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Non-Invasive Systemic Biomarker of E-cigarette or Vaping use-Associated Lung Injury (EVALI): A pilot study

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Short running title: Systemic biomarkers of EVALI

Abstract

Background: Electronic cigarettes (e-cig) vaping, containing nicotine and/or Δ^8, Δ^9 or Δ^{10} or Δ^o tetrahydrocannabinol (Δ^n-THC) are associated with an outbreak of e-cig, or vaping product use, associated lung injury (EVALI). Despite thousands hospitalized with EVALI, much remains unknown about diagnosis, treatment and disease pathogenesis. Biomarkers of inflammation, oxidative stress, and lipid mediators may help identify e-cig users with EVALI.

Methods: We collected plasma and urine along with demographic and vaping-related data of EVALI subjects (ages: 18-35 yrs) and non-users matched for sex and age in a pilot study. Biomarkers were assessed by ELISA/EIA and Luminex-based assays.

Results: Elevated levels of THC metabolite (11-nor-9-carboxy-THC) were found in plasma from EVALI subjects compared to non-users. Levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), an oxidative DNA damage biomarker, and 8-isoprostane, an oxidative stress marker, were slightly increased in urine samples from EVALI subjects compared to non-users. Conversely, plasma levels of lipid mediators, including Resolvin D_1 (RvD_1) and Prostaglandin E_2 (PGE_2), were significantly lower in EVALI subjects compared to non-users. Both pro-inflammatory biomarkers, such as TNF-α, MIP-1β, RANTES, and GM-CSF, as well as anti-inflammatory biomarkers, such as IL-9 and CC10/16, were decreased in plasma from EVALI subjects compared to non-users, supportive of a possible dysregulated inflammatory response in EVALI subjects.

Conclusions: Significant elevations in urine and plasma biomarkers of oxidative stress, as well as reductions in lipid mediators, were shown in EVALI subjects. These non-

invasive biomarkers of 8-OHdG, 8-isoprostane, RvD1, and CC10/16, either individually or collectively, may serve as biomarkers in diagnosing future EVALI subjects.

Keywords: EVALI, e-cigarettes, biomarkers, oxidative stress, inflammation, lipid mediators, growth factors

Introduction

Electronic cigarettes (e-cigs) or electronic nicotine delivery systems (ENDS), invented in 2003 [1], were originally conceived as smoking cessation aids but are now a major avenue of nicotine consumption that is rapidly rising in popularity amongst young people [2]. Cannabis e-cigs (CECs) are an adaptation of the same technology used to deliver Δ^9-tetrahydrocannabinol (Δ^9-THC) and have become popular among adolescents and young adults [3]. In 2019, the CDC described an outbreak of e-cigarette, or vaping, product use-associated lung injury (EVALI), which as of February 18, 2020, resulted in 2,807 hospitalizations/cases and 68 deaths in the US [4]. The disease presents with symptoms of cough, shortness of breath, chest pain, nausea, vomiting, diarrhea, abdominal pain, fever and chills [4-7]. The majority of patients/subjects with EVALI have reported the use of THC-based, counterfeit e-cig products [8]. In spite of thousands hospitalized with EVALI, there remains a debate on the harmfulness of e-cigs as well as their potential long-term health effects [9, 10].

Commonly used as a cutting agent in vaping products, vitamin E acetate (VEA) has been implicated as a key agent in the occurrence of EVALI since its discovery in the

bronchoalveolar lavage fluid (BALF) of 48 participants in a study of 51 known lung injury cases [11]. However, VEA was not found in the healthy participants, including 18 e-cig users [11]. Hence, there is some doubt over whether VEA is the sole causative agent. Chemical analyses of illicitly sourced CECs obtained from EVALI subjects demonstrated that these products can contain many different ingredients and adulterants, such as glycerol fatty acid esters, long-chain hydrocarbons, plasticizers, terpenes, metals and more [12, 13]. Aerosols generated from CECs contain concerning levels of irritants and carcinogens including benzene, isoprene, methyl vinyl ketone, butadiene, toluene, xylenes and more [3, 14]. Muthumalage *et al.* showed the molecular mechanisms of injurious responses by inhaled illicit cartridges that are shown to cause EVALI [13]. There is also debate on the protective effects of vitamin E acetate against toxicity *and vice-versa* [15-18]. Considering this, it is important to investigate further the mechanisms of EVALI disease development and progression among e-cig users.

Several potential mechanisms have been proposed for the pathogenesis of EVALI [19]. *In vivo* murine inhalation studies using EVALI subject-sourced CECs demonstrate that these products can cause cytotoxicity, epithelial barrier dysfunction, and inflammation [8]. Mice exposed to EVALI subject-sourced CECs showed increased levels of eicosanoid inflammatory mediators and leukotrienes in BALF, as compared to the mice exposed to VEA or other cutting agents [8]. To date, no specific biomarkers for EVALI have been identified and diagnoses are primarily achieved through a process of elimination with the appropriate history of recent e-cigarette use, new physical exam findings, and clinical imaging [4-7].

Systemic biomarkers of oxidative stress, inflammation, and lipid mediators have been studied in smokers [20-22], COPD patients [23-25] and ENDS users [8, 9, 26]. However, most studies at best can only imply and extrapolate to EVALI clinical cases.[8, 26-29] Little is known to assess potential biomarkers of EVALI. While attempting to bridge this gap in the research literature, the aim of this study is to identify potential biomarkers of oxidative stress, lipid mediators, and inflammatory responses that may play a role in the pathogenesis of EVALI.

Methods

Participants

This study was conducted at the University of Rochester Medical Center with the help of the Clinical Research Center (Rochester, NY). Participants were recruited through various local newspapers, magazine advertisements, and flyers around Monroe County along with word of mouth (IRB approval #CR00003968), or at the time of admission at Strong Memorial Hospital during initial diagnosis for EVALI. Subjects in the EVALI group met clinical criteria with: (1) recent use (within 90 days of hospitalization) of e-cigarette, or vaping, products, (2) bilateral ground-glass opacities on radiographic imaging, and (3) exclusion of other common causes for changes on chest imaging, including community-acquired pneumonia [30-31]. The majority (5 of 6) of samples from EVALI subjects were obtained prior to corticosteroid treatment. Participants were selected based on a self-reported questionnaire. To be eligible, all participants had to be between the ages of 18 and 35 years. Additionally, all participants were screened for a history of chronic illness such as heart and lung disease, diabetes, cancer, and/or current viral flu/pneumonia

infections. For non-users, participants also had to disclose if they were currently taking any anti-inflammatories or corticosteroid drugs and if so, were removed from the study. Participants were excluded if pregnant or breastfeeding. Participants in the non-user group were required to never have smoked any tobacco products. Participants in the EVALI group were screened for history of e-cigarette use/vaping in addition to their diagnosis. Written informed consent was obtained from all study participants.

Demography

During the questionnaire, participants provided their age, sex, and ethnicity. E-cigarette use duration, e-cigarette duration per session, e-cigarette smoking frequency, flavor type and approximate amount of nicotine (high/low) in each flavor were also disclosed. The participants were categorized into two groups: Non-user and EVALI subjects (Table 1).

Sample Collection

Whole venous blood (approximately 20-25 mL) was collected from participants in vacutainer tubes containing EDTA and spun at 1000 g for 10 min to obtain plasma and stored immediately at -80˚C until further use. Participants provided urine and samples were immediately stored at -80˚C for further use.

Measurement of Biomarkers by Multiplex Panel Assay

Cytokine/mediator levels in plasma from non-users and EVALI subjects were quantified by Bio-Plex Pro Human 27-Plex assay (M500KCAF0Y, Bio-Rad, Hercules, CA) assay with a 1:4 sample dilution per manufacturer’s instructions on a FLEXMAP 3D instrument (Luminex, Austin, TX).

Measurement of Biomarkers by ELISA/EIA

Commercially available kits were used for quantifying Resolvin D_1 (Cat #500380, Cayman Chemical, Ann Arbor, MI), Resolvin D_2 (Cat#501120, Cayman Chemical, Ann Arbor, MI), Resolvin E_1 (Cat#MBS286046, MyBioSource, San Diego, CA), Lipoxin B_4 (Cat#MBS9380211, MyBioSource, San Diego, CA), Prostaglandin E_2 (Cat#514010, Cayman Chemical, Ann Arbor, MI), Cotinine (Cat#1-2002, Salimetrics, Carlsbad, CA), and CC10/16 (Cat# DUGB00, R&D Systems, Minneapolis, MN), ,in plasma. In urine samples, 8-Isoprostane (Cat #516351 Cayman Chemical, Ann Arbor, MI), 8-OHdG (Cat#4380-096K, R&D systems, Minneapolis, MN) and THC metabolite, predominantly11-nor-9-carboxy-Δ^9-THC i.e. Δ^9-THC, (Cat#701570 Cayman Chemical, Ann Arbor, MI) were run according to the manufacturer's directions, respectively.

Statistical Analysis

Data from all assays were analyzed and graphed using GraphPad Prism9. An unpaired t-test as well as an outlier's test was used to determine statistical significance. A p-value less than 0.05 was considered significant.

Results

Six age- and sex-matched subjects with confirmed EVALI (n=6) and non-users (n=6) were included for biomarker testing. Baseline demographics, and if applicable, vaping status and hospitalizations details were obtained and summarized in Table 1. The average age of non-users and EVALI subjects was 22 years. The gender breakdown for both groups was 50% female to male. Non-users and EVALI subjects were mostly identified as

Caucasian (Table 1). The clinical presentation and median peak laboratory values for EVALI subjects were collected and summarized in Tables 2 and 3, respectively. All EVALI participants required hospitalization and met clinical criteria for 'confirmed' EVALI [4, 6]. Primary chief complaints were GI-related (3/6), respiratory (2/6), or combined (3/6) for EVALI subjects. Maximal respiratory support included supplemental oxygen (5/6) or invasive positive pressure (1/6). One EVALI subject disclosed known drug abuse and reported hypertriglyceridemia in the past medical history. All subjects received systemic steroids (median: 9 days). Based on the self-reported questionnaire, EVALI subjects had a preference for fruit-flavored e-cigs, followed by mint and candy flavors. Beverage and no flavor were also reported (Figure 1).

Plasma cotinine and THC metabolite levels in plasma and urine samples from EVALI subjects

Previous studies report high incidences of THC-based cartridges and associated e-cig products among those hospitalized with EVALI [7-9]. Considering this, all urine and plasma samples were evaluated for THC metabolite, i.e., (−)-11-nor-9-carboxy-Δ^9-THC and the nicotine metabolite, i.e., cotinine, respectively. Consistent with these prior reports, urinary levels of the THC metabolite were significantly elevated in EVALI subjects compared to non-users ($p=0.0112$, Figure 2). Plasma cotinine levels were also elevated in EVALI subjects, but not statistically different from non-users ($p=0.5815$), showing relatively low amount in EVALI users, but also suggesting that EVALI subjects are either dual users of e-cigs and tobacco cigarettes or vaped nicotine-containing e-cigs.

Oxidative Stress markers in plasma and urine from EVALI subjects

To determine if elevated reactive oxygen species (ROS)/oxidative stress secondary to vaping contributed to the hospitalization in EVALI subjects, we quantitated the levels of three oxidative stress markers- myeloperoxidase (MPO), 8-isoprostane, and 8-hydroxy-2′-deoxyguanosine (8-OHdG) - in the biological samples from all subjects. In plasma, MPO was not significantly different between groups (Figure 3a). Likewise, 8-isoprostane was insignificantly elevated in the urine of EVALI subjects when compared with non-users. However, the concentration of 8-OHdG ($p= 0.0258$) demonstrated a statistically significant increase in EVALI subjects as compared to non-users (Figure 3b).

Pro-Resolving Lipid Mediators in Plasma of EVALI subjects

Since many mediators of inflammation are derived from phospholipids or polyunsaturated fatty acids, levels of lipid-derived mediators, including Prostaglandin E_2 (PGE_2), Resolvin D_1 (RvD_1), Resolvin D_2 (RvD_2), Lipoxin B_4 (LXB_4), Thromboxane B_2 (TXB_2) and Resolvin E_1 (RvE_1), were measured in plasma from EVALI subjects and non-user. PGE_2 ($p=0.0342$) levels were significantly decreased in plasma from EVALI subjects compared with non-users (Figure 4). Similarly, RvD_1 (0.0062) demonstrated a significantly lower concentration in EVALI subjects compared with non-users. However, other mediators, such as RvD_2, RvE_1, LXB_4, and TXB_2 exhibited non-significant changes between the groups.

Inflammatory Biomarkers in Plasma

The levels of pro- and anti-inflammatory cytokines/chemokines were also measured in the plasma. Plasma levels of TNF-α, MIP-1β, RANTES, GM-CSF, IL-9 and CC10/16 demonstrated significantly lower levels in EVALI subjects than the non-users (Figure 5).

IL-1β levels, although not statistically significant, were lower in EVALI subjects compared to non-users (p=0.0509). Contrarily, we found non-significant increases between groups for MCP-1 (p=0.1528), IL-8 (p=0.3296), and IL-6 (p=0.3329). Other targets, like IL-1rα and eotaxin saw insignificant alterations in levels between EVALI subjects and non-users as well (Table 4).

Plasma Growth Factor Levels

PDGF-BB and B-FGF concentrations in plasma, while not statistically significant, were lower in EVALI subjects than their non-user counterparts (Figure 6). In contrast, VEGF levels were decreased, albeit insignificantly, for EVALI subjects when compared with non-users.

Discussion

In this study, plasma and urine from EVALI subjects and non-users were used in an effort to identify potential biomarkers of disease. We identified multiple inflammatory cytokines, pro-resolving lipid mediators, oxidative stress and DNA damage markers to be significantly different between EVALI subjects and non-user controls.

Evidence from the literature suggest that THC-based cartridge and associated e-cig product users are more susceptible to hospitalization due to EVALI [8]. In our self-reporting survey, only one of the subjects with EVALI admitted to using THC or CBD-based products for vaping. However, on urine analysis, multiple EVALI subjects, as well as non-user controls, demonstrated elevated levels of THC metabolite - (−)-11-nor-9-carboxy-Δ^9-THC. These findings are in agreement with a previous self-reported public-

survey that shows that EVALI subjects typically use THC-containing products more than e-cig users who did not develop lung injury [31]. They also found nicotine use to be more prevalent in those who did not develop EVALI [31]. However, multiple EVALI subjects in our study had slightly higher cotinine levels as compared to non-users. In fact, previous reports have also found significantly higher levels of cotinine amongst e-cig users when compared to non-users, with levels comparable to traditional smokers [26, 32, 33]. Our results confirm that dual users of both THC- and nicotine-containing products may place oneself at greater risk for hospitalization due to EVALI.

We found a significant increase in the levels of 8-OHdG in urine from EVALI subjects when compared to non-users. Similar findings were reported in e-cig users [26, 34] and cigarette smokers [35]. High levels of 8-OHdG indicate increased oxidative DNA damage and is a factor in the promotion of carcinogenesis [36]. Previous work by our group found an increase in the levels of 8-isoprostane in the urine from e-cig users as compared to non-users [26]. However, we did not see any significant change in the levels of MPO and 8-isoprostane (though there was a trend for increase) in the biological samples from EVALI subjects and non-users. The presence of increased levels of 8-OHdG supports that DNA damage plays a potentially important role in the pathogenesis of EVALI.

Resolvins D_1 (RvD_1) and D_2 (RvD_2) play a major role in dampening inflammation. Singh *et al*. previously demonstrated a significant decrease in the levels of RvD_1 in the plasma from e-cig users [26]. The levels of plasma RvD_1 were significantly decreased in EVALI subjects compared to non-user controls. RvD_1 is a lipid mediator that functions to dampen polymorphonuclear leukocytes infiltration and transmigration. Thus, lower levels of RvD_1 are in line with prominent serum neutrophilia seen in the majority of EVALI subjects

hospitalized with respiratory insufficiency, and may potentially contribute to failed lung repair after e-cig exposure and/or vaping THC products [26]. In contrast, we also found a significant decrease in PGE_2 levels in EVALI subjects compared to controls. PGE_2 induces inflammation through mast cell activation. One potential explanation for these dichotomous findings is the concurrent use of non-steroid anti-inflammatory drugs (NSAIDs) in those hospitalized with EVALI, which would falsely lower the PGE_2 levels seen in this group.

Cigarette smoke-induced oxidative stress is shown to activate the inflammatory response by upregulating cytokines, such as IL-6 and IL-8. IL-6 plays a key role in mediating acute phase response and is a prognostic biomarker in various acute organ injuries, including the lung. IL-8 functions in the chemotaxis and eventual phagocytosis of neutrophils and other granulocytes. Neutrophils are linked to inflammatory lung diseases, including COPD, asthma, bronchiolitis, respiratory distress syndrome, and interstitial pneumonia [37]. With multiple assays, we found IL-6 and IL-8 levels to be comparable between EVALI and non-users. When BEAS-2B cells were exposed to counterfeit cartridges, there were significantly higher levels of IL-6 and IL-8 compared to cells exposed to air, thus suggesting the eliciting of immune response of e-cig exposure [8]. Similarly, Singh *et al.* also reported a significant increase in IL-6 and IL-8 production among e-cig users when comparing the plasma inflammatory profiles with non-users [26]. Multiple explanations may account for these differences. One explanation is that immune suppression occurs through different cytokine pathways than that of e-cig exposure alone. A second possibility is that hospitalized EVALI subjects often presented later in the course of the

disease and after their treatments, peak IL-6 and IL-8 levels may have dissipated at the time of blood collection.

IL-9 is an activator of mast cells [38], and is significantly lowered in EVALI subjects compared to non-users. This interleukin has been linked to allergic asthma in mice [39], cancer [40], human allergic lung inflammation, and contribute to autoimmune disease [38]. IL-9 also has the ability to effect IL-13 and therefore affect epithelial cells of the lung and gut [38].

IL-1β, a pro-inflammatory cytokine, is linked to many acute and chronic inflammatory diseases, including acute lung injury. In this study, plasma IL-1β levels were lower, though not significant, for EVALI subjects when compared to the non-users. Our results are contrary to previous literature where IL-1β levels were significantly higher in BALF and saliva from e-cig users as compared to never smokers [9, 26]. However, when analyzed in plasma samples, there was a non-significant decrease in e-cig users [26]. This proves that the measurement of various pro-inflammatory mediators might vary based on the biological sample being tested as well as the time of collection.

MIP-1β, also known as CCL4, is a chemokine known to play a critical role in the chemotactic activity of monocytes through the CCR5 receptor, which has been connected to diverse immune responses. MIP-1β levels were found to be increased in natural killer cells, CD8+ T cells and CD4+ T cells in pregnant women exposed to the influenza A virus. Additionally, their levels correlate with the severity of influenza symptoms and viral replication along with a similar rise in kinetics after influenza infection like other chemokines. As a whole, the MIP family is implicated as important mediators of lung

disease. RANTES is a chemokine involved in leukocyte influx and bronchial hyper-responsiveness [41]. It has been established to play an important role in allergic lung inflammation and leukocyte infiltration [41]. In our study, MIP-1β and RANTES were significantly lowered in EVALI than non-users.

Club cell secreted protein (CC10/16) has several immune-regulatory activities, including inhibition of phospholipase A_2 [42, 43]. In past studies of COPD, asthma, idiopathic pulmonary fibrosis, sarcoidosis and other pulmonary issues, CC16 has shown promise as a potential biomarker of lung epithelial injury [42, 44]. Kropski *et al* found lower levels of CC16 in the plasma of acute lung injury patients when compared to acute cardiogenic pulmonary edema patients [42]. Not unexpectedly, when previously analyzed in e-cig users, there was only a minimal difference compared to normal subjects in both plasma and urine [26]. In our study, we found EVALI subjects to have significantly lower CC16 levels than their non-user counterparts. This is most likely due to the difference in severity at the time of presentation, with most EVALI subjects requiring hospitalization. It is possible that the lower levels of CC16 are secondary to alterations in alveolar epithelial permeability, club cell death, or changes in transcriptional activity within the remaining club cells[42]. However, CC16 remains a significant and biologically relevant biomarker for future investigation.

A potent inflammatory cytokine, TNF-α, has been implicated in various pulmonary diseases like asthma and COPD/emphysema. In a previous study, we found smaller concentrations of TNF-α in e-cig users compared to non-users, albeit non-significant [26]. This has concurred in the present study, where we found this to be true of EVALI subjects with the same level of non-significance.

MCP-1, which is also known as CCL2, is a chemokine that regulates the migration and infiltration of monocytes and macrophages in innate immunity [45]. This chemokine has been linked to atherosclerosis, inflammatory bowel disease, asthma and arthritis[45]. It was also connected to interstitial lung disease in pediatric patients with high levels, negatively correlating to restrictive lung function, forced vital capacity, total lung capacity and other lung disease severity scores [46]. In our previous study, the levels of MCP-1 in non-users and e-cigs users were indistinguishable from each other[26]. We observed non-significant higher concentrations in EVALI subjects compared to non-users in Luminex-based assays.

Platelet-derived growth factor (PDGF) mediates airway inflammation, remodeling in asthma, and plays a significant role in blood vessel formation [47]. Cucina *et al.* established that nicotine enhanced the release of PDGF-BB in endothelial cells [48]. Concurring with this, Singh *et al.* found the AA isoform to be significantly elevated in e-cig users, which established high levels of cotinine as well [26]. In contrast, our study found that the PDGF-BB isoform was insignificantly reduced in EVALI subjects compared to controls when we also showed an increase in nicotine amongst EVALI subjects.

To our knowledge, this is the first study to report changes in oxidative stress, lipid mediators, and inflammatory markers from blood and urine of EVALI subjects, and these biomarkers are differentially regulated as compared to e-cig users as shown by us recently [26]. There are some limitations. The first is that EVALI samples were collected after the EVALI subjects were admitted to the hospital, and nearly all subjects received drugs, including NSAIDs and antibiotics, prior to blood and urine collection. Most of the subjects received blood draws prior receiving treatment for EVALI with systemic steroids.

The second limitation is the small sample size. Furthermore, comparing the systemic biomarkers of acute lung injury (i.e. adult respiratory distress syndrome, ARDS) would have strengthened the study, however, such an investigation is beyond the scope of this pilot project. While thousands of young adults have been affected by EVALI, the incidence remains less after 2019. Subjects were recruited from a single institution, limiting sample size, however each sample was matched for age and sex.

Overall, this pilot study identified multiple potential EVALI biomarkers, including markers of oxidative stress, such as 8-OHdG and 8-isoprostane as well as reduced levels of pro-resolving lipid mediator, RvD_1 and anti-inflammatory airway epithelial marker, CC10/16. These findings provide a strong basis for the use of these potential biomarkers in the diagnosis of EVALI.

Author Contributions: SP, GK, TM, MDM and IR conceived and designed the experiments, wrote and edited the manuscript. IR obtained research funding and study design and experimental plans/assays. IR and MDM recruited the volunteers. SP, TM, GK performed the experiments and SP and TM analyzed data. All authors contributed to manuscript preparation and approved the final version before submission.

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List of Abbreviations:
E-cig – Electronic Cigarette
EVALI – E-cigarette, or Vaping, use-Associated Lung Injury
GI- Gastrointestinal
COPD- Chronic Obstructive Pulmonary Disease
8-OHdG – 8-Hydroxydeoxyguanosine

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Table 1. Demographic and vaping status characteristics of non-users and EVALI subjects

Characteristics	Non-users (n=6)	EVALI subjects (n=6)
Age, years		
Mean (SD)	22 (2.44)	22.3 (6.4)
Range	18 - 25	19 - 35
Sex, n (%)		
Male	3 (50)	3 (50)
Female	3 (50)	3 (50)
Demography, n		
Caucasian	5	3
African American	1	2
Asian	0	0
Not Hispanic/ Latino/Black (Not White)	0	1
E-cig use, n (%)		
Vaping Frequency	N/A	
10+ times/day		3 (50%)
3 - 5 times/day		2 (33.3%)
< 1 time/day		1 (16.6%)
Duration of Vape per Session	N/A	
20+ min		0
10 - 14 min		2 (33.3)
5 - 9 min		0
< 5 min		4 (66.6)
Vaping years	N/A	
>5+ yr.		0
2 - 5 yr.		2 (33.3)
1 - 2 yr.		2 (33.3)
< 1 yr.		2 (33.3)
Hospitalizations, n (%)	0	6(100)

N/A: Not Applicable

Table 2. Clinical Presentation of EVALI subjects (n=6)

Chief Complaint	
Gastrointestinal (GI)	3/6 (60%)
Respiratory	2/6 (33%)
Combination	3/6 (50%)
Respiratory support	
Positive pressure ventilation	1/6 (17%)
Supplemental oxygen	5/6 (83%)
No respiratory support	1/6 (17%)
Antibiotics	6/6 (100%)
Median duration of hospitalization (days, IQR)	5.5 (3.0-6.8)
Median duration of system steroids (days, IQR)	9.0 (6.5-15.7)

Table 3. Median peak laboratory values for EVALI subjects (n=6)

Lab Value	**Median (IQR)**	**Reference Units**
C-Reactive Protein (CRP)	332.5 (210.0 – 390.3)	<5.0 mg/L
Estimated Sedimentation Rate (ESR)	71.5 (39.0 – 95.3)	0 – 20 mm/hr
Total WBC count	15.3 (12.4 – 18.5)	3.8 – 10.5 K/µl
Neutrophils percentage	91.5 (89.8 – 92.3)	40-60 %
Neutrophil number	14.0 (11.1 – 17.1)	2.5 – 7.0 K/µl

Table 4: Mean values for various biomarkers quantitated in plasma or urine from EVALI subjects and healthy subjects.

Parameters	**Non-User (mean ± SD); pg/mL**	**EVALI (mean ± SD); pg/mL**	**p-value**
THC metabolite (11-nor-9-carboxy-THC)	(93.1 ± 123.4) X 10^3	(271.2 ± 35.5) X 10^3	0.0112*****
Cotinine	(54.6 ± 76.6) X 10^3	(86.3 ± 97.9) X 10^3	0.5815
Oxidative Stress			
MPO	98.3 ± 87.2	97.0 ± 69.7	0.9786
8-OHdG	(115.4 ± 49.0) X 10^3	(212.5 ± 66.9) X 10^3	0.0258*****
8-Isoprostane	(2.20 ± 2.62) X 10^3	(3.52 ± 1.77) X 10^3	0.3720
Lipid Mediators			
RvD_1	310.3 ± 120.1	88.8 ± 78.5	0.0062******
RvD_2	887.1 ± 366.3	790.2 ± 423.6	0.7071
RvE_1	(1.36 ± 2.32) X 10^3	(2.90 ± 4.17) X 10^3	0.4880
LXB_4	796.3 ± 129.4	766.0± 136.8	0.7264
PGE_2	379.3 ± 265.7	52.4 ± 24.2	0.0342*
TXB_2	937.3 ± 1179.6	266.8 ± 204.9	0.2390
Inflammatory Mediators			
TNFα	128.4± 41.5	83.0 ± 10.1	0.0389*****
IL-1β	8.66 ± 2.35	5.75 ± 1.76	0.0509
MCP-1	26.7 ± 9.1	167.0 ± 202.6	0.1528
IL-8	10.0 ± 7.1	15.8 ± 10.5	0.3296
IL-6	11.9 ± 13.1	32.0 ± 42.1	0.3329
CC10/16	(26.6 ± 4.9) X 10^3	(16. 3± 4.9) X 10^3	0.0083*****, #
MIP1β	455.4 ± 71.5	352.3 ± 31.3	0.0145*****
RANTES	16309.0 ± 9912.3	5507.1± 2573.93	0.0401*****
GM-CSF	12.2 ± 7. 7	2.8 ± 3.5	0.0330*****
Basic FGF	124.0 ± 14.3	99.2 ± 25.5	0.0861
VEGF	182.9 ± 356.6	2.1 ± 0	0.2833
PDGF-BB	4726.4 ± 3330.7	1419.5 ± 1414.1	0.0682
IL-1rα	193.1 ± 367.3	403.5 ± 502.3	0.4671
IL-2	5.39 ± 4.98	3.76 ± 2.35	0.5213
IL-4	5.54 ± 1.47	4.54 ± 1.06	0.2473
IL-5	24.6 ± 26.4	5.6± 6.5	0.1482
IL-7	50.9 ± 18.0	56.8 ± 4.5	0.4961
IL-9	635.2 ± 118.0	451.4 ± 53.8	0.0100*****
IL-10	1.26 ± 1.91	1.86 ± 1.33	0.5731
IL-13	3.58 ± 0.59	4.25 ± 0.93	0.2078
IL-15	219.4 ± 289.7	9.0 ± 0	0.1354

IL-17	22.2 ± 4.2	19.5 ± 5.1	0.3804
Eotaxin	71.3 ± 27.7	43.6 ± 23.0	0.1167
IFNγ	29.4 ± 11.3	24.2 ± 6.5	0.3979
G-CSF	163.1 ± 62.7	130.8 ± 22.9	0.3051
IL-12p70	8.21 ± 4.61	6.32 ± 4.09	0.5087
MIP-1α	3.47 ± 1.43	3.97 ± 1.79	0.6326
IP10	756.0 ± 267.9	2293.5 ± 2177.0	0.1481

THC metabolite, 8-OHdG, 8-isoprostane were measured in urine, whereas all other mediators were measured in plasma. *$p<0.05$; #$p<0.00125$ (FDR – $p<0.05/40$)

Figure 1

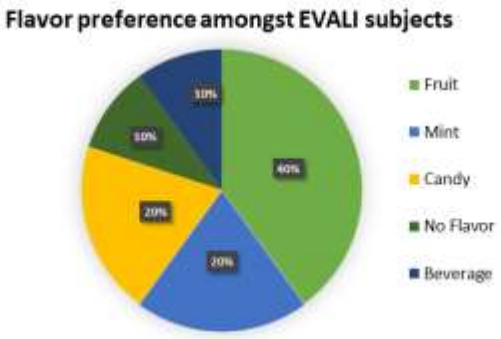

Figure 1. **Flavor preferences of EVALI subjects.** Self-reported survey data based on favorite flavor (n=6).

Figure 2

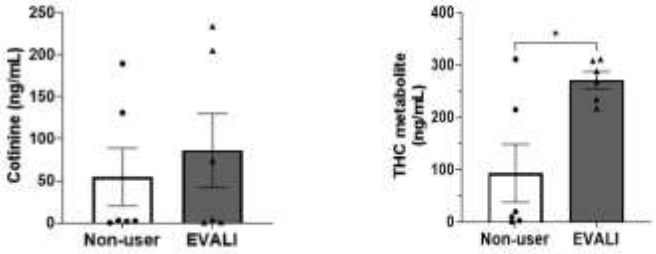

Figure 2: **Elevated plasma cotinine and THC levels in EVALI subjects.** The level of cotinine (in plasma) and THC metabolites (in urine) from non-users and EVALI subjects was quantified using ELISA. n=6/group. Data are shown as mean ± SEM. * $p<0.05$ as per unpaired t-test.

Figure 3

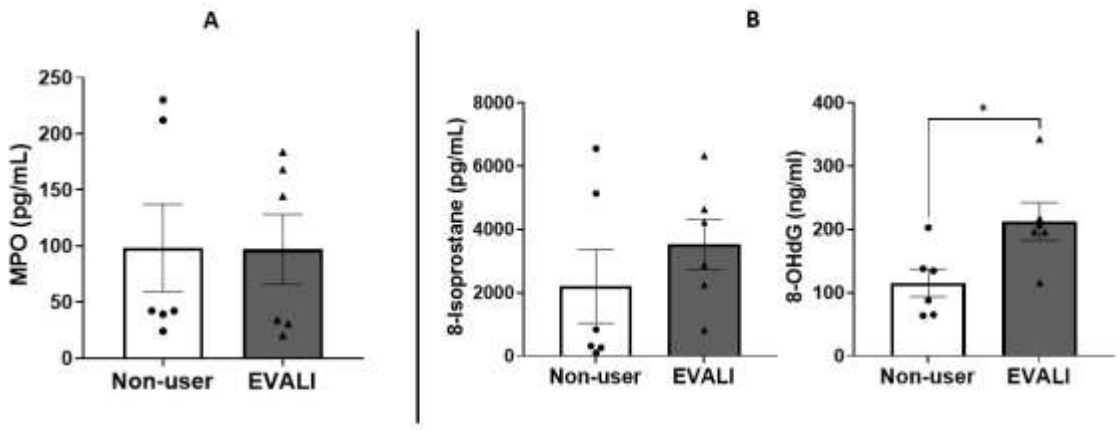

Figure 3: **Oxidative Stress markers in the plasma and urine from EVALI subjects.** The levels of myeloperoxidase (MPO), 8-isoprostane, and 8-OHdG were measured in (A) plasma and (B) urine from non-user and EVALI subjects using ELISA-based assays. n=6/group. Data are shown as mean ± SEM. *p<0.05 as per unpaired t-test.

Figure 4

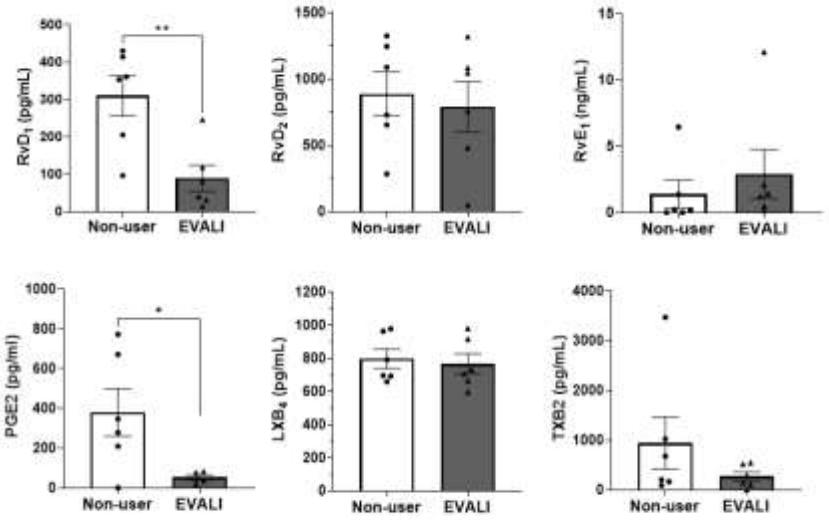

Figure 4: Changes in the levels of pro-resolving lipid mediators in plasma samples from EVALI subjects. Plasma levels of resolvins (RvD_1, RvD_2 and RvE_1), prostaglandin E_2 (PGE_2), lipoxin B_4 (LXB_4) and thromboxane B_2 (TXB_2) in non-users and EVALI subjects were quantitated using ELISA-based assays. n=6/group. Data are shown as mean ± SEM. * $p<0.05$, *** $p<0.001$ as per unpaired t-test.

Figure 5

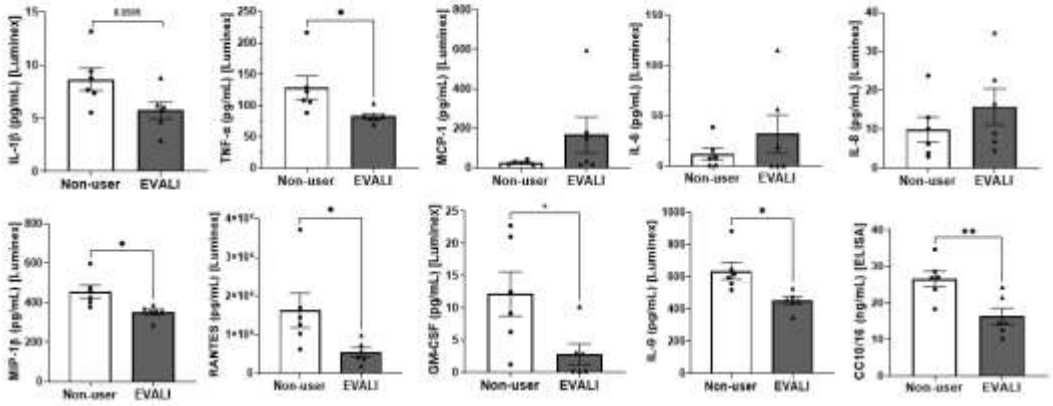

Figure 5: Dysregulated levels of inflammatory mediators in plasma from EVALI subjects. Plasma levels of inflammatory cytokines/chemokines/mediators in non-users and EVALI subjects were quantitated using Luminex (27-plex) and ELISA (CC10/16)-based assays. n=6/group. Data are shown as mean ± SEM. * $p<0.05$, ** $p<0.01$ as per unpaired t-test.

Figure 6

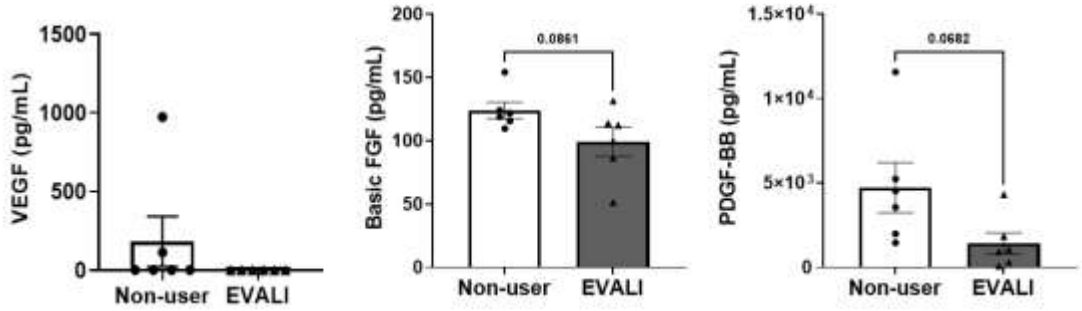

Figure 6: Altered levels of growth factors in subjects with EVALI. Plasma levels of growth factors- VEGF, FGF and PDGF-BB in non-users and EVALI subjects were determined using Luminex. n=6/group. Data are shown as mean ± SEM. P-value calculated per unpaired t-test.