

Heated Tobacco Products cause Pulmonary Injury and Inflammation: Rapid Elevation of Extracellular Vesicles Expressing ACE, C3, P4HB and CC16 in Healthy Young Individuals

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Abstract

Background

Heated tobacco products (HTPs) are increasingly popular alternative nicotine delivery systems. While marketed as lower-risk alternatives to combustible cigarettes, HTP aerosols still contain combustion byproducts. The acute systemic and pulmonary effects associated with these products have not yet been thoroughly examined. We aimed to evaluate the acute effects of HTP inhalation on the release of extracellular vesicles (EVs) bearing lung-specific and inflammatory biomarkers.

Methods

In a randomized, crossover study, 23 healthy occasional tobacco users underwent an active HTP exposure session (using an IQOS 3 Multi device) and a control session. Blood samples were collected at baseline and 4 hours post-exposure. Circulating EVs were isolated via ultracentrifugation and analyzed using flow cytometry. We quantified EVs expressing Angiotensin-Converting Enzyme (ACE), Aldehyde dehydrogenase 3B1 (ALDH3B1), Uteroglobin, Complement component 3 (C3), C-C motif chemokine ligand 3 (CCL3/MIP-1 α), Palate lung and nasal epithelial clone protein (PLUNC), and Prolyl 4-hydroxylase beta (P4HB).

Results

Acute inhalation of HTP aerosols induced a statistically significant increase in circulating EVs expressing ACE, P4HB, C3, and CC16/Uteroglobin compared to the non-exposure control. A trend toward elevated ALDH3B1 expression was observed, while levels of CCL3 and PLUNC remained unchanged.

Conclusion

Acute exposure to HTPs triggers a rapid release of EVs associated with cellular stress, pulmonary injury, and pro-inflammatory responses. Increases in biomarkers such as ACE, C3, and the cancer-associated protein P4HB indicate that HTPs could impair lung health and initiate pro-thrombotic and pro-inflammatory cascades, supporting the need for more stringent regulatory controls.

Introduction

Cigarette smoking remains a leading cause of preventable global mortality, contributing to approximately 8 million deaths annually (Collaborators 2021). Alongside e-cigarettes, heated tobacco products (HTPs) have rapidly gained global popularity as leading alternative nicotine delivery systems. Unlike traditional cigarettes, which burn at temperatures exceeding 850°C, HTPs operate by heating specially formulated tobacco inserts to roughly 350°C. Although manufacturers claim that this lower-temperature pyrolysis

reduces toxic emissions by 90–95%, independent aerosol analyses demonstrate that partial combustion still occurs. As a result, users continue to be exposed to hazardous combustion compounds, including high levels of reactive oxygen species, formaldehyde, and tobacco-specific nitrosamines (Davis et al. 2019; Upadhyay et al. 2023). Furthermore, HTPs deliver nicotine at 70–80% the yield of standard cigarettes, posing ongoing exposure risks, while their efficacy as smoking cessation aids remains highly questionable (Bekki et al. 2017; Mitova et al. 2016; Odani et al. 2023).

Emerging evidence highlights the detrimental cardiovascular and pulmonary effects of HTP consumption. Animal models have shown that HTP aerosols elicit oxidative stress, lung injury, and endothelial dysfunction similar to the physiological effects caused by conventional smoking (Husari et al. 2023; Koike et al. 2022). In our previous human studies, we have established that acute HTP inhalation induces measurable endothelial dysfunction indicated by elevated arterial stiffness, as well as promoting a pro-thrombotic state, as shown by increased ex-vivo thrombus formation (Lyytinen et al. 2023).

Central to understanding these systemic impacts is the role of extracellular vesicles (EVs). Far from being inactive cellular debris, these small, membrane-derived particles are integral to intercellular communication, tissue repair, and pro-thrombogenic processes (Chatterjee et al. 2020; Yáñez-Mó et al. 2015). By analyzing their specific surface markers, the cellular origin and activation state of EVs can be accurately mapped. We have previously demonstrated that respiratory exposure to e-cigarette aerosols significantly increases EV release. This release is not merely a marker of acute vascular injury, but a potential mechanistic driver for the long-term development of cardiovascular and pulmonary diseases upon repeated exposure (Mobarrez et al. 2020). Furthermore, our prior investigations confirm a specific increase in platelet-derived and activated EVs following acute HTP inhalation (Antoniewicz et al. 2024).

To comprehensively evaluate pulmonary inflammation and oxidative stress, we quantified a targeted panel of seven lung-relevant biomarkers (Kabéle et al. 2025). Our selection included Angiotensin Converting Enzyme 1 (ACE1), a localized pro-inflammatory mediator frequently elevated across various respiratory pathologies, alongside Aldehyde dehydrogenase 3B1 (ALDH3B1), an enzyme highly expressed in pulmonary tissue that actively mitigates oxidative stress (Imai et al. 2007; Jang et al. 2014; Kuba et al. 2006; Liu et al. 2020). We also assessed Club cell protein 16 (CC16/Uteroglobin), a critical protectant of the lung epithelium and a highly sensitive biomarker for acute lung injury and Prolyl 4-hydroxylase beta (P4HB), an indicator of endoplasmic reticulum stress and marker for tumor progression in malignant diseases (Almuntashiri et al. 2020; Kim et al. 2018; Wang et al. 2023). To capture the broader immune response, we included Complement component 3 (C3) and C-C motif chemokine ligand 3 (CCL3), both of which are central to pulmonary immune regulation and inflammatory cell recruitment (Robbins et al. 1991; Yuan et al. 2015). Finally, we measured palate, lung, and nasal epithelial clone protein (PLUNC), an integral component of airway mucosal defense known to exhibit altered expression profiles in smokers (Bartlett et al. 2011; Steiling et al. 2009).

Materials and Methods

Participants and Study Design

This investigation recruited 23 healthy volunteers (males and females, aged 18 to 40 years) characterized as occasional tobacco consumers, defined by a usage rate not exceeding 10 cigarettes or 10 snus pouches (Swedish moist snuff) per month. Prior to enrollment, all individuals provided written informed consent and completed a comprehensive health declaration.

A randomized, crossover design was employed. Participants were allocated to initially undergo either an HTP exposure session or a control session. The sessions were separated by a minimum one-week washout period. For seven days prior to each session, participants were instructed to completely abstain from all nicotine-containing products, anti-inflammatory medications, and vigorous physical exertion. Furthermore, dietary restrictions prohibiting the consumption of alcohol and caffeine were enforced 24 hours prior to clinical testing. Individuals were excluded if they presented with any chronic pathology, Body Mass Index (BMI) greater than 30 kg/m², confirmed pregnancy, or an acute infection/inflammatory state within the week preceding the trial. Following an overnight fast of eight hours, participants rested in a semi-supine position for 15 minutes in a climate-controlled laboratory (21–23°C) prior to the initiation of baseline vascular assessments and phlebotomy.

Exposure Protocol and Blood Sampling

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During the active exposure visit, participants inhaled from an IQOS 3 Multi device (Philip Morris Int.) under direct supervision. The standardized smoking protocol required 28 discrete inhalations over a 14-minute period (one puff every 30 seconds), with each inhalation maintained for 2 to 3 seconds. The HTP consumables (Sienna heat sticks, Philip Morris Int.) contained 203.3 mg of tobacco (25.6%), 47.1 mg of glycerol (5.9%), and 2.8 mg of propylene glycol (0.3%), designed to deliver an approximate nicotine yield of 1.4 mg per stick. During the control arm, participants were sitting and holding the inhaler without inhaling.

Venous blood was drawn from the antecubital vein at baseline and 4 hours following the exposure. The baseline draw facilitated routine hematological and biochemical profiling, which included a complete blood count, electrolyte panel, serum creatinine, lipid profile, and fasting glucose.

Extracellular Vesicle Measurement

For the assessment of extracellular vesicles, whole blood was collected in sodium citrate tubes and subjected to centrifugation at 2,000×g for 20 minutes at room temperature (RT) within 60 minutes of collection. The resulting platelet-poor plasma (PPP) was immediately aliquoted and cryopreserved at -80°C to guarantee sample stability prior to flow cytometric analysis. To isolate and concentrate circulating EVs, the frozen PPP aliquots were thawed in a water bath and processed via high-speed

ultracentrifugation at 20,160×g for 45 minutes at 21°C. The supernatant was subsequently discarded, and the remaining pellet was resuspended via vortexing to yield an EV-enriched fraction. For biomarker targeting, 20 µL of the processed suspension was transferred into a 96-well microtiter plate pre-loaded with 5 µL of specific fluorochrome-conjugated antibodies. Primary antibodies against ACE1, ALDH3B1, CC16/Uteroglobin, C3, CCL3 (MIP-1α), PLUNC and P4HB were conjugated in-house to FITC, R-Phycoerythrin (PE), Allophycocyanin (APC) or Peridinin Chlorophyll Protein (PerCP) using Lightning-Link® conjugation kits (Abcam, Cambridge, UK) according to the manufacturer's instructions. This mixture was incubated with the sample in the absence of light for 20 minutes. Following the incubation period, 120 µL of CytoFLEX sheath fluid was added to each well, and the samples were acquired on a CytoFLEX flow cytometer. The target EV population gate was calibrated using Spherotech Nano Fluorescent Yellow Particles across defined sizes (0.22 µm, 0.45 µm, 0.88 µm, and 1.35 µm). Vesicles falling within the 0.1 to 1 µm size range and exhibiting positive antibody fluorescence were definitively classified as EVs. The final data were expressed as the frequency percentage (%), calculated as the ratio of biomarker-specific EV events to the total number of events recorded within the established EV gate.

Statistical Analysis

Data were analyzed using GraphPad Prism version 11.0 (GraphPad Software Inc., US) and IBM SPSS Statistics version 30 (IBM Corp., US). The normality of the data distribution was assessed through visual inspection and the Shapiro-Wilk test. All initial data were significantly left-skewed. For further statistical evaluation, data were logarithmically transformed, after which no significant skewness was observed. Data were analyzed using a Linear Mixed Model. To account for the repeated-measures design, Subject ID was specified as the grouping variable. Time, Exposure, and the Time*Exposure interaction were entered as fixed effects. To account for baseline inter-individual differences, a random intercept was included for each subject using a variance components (VC) covariance structure. Statistical significance was set at $p < 0.05$. All statistical analyses were conducted by an investigator blinded to the group allocations.

Results

A total of 23 participants were included in the final analysis. Comprehensive demographic and baseline laboratory data for all subjects are summarized in Table 1.

Table 1
Baseline characteristics of study participants (N = 23)

Age, years	Mean $\pm$ SD
	26 $\pm$ 5
BMI, body mass index, kg/m ²	24.2 $\pm$ 3.4
Waist circumference, cm	81 $\pm$ 10
Hemoglobin, g/L	137 $\pm$ 11
White blood cells, x10 ⁹ /L	5.7 $\pm$ 1.9
Platelets, x10 ⁹ /L	242 $\pm$ 53
Creatinine, μ mol/L	72 $\pm$ 13
Glucose, mmol/L	5.2 $\pm$ 0.3
Cholesterol, mmol/L	4.4 $\pm$ 0.9
Low density lipoprotein, mmol/L	2.4 $\pm$ 0.8
Triglycerides, mmol/L	0.8 $\pm$ 0.3
Alanine transaminase, μ kat/L	0.3 $\pm$ 0.1
Sex, male/female	12/11

Analysis of circulating EVs revealed that populations expressing ACE1, P4HB, C3, and CC16/Uteroglobin increased significantly following acute inhalation of HTP compared to the control (Fig. 1). Additionally, there was a trend toward an increase in EVs expressing ALDH3B1 following HTP exposure (Fig. 2). Conversely, there were no significant changes in the expression of CCL3 or PLUNC following exposure (Fig. 2). The corresponding values for these biomarker expressions are detailed in Online Resource 1.

Discussion

In healthy young individuals, we demonstrate for the first time a statistically significant increase in circulating EVs expressing ACE, P4HB, C3, and Uteroglobin following acute inhalation of heated tobacco products.

Serum ACE1 serves as a circulating marker of renin-angiotensin-aldosterone system (RAAS) activity, a system central to blood pressure regulation and fluid homeostasis. While ACE (ACE1) is traditionally recognized for its high expression in the vascular endothelium of the lungs, kidneys, and heart, its counter-regulatory homolog, ACE2, is highly expressed in the epithelial cells of the respiratory tract (Hamming et al. 2004; Liu et al. 2020). Under normal physiological conditions, it is ACE2 – rather than ACE1 – that exerts a protective effect on the vasculature and lung parenchyma via vasodilation, anti-

inflammatory, and anti-proliferative properties (Wang et al. 2020). Tissue injury alters this balance. Acute endothelial damage causes a systemic shedding and pooling of ACE1 into the circulation (Gaddam et al. 2014; Orfanos et al. 2000). Concurrently, as highlighted during the SARS-CoV-2 pandemic, ACE2 serves as the primary viral entry receptor (Oz and Lorke 2021). Its expression is dynamically influenced by pro-inflammatory chemokines, including interleukins, interferons, and TNF. Consequently, while an acute spike in serum ACE1 reflects immediate vascular lung injury, a chronic depletion of epithelial ACE2 strips the lungs of their protective local factors, shifting the RAAS balance toward unchecked vasoconstriction and inflammation, thereby predisposing individuals to severe pulmonary infections and Acute Respiratory Distress Syndrome (ARDS) (Imai et al. 2007; Kuba et al. 2006). Importantly, smoking is a profound risk factor for ARDS, an association likely driven by this chronic down-regulation of epithelial ACE2 (Yuan et al. 2025).

Furthermore, this specific pathophysiological pathway of ACE1/ACE2 dysregulation and unchecked inflammation offers a compelling explanation for the development of E-cigarette or Vaping Use-Associated Lung Injury (EVALI). The acute vascular and epithelial damage induced by heated tobacco products (HTPs), compounded by inflammatory triggers such as reactive oxygen species (ROS) within the aerosol, can escalate local tissue damage. Pathologically elevated ACE1 activity accentuates this by suppressing the protective, anti-inflammatory ACE2 pathway, driving endothelial dysfunction and promoting systemic atherosclerosis (Liu et al. 2021).

Although not reaching statistical significance, we noted a subtle trend toward increased CCL3, a chemokine primarily secreted by alveolar macrophages; this suggests that HTP exposure may trigger both direct airway epithelial injury and a coordinated pro-inflammatory macrophage response (Menten et al. 2002). Systemic RAAS activation serves as a potent disease modulator in obstructive pulmonary conditions like asthma and COPD, potentiating chronic inflammation, driving ROS generation, and impairing mitochondrial redox signaling (Su et al. 2024; Teramoto et al. 2000). Previous studies show that ACE1-positive EVs are elevated in smokers and COPD patients, with levels decreasing upon smoking cessation unless permanent COPD pathology has been established, at which point they remain pathologically elevated (Strulovici-Barel et al. 2016). Furthermore, animal models indicate that repeated airway injury resulting in RAAS dysregulation can impair innate immune responses and fetal lung development (Dye et al. 2025).

Similarly, the significant rise in Uteroglobin-related protein (CC16) is highly indicative of respiratory distress. Secreted by club cells in the respiratory epithelium to facilitate local repair, Uteroglobin levels closely correlate with the severity of inhalation trauma, ARDS progression, and ICU admission rates (Almuntashiri et al. 2020; Henrich et al. 2021). With repeated daily toxicant exposure, this protective pulmonary reserve eventually becomes depleted. Therefore, COPD patients exhibit significantly lower baseline Uteroglobin levels, a deficiency also linked to the development of asthma and pulmonary fibrosis (Almuntashiri et al. 2020; Lacho-Contreras et al. 2016).

The complement system is critically affected by cigarette smoke (Robbins et al. 1991). While previous research on nicotine-containing e-cigarettes demonstrated only a trend toward elevated C3-positive EVs, our current data reveal a *significant* increase upon HTP inhalation (Kabéle et al. 2025). This is highly relevant; rather than representing a general increase in free circulating C3, the enrichment of C3 within extracellular vesicles suggests an active, cell-mediated shedding response triggered by acute vascular or epithelial stress. C3 plays a central role in immune response and inflammation. Elevated circulating C3 is a recognized cardiovascular risk factor, strongly associated with hypertension, renal damage, and the pathophysiology of emphysema, where C3 complexes physically deposit within the pulmonary tissue of smokers (Afzali et al. 2025; Wenzel et al. 2021; Yuan et al. 2015; Ząbczyk et al. 2023). The surge in C3-bearing EVs observed here may represent a novel, discrete vector for this pathway, potentially facilitating the targeted transport and deposition of complement factors into remote vascular beds. This reflects the multifaceted pathways through which inhaled toxins exert systemic harm.

Furthermore, we observed a direct and significant increase in EV-bound P4HB upon exposure. Released by the endoplasmic reticulum (ER), P4HB plays a pivotal role in regulating ROS production, mitigating cellular stress, and maintaining proteostasis (Kim et al. 2018). An abrupt systemic increase in P4HB-EVs is concerning, as it indicates that even brief HTP inhalation triggers a profound and immediate cellular ER stress response. While this acute spike represents an immediate compensatory mechanism to handle aerosol-induced oxidative stress, persistent dysregulation of this pathway carries long-term pathological implications. In chronic disease states, sustained upregulation of P4HB is strongly associated with cellular transformation and oncogenesis, which is why it is actively investigated as both a tumor prognostic marker and therapeutic target in various malignancies (Wang et al. 2023). While a single, brief exposure session is insufficient to induce oncogenesis, the fact that HTPs can acutely activate a concerted axis of stress-associated markers – including P4HB, ACE1, and C3 – is concerning. These findings suggest that long-term, habitual HTP use could chronically sustain this pro-inflammatory and pro-tumorigenic microenvironment (Afsar et al. 2021; Kleczko et al. 2019).

What specific factors within HTPs drive this acute vascular and cellular response? Although HTPs and nicotine-containing e-cigarettes are often grouped together as alternative tobacco products, they have different toxicological profiles. Our prior work on e-cigarettes demonstrated prominent elevations in ACE and PLUNC, but only minor trends in complement and macrophage markers (Kabéle et al. 2025; Mobarrez et al. 2020). Conversely, the robust statistical significance observed here for C3 and Uteroglobin following HTP exposure points to a distinct pattern of injury. We hypothesize that the pronounced response observed here is driven by the unique aerosol composition of HTPs. Unlike e-cigarette liquids, HTP aerosols inherently contain high concentrations of reactive oxygen species (ROS), tobacco-specific nitrosamines, ammonia, and acrolein due to the processing of real tobacco sheets (Upadhyay et al. 2023). The baseline cellular disruption caused by nicotine is likely heavily potentiated by these thermal degradation byproducts. This is supported by literature showing that highly toxic combustible cigarette smoke triggers a significantly larger release of endothelial microvesicles than e-cigarette vapor (Antoniewicz et al. 2016; Mobarrez et al. 2014; Mobarrez et al. 2020).

This nuanced difference is further highlighted by our subclinical findings. For instance, we noted a non-significant upward trend in ALDH3B1 – an aldehyde dehydrogenase crucial for protecting the bronchial epithelium from free radical cytotoxicity – reflecting an acute, localized wave of oxidative stress (Jang et al. 2014). Interestingly, while our previous e-cigarette cohorts showed significant elevations in the protective airway protein PLUNC, HTP exposure did not yield a detectable change in this marker (Bartlett et al. 2011; Steiling et al. 2009). This lack of response, coupled with the non-significant trend toward increased macrophage-derived CCL3, suggests that HTPs may bypass certain mucosal defenses, shifting the primary site of injury toward direct epithelial distress and systemic thromboinflammatory EV shedding.

Finally, the implications of our findings may extend beyond localized pulmonary and cardiovascular pathology. While RAAS dysregulation is classically viewed through a cardiorespiratory lens, the systemic release of ACE1-EVs introduces a potential mechanism for cross-organ communication, including central neurovascular effects. The vascular endothelium and the broader RAAS are known to play a crucial role in neurovascular coupling and the intrinsic regulation of cerebral blood flow, independent of systemic blood pressure dynamics (Bloch et al. 2015). Although speculative within the limits of the current study, it is conceivable that the acute disruption of this axis via HTP-induced ACE shedding and EV transport could influence cerebral microvascular integrity over time. These observations underscore the need for future translational studies to investigate whether circulating, lung-derived EVs contribute to downstream neurological or cognitive alterations associated with chronic alternative tobacco product use.

Strengths and Limitations

A notable strength of this study is its randomized, crossover design, which effectively minimizes inter-individual confounding variables by allowing participants to act as their own controls. The experimental setup also implemented strict baseline normalization protocols, enforcing a washout interval of at least one week between exposures.

Despite these methodological strengths, several limitations must be acknowledged. First, the investigation features a relatively small sample size of 23 healthy volunteers. Because the cohort was strictly limited to occasional tobacco consumers, the findings may not fully generalize to the physiological responses of chronic, heavy users or completely tobacco-naïve individuals.

Second, the study's design focused exclusively on acute physiological impacts, collecting blood samples only at baseline and precisely four hours post-exposure. This acute timeframe precludes the ability to map the long-term toxicological trajectory of repeated extracellular vesicle release. Finally, the active exposure protocol relied entirely on a single product type, meaning the observed biomarker responses may not completely generalize across the diverse range of heated tobacco products currently available on the market.

Conclusion

Collectively, our findings demonstrate that acute HTP inhalation induces an immediate cellular stress response characterized by the rapid release of phenotypic EVs carrying markers of vascular damage, complement activation, and endoplasmic reticulum stress. These data indicate that even brief exposure to HTP aerosols modifies circulating EV cargo in ways that align with pathways driving chronic pulmonary and cardiovascular diseases. Consequently, these findings support the need for careful regulatory consideration, comprehensive public health surveillance, and a reassessment of industry claims regarding the relative safety of heated tobacco products.

Declarations

Compliance with Ethical Standards

The research protocol was approved by the Swedish Ethical Review Authority in Stockholm (Reference No: 2020–03387) and adhered to the ethical guidelines established by the 1964 Declaration of Helsinki and its later amendments.

Competing interests

The authors declare no conflicts of interest.

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Author Contribution

ML, FM, JB and LA contributed to the study concept and experimental design. GL, ML, GM and FM managed sample processing and laboratory methodology. Statistical modeling and blinding were overseen by LA, GL and FM. All authors contributed to writing, critical manuscript revisions, and approved the final submitted version.

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Data Availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable academic request.

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Figures

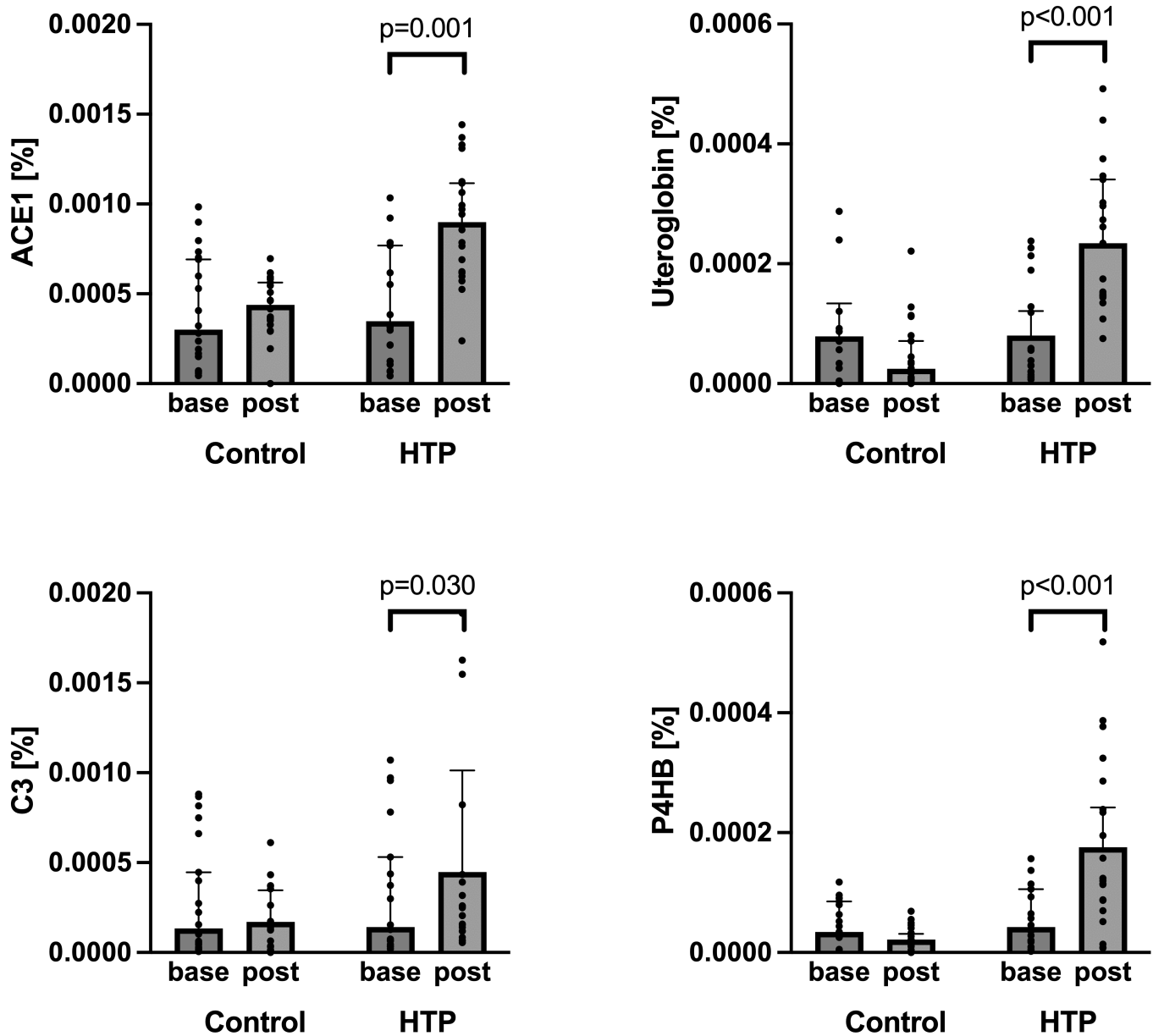


Figure 1

Acute effects of heated tobacco product (HTP) inhalation on circulating extracellular vesicle (EV) sub-populations. Bar graphs represent median frequency percentages (%) $\pm$ 95% Confidence Intervals (CI) of total EVs at baseline ("base") and 4 hours following exposure ("post") during both a non-exposure control session and an active HTP inhalation session (HTP). Quantified EV surface phenotypes include: Angiotensin-Converting Enzyme 1 (ACE1), Complement component 3 (C3), Prolyl 4-hydroxylase beta (P4HB), and Uteroglobin. Individual data points are indicated by black dots. Data are modeled using a Linear Mixed Model on logarithmically transformed values. Significant post-exposure elevations compared to baseline are indicated with brackets alongside their corresponding p -values

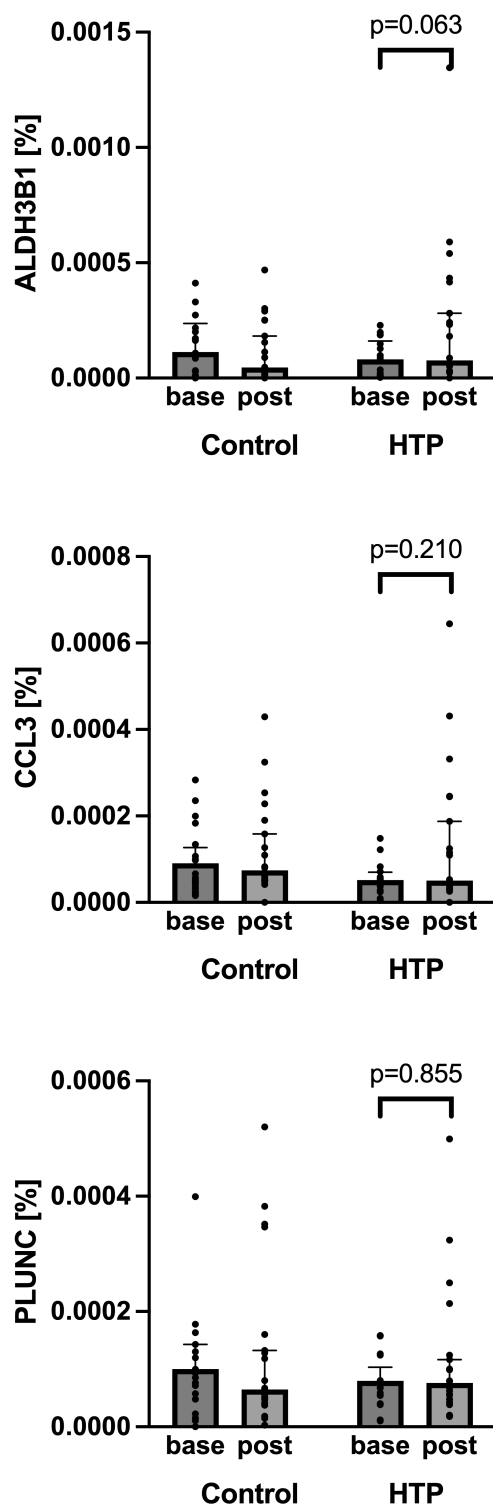


Figure 2

Unchanged or trend-level effects of heated tobacco product (HTP) inhalation on circulating extracellular vesicle (EV) sub-populations. Bar graphs represent median frequency percentages (%) $\pm$ 95% Confidence Intervals (CI) of total EVs at baseline ("base") and 4 hours following exposure ("post") during both a non-exposure control session and an active HTP inhalation session (HTP). Quantified EV surface phenotypes include: Aldehyde dehydrogenase 3B1 (ALDH3B1), C-C motif chemokine ligand 3 (CCL3) and Palate lung

and nasal epithelial clone protein (PLUNC). Individual data points are indicated by black dots. Data are modeled using a Linear Mixed Model on logarithmically transformed values. Brackets denote calculated interaction p -values

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