Effects of Different Odors on Rat Urinary Proteome

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Abstract

Do changes occur in the urinary proteome of rats when they smell odors? Do different odors produce different changes? This study collected urine samples from 6 rats on days 3 and 4 after exposure to sesame oil and essential balm for 3 days, and performed both group-based and individual before-after comparative analyses on the samples using LC-MS/MS technology. Simultaneously, the identified differential proteins were compared with those arising from growth and development in rats of the same age to exclude the influence of growth and development on the experimental results. The experimental results demonstrated that comparing the urinary proteomes of the sesame oil group between Day 0 and Day 4, 143 differential proteins could be identified after screening, with an average of 7.32 differential proteins arising randomly, indicating that at least 94.88% of the differential proteins were not randomly generated. Through group-based and individual before-after comparative analyses, the same odor exhibited relatively consistent changes. In the sesame oil group, differential proteins related to olfactory generation were identified, including low-density lipoprotein receptor-related protein 2 and fetuin B (a biomarker for COPD). Meanwhile, in the essential balm group, differential proteins with significant changes related to olfactory generation were identified, including uteroglobin, trefoil factor 3, and mucin 2. Notably, odorant binding protein 2A was again identified in the essential balm group, simultaneously present among the differential proteins from individual before-after comparisons in 4 rats, which is consistent with results presented in the e-cigarette model. This study demonstrates that odors can influence the urinary proteome of rats, and that different odors exert different effects. This provides a novel method for investigating the biological processes of olfaction.

Full Text

Effects of Different Odors on the Rat Urine Proteome

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Abstract: Do rats exhibit corresponding changes in their urinary proteome when exposed to different odors? And do different odors produce distinct proteomic signatures? To address these questions, we collected urine samples from six rats before and after exposure to sesame oil and essential balm for three consecutive days, analyzing them using LC-MS/MS technology through both group-based and individual before-after comparisons. To control for confounding effects, we compared identified differential proteins against those arising from normal growth and development in age-matched rats. Our results demonstrated that comparing Day 0 and Day 4 urine proteomes in the sesame oil group yielded 143 differential proteins, whereas random variation produced an average of only 7.32 differential proteins, indicating that at least 94.88% of the observed changes were non-random. Through group and individual comparative analyses, we found consistent patterns associated with each odorant. In the sesame oil group, we identified differential proteins related to olfactory processing, including low-density lipoprotein receptor-related protein 2 and fetuin B (a COPD biomarker). In the essential balm group, we identified significantly altered proteins associated with olfaction, including uteroglobin, trefoil factor 3, and mucin 2. Notably, we again identified odorant-binding protein 2A (OBP2A) in the essential balm group, which was present in differential protein sets from four individual rats in before-after comparisons, consistent with findings from our e-cigarette model. This study demonstrates that odors significantly impact the rat urinary proteome in an odor-specific manner, providing a novel approach for investigating the biological processes underlying olfaction.

Keywords: Urine, Proteomics, Odor

1.1 Urinary Biomarkers

Biomarkers are objective indicators that reflect normal and pathological physiological processes [1], enabling prediction, monitoring, and diagnosis of multifactorial diseases at various stages [2]. Compared to widely used blood-based biomarkers, the potential of urinary biomarkers remains underexplored, particularly for early disease diagnosis and state prediction. Due to homeostatic regulation in blood, disease-induced proteomic changes are often metabolized and eliminated before manifesting as detectable alterations in early disease stages. In contrast, urine is produced through glomerular filtration of plasma without homeostatic regulation, making it highly sensitive to subtle physiological changes that appear long before clinical symptoms, pathological changes, or even blood-based alterations. Animal models offer an ideal system for urinary

proteomics research because genetic and environmental factors can be controlled to minimize confounding influences beyond normal development. For example: (1) Zhang et al. [3] found 29 altered urinary proteins in an Alzheimer's disease transgenic mouse model before amyloid plaque deposition, 24 of which had been reported as Alzheimer's-related markers; (2) Wu et al. [4] identified 10 altered urinary proteins in a Walker-256 tumor-bearing rat model before tumors became palpable; (3) Zhang et al. [5] detected 15 differential urinary proteins in a chronic pancreatitis rat model at week 2, before pathological changes appeared; (4) Ni et al. [6] observed urinary protein changes in a glioma rat model before MRI detection; (5) Zhang et al. [7] identified 40 differential urinary proteins in a thioacetamide-induced liver fibrosis model before pathological changes, with 15 reported as fibrosis-related; (6) Yin et al. [8] found disordered urinary glucose levels before blood glucose elevation in type 2 diabetic rats; and (7) Huang et al. [9] identified reported COPD biomarkers in rats after only two weeks of cigarette smoke exposure. Comparative studies further revealed that urinary protein changes differ when tumor cells grow in different organs (subcutaneous [4], liver [7], bone [10], lung [11], brain [6]), demonstrating urine's potential to distinguish tumor location. Additionally, urine collection is non-invasive and readily obtainable [12], making it an excellent source of biomarkers and highlighting the importance of animal models in urinary proteomics research.

Currently, few studies have investigated how different odors affect the rat urinary proteome. Our previous research identified multiple odorant-binding protein isoforms in rat urine after jasmine-flavored e-cigarette exposure, demonstrating that urinary proteomics can reflect physiological changes following odor stimulation [13]. This raises an intriguing question: do different odors produce distinct proteomic changes? To address this, we selected sesame oil and essential balm as odorants and used 6-8-week-old male Wistar rats to investigate odor-specific effects on the urinary proteome [Figure 1: see original paper], aiming to provide novel methods for exploring olfactory biology.

[Figure 1: see original paper] Technical workflow for investigating the effects of different odors on the rat urine proteome

2.1 Animal Model Establishment

We purchased six specific-pathogen-free (SPF) male Wistar rats (6-8 weeks old, 180-200 g) from Beijing Vital River Laboratory Animal Technology Co., Ltd. (License No. SYXK(Beijing)2021-0011). All rats were housed under standard conditions (temperature 22$±$1°C, humidity 65-70%) and acclimatized for three days before experiments. All procedures were approved by the Ethics Committee of the College of Life Sciences, Beijing Normal University.

The odor exposure protocol was as follows: Six experimental rats were exposed to sesame oil once daily for three consecutive days. For each exposure, 15 mL of sesame oil was placed in a rat cage (36 × 20 × 28 cm) in a location inaccessible to the rats. Three rats were placed per cage for 1 hour under adequate oxygen

conditions, after which they were returned to their home cages. After a one-week washout period, the same rats were exposed to essential balm using an identical protocol (15 mL, 1 hour daily for 3 days). Behavioral changes were monitored throughout the experiment.

2.2 Urine Sample Collection

After three days of acclimatization, all rats were placed in metabolic cages to collect 12-hour urine samples (baseline, Day 0). Urine was similarly collected on Day 3 (after three days of sesame oil exposure) and Day 4 (one day post-exposure). The same collection schedule was applied for the essential balm group (Day 10: baseline; Day 13: after three days of exposure; Day 14: one day post-exposure). During collection, rats were deprived of food and water, and all samples were stored at -80°C.

2.3 Urine Sample Processing

Protein extraction and quantification: Urine samples were centrifuged at 12,000 × g for 40 min at 4°C. Supernatants were transferred to new Eppendorf tubes, mixed with three volumes of ice-cold ethanol, and precipitated overnight at -20°C. The following day, samples were centrifuged at 12,000 × g for 30 min at 4°C, supernatants were discarded, and protein pellets were resuspended in lysis buffer (8 M urea, 2 M thiourea, 25 mM DTT, 50 mM Tris). After centrifugation at 12,000 × g for 30 min at 4°C, supernatants were collected and protein concentrations were measured using the Bradford method.

Enzymatic digestion: For each sample, 100 g of urinary protein was loaded onto a 10 kDa ultrafiltration membrane (Pall, Port Washington, NY, USA) in an Eppendorf tube and diluted to 200 L with 25 mM NH_4HCO_3. Proteins were reduced with 20 mM DTT (Sigma) at 37°C for 1 hour, cooled to room temperature, and alkylated with 50 mM iodoacetamide (IAA, Sigma) for 40 min in the dark. The membrane was then washed as follows: (1) twice with 200 L UA solution (8 M urea, 0.1 M Tris-HCl, pH 8.5) at 14,000 × g for 5 min at 18°C; (2) sample loading and centrifugation at 14,000 × g for 40 min at 18°C; (3) twice with 200 L UA solution at 14,000 × g for 40 min at 18°C; (4) twice with 25 mM NH_4HCO_3 at 14,000 × g for 40 min at 18°C. Trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) was added at a 1:50 enzyme-to-protein ratio and incubated overnight at 37°C. Peptides were collected by centrifugation at 13,000 × g for 30 min at 4°C, desalted using HLB columns (Waters, Milford, MA), dried in a vacuum concentrator, and stored at -80°C.

2.4 LC-MS/MS Tandem Mass Spectrometry Analysis

Digested samples were dissolved in 0.1% formic acid, quantified using a BCA kit, and diluted to 0.5 g/ L. A pooled peptide sample was prepared by mixing 4 L from each sample and fractionated using a high-pH reverse-phase peptide

fractionation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Ten fractions were collected, dried, and reconstituted in 0.1% formic acid. iRT reagent (Biognosys, Switzerland) was added at a 10:1 sample-to-iRT ratio to calibrate peptide retention times. For analysis, 1 g of peptides per sample was analyzed using an EASY-nLC1200 chromatography system coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA).

For spectral library generation, the 10 fractions were analyzed in Data-Dependent Acquisition (DDA) mode with high-sensitivity settings. Full MS scans were acquired at 60,000 resolution over a 350-1500 m/z range. Individual samples were analyzed in Data-Independent Acquisition (DIA) mode using a 36-window method. Quality control was performed by analyzing a pooled peptide sample via DIA after every eight samples.

2.5 Database Searching and Label-Free DIA Quantification

Raw data files were processed in Proteome Discoverer (version 2.1, Thermo Scientific) and searched against the Swiss-Prot rat database (May 2019 release, 8,086 sequences) with iRT sequences appended. Search results were imported into Spectronaut Pulsar (Biognosys AG, Switzerland) for further analysis. Peptide abundance was calculated by summing peak areas of fragment ions in MS2 spectra, and protein intensity was determined by summing the abundances of constituent peptides.

2.6 Data Analysis

Each sample was analyzed in three technical replicates, with average values used for statistical analysis. Experimental groups were compared before and after odor exposure, with age-matched controls to exclude developmental effects. Differential proteins were identified using two criteria: (1) relaxed: fold change (FC) ≥ 1.5 or ≤ 0.67, two-tailed unpaired t-test $P < 0.05$; (2) stringent: FC ≥ 2 or ≤ 0.5, $P < 0.01$. Functional enrichment analysis was performed using the Wukong platform (https://www.omicsolution.org/wkomic/main/), UniProt (https://www.uniprot.org/), and DAVID database (https://david.ncifcrf.gov/). Literature searches in PubMed (https://pubmed.ncbi.nlm.nih.gov) were conducted for functional interpretation of differential proteins.

3.1 Animal Model Characteristics

Behavioral observations revealed distinct responses to the two odorants. Rats exposed to sesame oil exhibited excitement, hyperactivity, and apparent appetite, whereas those exposed to essential balm showed aversion, avoidance, and tended to huddle in corners.

3.2 Proteomic Identification in Rats Exposed to Different Odors

Urine samples were collected from six male Wistar rats (6-8 weeks old) at six time points (Day 0, 3, 4, 10, 13, 14). LC-MS/MS analysis of peptides from 36 urine samples identified a total of 1,145 proteins ($ $2 unique peptides per protein, protein-level FDR $< 1\%$).

3.3.1 Intra-Group Comparison of Urinary Proteome Changes After Sesame Oil Exposure

Comparative analysis of urinary proteins before and after sesame oil exposure revealed 52 differential proteins between Day 0 and Day 3 under relaxed criteria (FC ≥ 1.5 or ≤ 0.67, P < 0.05) [TABLE:S1]. Comparison with developmental differential proteins from age-matched rats over three days showed minimal overlap (only 4 of 52 proteins) [TABLE:S2, S3], suggesting that developmental effects contributed little to our observations. Comparison between Day 0 and Day 4 identified 143 differential proteins [TABLE:S1] that encompassed all 52 proteins from the Day 0 vs. Day 3 comparison [Figure 2: see original paper]. Under stringent criteria (FC ≥ 2 or ≤ 0.5, P < 0.01), we identified 1 differential protein (Cd99, FC = 0.03, P = 8.80E-03) between Day 0 and Day 3, and 17 differential proteins between Day 0 and Day 4 .

Several identified proteins have reported roles in olfaction. Ekström et al. [14] reported that serum C-reactive protein (CRP) levels negatively correlate with olfactory identification ability in aging, while Proft et al. [15] found that olfactory impairment in granulomatosis with polyangiitis patients correlates with elevated CRP. Frizzled receptors, atypical GPCRs with seven transmembrane domains, mediate olfactory sensory neuron axon extension via Wnt/Frizzled signaling [16], and Yue et al. [17] demonstrated that Wnt-activated olfactory ensheathing cells stimulate neural stem cell proliferation and differentiation. Strotmann and Breer [18] showed that low-density lipoprotein receptor-related protein 2 (LRP2) mediates internalization of odorant-binding protein/odorant complexes into sustentacular cells, representing a potential mechanism for rapid odorant clearance. Gajera et al. [19] demonstrated that LRP2 deficiency impairs neuroprogenitor proliferation in the subventricular zone, reducing neuroblast migration to the olfactory bulb. Diao et al. [20] identified fetuin B as a plasma biomarker candidate correlating with COPD severity. We also identified apolipoprotein E (ApoE) under relaxed criteria [TABLE:S1], which is highly expressed in the central nervous system including olfactory epithelium and bulb, playing critical roles in olfactory information processing and affecting the olfactory system in early Alzheimer's disease [21][22]. Struble et al. [23] also linked ApoE to ongoing degeneration and regeneration in the olfactory nerve.

Functional enrichment analysis of Day 0 vs. Day 3 differential proteins (relaxed criteria) revealed 73 biological processes (e.g., cellular response to cytochalasin B, negative regulation of endopeptidase activity, regulation of norepinephrine uptake, protein localization to adherens junctions) [TABLE:S4] and 31 signal-

ing pathways (e.g., apoptosis, phagocytosis, proteoglycans in cancer, leukocyte transendothelial migration) [TABLE:S5]. Analysis of Day 0 vs. Day 4 differential proteins identified 134 biological processes (e.g., negative regulation of endopeptidase activity, proteolysis, cellular response to cytochalasin B, aging, acute-phase response, regulation of norepinephrine uptake) [TABLE:S6] and 34 signaling pathways (e.g., proteoglycans in cancer, complement and coagulation cascades, leukocyte transendothelial migration) [TABLE:S7].

3.3.2 Intra-Group Comparison of Urinary Proteome Changes After Essential Balm Exposure

Under relaxed criteria, comparison of Day 10 vs. Day 13 identified 66 differential proteins [TABLE:S8], with minimal overlap with developmental changes (only 2 of 66 proteins) [TABLE:S2, S3]. Day 10 vs. Day 14 comparison identified 90 differential proteins [TABLE:S8]. Under stringent criteria, Day 10 vs. Day 13 yielded 14 differential proteins , while Day 10 vs. Day 14 yielded 20 differential proteins , with 10 proteins overlapping between the two comparisons.

Stringent comparisons identified mucin 1 (Muc1), protein kinase C substrate 80K-H, mucin 19, trefoil factor 3, and β-defensin 1 (BD-1) in both Day 10 vs. Day 13 and Day 10 vs. Day 14 analyses . Among 21 known mucin isoforms, Muc1, 4, 5AC, and 8 are found in human sinus epithelium, while Muc1, 5B, and 8 localize to sinus glands [24][25][26][27]. Some mucins, including Muc1, contain transmembrane domains that anchor them to the cell membrane, while others are secreted [28], suggesting Muc1 may protect sensitive olfactory structures [29]. Bruch et al. [30][31] demonstrated that protein kinase C (PKC) mediates signal termination and desensitization in olfaction through phosphorylation of odorant receptors and other substrates. Li et al. [32] found that trefoil factor 3 reverses depression-like behaviors in olfactory bulbectomized rats via BDNF-ERK-CREB signaling activation. Baines et al. [33] reported that elevated β-defensin-1 characterizes COPD and severe asthma, with dysregulated production in COPD epithelial cells, suggesting its potential as a biomarker and therapeutic target.

Aquaporin-1 (AQP-1) was identified in both stringent Day 10 vs. Day 13 and relaxed Day 10 vs. Day 14 comparisons [TABLE:2, S8]. AQP-1 is expressed in olfactory ensheathing glia, vascular endothelial cells of olfactory and respiratory mucosa, and surrounding connective tissue [34][35], though its specific role in olfaction remains unclear. Gliomedin and insulin-like growth factor binding protein (IGFBP) were identified in stringent Day 10 vs. Day 14 and relaxed Day 10 vs. Day 13 comparisons [TABLE:2, S8]. Gliomedin, an olfactomedin family member, is required for molecular assembly at peripheral nervous system nodes of Ranvier [36][37], while IGFBP-2 binds proteoglycans in rat olfactory bulb membranes and regulates chitosan-mediated differentiation of human olfactory receptor neurons [38][39]. Pro-epidermal growth factor (EGF) identified in relaxed Day 10 vs. Day 14 comparison [TABLE:S8] may regulate mitosis in olfactory epithelium [40], and reduced EGF receptors in aging mouse olfactory

epithelium may inhibit cell proliferation and cause epithelial atrophy [41].

Functional enrichment of Day 10 vs. Day 13 differential proteins (relaxed criteria) revealed 29 biological processes (e.g., intermediate filament organization, wound healing, response to nutrient levels) [TABLE:S9] and 3 signaling pathways (Staphylococcus aureus infection, ECM-receptor interaction, TGF-β signaling) [TABLE:S10]. Day 10 vs. Day 14 analysis identified 133 biological processes (e.g., positive regulation of cell migration, response to xenobiotic stimulus) [TABLE:S11] and 8 signaling pathways (focal adhesion, fluid shear stress and atherosclerosis, proteoglycans in cancer) [TABLE:S12].

3.4 Inter-Group Comparison of Urinary Proteome Changes After Odor Exposure

Venn diagram analysis of differential proteins from the four comparisons under relaxed criteria showed only 13 shared proteins between sesame oil and essential balm groups [FIGURE:2, TABLE:3], with most differential proteins being odor-specific, indicating minimal consistency in proteomic responses to different odors.

[Figure 2: see original paper] Venn diagram of differential proteins identified at different time points after sesame oil and essential balm exposure

Differential proteins shared between sesame oil and essential balm groups (FC ≥ 1.5 or ≤ 0.67, $P < 0.05$)

Interestingly, we repeatedly identified serine-related proteins in both groups . Serine protease inhibitor Spi2 overexpression produces secreted signals causing olfactory neuron death [42], while calcium/calmodulin-dependent serine protein kinase CASK localizes to olfactory cilia and may function in odor transduction [43]. The serine-related proteins identified in our study have not been previously linked to olfaction, potentially offering new avenues for investigating olfactory mechanisms.

3.5.1 Individual Rat Analysis: Sesame Oil Group

Under relaxed criteria, Venn diagrams of differential proteins from individual rats comparing Day 0 vs. Day 3 [Figure 3A: see original paper] and Day 0 vs. Day 4 [Figure 3B: see original paper] showed no proteins shared by five or more rats, with substantial variation in the number of differential proteins between individuals (e.g., Rat #1 vs. Rat #6), indicating strong individual variability in urinary proteomic responses to sesame oil.

[Figure 3: see original paper] Venn diagrams of differential proteins from six individual rats in before-after comparisons (FC ≥ 1.5 or ≤ 0.67, $P < 0.05$). (A) Day 0 vs. Day 3. (B) Day 0 vs. Day 4. (C) Day 10 vs. Day 13. (D) Day 10 vs. Day 14.

Differential proteins identified in $ $3 rats in individual before-after comparisons in the sesame oil group (FC $\geq$ 1.5 or $\leq$ 0.67, P $<$ 0.05)

3.5.2 Individual Rat Analysis: Essential Balm Group

In contrast to sesame oil, essential balm exposure produced more consistent proteomic changes across individuals. Venn diagrams revealed 18 proteins shared among all six rats for Day 10 vs. Day 13 [Figure 3C: see original paper] and 63 proteins shared by $ $5 rats [TABLE:S13]. Similar consistency was observed for Day 10 vs. Day 14 [Figure 3D: see original paper]. Notably, trefoil factor 3, hepcidin, and mucins identified in individual analyses overlapped with group analysis results [TABLE:2, TABLE:6]. We also identified three glypican isoforms (glypican-1, 3, 4) in multiple rats. Saroja et al. [44] demonstrated that glypican-4 is an ApoE4 binding partner that mediates ApoE4-induced tau hyperphosphorylation. Since ApoE4 plays critical roles in olfactory information processing and affects the olfactory system in early Alzheimer's disease [21][22], our identification of glypican-4 may provide clues for investigating its role in olfaction.

Importantly, odorant-binding protein 2A (OBP2A) was identified in four rats for Day 10 vs. Day 13 (with three showing downregulation) and in three rats for Day 10 vs. Day 14 (with two showing downregulation). This protein was also identified in our rat e-cigarette model with consistent expression changes across six rats [13].

Differential proteins identified in $ $6 rats in individual before-after comparisons in the essential balm group (FC $\geq$ 1.5 or $\leq$ 0.67, P $<$ 0.05)

3.6 Random Grouping Validation

To assess the likelihood that identified differential proteins arose by chance, we performed random grouping validation. For Day 0 vs. Day 3 samples (FC $\geq$ 1.5 or $\leq$ 0.67, P $<$ 0.05), 462 random combinations yielded an average of 9.79 differential proteins, indicating at least 81.17% of our identified proteins were non-random . For Day 0 vs. Day 4, random combinations averaged 7.42 differential proteins, indicating 94.88% reliability . Using stringent criteria (FC $\geq$ 2 or $\leq$ 0.5, P $<$ 0.01), random combinations averaged 0.16 proteins, indicating 99.06% reliability . For Day 10 vs. Day 13, random combinations averaged 19.91 proteins (relaxed criteria) and 1.41 proteins (stringent criteria), indicating 69.83% and 89.93% reliability, respectively . For Day 10 vs. Day 14, random combinations averaged 21.12 proteins (relaxed criteria) and 1.49 proteins (stringent criteria), indicating 76.53% and 92.55% reliability, respectively .

Results of random grouping validation

4. Conclusion

This study demonstrates that odors significantly and differentially affect the rat urinary proteome. Different odorants, such as sesame oil and essential balm, produce distinct proteomic signatures, providing a novel approach for investigating the biological mechanisms of olfaction.

References

[1] Kyle Strimbu, Jorge A Tavel. What are biomarkers? Current Opinion in HIV and AIDS. 2010, 5(6). [2] Gerszten Robert E, Wang Thomas J. The search for new cardiovascular biomarkers. Nature. 2008, 451(7181). [3] Zhang F, Wei J, Li X, Ma C, Gao Y. Early Candidate Urine Biomarkers for Detecting Alzheimer's Disease Before Amyloid-β Plaque Deposition in an APP (swe)/PSEN1dE9 Transgenic Mouse Model. J Alzheimers Dis. 2018, 66(2):613-637. [4] Wu J, Guo Z, Gao Y. Dynamic changes of urine proteome in a Walker 256 tumor-bearing rat model. Cancer Med. 2017, 6(11):2713-2722. [5] Zhang Y, Xie X, Zhao X, Tian F, Lv J, Ying W, Qian X. Systems analysis of singly and multiply O-glycosylated peptides in the human serum glycoproteome via EThcD and HCD mass spectrometry. J Proteomics. 2018, 170:14-27. [6] Ni Y, Zhang F, An M, Yin W, Gao Y. Early candidate biomarkers found from urine of glioblastoma multiforme rat before changes in MRI. Sci China Life Sci. 2018, 61(8):982-987. [7] Zhang F, Ni Y, Yuan Y, Yin W, Gao Y. Early urinary candidate biomarker discovery in a rat thioacetamide-induced liver fibrosis model. Sci China Life Sci. 2018, 61(11):1369-1381. [8] Yin W, Qin W, Gao Y. Urine glucose levels are disordered before blood glucose level increase was observed in Zucker diabetic fatty rats. Sci China Life Sci. 2018, 61(7):844-848. [9] Qin W, Huang H, Dai Y, et al. Proteome analysis of urinary biomarkers in a cigarette smoke-induced COPD rat model. Respir Res. 2022, 23: 156. [10] Wang T, Li L, Qin W, Gao Y. Early urine proteome changes in an implanted bone cancer rat model. Bone Rep. 2019, 12:100238. [11] Wei J, Ni N, Meng W, Gao Y. Early urine proteome changes in the Walker-256 tail-vein injection rat model. Sci Rep. 2019, 9(1):13804. [12] Zou Lili, Sun Wei. Human urine proteome: a powerful source for clinical research. Adv Exp Med Biol. 2015, 845:31-42. [13] Liu Y, Shen Z, Gao Y, et al. Urine proteomic analysis of the rat e-cigarette model. PeerJ. 2023, 11:e16041. [14] Ekström I, Vetrano DL, Laukka EJ, et al. Serum C-Reactive Protein Is Negatively Associated With Olfactory Identification Ability in Older Adults. Iperception. 2021, 12(2):20416695211009928. [15] Proft F, Steinbach S, Grunke M, et al. Gustatory and olfactory function in patients with granulomatosis with polyangiitis (Wegener's). Scand J Rheumatol. 2014, 43(6):512-8. [16] Rodriguez-Gil DJ, Greer CA. Wnt/Frizzled family members mediate olfactory sensory neuron axon extension. J Comp Neurol. 2008, 511(3):301-17. [17] Yue Y, Xue Q, Zhang L, et al. Wnt-activated olfactory ensheathing cells stimulate neural stem cell proliferation and neuronal differentiation. Brain Res. 2020, 1735:146726. [18] Strotmann J, Breer H. Internalization of odorant-binding proteins into the mouse olfactory epithelium. Histochem Cell

Biol. 2011, 136(3):357-69. [19] Gajera CR, Emich H, Hammes A, et al. LRP2 in ependymal cells regulates BMP signaling in the adult neurogenic niche. J Cell Sci. 2010, 123(Pt 11):1922-30. [20] Diao WQ, Shen N, He B, et al. Fetuin-B (FETUB): a Plasma Biomarker Candidate Related to the Severity of Lung Function in COPD. Sci Rep. 2016, 6:30045. [21] East BS, Fleming G, Wilson DA. Human Apolipoprotein E Genotype Differentially Affects Olfactory Behavior and Sensory Physiology in Mice. Neuroscience. 2018, 380:103-110. [22] Zhang J, Hao C, Yang L, et al. The mechanisms underlying olfactory deficits in apolipoprotein E-deficient mice: focus on olfactory epithelium and olfactory bulb. Neurobiol Aging. 2018, 62:20-33. [23] Struble RG, Short J, Nathan BP, et al. Apolipoprotein E immunoreactivity in human and mouse olfactory bulb. Neurosci Lett. 1999, 267(2):137-40. [24] Richard A. Cone. Chapter 4 - Mucus. Mucosal immunology (Third Edition). 2005, 49-72. [25] Martínez-Antón A, Debolós C, Mullol J, et al. Mucin genes have different expression patterns in healthy and diseased upper airway mucosa. Clin Exp Allergy. 2006, 36:448-457. [26] Ali MS, Pearson JP. Upper airway mucin gene expression: a review. Laryngoscope. 2007, 117:932-938. [27] Rubin BK. Mucus and mucins. Otolaryngol Clin North Am. 2010, 43(1):27-34, vii-viii. [28] Hattrup CL, Gendler SJ. Structure and function of the cell surface (tethered) mucins. Annu Rev Physiol. 2008, 70:431–457. [29] Kennel C, Gould EA, Ramakrishnan VR, et al. Differential Expression of Mucins in Murine Olfactory Versus Respiratory Epithelium. Chem Senses. 2019, 44(7):511-521. [30] Bruch RC. Phosphoinositide second messengers in olfaction. Comp Biochem Physiol B Biochem Mol Biol. 1996, 113(3):451-9. [31] Bruch RC, Kang J, Medler KF, et al. Protein kinase C and receptor kinase gene expression in olfactory receptor neurons. J Neurobiol. 1997, 33(4):387-94. [32] Li J, Luo Y, Shi J, et al. Neuropeptide Trefoil Factor 3 Reverses Depressive-Like Behaviors by Activation of BDNF-ERK-CREB Signaling in Olfactory Bulbectomized Rats. Int J Mol Sci. 2015, 16(12):28386-400. [33] Baines KJ, Wright TK, Gibson PG, et al. Airway β-Defensin-1 Protein Is Elevated in COPD and Severe Asthma. Mediators Inflamm. 2015, 2015:407271. [34] Shields SD, Moore KD, Basbaum AI, et al. Olfactory ensheathing glia express aquaporin 1. J Comp Neurol. 2010, 518(21):4329-41. [35] Ablimit A, Matsuzaki T, Takata K, et al. Immunolocalization of water channel aquaporins in the nasal olfactory mucosa. Arch Histol Cytol. 2006, 69(1):1-12. [36] Eshed Y, Feinberg K, Peles E, et al. Gliomedin mediates Schwann cell-axon interaction and the molecular assembly of the nodes of Ranvier. Neuron. 2005, 47(2):215-29. [37] Maertens B, Hopkins D, Koch M, et al. Cleavage and oligomerization of gliomedin, a transmembrane collagen required for node of ranvier formation. J Biol Chem. 2007, 282:10647–59. [38] Russo VC, Bach LA, Werther GA, et al. Insulin-like growth factor binding protein-2 binds to cell surface proteoglycans in the rat brain olfactory bulb. Endocrinology. 1997, 138(11):4858-67. [39] Huang TW, Li ST, Young TH, et al. Regulation of chitosan-mediated differentiation of human olfactory receptor neurons by insulin-like growth factor binding protein-2. Acta Biomater. 2019, 97:399-408. [40] Farbman AI, Buchholz JA. Transforming growth factor-alpha and other growth factors stimulate cell division in olfactory epithelium in vitro. J Neurobiol. 1996, 30(2):267-80. [41] Ohta Y, Ichimura K.

Changes in epidermal growth factor receptors in olfactory epithelium associated with aging. Ann Otol Rhinol Laryngol. 2000, 109(1):95-8. [42] Thiemmara V, Pays L, Mehlen P. et al. Serine protease inhibitor Spi2 mediated apoptosis of olfactory neurons. Cell Death Differ. 2002, 9(12):1343-51. [43] Saavedra MV, Smalla KH, Wyneken U. et al. Scaffolding proteins in highly purified rat olfactory cilia membranes. Neuroreport. 2008, 19(11):1123-6. [44] Saroja SR, Gorbachev K, Pereira AC, et al. Astrocyte-secreted glypican-4 drives APOE4-dependent tau hyperphosphorylation. Proc Natl Acad Sci USA. 2022, 119(34): e2108870119.

Note: Figure translations are in progress. See original paper for figures.