LMs were eluted in a gradient of methanol/water (50/50, vol/vol) from 0 to 2 minutes, and then changed to methanol/water 80/20, vol/vol from 2 to 14.50 minutes, and the third segment was increased to methanol/water 98/2, vol/vol from 14.60 minutes to 20 minutes (0.5 mL/min flow rate).

Targeted MRM (multiple reaction monitoring) and EPI (enhanced product ion) were utilized in order to quantify the mediator levels. The criteria used for identification included MS-MS matching to at least 6 diagnostic and signature ion fragments per molecule that match those

of authentic and synthetic standards [4]. The limits of detection for this instrumentation were ~0.1 picograms, and therefore lipid mediators that were below this value were deemed to be non-detectable. For EPI, the scan rate was set at 10,000 Da/s. The operating polarity for the targeted MS/MS was set in negative mode, with a MRM detection window of 90 seconds and targeted scan time of 0.5 seconds per lipid mediator. Further specific parameters of EPI were as follows: Declustering Potential (DP) set at -80.0; Entrance Potential (EP) set at -10.0; Collision Energy (CE) set at -24.0; and Collision Energy Spread (CES) set at 0.0. These settings were used throughout. Also, it is important to note that each LM targeted by MRM has a unique CE, target retention time (RT), and specific Q1 and Q3 mass, all of which are reported in Table 1.

2.2 Data Analysis & Recovery

A quantitation and recovery were determined using the deuterium labeled internal standards (d8-5-HETE, d5-RvD2, d5-LXA₄, d4-LTB₄, d4-PGE₂; 500 pg/μL), and a LM-SPM profile was constructed for each donor. Data analysis was performed on the Sciex software platform, Analyst version 1.6. Principal component analysis (PCA) was carried out as in ref. [4] using SIMCA software, version 13.0.3 (Umetrics, Umea, Sweden).

3. Results and Discussion

3.1 SPM identification & profiles in human emotional tears

Three main polyunsaturated fatty acids (PUFA) metabolomes, i.e. Arachidonic Acid (AA), Eicosapentaenoic Acid (EPA), and Docosahexaenoic Acid (DHA), and the biosynthetic pathways that give rise to the LM-SPM profiles in inflammation-resolution are illustrated in Figure 1. This illustration outlines the temporal relationships for LM profiles in the acute inflammatory response in leukocyte-rich exudates, along with key roles these mediators play in the initiation and resolution of inflammation. The pathways (Figure 1 left to right) for the pro-inflammatory Prostaglandins and Leukotrienes, and the pathways for the pro-resolving Lipoxins, Resolvins, Protectins, and Maresins are each depicted. Utilizing a targeted lipid mediator metabololipidomics approach with LC-MS-MS based identification system [4], human tears provoked through an emotional response (100 μL aliquots per donor sample) were each extracted and profiled for their LM-SPM. The demographics from the 12 healthy donors are listed in Table 1.

Table 2 reports the optimized acquisition parameters utilized to monitor the mass spectrometry of each of the LM, SPM, and their respective pathway markers. Included in this table are Analyte ID and Scan Time, which represent the timeframe in which each analyte (LM-SPM) passed through the liquid chromatography column and detected. Also included in this Table of parameters are Q1 parent mass and Q3 diagnostic daughter fragmentation mass, Declustering Potential (DP), Collision Energy (CE), and Collision Exit Potential (CXP). It is important to note that these parameters are all required for the rigorous identification of specific SPMs and LMs within the samples profiled.

A key component of this profiling analysis, which serves as a criterion for rigorous identification, is matching for chromatographic retention time behaviors of authentic as well

as synthetic standards [4, 26]. For human tears this is depicted in Figure 2, where we present representative chromatograms obtained for the bioactive LM-SPMs identified in the tear samples. Each chromatogram plot from the accompanying SPM data was obtained from a male donor T4646, where all products could be quantified and matched to the published criteria. The next component that we employed for identification of LM-SPM in the human tear samples is identification of individual MS-MS spectrum. This is based upon matching of at least 6 diagnostic ions, and targeted MRM (Multiple Reaction Monitoring) of ion pairs Q1 (parent mass) and Q3 (diagnostic daughter ion).

We were able to confirm identified LM-SPM in human emotional tears via obtaining the MS-MS spectra shown in Figure 3–6. For example, in Figure 3, the spectra for several of the main pro-resolving mediators from the DHA bioactive metabolome is displayed, with diagnostic ions and fragmentation patterns for each which are reported as follows: RvD1 (375=M-H; 357=M-H-H₂O; 339=M-H-2H₂O; 331=M-H-CO₂; 313=M-H-CO₂; 295=M-H-2H₂O-CO₂; 261=305-CO₂; 259=277-H₂O; 243=305-H₂O-CO₂; 241=277-2H₂O; 215=233-H₂O; 197=233-2H₂O; 185=203-H₂O; 123=141-H₂O) RvD2 (375=M-H; 357=M-H-H₂O; 339=M-H-2H₂O; 331=M-H-CO₂; 313=M-H-H₂O-CO₂; 295=M-H-2 H₂O-CO₂; 287=305-H₂O; 215=233-H₂O; 203=247-CO₂) RvD5 (359=M-H; 341=M-H-H₂O; 323=M-H-2 H₂O; 315=M-H-CO₂; 297=M-H-H₂O-CO₂; 279=M-H-2H₂O-CO₂; 245=289-CO₂; 243=261-H₂O; 227=289-H₂O-CO₂; 199=217-H₂O) and PD1 (359=M-H; 341=M-H-H₂O; 341=M-H-H₂O; 323=M-H-2H₂O; 315=M-H-CO₂; 297=M-H-H₂O-CO₂; 279=M-H-2H₂O-CO₂; 243=261-H₂O; 217=261-H₂O; 199=261-H₂O-CO₂; 188=206-H₂O; 159=177-H₂O; 137=181-CO₂). These ions were each matched to those of authentic and synthetic standards for these SPM [4, 26].

Results in Figure 4 reports spectra for SPM derived from the AA bioactive pathway, and the diagnostic ions and fragmentation patterns are as follows: LXA₄ (351=M-H; 333=M-H-H₂O; 315=M-H-2H₂O; 307=M-H-CO₂; 289=M-H-H₂O-CO₂; 271=M-H-2H₂O-CO₂; 261=279-H₂O; 207=251-CO₂; 227=289-H₂O-CO₂; 199=235-2H₂O; 127=145-H₂O) AT-LXA₄ (351=M-H; 333=M-H-H₂O; 315=M-H-2H₂O; 307=M-H-CO₂; 289=M-H-H₂O-CO₂; 271=M-H-2H₂O-CO₂; 261=279-H₂O; 207=251-CO₂; 217=235-H₂O; 215=251-2H₂O; 199=235-2H₂O; 189=251-H₂O-CO₂; 171=251-2H₂O-CO₂) and 5S,15S-diHETE (335=M-H; 317=M-H-H₂O; 299=M-H-2H₂O; 291=M-H-CO₂; 273=M-H-H₂O-CO₂; 255=M-H-2H₂O-CO₂; 227=263-2H₂O; 217=235-CO₂; 201=219-H₂O; 191=235-CO₂; 173=235-H₂O-CO₂) a marker of lipoxin biosynthesis [28].

Figure 5 displays spectra and diagnostic fragmentation for pro-inflammatory mediators from the AA metabolome. These included, PGD₂ (351=M-H; 333=M-H-H₂O; 315=M-H-2H₂O; 289=M-H-H₂O-CO₂; 271=M-H-2H₂O-CO₂; 233=251-H₂O; 217=279-H₂O-CO₂; 189=251-H₂O-CO₂) PGE₂ (351=M-H; 333=M-H-H₂O; 315=M-H-2H₂O; 289=M-H-H₂O-CO₂; 271=M-H-2H₂O-CO₂; 243=279-2H₂O; 235=279-CO₂; 233=251-H₂O; 217=279-H₂O-CO₂; 207=251-CO₂; 189=251-H₂O-CO₂) PGF₂α (353=M-H; 335=M-H-H₂O; 317=M-H-2H₂O; 299=M-H-3H₂O; 291=309-H₂O; 273=309-2H₂O; 263=281-H₂O; 209=253-CO₂; 193=253-CO₂; 173=253-2H₂O-CO₂) and LTB₄ (335=M-H; 317=M-H-H₂O; 299=M-H-2H₂O; 273=M-H-H₂O-CO₂; 255=M-H-2H₂O-CO₂; 205=233-CO₂; 177=195-H₂O; 161=233-H₂O-

CO₂; 133=195-H₂O-CO₂). These results document the presence of both prostaglandins and leukotriene B₄ in human emotional tears.

SPM biosynthesis pathway markers were also identified, from both the DHA and EPA pathways (Figure 6). The fragmentation patterns for each were reported as such: 17-HDHA (343=M-H; 325=M-H-H₂O; 299=M-H-CO₂; 281=M-H-H₂O-CO₂; 229=273-CO₂; 211=273-H₂O-CO₂; 201=245-CO₂) 14-HDHA (343=M-H; 325=M-H-H₂O; 299=M-H-CO₂; 281=M-H-H₂O-CO₂; 215=233-H₂O; 189=233-CO₂; 161=205-CO₂) 18-HEPE (317=M-H; 299=M-H-H₂O; 273=M-H-CO₂; 255=M-H-H₂O - CO₂; 215=259-CO₂). As recently reported in Ref. [29], 17-HDHA was found to have an anti-hyperalgesic impact in a rat arthritis model; this demonstration of bioactivity could suggest that this LM may have similar anti-inflammatory and pain reducing actions in the eye via its presence in human tears. Similarly, in Ref. [30], it was demonstrated that 18-HEPE inhibited pro-inflammatory fibroblast activation, and when administered in vivo exhibited a therapeutic effect on cardiac fibrosis and inflammation. These reported biologic actions suggest a potential physiologic impact for 18-HEPE present in tears for ocular physiology. Also, the identification of both endogenous 17-HDHA and 18-HEPE in human tears suggests that both D-series and E-series Resolvins may also be in high amounts locally than quantitated in the present analyses, because both series of resolvins are known to be further metabolized to oxo- and dehydro-resolvin products [1] that were not profiled in the present LC-MS-MS method. In this context, we did not obtain evidence for E-series resolvins, specifically RvE1, RvE2 or RvE3, in emotional tears, whereas resolvin E1 is clearly bioactive in the eye [16].

The lipid mediators and their associated pathway markers from the DHA, EPA, and AA bioactive metabolomes were each identified in these human samples at picogram amounts per 100 microliters, giving a concentration range from 27 pM to 2.7 nM. The values obtained for each mediator identified for the de-identified donors is reported in Table 3. From the AA bioactive metabolome we identified lipoxins A₄ (LXA₄) along with its 17R-epimer (also known as the aspirin triggered form denoted AT-LXA₄), the distinct lipoxin pathway marker and double dioxygenation product 5S, 15S-diHETE. Also, present in these samples were the prostaglandins PGE₂, PGD₂, & PGF₂α, and the leukotriene LTB₄ along with its double dioxygenation isomer, 5S, 12S-diHETE. Note that lipoxygenase pathway markers from the AA metabolome were identified, these included 15-HETE, 12-HETE, and 5-HETE. From the EPA bioactive metabolome, the bioactive precursor 18-HEPE was identified as indicated above. From the DHA bioactive metabolome, we identified the resolvins RvD1, RvD2, and RvD5, the protectin PD1 and its double dioxygenation isomer 10S, 17S-diHDHA (PDx), as well as important DHA-pathway precursors 17-HDHA and a maresin pathway marker 14-HDHA (Table 3). Overall, the quantitative results is displayed a gender dependence in that the production of LM-SPMs favors the male donors or at least were in a range that were detected and identified by this profiling approach. For example, resolvin D1 (RvD1) was identified in 3 of the 6 males, while it could not be detected in the samples from female donors. Also, resolvin D5 (RvD5) was clearly identified in 4 of the 6 male donors while it was not detected in the samples from female donors.

3.2 Signature LM-SPM gender profile of human emotional tears

In order to examine the potential gender differences in the mediator profiles obtained from human emotional tears, we employed Principal Component Analysis (PCA) on the LC-MS-MS results from the 12 donors. The lipid mediators are displayed 2-dimensionally, consisting of two components (represented on the axes shown). The gray ellipse seen in Figure 7A is representative of the 95% confidence interval, with anything outside of that region being considered an outlier. This is based off of Hotelling's T-squared equation. Within this score plot (Figure 7A), we were able to diagnose a gender difference in the donors included for this study. The female donors, seen in green, group together tightly on the left side of the plot, while the male donors were more dispersed (in the blue to the right side of the plot, Figure 7A). In the loading plot, Figure 7B, an apparent gender difference was also revealed in the specific mediators identified in human tears between males and females. The blue cluster on the right highlights those SPM that are in association with males (RvD1, RvD2, RvD5, & PD1) while the two green circles on the left represent an association with females (LXA₄ & AT-LXA₄). In the bar graph (Figure 7C), the summation ratio of SPMs, 17-HDHA, and 18 HEPE vs. LTB₄ was calculated, separated by gender. As is depicted, the ratio is much higher in males, demonstrating a potential lack of SPM in female tears as well as an increase in the pro-inflammatory mediators in females. A lower ratio in females was also found to be statistically significant, with a p-value of 0.04.

3.3 SPM and Eicosanoid Ocular Functions

Table 4 highlights the LM and SPM identified in human tears that have known functions within the eye, and have been communicated in the literature. Shown in this table are the main bioactions and LM-SPM dose implemented, along with each reference in which the two are reported. Also, the last column in the table outlines the concentration we calculated for the LM-SPMs in male donor T4646, for all of the LM-SPMs were detected in this individual's tear sample. All of the calculated concentrations falling in the bioactive picomolar to nanomolar range. These results further exemplify the potential physiologic relevance of these mediators in the eye, as well as highlighting the importance they could have in the resolution of inflammation in ocular diseases.

For each SPM identified herein, their key diagnostic ions were confirmed with deuterium-labeled fragments prepared by total organic synthesis of the SPM [26]. Thus, the profiling of SPM and these lipid mediators as in Figures 1–7 are definitive in both identity and relative relationships for each SPM-LM signature. Recently, sex differences for resolvins in skin blisters of human exudates have been demonstrated [31]. In the present report, male emotional tears appear to display both SPM and pro-inflammatory eicosanoid mediators, whereas emotional tears from women demonstrated mainly arachidonic acid-derived mediators including the proresolving mediators from this metabolome, namely lipoxins as well as 17-HDHA from DHA and prostaglandins (Table 3). Women are known to have a higher prevalence of dry eye syndrome that may reflect lower utilization of DHA for SPM production over the age of 45 [32, 33]. Whether this relationship is related to n-3 differences remains to be determined.

Taken together the results of the present study indicate that human tears possess lipid mediators in concentrations that are known to be bioactive in the eye and immune system. Both pro-inflammatory mediators and SPM were identified and profiled in the human tears obtained from male donors while female donors appear not to have detectable quantities of SPM. This finding, while at an early stage given the small number of donors in the present data set (n=12), suggests that human tears can serve as a useful source of tissue to profile both eicosanoids and SPM as well as determine their quantitative relationships. The tear SPM rigorously identified herein were present in male donor tears at concentrations commensurate with their reported stereoselective bioactions in ocular systems as well as other organs in vivo in animal disease models. Hence, it maybe possible to identify and profile SPM in human tears by this LC-MS-MS based approach and methodology to obtain information on systemic events as well as nutritional status in humans.

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Abbreviations

AA	arachidonic acid
COX	cyclooxygenase
d	deuterated
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
HDHA	hydroxy-docosahexaenoic acid
HEPE	hydroxy-eicosapentaenoic acid
HETE	hydroxy-eicosatetraenoic acid
HpETE	hydroperoxy-eicosatetraenoic acid
LC-MS-MS	liquid chromatography tandem mass spectrometry
LM	lipid mediators
LOX	lipoxygenase
LT	leukotriene
LTB₄	leukotriene B ₄ (5S, 12R-dihydroxy-eicosa-6Z, 8E, 10E, 14Z-tetraenoic acid)
LX	lipoxin
LXA₄	lipoxin A ₄ (5S, 6R, 15S-trihydroxy-eicosa-7E, 9E, 11Z, 13E-tetraenoic acid)

LXA₅	lipoxin A ₅ (5S, 6R, 15S-trihydroxy-eicosa-7E, 9E, 11Z, 13E, 17Z-pentaenoic acid)
LXB₄	lipoxin B ₄ , (5S, 14R, 15S-trihydroxy-eicosa-6E, 8Z, 10E, 12E-tetraenoic acid)
MaR1	maresin 1 (7R, 14S-dihydroxy-docosa-4Z, 8E, 10E, 12Z, 16Z, 19Z-hexaenoic acid)
MRM	multiple reaction monitoring
PCA	principal component analysis
PD	protectin
PD1	protectin D1 (10R, 17S-dihydroxy-docosa-4Z, 7Z, 11E, 13E, 15Z, 19Z-hexaenoic acid), also known as neuroprotectin D1 (NPD1)
PG	prostaglandin
PGD₂	11-oxo-9 α , 15S-dihydroxy-prosta-5Z, 13E-dien-1-oic acid
PGE₂	9-oxo-11 α , 15S-dihydroxy-prosta-5Z, 13E-dien-1-oic acid
PGF_{2α}	9 α , 11 α , 15S-trihydroxy-prosta-5Z, 13E-dienoic acid
Rv	resolvin
RvD1	Resolvin D1 (7S, 8R, 17S-trihydroxy-docosa-4Z, 9E, 11E, 13Z, 15E, 19Zhexaenoic acid)
RvD2	Resolvin D2 (7S, 16R, 17S-trihydroxy-docosa-4Z, 8E, 10Z, 12E, 14E, 19Zhexaenoic acid)
RvD3	Resolvin D3 (4S, 11R, 17S- trihydroxy-docosa-5Z, 7E, 9E, 13Z, 15E, 19Z hexaenoic acid)
RvD5	Resolvin D5 (7S, 17S-dihydroxy-docosa-4Z, 8E, 10Z, 13Z, 15E, 19Z-hexaenoic acid)
RvE1	Resolvin E1 (5S, 12R, 18R-trihydroxy-eicosa-6Z, 8E, 10E, 14Z, 16E-pentaenoic acid)
RvE2	Resolvin E2 (5S, 18R-dihydroxy-eicosa-6E, 8Z, 11Z, 14Z, 16E-pentaenoic acid)
RvE3	Resolvin E3 (17R,18R-dihydroxy-eicosa-5Z, 8Z, 11Z, 13E, 15E-pentaenoic acid)
SPM	specialized pro-resolving mediator
5S	15S-diHETE, 5S,15S-dihydroxy-eicosa-6E, 8Z, 11Z, 13E-tetraenoic acid

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Highlights

- Human emotional tears contain both eicosanoids and specialized proresolving mediators (SPM; i.e. lipoxins, resolvins and protectins).
- Lipid mediators in human tears were identified using a new detailed LC-MS-MS profiling approach with 6 diagnostic ions from MS-MS for rigorous identification of each lipid mediator (LM).
- LM-SPM signature profiles for human emotional tears were distinct for each donor.
- This approach and LC-MS-MS profiling of human tear SPM can provide a non-invasive means to assess nutritional status and resolution of inflammation pathways.

Figure 1.

Overview of eicosanoids and specialized pro-resolving mediators pathways. Each are derived from three PUFA namely AA, EPA, and DHA. Known biologic functions from the pro-inflammatory mediators involved in the initiation of inflammation and those pro-resolution families of mediators involved in the resolution of inflammation are included. Mediators detected in tear samples are highlighted in bold text.

Figure 2.

Representative MS-MS identification of human emotional tears LM. Bioactive LM, isomers, and pathway markers, with representatives from the DHA and AA metabolomes, as well as Docosahexaenoic and Eicosapentaenoic Monohydroxy pathway markers and Arachidonic Monohydroxy Acid pathway markers. Results are representative of a single individual (from n=12) where all mediators were identified, quantified, and matched to MS-MS spectra.

Resolvin and Protectin SPM MS-MS spectra identified in human emotional tears. Identification is based on presence of at least 6 diagnostic ion fragmentation patterns, denoted in the insets and identification criteria (see text for details). MS-MS data are representative of the major lipid mediators shown at the picogram level in Table 2.

Figure 4.
MS-MS spectra for Lipoxins from the AA-bioactive metabolome.

Figure 5. MS-MS spectra for Prostaglandins and a Leukotriene from the AA-bioactive metabolome.

Figure 6.
MS-MS spectra for DHA & EPA pathway markers.

Figure 7.

PCA and quantitative ratio by gender for LM-SPMs identified in human emotional tears. (A) 2-dimensional score plot of human emotional tear donors; blue circles (n=6) are representative of males, while green circles (n=6) are representative of females. Gray ellipse denotes 95% confidence interval. (B) 2-dimensional loading plot of LM-SPMs identified in human emotional tears; blue circles are those mediators associated with male donors & green circles are associated with female donors. (C) Bar graph depicting the ratio of total SPMs including RvD1, RvD2, RvD5, PD1, LXA, AT-LXA, 17-HDHA, and 18-HEPE compared to LTB₄, in males compared females (n=6 for each gender; #P <0.05 for male donors vs. female donors; *P<0.05, females vs. males).

Table 1

Donor Demographics

Donor Identifier	Age	Sex
T5983	2	F
T5975	18	M
T5972	20	F
T4665	23	M
T5711	25	M
T4389	28	F
T4646	29	M
T5129	30	M
T2407	36	F
T5640	46	M
T5846	47	F
T5701	50	F

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Table 2

Acquisition Parameters^{*}

Analyte	Q1	Q3	Scan Time	DP (volts)	CE (volts)	CXP (volts)
RvD1	375.2	121.1	11.5	-80	-40	-13
RvD2	375.2	141.1	10.9	-80	-21	-13
RvD3	375.2	147.1	10.8	-80	-25	-13
RvD4	375.2	101.1	12.5	-80	-22	-10
RvD5	359.2	199.1	13.5	-80	-21	-13
RvD6/4,14	359.2	101.1	14.3	-80	-22	-16
RvE1	349.2	195.1	8.6	-80	-22	-12
RvE2	333.3	253.1	12.1	-80	-20	-12
RvE3	333.3	201.2	13.5	-80	-20	-12
MaR1	359.2	221.1	13.7	-80	-20	-16
MaR2	359.1	221.2	14.4	-80	-20	-12
22-OH-MaR1	375.3	221.1	10	-80	-24	-15
22-COOH-MaR1	389.3	221.1	9.4	-80	-24	-15
PD1	359.2	153.1	13.4	-80	-21	-9
22-OH-PD1	375.3	153.1	10	-80	-24	-15
22-COOH-PD1	389.3	153.1	9.4	-80	-24	-15
LXA ₄	351.2	115.1	11.5	-80	-20	-13
LXB ₄	351.2	221.1	10.9	-80	-20	-13
5,15-diHETE	335.3	115.1	13.4	-80	-22	-13
PGF ₂	351.3	189.1	10.7	-80	-25	-14
PGD ₂	351.3	233.1	10.8	-80	-16	-15
PGF ₂ -α	353.3	193.1	10.9	-80	-34	-11
TXB ₂	369.3	169.1	10.2	-80	-22	-15

^{*} Q1 and Q3 are parent mass and diagnostic daughter ion respectively; Scan time is the relative time window in which each analyte passes through the chromatography column and is detected; DP=Declustering Potential; CE=Collision Energy; CXP=Collision Exit Potential

Table 3

Human Emotional Tear LM-SPM Quantitation

Mediators	Male						Female					
	1	2	3	4	5	6	1	2	3	4	5	6
SPM Metabolome												
RvD1	38	18.8	-	39.6	-	-	-	-	-	-	-	-
RvD2	-	690	700	760	-	-	-	-	-	860	-	-
RvD3	-	-	-	-	-	-	-	-	-	-	-	-
RvD4	-	-	-	-	-	-	-	-	-	-	-	-
RvD5	225	170.7	245.1	586	-	-	-	-	-	-	-	-
PD1	-	-	-	210	20	-	-	-	-	-	-	-
10S,17S-diHDHA	-	-	-	-	-	-	-	-	-	-	-	-
LXA ₄	12.7	-	-	32.9	-	-	36.2	-	10.8	-	52.2	-
LXB ₄	-	-	-	-	-	-	-	-	-	-	-	-
5S,15S-diHETE	628.4	70.6	155.5	596.2	22.2	31.8	145.7	-	16.4	17.5	57.9	31.9
AT-LXA ₄	52.1	14.3	-	39.2	-	-	39.6	-	25.8	-	57	-
Eicosanoid Metabolome LTB ₄	204.1	25.4	77.8	551.1	20.4	120.1	86.1	-	-	56.3	180.7	-
20-OH-LTB ₄	13.4	10.9	54.5	29.8	-	-	-	-	-	21.9	-	-
5S,12S-diHETE	54.6	35	19.4	120.7	-	23.4	-	-	-	-	-	-
PGD ₂	35	20.9	12.8	52.2	-	11.8	27.2	-	-	11.9	-	12.7
PGE ₂	60.6	34.2	209.1	121.7	24.8	135.1	-	-	-	36.3	70.5	46.1
PGF _{2α}	61.3	30.3	198.7	133.7	-	51.5	-	-	-	-	-	-
Pathway Markers & Precursors												
17-HDHA	300.2	213.5	1094.1	926.8	38	69	54.3	19.8	13.8	29.2	130.5	170.8
14-HDHA	248.4	69.6	141	612	41.4	69.7	37.6	17.2	10.6	178.8	-	101.4
18-HEPE	70.3	24.2	-	311.7	-	148.5	-	-	-	-	-	-
15-HETE	3300	2960	7100	12020	150	360	1050	160	110	160	1730	1380
12-HETE	8054.3	1494.9	1793.9	14297.2	454.5	468.8	1416.5	443.1	225.5	513.9	2595.5	507.7
5-HETE	990	140	200	1160	-	150	190	60	30	90	200	100

=limit of detection; limit ~0.1 pg; see methods and text for details *Results expressed in pg/100 µL

Table 4**SPM and Eicosanoid Ocular Functions**

LM	Main Bioactions	Dose	References	Concentration within Human Tears*
RvD1	Reduces vaso-obliteration and neovascularization	10 ng/day	Connor et al. [13]	1.1 nM
	Reduction of inflammatory cytokines and mediators; reduction of PMN transmigration	50 nM; 50–200nM	Tian et al. [20]	
	Block LTD ₄ stimulated goblet cell secretions	10 ⁻⁹ –10 ⁻⁸ M	Dartt et al. [11]	
	Regulation of histamine-stimulated goblet cell secretions	10 ⁻⁹ M	Li et al. [22]; Hodges et al. [34, 35]	
	Block angiogenesis in inflamed cornea	100 ng/10μL/mouse	Hua et al. [14]	
	Reduction of inflammation in rat eye following uveitis	10-100-1000 ng/kg	Settimio et al. [17]	
RvE1	Reduces vaso-obliteration and neovascularization	10 ng/day	Connor et al. [13]	N/A
	Reduction of inflammatory cytokines and mediators; reduction of PMN transmigration	50 nM; 50–200nM	Tian et al. [20]	
	Block LTD ₄ stimulated goblet cell secretions	10 ⁻⁹ –10 ⁻⁸ M	Dartt et al. [11]	
	Block angiogenesis in inflamed cornea	100 ng/10μL/mouse	Jin et al. [15]	
	Inhibition of corneal inflammation induced by LPS and bacteria	2 μg/2 μL PBS	Lee et al. [16]	
	Reduces vaso-obliteration and neovascularization	10 ng/day	Connor et al. [13]	
PD1	Increased corneal re-epithelialization and attenuation of thermal injury	1 μg	Gronert et al. [10]	5.8 nM
	Provides protection from oxidative-stress induced apoptotic damage in the retina	50 nM	Mukherjee et al. [36]	
	Restores corneal nerve integrity and function after surgery	100 ng, dropwise (topical)	Cortina et al. [37]	
	Decrease in inflammation, increase in wound healing; overall corneal & stroma protection	1 μg/eye in 10 μL PBS (mice)	Kakazu et al. [19]	
LXA ₄	Effector T cell inhibition, regulatory T cell amplification, dry eye pathogenesis reduction	100 ng topically (3x/day), 1 μg systemically (daily)	Gao et al. [12]; Hodges et al. [34, 35]	935 pM
	Reduction of VEGF-A and FLT4 expression and inflammatory angiogenesis	100 ng (3x daily)	Leedom et al. [38]	
	Attenuation of chemokine formation	1–1000 ng (3x daily)	Biteman et al. [18]	
	Increased wound healing/re-epithelialization in cornea	1 μg (3x daily)	Biteman et al. [18]	
	Suppression of VEGF-A induced hemoangiogenesis; control innate immune cell infiltration	100 ng/mouse	He et al. [39]	
AT-LXA ₄	Enhancement of endothelial cell wound closure and healing in cornea;	100 nM	He et al. [39]	1.1 nM
LTB ₄	Protection of corneal integrity during wound healing	100 nM	He et al. [40]; Hodges et al. [34]	16.4 nM
	Stimulation of conjunctival goblet cell mucous secretion	10 ⁻¹⁰ M	Dartt et al. [11]	
PGE ₂	Increase in inflammatory cytokines (IL-1β & IL-6) and intraoperative miosis following cataract surgery	25.6 pg/mL– 64.2 pg/mL	Wang et al. [41]	3.5 nM

LM	Main Bioactions	Dose	References	Concentration within Human Tears [*]
PGF ₂ α	Significantly higher presence in Dry Eye (DE) disease patient tears, compared to healthy control tears	2.72 $\pm$ 3.42 ng/mL (average; n=23)	Shim et al. [42]	3.8 nM
	Correlation with low tear osmolarity, meibomian gland plugging, and corneal staining	13.7 $\pm$ 3.1 pg/tear sample	Walter et al. [24]	
	Reduction in proliferation and adipogenesis	10 ⁻⁸ –10 ⁻⁶ M	Draman et al. [43]	
	Reduction in intraocular pressure (IOP)	50 μ g/mL topical	Lusky et al. [44]	

^{*} values from a representative donor