Changes in the Lipidomic Signature in Response to Burn Injury and the Effects of Elevated 15-HETE on Skeletal Muscle Wasting and Metabolism

Research Thesis

Presented in partial fulfillment of the requirements for graduation with research distinction in the undergraduate colleges of The Ohio State University

by

Ashley Keenan

The Ohio State University

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Project Advisor: Professor Kristin Stanford, Department of Physiology and Cell Biology

Abstract:

Patients with severe burn injuries experience dramatic changes in metabolism that are responsible for much of the pathophysiology of severe burns. These pathological changes begin with an ebb phase, which is an initial hypometabolic state, followed by a flow phase, which is a hypermetabolic state that typically starts on the third day post burn injury. Among the metabolic alterations following severe burn injury, massive lipolysis of the white adipose tissue (WAT) results in a dramatic increase in circulating lipids. In our lab, we have seen that this WAT lipolysis precedes other deleterious effects that are seen in response to burn injury including severe muscle wasting. Thus, we investigated the effect of severe burn injury on the concentration of signaling lipids, or lipokines, in plasma samples collected from 9 adult male and female burn patients with over 60% TBSA (Total Body Surface Area) burn injuries at each consecutive surgery following their admission to the hospital. Control samples were obtained from 9 age-matched male subjects. The serum samples were analyzed via MS/MS^{ALL} shotgun lipidomics. One-way ANOVAs determined which lipid species were significantly increased in circulation in response to the severe burn injury at each sequential surgery compared to normal subjects. There were significant increases in the circulation of eighteen different lipid species throughout specific time points post burn injury including eleven arachidonic acid metabolites (18-HETE, 5-HETrE, 11-dehydro TxB2, 15-HETE, 8-HETE, 18-HETE, 18-HEPE, 13,14-dihydro-15-keto PGE2, 13,14-dihydro-15-keto PGF2a, PGD2, and PGB2). Arachidonic acid metabolites have been seen to induce skeletal muscle wasting in myocytes, thus, the upregulation of arachidonic acid metabolites suggests that these signaling lipids may play a role in the muscle wasting seen in burn patients. To investigate this, the arachidonic acid metabolite that was most significantly elevated across all 9 burn patients, 15-HETE, was incubated with C2C12 myotubes for 4 hours at 0nM, 30nM, 100nM, and 300nM concentrations and qPCR was done to determine the expression of 3 ubiquitin-proteasome pathway genes, Fbxo32, Psma7, and Psmd4. The

results showed that 15-HETE degreased expression of these genes indicating that it may down-regulate this pathway.

Introduction:

Patients with severe burn injuries experience dramatic metabolic changes that greatly influence the pathophysiology of the response to burn injury (Caldwell et Al., 1960). These metabolic changes involve an initial ebb phase which is a hypometabolic state lasting throughout the first three days post burn injury and is referred to as the initial shock response. During the ebb phase, there is a reduced demand for nutrients. It has been seen that the longer this state lasts, the better the outcome (Brenznock, 1980). The ebb phase is followed by a flow phase which is a hypermetabolic state starting on the third day post burn injury and lasting up to several weeks. (Wolfe, 1981). This increased metabolism is due in part to the increased demand of the injured tissues for nutrients and energy sources for healing and due to the loss of insulation normally provided by intact skin (Wade, 2013).

The flow phase results in a massive loss of body mass including both fat mass and lean body mass (Wade, 2013). During this time there is massive lipolysis resulting in an increase in circulating signaling lipids (Yo et al., 2013), which are lipid molecules that bind to receptors and induce cellular responses. One particularly devastating outcome of these metabolic alterations during the flow phase is skeletal muscle atrophy (Cao et al., 2018). This skeletal muscle atrophy greatly compromises the patient's ability to resume normal activities following recovery from a burn injury (Wade, 2013). In our lab, we have seen that this lipolysis precedes skeletal muscle atrophy associated with severe burn injury in rats. Therefore, we hypothesized that signaling lipids released during this massive lipolysis may be responsible for other deleterious effects that are seen in response to severe burn injury including skeletal muscle wasting.

The signaling lipids we investigated fall into 3 different classes. Eicosanoids are derivatives of 20 carbon polyunsaturated fatty acids called eicosapolyenoic acids. Eicosanoids include prostaglandins, thromboxanes, leukotrienes, endocannabinoids, and isoecosanoids and have strong pro-inflammatory effects. Octadecanoids are derivatives of 18 carbon unsaturated fatty acids. Docosanoids are derivatives of 22 carbon fatty acids such as the omega-3 fatty acid, docosahexaenoic acid, and many have anti-inflammatory properties.

The pathway of protein catabolism that is proposed to be responsible for the skeletal muscle wasting seen in response to severe burn injury is the ubiquitin-proteasome pathway. Most proteins in the cytosol and nucleus of eukaryotic cells are degraded via the ubiquitin-proteasome pathway (Vogus et al., 1991). Additionally, studies have also shown that genes involved in regulating the ubiquitin-proteasome pathway are upregulated in response to severe burn injury (Padfield, 2005). Of the ubiquitin-proteasome pathway genes that are upregulated in response to burn injury, the most dramatic increases were seen in Fbxo32, Psmd4, and Psma7 (Padfield, 2005). Fbxo32 is a skeletal muscle specific gene that has been shown to be required for skeletal muscle atrophy (Bodine, 2001). Fbxo32 codes for a protein that functions as a subunit of the SCF (Skp1, Cullin, F-box protein) ubiquitin ligase complex, also called E3 ligase. The function of E3 ligase is to transfer ubiquitin to the protein substrate and thus tag the protein for degradation by the proteasome. Fbxo32 has also been seen to be dramatically upregulated during skeletal muscle wasting (Mei, 2015). Psmd4 and Psma7 are both are subunits of the 26S proteasome. The Proteasome functions to degrade proteins that have been tagged with ubiquitin.

Materials and Methods:

Lipidomics and Data Analysis:

We investigated the effect of severe burn injury on the concentration of 88 signaling lipids in circulation throughout the first 33 days following severe burn injury. Plasma samples were taken from 9

adult male and female patients with at least 60% total body surface area burn injuries at each consecutive surgery following their admission to the hospital. Control samples were obtained from 9 age-matched male subjects. The serum samples were analyzed via MS/MS^{ALL} shotgun lipidomics to determine the concentration of 88 signaling lipid species in each plasma sample collected. Lipidomics was performed using a mixture of deuterium-labeled internal standards added to aliquots of thawed serum, followed by cold methanol for Solid Phase Extraction. The supernatant was then acidified and Solid Phase Extraction was performed. The methyl formate fractions were collected, dried under nitrogen, reconstituted in MeOH:H2O, centrifuged, and the supernatant was then analyzed using the LC–MS/MS mediator lipidomics platform (Stanford, 2018).

The data were pooled according to time post injury to generate six time points for comparison. The first time point groups samples taken between days 2 and 4 post burn injury. The second time point corresponds to samples taken between days 6 and 8 post burn injury. The third time point groups samples taken between days 10 and 12 post burn injury. The fourth time point groups samples taken between days 15 and 19 post burn injury. The fifth time point includes samples taken between days 25 and 26 post burn injury. Finally, the sixth time point groups samples taken between days 29 and 33 post burn injury.

Statistical analysis:

One-way ANOVAs determined which lipid species were significantly increased in circulation in response to the severe burn injury at each sequential time point compared to normal subjects.

Cell Culture and Incubations:

C2C12 myoblasts were cultured in growth medium (DMEM high glucose, 10% fetal bovine serum and 1% penicillin/streptomycin) at 37°C in an atmosphere of 5% CO2 in air on treated 6 well plates. Differentiation of the C2C12 myoblasts into myofibers was initiated when the cells reached 90-100%

confluence. To differentiate the cells, they were washed twice with PBS and replenished with differentiation media (DMEM with high glucose, 2% horse serum, and 1% penicillin/streptomycin) and then incubated at 37°C in air.

Differentiated cells were incubated with dilutions of 15-HETE in differentiation media. The dilutions used were 0 nM 15-HETE as a control, 30 nM 15-HETE, 100 nM 15-HETE, and 300 nM 15-HETE. The myocytes were incubated with these dilutions and the samples were collected for analysis of gene expression after 4 hours of incubation.

RNA extraction and qPCR:

At each time point, incubation was terminated via aspiration of differentiation media. Each well was washed with PBS prior to lysis with TRIzol reagent. RNA was extracted from the samples and qPCR was performed to determine the expression of three genes involved in protein degradation, Fbxo32, Psmd4, Psma7. Expression of GAPDH was used as a control. Primer sequences are included in the supplementary table (table 2).

Results:

There was a peak increase in the total concentration of signaling lipids during the flow (hyperdynamic) phase between days 10 and 12 post burn injury (figure 1). We performed one-way hierarchical clustering of the average concentrations of individual signaling lipid species at each time point post burn injury and the average signaling lipid concentration in normal unburned subjects which indicated that the signaling lipid signature in the burn patients was different than that of the normal unburned subjects (figure 2). Furthermore, the signaling lipid signature changed over time post burn injury (figure 2).

Signaling lipids species belonging to different classes peaked in concentration at specific time points post burn injury. Specific docosanoid signaling lipid species fluctuated in concentration with different lipids increasing in concentration at specific time points starting on day 6 post burn injury and ending after day 26 post burn injury (figure 4 A). Octadecanoid signaling lipid species showed a peak increase starting on day 15 post burn injury and falling after day 26 post burn injury (figure 4 B). Eicosanoid signaling lipid species contributed the most to the dramatic increase in signaling lipids seen between days 10 and 12 post burn injury. Dramatic increases were seen in eicosanoid signaling lipids beginning on day 10 post burn injury and falling after day 28 post burn injury (figure 4 C).

Eighteen different lipid species were significantly increased throughout specific time points post burn injury (figure 3) including eleven arachidonic acid metabolites (18-HETE, 5-HETrE, 11-dehydro TxB2, 15-HETE, 8-HETE, 18-HETE, 18-HEPE, 13,14-dihydro-15-keto PGE2, 13,14-dihydro-15-keto PGF2a, PGD2, and PGB2). Arachidonic acid metabolites belong to the eicosanoid class of lipids, of which many are known to have a pro-inflammatory function. Additionally, arachidonic acid and its metabolites have been shown to increase protein catabolism in C2C12 myocytes (Prisk et al., 2013), suggesting that arachidonic acid and its metabolites could contribute to skeletal muscle wasting.

Compared to the average level of lipokines in the unburned patients, the burn patients showed a significant increase in the lipokines 11-dehydro TxB and 5-HETrE during days 2 to 4 post injury (figure 4 D and E). 13, 14-dihydro-15-keto PEG2 and 15-HETE increased significantly compared to the unburned patients days 2-8 post injury (figure 4 H and K). PD1, PGB2, and Tetramor-PGFM increased significantly during days 6 to 8 compared to unburned patients (figure 4 E, F, and M). 8-HETE, RvD1, 13-HOTrE/13-HOTrE(r), and 13-HODE were significantly increased days 10 to 19 post injury compared to unburned patients (figure 4 B, C, L, and O). 6-keto-PGF1a and 18-HEPE increased significantly in circulation during days 15 to 19 compared to unburned patients (figure 4 G and P). 13, 14-dehydro-15-keto PGF2a, 9(10)-EpOME, and 19, 20-diHDPA increased significantly during days 25-26 compared to unburned patients

(figure 4 A, Q, and R). PGD2 and 18-HETE increased significantly during days 29 to 33 compared to unburned patients (figure 4 J and N). This data is summarized in table 1. These fluctuations of signaling lipids suggest that the various lipokines upregulated during the ebb and flow phase of the response to burn injury may contribute to the timing of the metabolic changes.

The dramatic increase in the metabolites of arachidonic acid during the flow phase of the response to severe burn injury suggests that one or several of these metabolites may be involved the skeletal muscle atrophy seen in response to burn trauma. Of these metabolites, 15-HETE was consistently elevated in all 9 burn patients in the first 7 days post burn injury. This suggests that 15-HETE may be an important metabolite involved in regulating protein catabolism. We hypothesized that 15-HETE may regulate skeletal muscle wasting through inducing the upregulation or down regulation of genes in the ubiquitin-proteasome pathway. To test this hypothesis, we incubated C2C12 myocytes with 0nM, 30nM, 100nM and 300nM concentrations of 15-HETE for 4 hours and then performed qPCR to determine if stimulation with 15-HETE altered the expression of Fbxo32, Psmd4, and Psma7 in these skeletal muscle cells.

When testing the primers, the Fbxo32 primer did not work and was therefore omitted from the results. Both Psma7 and Psmd4 gene expression showed an inverse relationship to 15-HETE concentration. There were significant increases in both Psmd4 and Psma7 gene expression with decreasing concentration of 15-HETE and the highest expression of both Psmd4 and Psma7 were seen in the 0nM 15-HETE control samples after 4 hours of incubation (figure 6).

Figure 1:

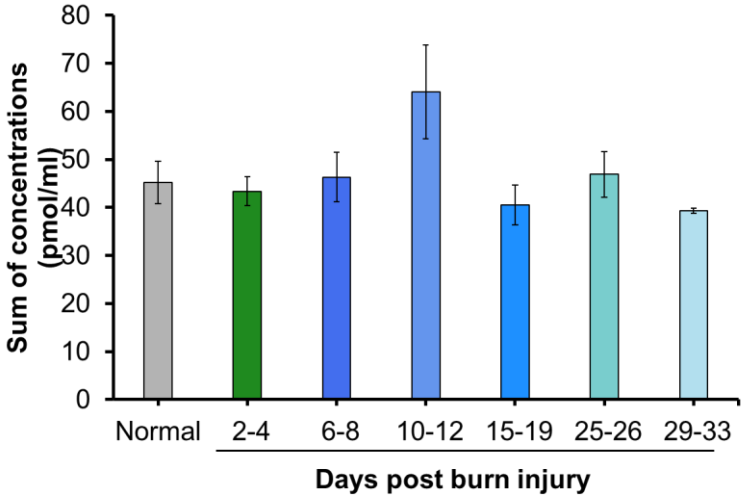


Figure 1: Average total signaling lipid concentration at consecutive time points post burn injury. Normal represents the average signaling lipid concentration in 9 age-matched unburned subjects. Time points are 2-4 days, 6-8 days, 10-12 days, 15-19 days, 25-26 days, and 29-33 days post burn injury.

Figure 2:

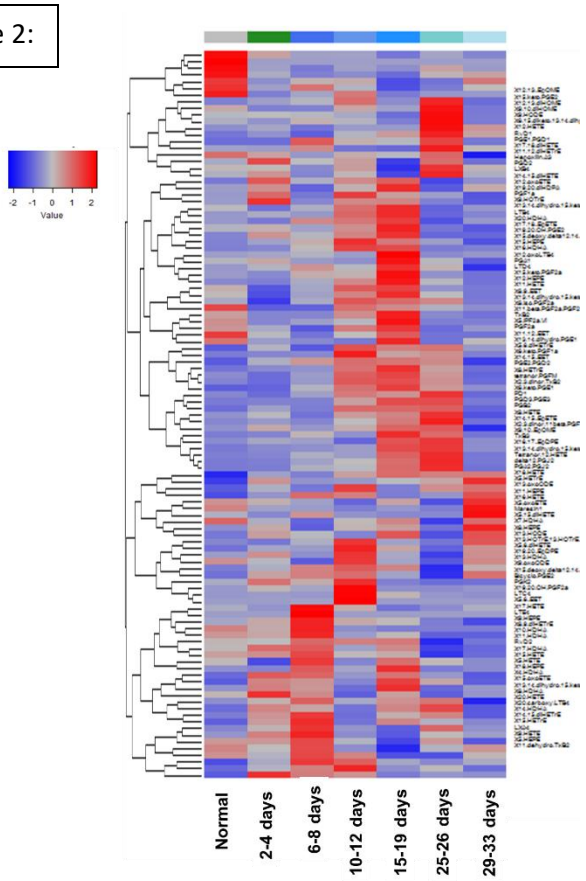


Figure 2: Heatmap representing the one-way hierarchical clustering of average concentrations of individual signaling lipid species at each time point post burn injury and the average signaling lipid concentration in normal unburned subjects.

Figure 3:

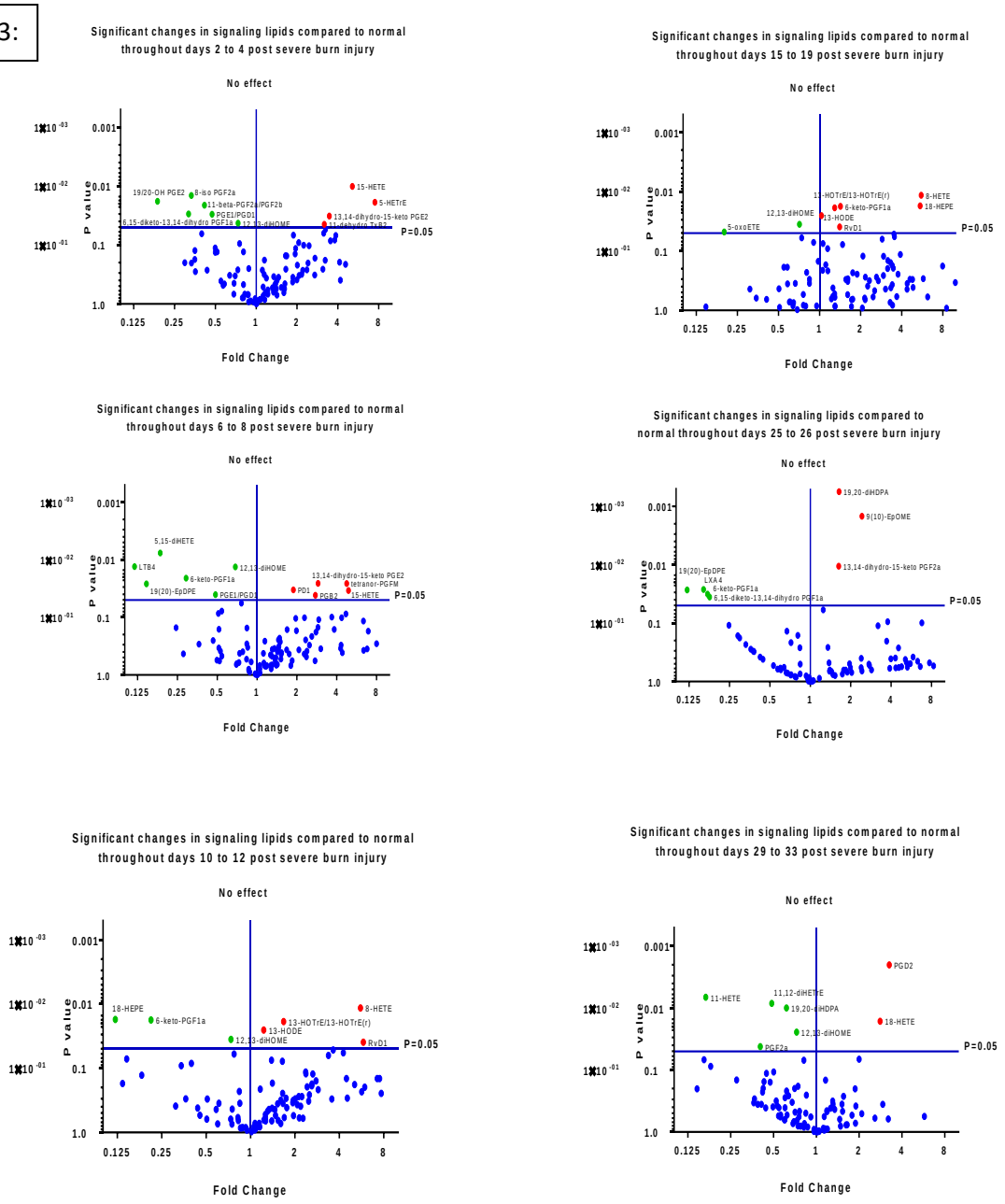
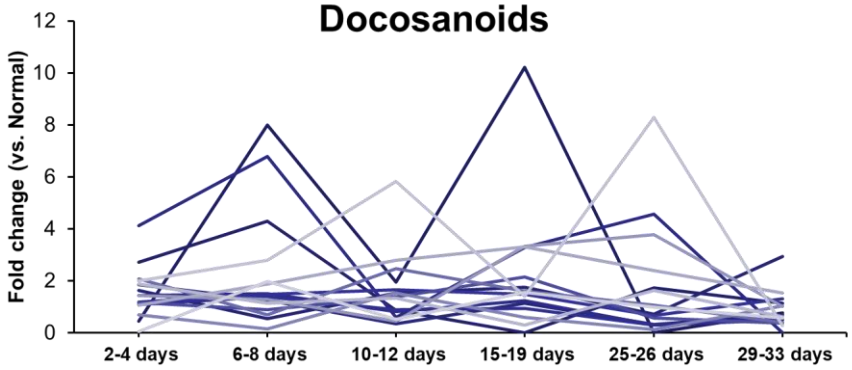


Figure 3: Volcano plots comparing the fold change in concentration of each signaling lipid species in plasma samples from 2 to 4 days post burn injury, 6 to 8 days post burn injury, 10 to 12 days post burn injury, 15 to 19 days post burn injury, 25 to 26 days post burn injury, and 29 to 33 days post burn injury to the average concentrations in plasma samples from normal unburned subjects. Red dots indicate an increase ($p < 0.05$) and green dots depict a decrease ($p < 0.05$) in serum concentration vs. Normal.

Figure 4:

A



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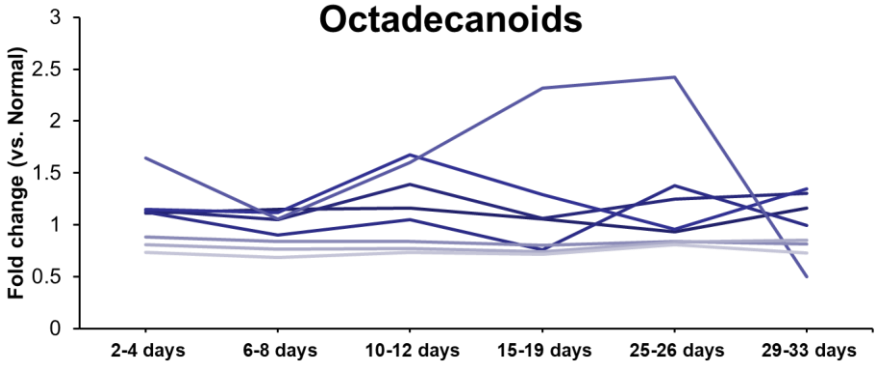

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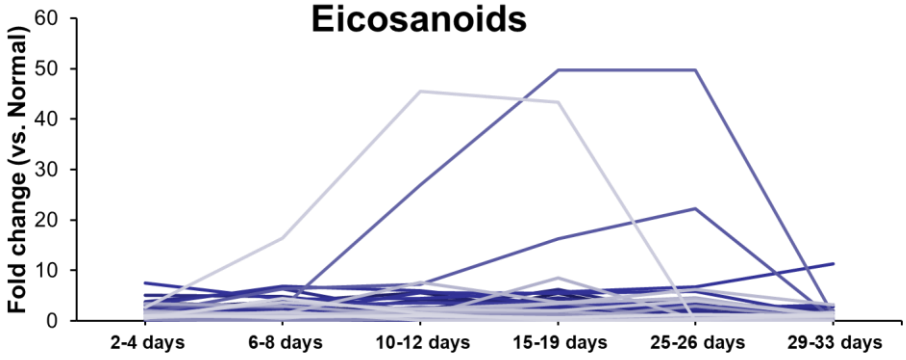
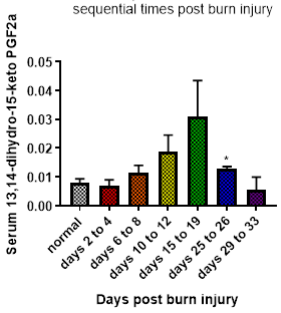


Figure 4: Fold change of the average concentration of docosanoid, octadecanoid, eicosanoid signaling lipid species in serum of patients at consecutive time points post burn injury compared to the average plasma concentration in 9 normal unburned subjects. Lines represent an individual signaling lipid species.

Figure 5:

A



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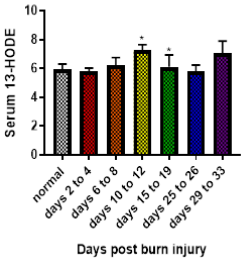

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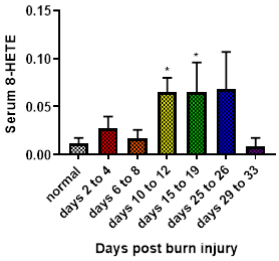

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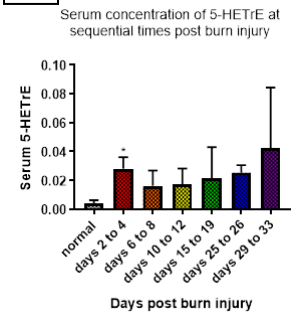

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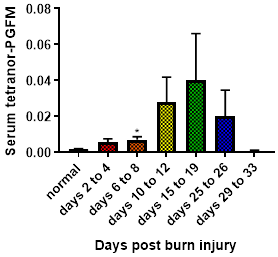

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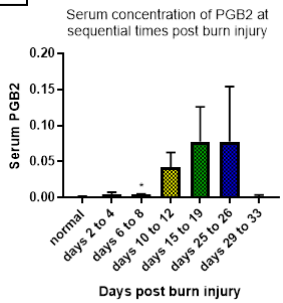

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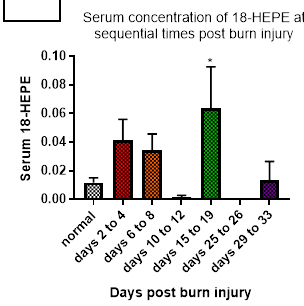

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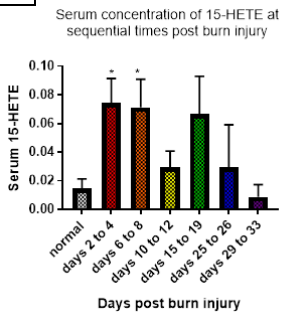

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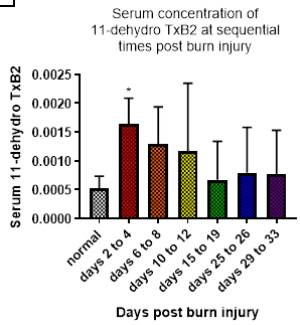

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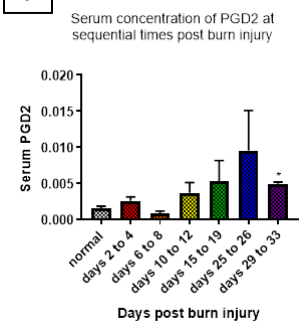

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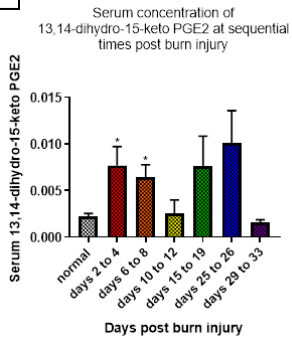

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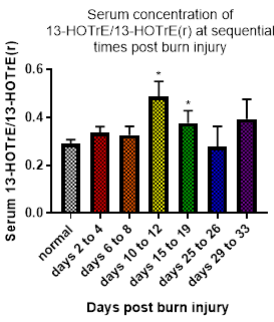

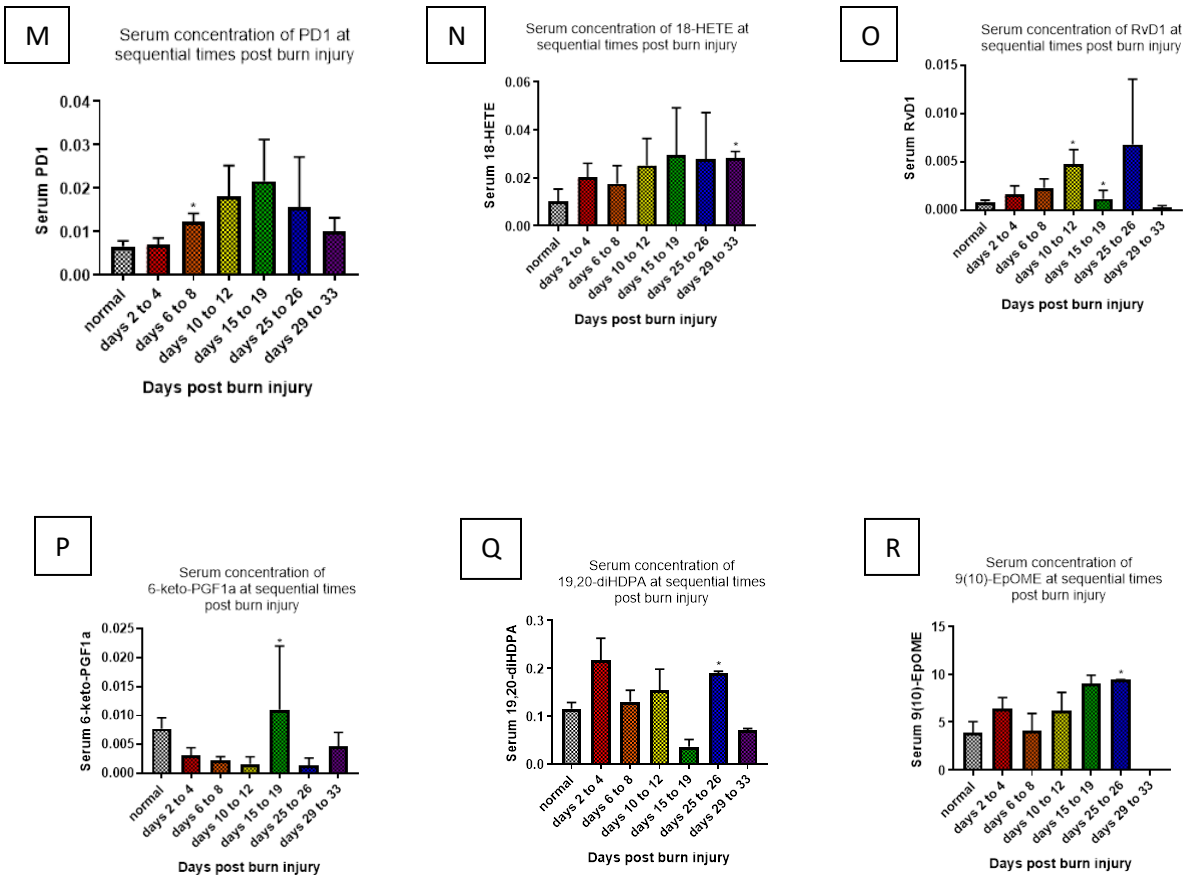

Figure 5: Average plasma concentration at each consecutive time point post burn injury of the signaling lipid species that significantly increased at one or more time points post burn injury and the average plasma concentration of 9 normal unburned subjects.

Figure 6:

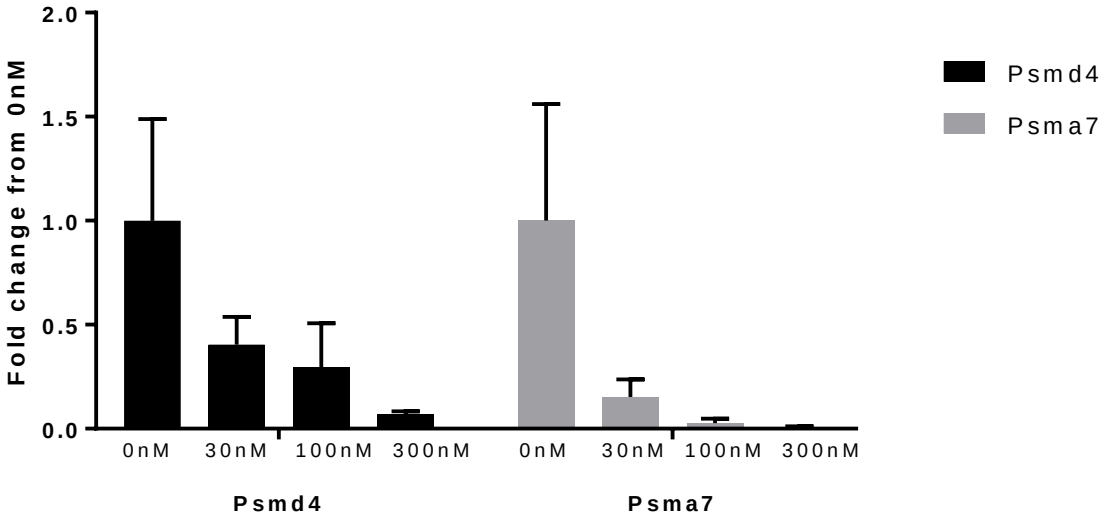

Figure 6: Average gene expression of Psmd4 and Psma7 after 4 hours of incubation with 0nM, 30nM, 100nM, and 300nM 15-HETE.

Table 1:

Lipid Species	2-4 days	6-8 days	10-12 days	15-19 days	25-26 days	29-33 days
11-dehydro TxB	↑					
5-HETrE	↑					
13, 14-dihydro-15-keto PEG2	↑	↑				
15-HETE	↑	↑				
Tetramor-PGFM		↑				
PD1		↑				
PGB2		↑				
8-HETE			↑	↑		
RvD1			↑	↑		
13-HOTrE/13-HOTrE(r)			↑	↑		
13-HODE			↑	↑		
6-keto-PGF1a				↑		
18-HEPE				↑		
13, 14-dehydro-15-keto PGF2a					↑	
9(10)-EpOME					↑	
19, 20-diHDPA					↑	
PGD2						↑
18-HETE						↑

Table 1: Signaling lipid species increased at individual time points post burn injury.

Discussion:

In this study, we investigated the effects of severe burn injury on the concentration of circulating lipokines in 9 adult male and female burn patients with over 60 % TBSA burn injuries. Plasma samples were collected at each sequential surgery following their admission to the hospital and the concentration of 88 signaling lipid species was determined via MS/MS^{ALL} Lipidomics. The concentrations of each lipid species in plasma samples taken at each sequential surgery post admission to the hospital were compared to the average values in 9 age-matched unburned male and female subjects. Significant increases were seen in 18 signaling lipid species at varying times post burn injury.

To evaluate the potential roles of these signaling lipids on skeletal muscle wasting, we assessed the expression of 3 genes involved in protein degradation (Fbxo32, Psma7, and Psmd4) upon stimulation of C2C12 myocytes with the predominant signaling lipid that was found to be increased across all burn patients in the first 7 days post burn injury, 15-HETE. The consistent elevation of this signaling lipid across all burn patients in the first 7 days post burn injury suggests that this arachidonic acid metabolite may have an important role in regulating the metabolic changes involved in the ebb and flow phases of severe burn injury. The down regulation of these genes of the ubiquitin-proteasome pathway in C2C12 myotubes in response to stimulation with 15-HETE suggests that 15-HETE may be responsible for the delay of protein catabolism seen during the ebb phase of the response to burn injury. The decrease in 15-HETE concentration concurrent with an increase in its down stream metabolites during the flow phase suggests that further metabolism of 15-HETE into different signaling lipids or other arachidonic acid metabolites may be responsible for initiating the increase in skeletal muscle wasting seen during the flow phase of the response to burn injury.

The peak increase that was seen in the total concentration of signaling lipids between days 10 and 12 post burn injury suggests that these signaling lipids may be responsible for the metabolic

changes seen during the hypermetabolic flow phase of the response to burn injury, including increased protein catabolism. During the flow phase following burn injury carbohydrate metabolism increases and skeletal muscle wasting begins (Tredget, 1992). This skeletal muscle wasting is thought to be due to the increased demand for amino acids as precursors to produce acute phase proteins and to be utilized in wound healing (Malagaris, 2018). Further studies to investigate the roles of each of these signaling lipids in protein catabolism and other metabolic effects may provide a way to anticipate metabolic changes in response to serum signaling lipid signature following injury. Further studies into the roles of these lipids may also elucidate potential targets for therapy by manipulation of the serum concentration of these signaling lipids either directly or through alteration of the function of enzymes involved in the production of these signaling lipids.

The class of lipids that contributed most dramatically to the spike in serum lipid concentration were eicosanoids, a class of lipids known to have pro-inflammatory functions. Dramatic increases were seen in eicosanoid signaling lipids beginning on day 10 post burn injury and falling after day 28 post burn injury. Included in the class of eicosanoids are the arachidonic acid metabolites of which eleven species were found to be significantly upregulated in circulation following burn injury (18-HETE, 5-HETrE, 11-dehydro TxB2, 15-HETE, 8-HETE, 18-HETE, 18-HEPE, 13,14-dihydro-15-keto PGE2, 13,14-dihydro-15-keto PGF2a, PGD2, and PGB2). Further studies into the individual roles of these signaling lipids on protein catabolism and skeletal muscle wasting may provide useful information for generating therapies to alter signaling lipid signature in repose to burn injury and provide a means to avoid the deleterious metabolic effects that may be regulated by these signaling lipids, including skeletal muscle wasting.

Following 4-hour incubation with 0nM, 30nM, 100nM, and 300nM 15-HETE, C2C12 myotubes showed degreased expression of Psma7 and Psmd4, indicating that these genes were down-regulated in response to stimulation with 15-HETE in skeletal muscle cells. This suggests that 15-HETE may have a role in down regulating these genes in the ubiquitin-proteasome pathway and thus down-regulate this

method of protein catabolism. Further studies investigating protein expression would also be beneficial to determine the effect of 15-HETE and other signaling lipids on the regulation of the ubiquitin-proteasome pathway in skeletal muscle cells.

These findings suggest that specific signaling lipids are increased in circulation in response to burn injury that may contribute to the pathophysiology of thermal injury. The identification of specific signaling lipids that may be responsible for metabolic changes associated with the ebb and flow phases of burn injury could provide potential targets for therapy directed at preserving skeletal muscle in burn injured patients. Additionally, the signaling lipid signature correlated with the metabolic changes seen at sequential time points post injury may assist in tailoring treatments and predicting pathological outcomes associated with these metabolic changes.

Citations:

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Table 2:

Gene	Forward	Reverse
Fbxo32	CTTCTCGACTGCCATCCTGGAT	TCTTTTGGGCGATGCCACTCAG
Psma7	ATCTGCGCCTTGGACGATAACG	TCAGACTCGCAATGTAGCGGGT
Psmd4	CCATCAACCAGCAGGAGTTTGG	CTGGCATCCATGTCAGCCGATT
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA