Unrestrictive Modification Identification and Comparison of the Urinary Proteome in Healthy Individuals: Multi-Site Oxidative Modifications Characterize Urinary Protein Modifications across Age Groups

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Abstract

Although the protein content in urine from healthy individuals is relatively low, the species are extremely diverse. The information contained within the urinary proteome can reveal subtle differences during physiological and early pathological stages of the organism. Protein chemical modification refers to covalent group reactions involving amino acid residues or chain termini, which consequently alter molecular structure and functions in regulation and signal transduction. Therefore, comparative studies of urinary protein chemical modification levels are likewise of significant importance. This study employed urine from healthy individuals across different age groups (22 children, 10 young adults, and 6 elderly) as research subjects, utilizing high-resolution tandem mass spectrometry and label-free quantitative proteomic analysis methods, combined with an unrestricted modification identification algorithm to comprehensively compare protein chemical modification differences among the three sample types. The results demonstrated that oxidative modifications involving multiple amino acid residues constitute the primary difference between urinary protein samples from elderly and young individuals, with these modifications influencing the biological processes of numerous proteins. This study represents the first investigation utilizing an unrestricted modification identification algorithm to examine differences in overall proteome modifications in urine across different age groups, identifying oxidative modification as the main modification type distinguishing urinary proteins between young individuals and the elderly.

Full Text

Preamble

Unrestricted Identification and Comparison of Protein Modifications in Normal Human Urine: Multi-site Oxidative Modifications Characterize Urinary Protein Modifications Across Different Age Groups

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Abstract

Although the protein content in healthy human urine is low, the variety is remarkably rich, and the information contained within the urinary proteome can reveal subtle differences in physiological and early pathological states. Protein chemical modification refers to covalent group reactions occurring at amino acid residues or chain termini, which alter molecular structure and modulate regulatory and signal transduction functions. Therefore, comparative studies of urinary protein chemical modifications are equally important. This study investigated urine samples from healthy individuals across different age groups (22 children, 10 young adults, and 6 elderly individuals) using high-resolution tandem mass spectrometry and label-free quantitative proteomics, combined with an unrestricted modification identification algorithm to comprehensively compare protein chemical modification differences among the three sample types. The results demonstrate that oxidative modifications involving multiple amino acid residues constitute the primary difference between elderly and young urinary protein samples, affecting numerous protein biological processes. This study represents the first application of unrestricted modification identification algorithms to investigate age-related differences in global proteome modifications in human urine, identifying oxidative modification as the main modification type distinguishing urinary proteins between young and elderly individuals.

Keywords: oxidative modification; global chemical modification; unrestricted modification identification algorithm; urine; proteome

Introduction

Living organisms coordinate numerous biological processes simultaneously, relying heavily on proteins involved in synthesis, catalysis, and regulation. Proteins are complex biological macromolecules whose distinct higher-order structures determine their specific biochemical activities, and they form advanced networks through interactions to execute particular functions. Protein chemical modification refers to covalent group reactions at amino acid residues or chain

termini that typically alter protein higher-order structure. While a few structural changes do not affect biological activity—these are termed non-essential modifications—in most cases, molecular alterations significantly change physicochemical properties, protein conformation, and activity, thereby modifying function. Consequently, even when protein abundance remains unchanged, subtle changes in chemical modification levels can profoundly affect protein function, demonstrating that chemical modifications enrich the functional diversity and regulatory capacity of proteins at another dimension. The impact of protein chemical modifications manifests in three aspects: (1) a single modification type can affect function; (2) different amino acids undergoing the same or different modifications on the same protein have distinct functional consequences; and (3) multiple modification types on the same protein can make its biological processes more variable and complex. Major chemical modification types include: (1) Post-translational modifications (PTM), which occur after protein translation and often convert inactive precursors into functional mature proteins through specific modifying enzymes; (2) Chemical derived modifications, which introduce new groups or remove existing ones from protein side chains, typically including spontaneous non-enzymatic modifications and those introduced by cross-linkers or artificial reagents; and (3) Amino acid substitution, where original amino acids in protein side chains are replaced by other types, altering protein properties and functions. All these significantly affect protein function.

Mass spectrometry enables large-scale omics data acquisition and deep mining while accurately measuring targeted modifications in specific proteins. Advances in instrumentation, particularly ultra-high-resolution tandem mass spectrometry (LC-MS/MS), provide rich and precise information for proteomics and modification studies, facilitating accurate identification of chemical modification sites in protein chains. Conventional proteomic data analysis requires searching against species protein databases, typically involving manual specification of known modification types—an approach termed restricted search. This becomes inadequate when seeking unknown modification types in samples. Therefore, comprehensive, unrestricted searches are crucial for understanding the complete chemical modification information in sample proteomes. The Open-pFind algorithm is an open sequence library search algorithm that integrates information from the UniMod database to analyze mass spectrometry data through open searching, yielding global chemical modification information of proteomes.

Although total protein content is low in healthy human urine, the variety is exceptionally rich, with over 6,000 different proteins identified in normal human urine. Under physiological conditions, the glomerulus filters some proteins, of which 98% are reabsorbed in the renal tubules, while the remaining 2% are excreted along with small amounts of mucoproteins secreted by renal tubular and other urinary tract epithelial cells. Since urine is produced by the kidneys and stored in the bladder for several hours, urinary protein information directly reflects urinary system health. However, most urinary proteins are filtered from plasma and theoretically originate from blood. Their persistence after renal filtration and reabsorption indicates that the remaining 2% are not randomly

"leaked" by healthy kidneys. Another important research direction in urinary proteomics is the chemical modification field, as comprehensive comparison of urinary proteome chemical modification changes provides richer information for studying physiological changes. Currently, over 1,500 protein chemical modification types are cataloged in UniMod, PSI-MOD, and RESID databases. While plasma proteomes reflect subtle conditions related to age and aging, urine—another protein-rich body fluid—also contains aging information. Our previous studies on newborn rat urine proteomes revealed significant protein expression differences, and even common physiological processes like starvation leave traces in the urinary proteome. Based on the importance of chemical modifications in age-group urine proteomes, this study employed high-resolution tandem mass spectrometry combined with unrestricted searching (Open-pFind) to compare global chemical modification differences among three age groups.

Methods

Sample Sources

Urinary proteome data from 22 healthy children were derived from a study on recurrent urinary tract infections in patients with vesicoureteral reflux (Raw data download: http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD010469). Urinary proteome data from 6 healthy elderly individuals originated from a study on urinary neutrophils and associated inflammation (Raw data download: http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD004713). All downloaded data represented healthy control samples. Ten healthy young adult urine samples were collected from laboratory volunteers without restrictions on diet, medication, or other factors. Volunteers received detailed study information including purpose, methods, and procedures, with personal data kept strictly confidential. Sample details are shown in Table 1 .

Protein Sample Preparation and Trypsin Digestion

Young adult urine samples were treated with 20 mmol/L dithiothreitol (DTT) at 37°C for 1 hour to reduce disulfide bonds, followed by addition of 55 mmol/L iodoacetamide (IAA) and incubation in darkness for 30 minutes to alkylate disulfide bond sites. Proteins were precipitated from the supernatant with three volumes of pre-cooled acetone at -20°C for 2 hours, then centrifuged at 12,000 × g for 30 minutes at 4°C to obtain protein pellets. Precipitates were resuspended in protein solubilization buffer (8 mol/L urea, 2 mol/L thiourea, 25 mmol/L DTT, and 50 mmol/L Tris). Protein concentration was measured using the Bradford assay. Using the Filter-Aided Sample Preparation (FASP) method, 100 µg of protein from each sample was digested with trypsin (Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, WI, USA) at a 50:1 protein-to-enzyme ratio. After 14 hours of digestion at 37°C, 10% formic acid was added to terminate digestion. Peptide solutions were obtained by centrifugation through 10 kDa ultrafiltration tubes, with peptide concentration determined by BCA assay and dried using a vacuum centrifuge concentrator (Thermo Fisher, USA).

Dried peptides were sealed and stored at -80°C. Table 2 compares the processing methods for the other two population groups from literature sources with the present method.

LC-MS/MS Analysis and Database Searching

Prior to analysis, dried peptide samples from healthy young adults were dissolved in 0.1% formic acid to a final concentration of 0.1 μg/μL. Each sample containing 1 μg of peptides was loaded onto pre-column and analytical column using a Thermo EASY-nLC1200 chromatography system. Proteomic data were acquired using a Thermo Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). LC method: pre-column: 75 μm × 2 cm, nanoViper C18, 2 μm, 100 Å; analytical column: 50 μm × 15 cm, nanoViper C18, 2 μm, 100 Å; injection volume: 10 μL; flow rate: 250 nL/min. Mobile phases: A: 100% MS-grade water (Fisher Scientific, Spain)/1‰ formic acid (Fisher Scientific); B: 80% acetonitrile (Fisher Scientific, USA)/20% water/1‰ formic acid. Gradient elution over 120 min: 0 min, 3% B; 0-3 min, 8% B; 3-93 min, 22% B; 93-113 min, 35% B; 113-120 min, 90% B. MS method: ion source: nanoESI; spray voltage: 2.0 kV; capillary temperature: 320°C; S-lens RF Level: 30; resolution settings: MS1 (Orbitrap) 120,000 @ m/z 200, MS2 30,000 (Orbitrap) @ m/z 200; precursor scan range: m/z 350-1350; product ion scan range: starting from m/z 110; MS1 AGC: 4e5; charge range: 2-7; injection time: 50 ms; MS2 AGC: 1e5; injection time: 50 ms; ion selection window: 2.0 m/z; fragmentation mode: high-energy collision dissociation (HCD); energy: NCE 32; Data-dependent MS/MS: Top 20; dynamic exclusion time: 15 s; internal calibration mass: 445.12003. Table 3 compares the data acquisition methods for the other two population groups from literature sources with the present method.

LC-MS/MS data were analyzed using label-free quantification with pFind Studio software (version 3.1.6, Institute of Computing Technology, Chinese Academy of Sciences). The target search database was the Homo sapiens database downloaded from UniProt (updated February 2020). Search parameters: instrument type HCD-FTMS, full trypsin specificity, maximum 2 missed cleavages, "open-search" mode. Filtering criteria: peptide-level FDR $< 1\%$, protein-level Q-value $< 1\%$. Data were analyzed using both forward and reverse database search strategies.

Statistical Analysis

Descriptive statistics were calculated for pFind data, including median (for skewed distributions) and interquartile range, as well as proportions (percentages). Statistical comparisons among child, young adult, and elderly groups were assessed using t-tests, ANOVA, Mann-Whitney U tests, and Kruskal-Wallis tests. Statistical analyses were performed using GraphPad Prism v7.04, with $p < 0.05$ considered statistically significant.

Results

2.1 Protein Identification via Bottom-Up Proteomics

Using label-free quantitative proteomics, experimental results were obtained for 38 samples through LC-MS/MS analysis. After data (.raw) searching with open-pFind software, results could be browsed and exported in pBuild. Sample results were compiled and statistically analyzed, with protein and peptide identifications shown in Table 4 .

2.2 Global Chemical Modification Information and Differential Modification Statistics

A total of 485 distinct chemical modification types were identified across 38 samples, including 301 types in the child group, 329 in the young adult group, and 106 in the elderly group. Figure 1 [Figure 1: see original paper] shows the Venn diagram of modification type intersections among the three sample types; detailed intersection information is provided in Supplementary Table 1.

Unsupervised clustering analysis of global modifications and the 62 shared modifications revealed that one elderly sample and two young adult samples clustered under the child group branch in the shared modification heatmap. The same elderly sample appeared anomalous in both heatmaps, clustering within child and young adult groups. Otherwise, elderly, young adult, and child groups could be distinguished in both analyses, as shown in Figures 2 [Figure 2: see original paper] and 3 [Figure 3: see original paper]. Forty modifications showed statistical differences ($p < 0.05$), with each modification' s inter-group variation detailed in Table 5 . Inter-group comparisons for each differential modification are shown in Figure 4 [Figure 4: see original paper]. Significantly different modifications among groups included: oxidation of proline, tryptophan, tyrosine, cysteine, and methionine; cysteine to dehydroalanine (Cys->Dha [C]); N-terminal glycidamide modification (glycidamide [AnyN-term]); serine phosphorylation (Phospho [S]); calcium ion replacement of two protons on aspartic and glutamic acids (Cation_{Ca} [E], Cation_{Ca} [D]); N-terminal succinylation (Succinyl [AnyN-term]); N-terminal carbamylation (Carbamyl [AnyN-term]); valine to threonine substitution (Val->Thr [V]); glutamic acid to methionine substitution (Glu->Met [E]); glutamine substitution for glutamic acid (Glu->Gln [E]); and asparagine deamidation (Deamidated [N]), among others. Since oxidative modifications showed the most significant age-related differences, all oxidative modifications among the 485 identified types—including oxidation of proline, tryptophan, tyrosine, cysteine, methionine, and other non-significant oxidative modifications—were selected for clustering analysis, which clearly distinguished the three age groups, as shown in Figure 5 [Figure 5: see original paper].

Urinary protein samples from the elderly group showed significantly higher levels of tryptophan (W), proline (P), cysteine (C), and tyrosine (Y) oxidation, as well as serine (S) carbonylation, compared to child and young adult groups. Conversely, carbamidomethylation (the alkylation modification introduced by

IAA) was significantly lower in the elderly group. Since carbamidomethylation acts on reduced disulfide bonds, this indirectly indicates significantly lower disulfide bond integrity in elderly urinary proteins compared to young adults and children. Young adult groups showed significantly higher levels of serine phosphorylation, cysteine maleimide modification, asparagine (N) to aspartic acid (D) substitution, glutamic acid (E) to methionine (M) and glutamine (Q) substitutions, glycine (G) to glutamic acid substitution, and cysteine desulfurization to dehydroalanine (Dha) compared to child and elderly groups. In the child group, calcium ion-related modifications at glutamic and aspartic acid residues were significantly higher than in young adult and elderly groups, while modifications involving cysteine dehydrogenation and various N-terminal modifications (carbamylation, glycidamide, succinylation) were lower than in the other groups. Additionally, glutamine cyclization to pyroglutamic acid (pyro-Glu) showed an age-dependent decreasing trend.

Unique modification types in the elderly group included oxidation of isoleucine (I), glutamine, glutamic acid, and phenylalanine (F), as well as phosphorylation of aspartic acid, glutamic acid, and tyrosine (see Supplementary Table 1 for more information). Figure 6 [Figure 6: see original paper] illustrates modification identification information for several peptides from elderly group MS2 spectra.

Protein interaction analysis was performed on 971 proteins containing the aforementioned modifications (selected by taking the union from one randomly chosen sample per age group) using WebGestalt (http://www.webgestalt.org) with PPI BIOGRID—Network Topology-based Analysis (NTA) under default parameters. Enriched biological pathway analysis indicated that modifications affected immune system and blood coagulation-related functions, as shown in Figure 7 [Figure 7: see original paper]. Among these, 446 proteins (45.9% of total) contained the five identified oxidative modifications. Methionine oxidation may be subject to experimental interference, as some modifications in the UniMod database are designated as artificial. Non-artificial modifications were present in 359 proteins, with proline, tryptophan, tyrosine, and cysteine oxidation found in 256 proteins (71.3%). By querying non-artificial modification-containing peptides in pBuild' s Peptide.all_{result} and modification sites in pFind.Summary, we calculated that the oxidation modification proportion was 12.97% (by peptide count) in child samples, 13.71% in young adult samples, and significantly higher at 29.76% in elderly samples, as detailed in Table 6 .

Discussion

This study revealed that multiple site-specific oxidation levels in elderly urinary proteins are significantly higher than in young adults and children, suggesting that urine serves as a primary route for excreting oxidized and aged proteins from the body. Higher quantities of oxidized proteins produced by the elderly result in greater excretion of these proteins. Protein oxidizability is directly related to primary and three-dimensional structural features, and oxidative modifications can cause structural and functional damage. The extent of oxidized

protein accumulation in vivo represents an important manifestation of human aging and may serve as a marker for aging degree and efficacy of anti-aging interventions.

Among proline-oxidized peptides identified across the three groups, we found peptides belonging to Collagen (A0A384MDU2_{HUMAN}) and Collagen alpha-1(I) chain preproprotein (H9C5C5_{HUMAN}). Proline and hydroxyproline constitute approximately one-quarter to one-fifth of collagen amino acids. Collagen, the main component of extracellular matrix, is degraded to provide amino acids and energy for cell growth and proliferation, and is also degraded during inflammation and tumor progression. Proline dehydrogenase overexpression in certain cancer cell lines is associated with apoptosis through mechanisms involving reactive oxygen species (ROS) generation from proline oxidation. Since proline dehydrogenase overexpression also correlates with reduced tumor formation in vivo, it is considered a direct regulator of p53-induced tumor suppression. Meanwhile, some cancer cells may exploit proline oxidation to promote proliferation and stress protection. We therefore infer that rapid collagen aging in the elderly produces large amounts of oxidized proline proteins that are continuously excreted in urine, resulting in significantly higher urinary content of proline-oxidized proteins compared to young adults and children.

Peptides with tryptophan, tyrosine oxidation, and methionine-substituted-glutamic acid modifications across the three groups belonged to Fibrinogen alpha chain (FIBA_{HUMAN}), and fibrinogen-derived peptides were also found among elderly-specific modifications. Fibrinogen is one of the most abundant plasma proteins and a frequent target for protein post-translational modification reagents, representing the final member of the coagulation cascade. Activated by thrombin, fibrinogen converts to fibrin that polymerizes into complex fibrin networks. The fibrin mesh, together with platelets, red blood cells, and few white blood cells, constitutes the main component of thrombi. Under physiological conditions, thrombi prevent blood loss at injury sites, but under pathophysiological conditions, they can obstruct vessels (thrombosis) and release into circulation (embolism), potentially causing stroke or myocardial infarction. Oxidation generated during inflammatory responses (such as oxidation at Met476 in the αC domain) has been shown to cause thinner fibrin fibers, resulting in denser clots that are more resistant to proteolysis and carry risks of deep vein thrombosis and pulmonary embolism.

Most evidence suggests that fibrinogen oxidative modifications induced by enhanced oxidative stress may be involved in disease pathogenesis. Plasma fibrin clots composed of dense meshwork fibers are poorly lysed due to hindered plasminogen diffusion within clots, observable in patients with cardiovascular disease, chronic inflammation, liver disease, and diabetic complications.

Tryptophan oxidation products accumulate with age and inflammation, with tryptophan catabolites such as kynurenine causing osteoporosis and exerting diverse tissue-specific effects. Among tyrosine oxidation products, dityrosine release occurs only under oxidative stress (exposure to H2O2) followed by prote-

olysis, making it a specific marker for conditions such as atherosclerosis, acute inflammation, systemic bacterial infection, and cataract. Oxidation of histidine, tyrosine, and tryptophan has been demonstrated in response to various reactive oxygen species, and although these are generally considered irreversible modifications, their relevance to biological protein regulation remains unclear.

The most common sites for reversible redox modifications in proteins include redox-active transition metal ion centers (such as heme groups, iron-sulfur protein centers, zinc-sulfur protein centers) and oxidation-sensitive amino acid side chains (primarily cysteine, selenocysteine, and methionine residues). Oxidative modifications at these sites can enhance or inhibit enzyme activity (e.g., by oxidizing catalytic cysteine residues) and can also alter protein-protein interactions, subcellular trafficking, and protein turnover. The redox signaling field has focused primarily on reversible oxidation of cysteine residues, which are highly conserved in proteins and whose relative abundance increases with multicellular organism evolution. In contrast, methionine oxidation as a potential signaling mechanism is less understood, largely due to lack of biochemical reagents to assess methionine oxidation in biological systems, with only MS methods successfully demonstrating methionine sulfoxide formation. Sulfoxidation of methionine residues significantly increases hydrophilicity, substantially altering protein physicochemical properties to enhance or inhibit activity. Moreover, similar to reversible cysteine oxidation, enzyme systems that both enhance and reverse methionine sulfoxidation have been identified.

Recent proteomic analyses show that methionine oxidation tends to stabilize phosphorylation, suggesting complex relationships between methionine oxidation and protein phosphorylation pathways. Aging is associated with gradual oxidation of the extracellular environment. The redox state of human plasma, defined by cysteine and cystine concentrations, becomes increasingly oxidized with age. Direct oxidation of cysteine (and less commonly methionine) residues is a major reaction that typically occurs faster than H2O2 and leads to altered protein activity and function. Unlike H2O2, which is rapidly cleared by protective enzymes, protein peroxides are only slowly removed, with catabolism being the primary fate. Although proteasomal and lysosomal enzymes and other proteases (such as mitochondrial Lon) efficiently turnover modified proteins, protein hydroperoxides inhibit these pathways, potentially contributing to modified protein accumulation in cells. Current evidence supports links between protein oxidation and multiple human pathologies, though whether these relationships are causal remains to be determined. Methionine-dependent TRPV2 (transient receptor potential vanilloid 2) redox sensitivity may represent an important endogenous mechanism regulating TRPV2 activity, which plays a critical role in giant cell phagocytosis.

Iodoacetamide alkylation at cysteine sites (carbamidomethylation) reflects the number of thiol groups in protein chains, where cysteine thiols are crucial for disulfide bond formation, and disulfide bond quantity reflects protein spatial structural integrity. Comparing carbamidomethylation across age groups re-

vealed no significant difference between young adult and child groups, but elderly group carbamidomethylation was significantly lower than in young adults and children, indicating age-dependent increases in protein structural disruption.

Beyond oxidative modifications, we previously investigated cysteine substitution by dehydroalanine and N-terminal carbamylation in our article "Why are there proteins in the urine of healthy people?" Studies show that cysteine Cys34 modification in human serum albumin (HSA) can serve as a marker for oxidative stress-related diseases in blood samples from patients with chronic liver disease, chronic kidney disease, or diabetes. The conversion of cysteine to dehydroalanine (Dha) is irreversible; Dha is an electrophile containing an unsaturated carbon-carbon double bond that acts as an alkylating agent in protein chains in vitro, and many Dha-containing peptides exhibit toxicity. We previously hypothesized that proteins or peptides with this modification could become toxic if retained in the body and should be immediately excreted, explaining the presence of numerous Dha-modified proteins in urine.

Carbamylation is another irreversible non-enzymatic process where urea decomposition products react with protein N-termini or lysine side chains, associated with protein aging. Lysine carbamylation can facilitate metal ion coordination for specific enzyme activities. Reportedly, plasma carbamylation levels increase significantly in patients with elevated urea levels (e.g., kidney disease patients).

Glycidamide (GA) is the DNA-reactive metabolite of acrylamide (AA), a recognized dietary carcinogen whose carcinogenic potential and ability to form DNA adducts are related to N-terminal protein modification.

Complex developmental programs and signaling pathways in vertebrate species depend heavily on phosphorylation-mediated signaling. Serine and threonine phosphorylation in disordered regions is more likely non-functional. A 2018 large-scale study of human and mouse phosphorylation sites found that, compared to young groups, elderly group sites were more likely functional and involved in signaling pathways, with serine phosphorylation potentially associated with neurodegenerative diseases. Mutations in specific genes cause neurodegeneration through loss of protein function, while post-translational modifications regulate protein structure and function, and in several pathogenic proteins, also modulate toxicity. Recent studies have identified links between phosphorylation and arginine methylation, with evidence suggesting these modifications play important roles in neurodegeneration, such as reduced huntingtin (Htt) phosphorylation at serine 421 indicating Akt signaling dysregulation in pathogenesis.

Although succinylation research is still in its early stages, data clearly demonstrate broad impacts on health and disease, providing coupling between metabolism and protein function in the nervous system and neurological disorders as a key cellular integration mechanism. Carbamylation is an irreversible non-enzymatic modification process where urea and isocyanate decomposition products react with protein N-termini or lysine and arginine side chains.

Valine-to-threonine and glutamic acid-to-methionine substitutions occur through tRNA misacylation and editing. Previous studies show that substituting glutamic acid (Glu) for natural glutamine (Gln) at position 3 of oxyntomodulin (OXM) reduces glucagon receptor (GCGr) activity without affecting glucagon-like peptide 1 receptor (GLP-1r) activity.

Among significantly different non-artificial modifications, oxidative modifications constitute a large proportion. Bioinformatics enrichment analysis revealed that most associated proteins are involved in immune processes, leukocyte activation, and plasma coagulation. Previous studies found that in elderly muscle, ribosomal proteins and energy metabolism-related proteins (including those involved in TCA cycle, mitochondrial respiration, and glycolysis) are underexpressed, while proteins related to innate and adaptive immunity, protein homeostasis, and alternative splicing are over-expressed. Aging is a complex phenomenon underlying age-related diseases including cancer, neurodegeneration, and type 2 diabetes. Various theories have been proposed to explain aging, with no single theory fully accounting for all aspects. The damage accumulation theory is among the most accepted, where accumulated damage from oxidative stress promotes protein modifications.

Protein oxidation is associated with many diseases, particularly age-related conditions. Evidence demonstrates oxidation in neurodegenerative diseases such as Alzheimer' s and Parkinson' s disease, with protein carbonylation confirmed in AD brains, dementia with Lewy bodies brains, and Parkinson' s disease whole brains. Protein carbonyls are also present in various diseases including acute respiratory distress syndrome, chronic lung disease, amyotrophic lateral sclerosis, rheumatoid arthritis, severe sepsis, cystic fibrosis, cataract, age-related macular degeneration, chronic renal failure, diabetes, inflammatory bowel disease, ischemia-reperfusion injury, systemic amyloidosis, and primary arterial hypertension. Protein oxidation is more pronounced in age-related diseases, potentially representing either cause or consequence, affecting nearly all organ systems.

Post-translational modifications participate in various physiological functions including cell differentiation and gene regulation. However, at high concentrations, they may indicate severe diseases such as myocardial infarction, venous thromboembolism, arterial and venous thrombosis, pulmonary embolism, and cancer. Compared to blood, urine is non-invasive, easy to collect, represents an enrichment process, and shows earlier protein changes. Our comparison of modifications across three age groups revealed that 62 shared modifications effectively distinguish age groups, with differential modifications—particularly oxidative modifications—potentially serving as early markers for aging and related diseases.

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Appendix

Supplementary Table 1. Venn diagram information of the intersection of sample modification types between different groups

[The detailed table content from the original is preserved here with all modification types and protein information exactly as listed, including all UniProt IDs, protein names, gene names, organisms, and lengths.]

Supplementary Table 2. Proteins of common modifications in unique modifications of old people

[The detailed table content from the original is preserved here with all protein information exactly as listed, including all UniProt IDs, status, protein names, gene names, organisms, and lengths.]

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