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# Urine proteome profile of firefighters with exposure to emergency fire-induced smoke: A pilot study to identify potential carcinogenic effects

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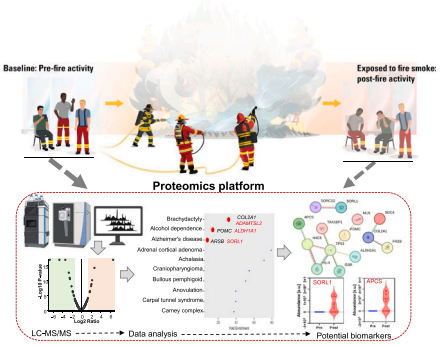
## HIGHLIGHTS

- First urinary proteome profiles of firefighters exposed to smoke in emergency fires
- Potential urological cancer biomarkers were identified.
- Urine proteomics are suitable for screening and early cancer detection.
- A larger sample cohort with different age groups could potentially enhance cancer biomarker discovery.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Firefighters are frequently exposed to a variety of chemicals formed from smoke, which pose a risk for numerous diseases, including cancer. Comparative urine proteome profiling could significantly improve our understanding of the early detection of potential cancer biomarkers. In this study, for the first time, we conducted a comparative protein profile analysis of 20 urine samples collected from ten real-life firefighters prior to and following emergency fire-induced smoke. Using a label-free quantitative proteomics platform, we identified and quantified 1325 unique protein groups, of which 45 proteins showed differential expressions in abundance in response to fire-smoke exposure (post) compared to the control (pre). Pathway analysis showed proteins associated with

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Fire smoke
Urine

epithelium development (e.g., RHCG, HEG1, ADAMTSL2) and Alzheimer's disease (SORL1) were significantly increased in response to smoke exposure samples. A protein-protein-network study showed a possible link between these differentially abundant proteins and the known cancer gene (TP53). Moreover, a cross-comparison analysis revealed that seven proteins—ALDH1A1, APCS, POMC, COL2A1, RDX, DDAH2, and SDC4 overlapped with the previously published urine cancer proteome datasets, suggesting a potential cancer risk. Our findings demonstrated that the discovery proteomic platform is a promising analytical technique for identifying potential non-invasive biomarkers associated with fire-smoke exposure in firefighters that may be related to cancer.

## 1. Introduction

Firefighting is one of the most complex and dynamic works, and exposure to fire smoke adversely affects firefighters' health. As a consequence, firefighters are at a heightened risk for various cancers. The International Agency for Research on Cancer (IARC), which classified occupational exposure for firefighters as possibly carcinogenic to humans (Group 2B) in 2010, reclassified it as carcinogenic to humans (Group 1) in 2022 on the basis of sufficient evidence for cancer in humans (Demers et al., 2022). For urologic cancers, in particular, the most common sites are the kidney, bladder, and prostate. A group led by the National Institute for Occupational Safety and Health (NIOSH) examined mortality risk in a large cohort of 30,000 firefighters (Pinkerton et al., 2020). The study results indicate that firefighters had an elevated risk of kidney (standardized mortality ratio [SMR] = 1.22, 95 % CI = 1.00–1.47) and prostate (SMR = 1.08, 95 % CI = 0.97–1.20) cancers, but not bladder (SMR = 0.98, 95 % CI = 0.80–1.18) cancer compared to the U.S. general population. In a study of a cohort of firefighters in Indiana (Muegge et al., 2018), the odds of death due to kidney cancer (OR = 1.84, 95 % CI = 1.17–2.83) were significantly higher for firefighters than for a group of non-firefighters. Similarly, occupational exposure for Nordic firefighters was significantly associated with early-onset prostate cancer (SIR = 1.71, 95 % CI = 1.23–2.31) (Barry et al., 2017). A meta-analysis of 25 cancer mortality cohort studies revealed elevated meta-relative risk estimates for each type of cancer (kidney SMR = 1.18, 95 % CI = 0.42–1.94; bladder SMR = 1.72, 95 % CI = 1.05–2.38; prostate SMR = 1.04, 95 % CI = 0.86–1.22) (Casjens et al., 2020).

The detection of early-stage tumors is crucial for reducing the mortality rate of these cancers. To that end, the collection and analysis of urine, a good source of biomarkers, has several advantages. Specifically, a urine sample is self-collected and noninvasive; the protein and peptides are already solubilized; the proteolytic processing is finished; and urine proteins and peptides are more stable than plasma (Trindade et al., 2021). Finally, 70 % of urinary proteins are derived from the kidney and urinary tract and, as such, are closer to a potential cancer sites (Desiderio and Loo, 2018). Although larger fire departments in metropolitan areas that are staffed by career firefighters have the capacity to conduct annual health assessments, these departments represent only a small percentage of all fire departments in the U.S. (Hwang et al., 2019). On average, 80 % of the U.S. fire departments are staffed by volunteer or mostly volunteer firefighters (USFA, 2023), with this pattern being more predominant in rural areas that experience a lack of health assessment resources.

We have previously reported on the different protein expression in pre- versus post-simulated firefighting rescues (Zhu et al., 2021). Using Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, we found that changes in protein expression were significantly related to acute inflammatory responses, immune responses, complement activation, and oxidative stress. Yet, the results of this simulated study were not tested in a real-world situation. In the present study, we used a proteomic analysis to identify alterations in protein and peptide expression patterns in response to emergency fire suppression activity. Our hypothesis was that a proteomic evaluation of urine sample would reveal signaling pathways that are critical for cancer candidate response and cell fate. Therefore, we identified and quantified urine protein and peptide samples from healthy firefighters pre- and post-fire suppression using liquid chromatography (LC)-based with tandem mass spectrometers (MS/MS) and label-free approach, which aid in the elucidation of proteomic analysis.

## 2. Materials and methods

### 2.1. Study design

Using a repeated measures design, urine samples were obtained from firefighters pre- and post-emergency firefighting incidents. Due to the unpredictable field environment, we began with a post-fire sampling collection, unlike a traditional pre-post study design. From December 2021 to June 2022, we collected 50 urine samples (30 pre-fire and 20 post-fire) from 30 firefighters in seven fire departments throughout Oklahoma. The pandemic resulted in an unequal number of pre- and post-fire samples. In this exploratory study, we selected 20 convenience samples (white male) from a pool of 50 urine, comprising ten before (Pre) and ten after (Post) an emergency fire activity. The methodologies for assessing environmental exposures and characterizing fire smoke will be described in subsequent publications.

### 2.2. Study participants

Each firefighter had at least one year of experience subsequent to probation and had not participated in a fire activity at least seven days prior to the baseline sampling. In our analysis of 20 urine samples from participants, the average age was 29.17 years, with 6.63 (SD). Only one participant was identified as a smoker. Only two had a BMI considered normal (18.5–24.9), with the remaining participants being either overweight (BMI: 25–29.9) or obese (BMI $\geq$ 30). Participants also signed an informed consent form approved by the Institutional Review Board (IRB No. 11466) of the University of Oklahoma Health Sciences Center. For the pre-sampling, each participant collected his own urine in a sterile urine specimen cup (BD Vacutainer, Franklin Lakes, NJ) as a baseline measurement before the fire activities. For the post-sampling, each participant collected his urine within 24 h after the fire activities. The collected urine samples were transported on ice and stored at −80 °C until they were further analyzed at the University of Oklahoma Proteomics and Metabolomics Core Facility.

### 2.3. Protein extraction and sample processing for bottom-up proteomic analysis

To concentrate the urine proteins, 1 ml of urine from 20 individual samples were subjected to ProteoSpin column (ProteoSpin™ Urine Protein Concentration Kits, Cat. 17400, Norgen Biotek, Canada) (Fig. 1, Step 1). Protein concentration was measured at 280 nm by a NanoDrop™ One/OneC microvolume UV–Vis spectrophotometer (Thermo Fisher Scientific, IL, USA). Proteins were resuspended with freshly prepared 8 M urea buffer and a total of 100 μg of protein per sample was subjected for in-solution proteolysis. To investigate the proper enzyme digestion performance and downstream LC-MS/MS analysis, equal amount (1 μg) of bovine serum albumin (BSA, Cat#23225, Thermo Scientific, USA) was included in each sample prior to in-solution digestion (Fig. 1, Step 1). In-solution digestion with trypsin/LysC (Cat

# V5071, Promega, WI, USA) was performed according to the manufacturer's protocol (Fig. 1, Step 2). After overnight incubation at 37 °C, tryptic peptides were desalted using C18 Sep-Pak plus cartridges (Cat # WAT023590, Waters, MA, USA) and were lyophilized for 8 h to dryness (Fig. 1, Step 3). The dried peptides were reconstituted with 100 µl buffer A (0.1 % formic acid, *v*/v) and an equal amount (2 µg) of peptides/sample was injected for LC-MS/MS analysis (Fig. 1, Step 4).

### 2.4. LC-MS/MS analysis

The LC-MS/MS analysis was performed as described previously (Ahsan et al., 2023). Briefly, resuspended tryptic peptides were loaded onto an in-house packed trap column (150 µm × 3 cm, packed with Bio-C18 3 µm resin, Sepax Technologies, DE, USA) using mobile phase A (0.1 % formic acid in LC-MS grade water) at a flow rate of 3 µl/min for 10 min and separated on an analytical column (75 µm × 30 cm, packed in house with Bio-C18 3 µm resin, Sepax Technologies) at 350 nl/min. The analytical column was heated to 55 °C and the peptides were separated through a 70 min-long linear gradient from 0 % to 40 % mobile phase B (0.1 % formic acid in acetonitrile). Nanoelectrospray was obtained using a fused silica emitter (PicoTip emitter, New Objective, Woburn, MA) on a custom-built ionization source. LC-MS/MS was performed by using a Dionex UltiMate® 3000 UHPLC system (Thermo Fisher Scientific, CA, USA) connected to a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The total run time was 90 min including column wash and re-equilibration.

### 2.5. Data analysis for bottom-up proteomics

RAW files were searched against the UniProt reviewed Human protein database (TaxID: UP000005640) using the Sequest algorithm within Proteome Discoverer v 2.4 (Thermo Fisher Scientific, San Jose, CA) (Fig. 1, Step 5). The Sequest database search was performed with the following parameters: trypsin enzyme cleavage specificity, two possible missed cleavages, 10 ppm mass tolerance for precursor ions, and 0.02 Da mass tolerance for fragment ions. Search parameters permitted dynamic modification of methionine oxidation (+15.9949 Da) and static modification of carbamidomethylation (+57.0215 Da) on cysteine. Peptide assignments from the database search were filtered down to a 1 % false discovery rate (FDR). Label-free quantitation across the samples employed the Minora algorithm and the adjoining bioinformatics tools available in Proteome Discoverer (PD) 2.4. A $p$-value$<0.05$ was used as the cut-off for all statistical analyses (Fig. 1, Step 6).

### 2.6. Bioinformatic analysis of bottom-up proteomic results

Functional and pathway analyses were performed using ShinyGO 0.76 (Ge et al., 2020), an open source bioinformatic platforms. Heatmaps, volcano, and UpSet plots were plotted by SRplot (https://www.bioinformatics.com.cn/en), a free online platform for data analysis and visualization. Venn diagrams were generated using Venn-Diagram-Plotter (PNNL, https://pnnl-comp-mass-spec.github.io/Venn-Diagram-Plotter/). Protein-protein network was generated using STRING (https://string-db.org/). Potential protein pathway was searched through KEGG (https://www.genome.jp/kegg/pathway.html). All the violin plots were generated with GraphPad Prism version 7.0 for Windows, GraphPad Software, Boston, Massachusetts, USA (https://www.graphpad.com).

## 3. Results

### 3.1. Quality control of the bottom-up proteomics pipeline

To evaluate the sample-to-sample variation which, may occur in various sample processing steps (e.g. depletion of abundant urine proteins, trypsin digestion, de-saltation, tryptic peptide enrichment, and LC-MS/MS), 1 µg of internal protein standard bovine serum albumin (BSA) was spiked in each sample prior to the urine protein enrichment processing step (Fig. 1). As expected, all possible tryptic peptides (87 % sequence coverage) of BSA were successfully identified (Fig. SI-1A). Peak-area based quantitation analysis of the Internal control BSA protein showed no significant quantitative variation across the samples

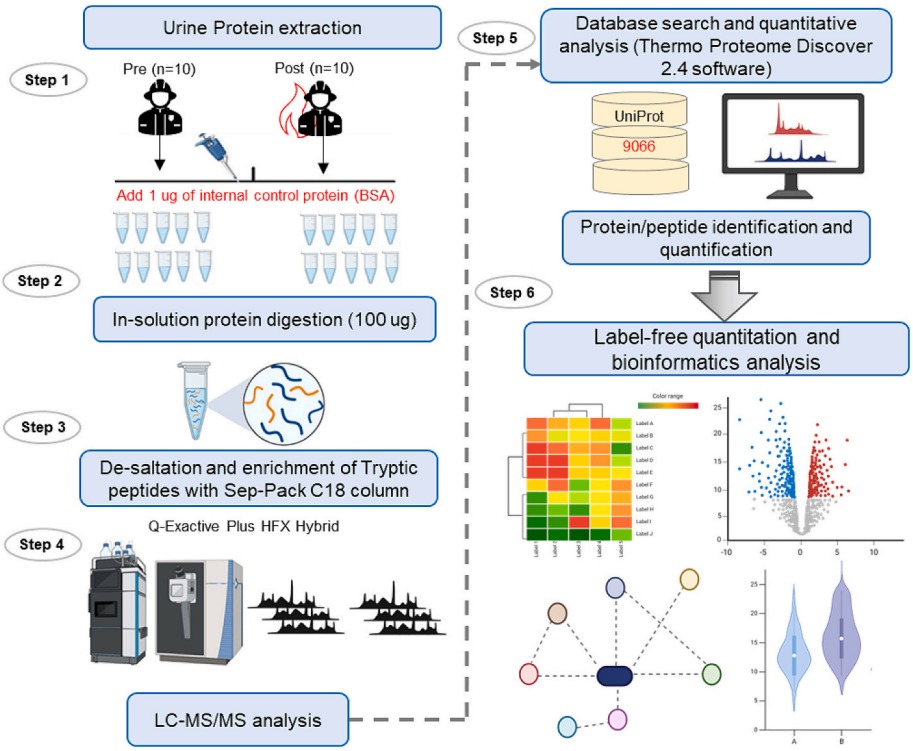


**Fig. 1.** Sample processing and LC-MS/MS pipeline of firefighter urine proteomic analysis.

suggesting uniform sample processing throughout the analysis (Fig. SI-1B). Similarly, human albumin, one of the major proteins in urine, was also identified with 88 % sequence coverage and no significant differences, indicating complete tryptic digestion and no technical variation was observed across the sample (Fig. SI-1C and -1D).

### 3.2. Firefighter urine proteome profile

In this study, a total of 184,480 peptide-matched spectra (PSM) and 5465 unique peptides corresponding to 1325 unique protein groups were identified and quantified from the firefighter urine samples (Tables SI-1 and SI-2). The physicochemical properties, such as theoretical MW of the urine proteome show the majority (>89 %) of the identified proteins ranged from 10 to 100 kDa (Fig. 2A). Similarly, 67 % of proteins were neutral (pI 5–8) wherein 9 % were acidic (pI 4–5) and 25 % were very basic (pI 8–13) (Fig. 2A). As expected, many known urine marker proteins, such as albumin, uromodulin, serotransferrin, alpha-amylase, AMBP, and Kininogen 1, were identified as abundant proteins with high sequence coverages (Fig. 2B–C). Functional analysis of the entire proteome set was cross-validated by two different bioinformatics platforms – GO Cellular and JENSEN Compartments analysis (Fig. 2D–E, Table SI-3). Both analyses revealed that proteins associated with secrettory granule, secrettory vesicle, vesicle lumen, and extracellular space were enriched (Fig. 2D–E). KEGG pathway analysis further showed the highest number of proteins associated with PI3K-Akt signaling pathway and Lysosome processes (Fig. SI-2, Table SI-3). Overall, the results show that the primary urinary proteins have been successfully identified, indicating that the urine proteome quality is suitable for additional quantitative comparative study.

### 3.3. Quantitative analysis of post vs pre firefighter urine proteome

A label-free quantitative proteomic analysis of the two group of urine samples successfully identified and quantified a total of 1325 unique protein groups. A principal component analysis (PCA) of total protein abundance shows overlapped clustering among the two groups of samples, indicating the high biological variability among the urine samples (Fig. 3A). Similarly, heat map clustering of the protein abundance of the total unique identified proteins further demonstrates no distinct cluster between the two groups (Fig. 3B). However, volcano plot analysis revealed that between the post- and pre-samples, the abundance of several proteins significantly (at least 1.5-fold up/down with an adjusted $p$-value <0.05) altered (Fig. 3C, Table SI-1). A total of 19 and 26 proteins were significantly increased and decreased, respectively, in the post-group (Fig. 3C, Table SI-1).

### 3.4. Identification of potential urine biomarkers in response to emergency smoke

One of our particular interests of this study is to identify some potential target proteins in response to fire smoke exposure. To achieve a highly stringent target protein list, we further applied a threshold to each significant protein that should be identified and quantified at least 50 % (five out of 10) of the sample in each group. This criterion facilitated the identification of a total of thirteen highly confident potential proteins that were suitable for consideration as biomarkers (Fig. SI-3). Among these, thirteen proteins [i.e. cDNA FLJ78262, highly similar to semenogelin II (SEMG2), Syndecan (SDC4), retinal dehydrogenase 1 (ALDH1A1), serum amyloid P-component (APCS), sortilin-related receptor (SORL1), CNDP dipeptidase 2 (CNDP2), D-dopachrome decarboxylase (DDT), NSFL1 cofactor p47 (NSFL1C), secretoglobin family 1D member 2 (SCGB1D2), putative transmembrane protein INAFM1 (INAFM1), C-type lectin domain family 7 (CLEC7A), ammonium transporter Rh type C (RHCG), and gamma-glutamylcyclotransferase], were increased abundance in the post-group. Similarly, three proteins [i.e. adrenocorticotropic hormone (POMC), multimerin-1 (MMRN1), and VPS10 domain-containing receptor SorCS2 (SORCS2)] decreased in the pre-group. Among these proteins, APCS, SORL1, NSFL1C, SCGB1D2, and CLEC7A are only identified in the post group. On the other hand, SEMG2, SDC4, CNDP2, and INFAM1 were identified in both group of samples but their abundance in post group is >10-fold higher compared with the pre group. Gene ontology biological pathway analysis of these proteins shows proteins associated with epithelium development, such as ADAMTSL2 and SORL1, which are involved in many other functions were significantly increased (Fig. 4A and B). Similarly, disease pathway analysis of these differentially expressed proteins shows SORL1, which is associated with Alzheimer's disease, was significantly increased in the post group sample (Fig. 4C, Table SI-1). A protein-protein network analysis further elucidated the association between these differentially expressed proteins and the most prevalent human cancer protein TP53. (Fig. 4D).

### 3.5. Cross-comparison analysis with urine cancer proteome dataset

Another major goal of the current study was to determine whether firefighters' exposure to fire smoke increases their risk of developing cancer. We therefore performed a pairwise comparison of the significantly regulated proteins identified in this study against four curated panels of urine proteins resulting from important cancer research initiatives. The first panel includes a total of 572 proteins that are considered as potential cancer biomarkers in Human Protein Atlas (https://www.proteinatlas.org/). The second set comprises 349 urine proteins are associated with various renal conditions and prostate cancer (Swensen et al., 2021). The third and fourth datasets contained 69 and 41 urine proteins, respectively, that showed differential regulation in response to lung (Zhang et al., 2018) and colorectal cancer (Sun et al., 2022). Our pairwise comparison analysis showed that a total of seven proteins (i.e. COL2A1, SDC4, RDX, POMC, APCS, ALDH1A1, and DDAH2) overlapped with these known cancer proteins and/or biomarkers (Fig. 5A). Out of these seven proteins, ALDH1A1, APCS, RDX, COL2A1 and SDC4 were increased and POMC and DDAH2 were decreased in abundance in the fire smoke exposure group (Fig. 5B).

## 4. Discussion

Each emergency fire presents a unique situation, especially in terms of duration, fuel, and type of fire. In this study, participants were exposed to wildland (e.g., grass) and structural (e.g., residential, trailer house, warehouse) fires for an average duration of 1.5 h. Despite the wide range of variability in fire smoke exposure during these emergencies, results of the current study support our hypothesis that exposure to fire smoke at an emergency fire alters the expression of protein and peptide patterns in the urine of male firefighters. Similar proof-of-concept research on the systemic molecular changes of the plasma proteome in response to ambient environmental exposure revealed that a number of proteins had significantly altered in abundance and were considered potential candidates for heightened cardiovascular disease risk markers (Mookherjee et al., 2022).

Because proteins serve as the primary players in cellular functions, carrying out essential tasks within our cells, the proteins' altered levels benefit cells and tissues in disease states, specifically cancers (Ding et al., 2022). Furthermore, significant expressions of the identified proteins have the potential to serve as markers for therapeutic and diagnostic purposes in cancer cases (Madda et al., 2020), particularly within the firefighter cohort, known for elevated cancer incidence.

Our comparative proteomic analysis identified a total of 45 differentially expressed proteins (DEPs), 19 of which were up-regulated (2.8-folds, $p < 0.05$) and 26 of which were down-regulated (0.35-folds, $p < 0.05$). Using the criterion that >50 % of both the pre- and post-fire samples were quantified, we identified 16 urine proteins that respond to exposure to fire smoke as potential biomarkers with high confidence. The potential role of these proteins was discussed further in relation to

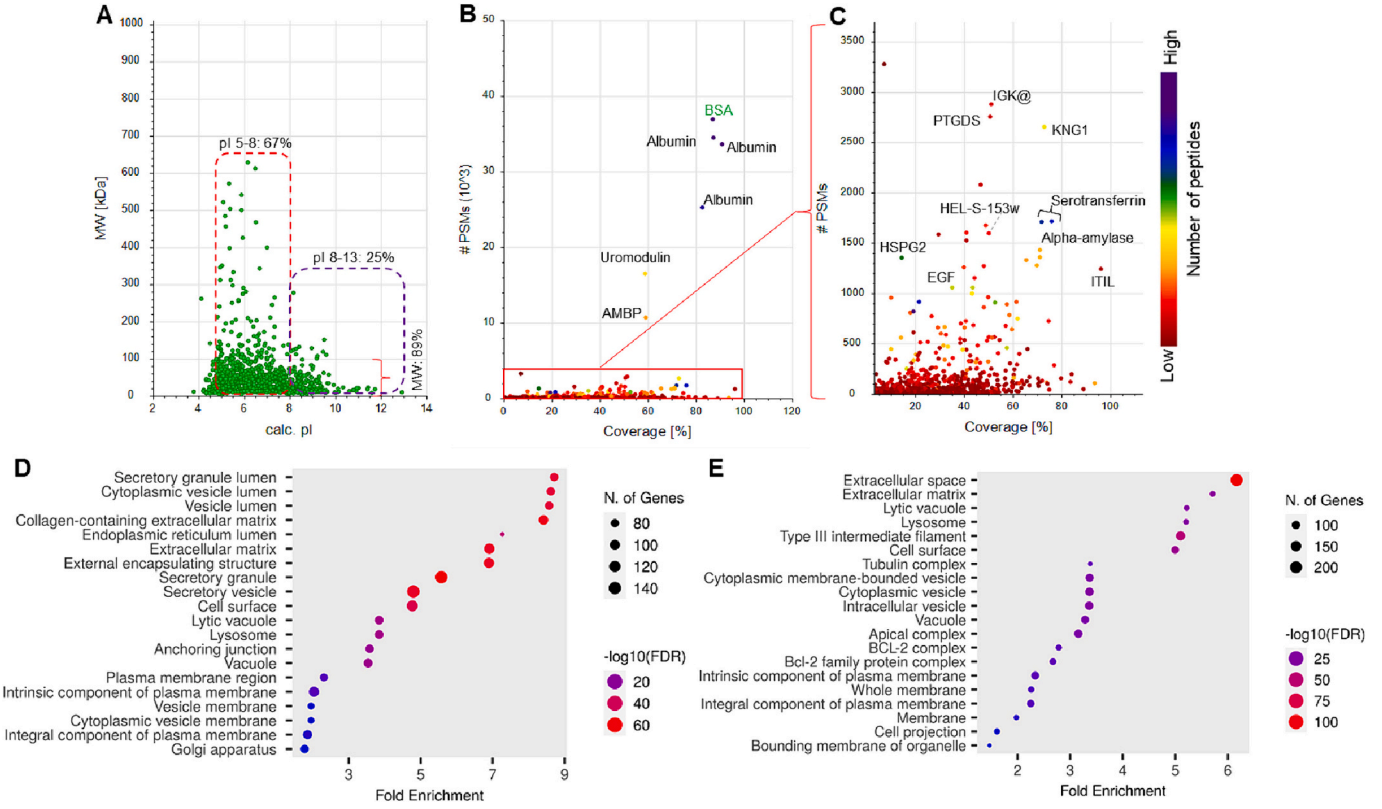


**Fig. 2.** Physiochemical and qualitative properties of urine proteome. (A) Scatter plot analysis shows the pI and MW of the identified proteins. The red and purple dotted box shows the pI range of the majority of the proteins. The red brace shows 89 % of proteins MW ranged 10–100 Da. (B–C) Shows the relationship of the number of identified peptides, peptide spectral match (PSM) and sequence coverage (%) of the proteins identified from urine samples. Known urine protein markers such as Albumin, Uromodulin, Serotransferrin, etc. are labeled. (D–E) Represent the GO Cellular and JENSEN Compartments analysis of the urine proteome. ShinyGO 0.76 (Ge et al., 2020) was used to perform the functional analysis.

cancer and other disorders. It is interesting to note that a protein-protein networks investigation reveals some of these proteins are associated to TP53 (Fig. 4D), the most prevalent cancer gene (Donehower et al., 2019; Hernández Borrero and El-Deiry, 2021; Hassin and Oren, 2023), suggesting a possible direct or indirect association in the regulation of TP53 pathways.

Both gene ontology and the diseases pathway analyses showed that sortilin-related receptor 1 (SORL1) protein was involved in various biological functions, particularly with Alzheimer's disease (Fig. 4C). Additionally, SORL1 shows strong association with VPS10 domain-containing receptor (SORCS2), wherein SORL1 and SORCS2 were increased and decreased, respectively, in the post group (Figs. 4D, SI-3). The biological function The sortilin-related receptor 1 (SORL1) gene belongs to two distinct receptor families: the low-density lipoprotein receptor (LDLR) family of ApoE receptors and the vacuolar protein sorting 10 (VPS10) domain receptor family (NCBI, 2023). While the mutation of this gene is primarily linked to Alzheimer's disease, recent studies have uncovered its significance in relation to prognostic biomarkers of colorectal cancer (Li et al., 2023), bladder urothelial carcinoma (Xu et al., 2021), and renal cancer (Uhlen et al., 2017). The secretoglobin ecretoglobin family 1D member 2 (SCGB1D2) gene is a member of the secretoglobin superfamily and is highly expressed in mammary tissues (NCBI, 2023). A study by Lu et al. (2011) found that SCGB1D2 mRNA was not expressed in nasal mucosa from chronic rhinosinusitis patients. The absence of SCGB1D2 suggests that it is not a significant contributor to airway function in this specific pathological condition. Furthermore, an earlier cancer study of pancreatic ductal adenocarcinoma patients found that the SCGB1D2 in serum may not be useful as a diagnostic biomarker (Taniuchi et al., 2018). However, further studies need to be conducted with urinary samples. The C-type lectin domain family 7 (CLEC7A) gene is a member of the C-type lectin/C-type lectin-like domain (CTL/CTLD) superfamily and plays a role in the innate immune response against pathogenic fungi (NCBI, 2023). CLEC7A expression was observed in myeloid-derived suppressor cells of colorectal cancer patients and correlated with death rate and tumor severity (Tang et al., 2023).

To further investigate proteins that show significant regulation in response to fire-smoke, we compared them with the protein panels from urine proteome cancer-focused studies (Fig. 5). This comparison revealed an overlap of seven proteins with known cancer proteins and biomarkers. Four proteins (i.e. APCS, ALDH1A1, RDX, and POMC) overlapped with Swensen et al. (2021), two (COL2A1 and SDC4) proteins with Human Protein Atlas, and DDAH2 with Zhang et al. (2018).

It is also important to note that APCS, ALDH1A1, and POMC showed potential interactions with TP53 (Fig. 4D). The serum amyloid P-component (APCS), also known as SAP, is a universal constituent of human amyloid deposits. APCS is associated with many human diseases by mediating the action of antidepressant medications (Yang et al., 2022), causing subsequent neurodegeneration in the brain (Yip et al., 2023), and predicting overall survival in non-small cell lung cancer (NSCLC) patients (Zhao et al., 2016). Furthermore, Aldehyde dehydrogenase 1A1 (ALDH1A1), CD133, and mutant TP53 are promising candidates for NSCLC. Patients without ALDH1A1, CD133, TP53 show a much better prognosis than others (Yamashita et al., 2022). It has been reported that UV radiation induced p53 activation to promote cutaneous pigmentation by upregulating opiomelanocortin (POMC) transcriptional activity in the skin (Rizzato et al., 2011). Similarly, SDC4 and COL2A1 also showed potential association to human diseases (Fig. 4D). Syndecan 4 (SDC4), a protein-coding gene associated with thyroid cancer and achromatopsia 7, shows a direct correlation with COL2A1. A study

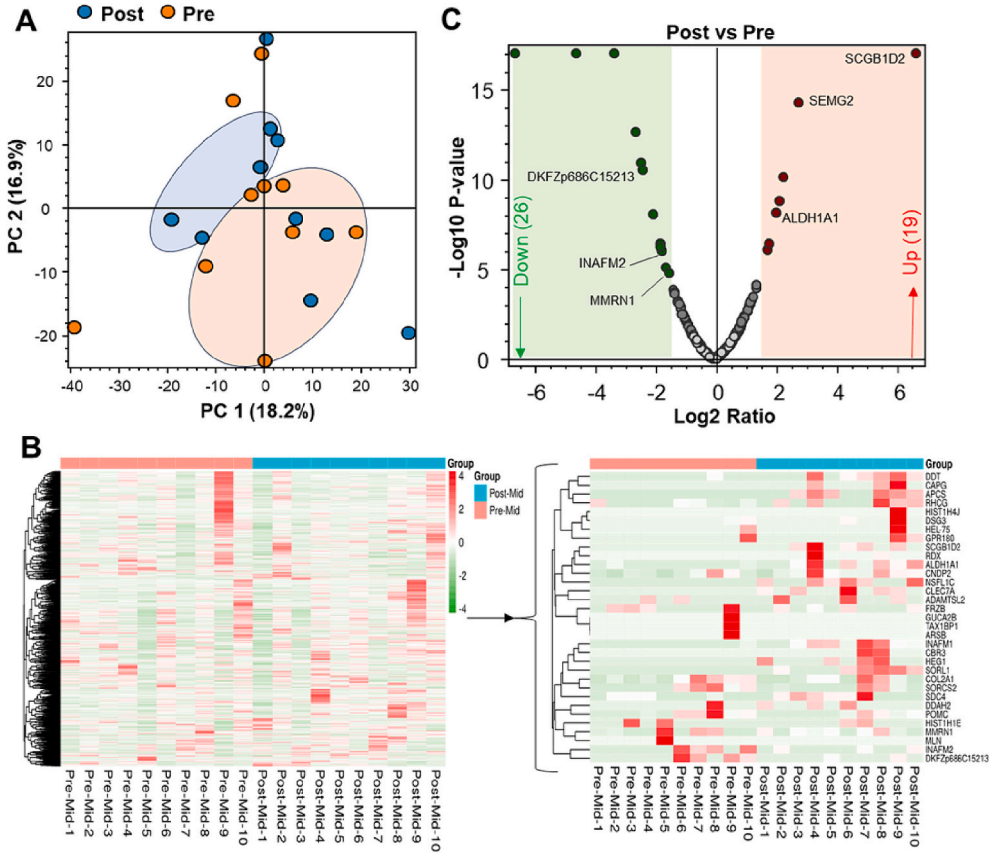


**Fig. 3.** Comparative quantitative (label-free) proteomic analysis of pre and post exposure firefighter urine samples. Total proteome (1325 proteins) identified from 20 firefighter urine samples were subjected to quantitative analysis. (A–B) Represent the principal component analysis (PCA) and heat map clustering of total the protein abundance of each sample identified and quantified from urine samples. (C) Shows the volcano plot analysis of the significantly increased (dark red circle) and decreased (dark green circle) proteins in post sample compared to pre sample. Gray circles are non-significant (below threshold |log2(fold change)| <1.5 fold and/or $p > 0.05$).

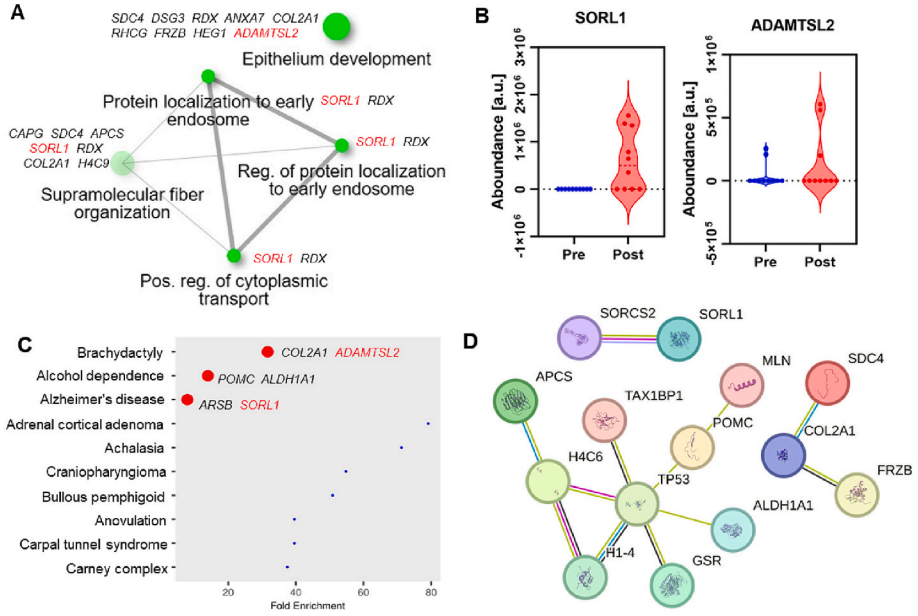


**Fig. 4.** Pathway and protein-protein analysis of differentially expressed proteins. A, Biological pathway analysis of the significantly increased and decreased proteins. Node size indicates the number of the genes and edge thickness indicates relative fold-enrichment. B, violine plot analysis (expression pattern) of two target proteins (red color genes in panels A and C) were shown as potential urine biomarkers for firefighters exposed to emergency fire smoke. C, Jensen disease pathway analysis. D, a protein-protein network of the differentially expressed proteins in connection to the most prevalent human cancer gene (TP53) was generated by using STRING (https://string-db.org/).

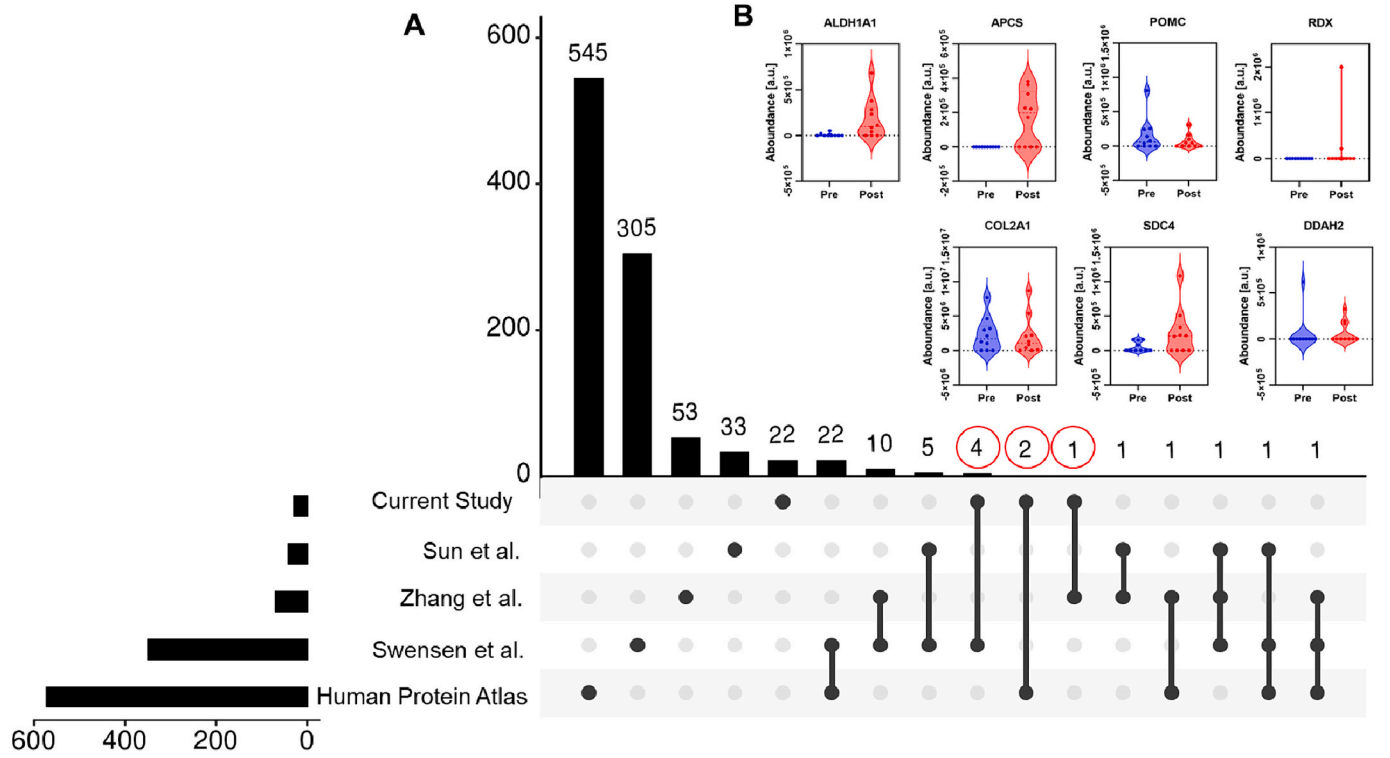


**Fig. 5.** Cross comparison between proteins from this work and from urine proteome cancer-focused studies. (A) Upset chart showing the overlapping proteins with other urine proteome dataset. Numbers of identified proteins shared between different sets of are indicated in the top bar chart and the specific study in each set are indicated with solid points below the bar chart. Figure generated using Upset R package as described in Materials and methods section. (B) Violin plot analysis of the seven overlapping proteins (red circled in panel A).

showed that by knocking down SDC4 in TNF-α-induced OA rats, COL2A1 and ACAN were increased (Cao et al., 2022).

Overall, the importance of our findings is reinforced by the overall overlaps of these urine proteins with previously reported cancer protein datasets, which improves our understanding of the development and progression of malignant tumors as possible cancer biomarkers. In addition, some of these discovered urine proteins have been validated as biomarkers for colorectal, bladder, kidney, and prostate cancers, which are especially suitable for screening and early detection. Although limited, previous studies attempting to identify candidate cancer biomarkers by employing urine samples have detected alterations in protein concentrations when comparing cancer patients to healthy controls. Urine from bladder cancer patients showed elevated protein abundance in FGB (fibrinogen b chain), APOE (apolipoprotein E), SERPINA1 (α-1-antitrypsin), and LRG1 (leucine-rich α-2-glycoprotein 1) when compared to urine from healthy control groups, identifying those proteins as potential biomarkers (Lindén et al., 2012). A case-control study (Fan et al., 2022) compared 255 subjects, some with gastric cancer and others in different stages of gastric lesions. The study identified 43 differentially expressed urine proteins in the subjects with gastric cancer as compared to those with mild or advanced gastric lesions. The ANXA11, CDC42, and NAPA proteins showed positive associations with the risk of gastric lesion progression. However, the identification of tumor origin for cancer in the urine proteomic is controversial. Ding et al. (2022) argue that protein profiles derived from liquid biopsy samples are likely to provide more organ-specific information. Yet, Fan et al. (2022) contend that it becomes challenging to precisely determine the primary source of changes in protein abundance as organ- or tissue-specific.

In clinical settings, the potential translational significance of non-invasive urine biomarkers becomes evident in the identification of high-risk populations for therapeutic purposes and the prognosis of cancer. In order to offer patients a range of effective therapeutic options, it is crucial to identify specific molecular alterations that can help categorize them and to expand our knowledge of genotype (from genomics alone) to phenotype (from multi-omics including proteomics) relationships (Gonçalves et al., 2022). This need includes understanding how signaling pathways in tumor cells are modified and determining how to effectively target these pathways for cancer therapy (Kwon et al., 2021). Thus, in the context of occupational preventive medicine, it is essential to compare the alterations at molecular levels through the detection of urine proteins, which provides a promising approach to investigating biomarkers associated with the progression of carcinogenesis and the risk of early-stage cancer.

During the initial recruitment process for firefighters, most fire departments enforce a mandatory physical examination. Additionally, newly recruited firefighters are typically obligated to engage in a comprehensive occupational medical program that is mandated by the fire department. However, it remains uncertain how many firefighters will continue to receive annual physical and medical exams once the initial requirements have been fulfilled. Presently, the National Fire Protection Association (NFPA) 1582 Standard on Comprehensive Occupational Medical Program for Fire Departments (NFPA, 2022) is the only standard that addresses medical programs for firefighters, including cancer screening (in particular, colon, prostate, lung, testicular, bladder, oral, thyroid, and skin). However, compliance with this standard is voluntary. As a diagnostic technique, MS-based proteomics can accurately identify and quantitively measure biomarkers to proactively prevent cancer. Our recommendation is to adapt this technology for incorporation into annual occupational medical exams. Recently, the House of Representatives introduced a bill that provides medical testing and related services to detect and prevent certain cancers to firefighters in the Department of Defense (DoD) (U.S. Congress, 2023). This bill mainly focuses on breast, colon, and prostate cancers among DoD firefighters and would extend these services to municipal and independent volunteer firefighters across the U.S.

The present study's strength lies in three aspects. First, this preliminary investigation was designed so that each participant was his own control (pre-fire), thus mitigating the potential effects of confounding covariates. This approach reduces error terms and enhances the statistical power of the analysis. Second, we purposely selected a subset of samples collected from white males from a larger cohort population to minimize any other human physiological changes. Although sample size is a limiting factor for this study, we demonstrated the feasibility of generating fire smoke-related proteome profiles of healthy firefighters. Third, most applications in proteomic research have been conducted with clinical samples from patients. The proteome profile of our healthy cohort using pre-fire as a baseline provided a unique and stable set of proteins, indicating that a panel of proteins could potentially be used for early diagnosis of cancer, especially within a longitudinal study design.

On the other hand, we also acknowledge the limitations of this study. Due to the small number of participants and biological sample types, we were unable to consider other exposure factors such as years of experience as a firefighter, quantity of exposure to fire smoke, and hours spent at the fire suppression. Our findings cannot be generalized to a larger firefighter population. Therefore, a research plan should be prioritized with a larger cohort of samples across a range of age groups using a number of biological samples, such as urine and plasma, in order to uncover the potential cancer biomarkers for firefighters.

## 5. Conclusion

In summary, this pilot study sought to gain insight into the urinary proteome of firefighters between two time points – before (pre) and after (post) a fire suppression. We identified seven potential target proteins that respond to exposure to fire smoke, all of which are associated with urological cancers. Given the potential use of these proteins as biomarkers, we recommend integrating cancer screening into current physical and medical exams for both new recruits and incumbent firefighters across fire departments. In the future, this work also has the potential to make a broader impact on other populations through the elucidation and application of this technique. This study confirmed the clinical significance of alterations in the urine proteome with respect to urological tumors and identified several urinary biomarkers for detecting bladder cancer. By promoting health screening such as urine biomarker tests, we can identify key proteins that regulate pathways associated with tumor cell growth and metastasis. Furthermore, using a healthy firefighter cohort, we can identify prognostic biomarkers in untargeted proteomics experiments based on urine proteins and peptides. Further experiments will be required to validate clinical utility using independent sets of larger numbers of clinical samples and a prospective study design.

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## CRediT authorship contribution statement

**Jooyeon Hwang:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Zongkai Peng:** Writing – review & editing, Formal analysis. **Fares Z. Najar:** Writing – review & editing, Formal analysis. **Chao Xu:** Writing – review & editing. **Robert J. Agnew:** Writing – review & editing, Investigation. **Xin Xu:** Writing – review & editing. **Zhibo Yang:** Writing – review & editing, Formal analysis. **Nagib Ahsan:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

All mass spectrometry RAW files, and comparative proteome analysis results files were deposited in the online repository platform MassIVE (https://massive.ucsd.edu/). Proteomics data can be found at MassIVE database via (MSV000093896).

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