Changes of Serum Bilirubin and Urine Peptides Related to Bilirubin Metabolism in Patients with Allergic Rhinitis

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Abstract

Background: The incidence rate of allergic rhinitis (AR) has been increasing, which has become a global health problem. As a kind of inflammatory airway disease, allergic rhinitis has a large number of inflammatory cells and inflammatory mediators that participate. Bilirubin is an effective endogenous antioxidant and anti-inflammatory molecule. This study aims to explore the relationship between bilirubin and allergic rhinitis, and to explore the potential value of bilirubin-related metabolites in blood and urine in the assessment of AR disease.

Methods: A total of 63 allergic rhinitis (AR-S group) patients and 86 healthy controls (NC-S group) were enrolled. Venous blood was obtained for measurement of serum total IgE levels and bilirubin parameters. Patients were classified into normal IgE level group (AR2-S group) and elevated IgE level group (AR1-S group). Ten subjects were randomly selected from each group, which were AR2 group, AR1 group and NC group. Urine samples were measured with the nano UPLC-MS/MS system consisting of a Nanoflow HPLC system (EASY-nLC 1000 system from Thermo Scientific) and Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific).

Results: The mean total TBIL level (12.5 vs 15.7 μmol /L, p <0.001), median total DBIL level (4.4 vs 5.3 μmol /L, p <0.001) and mean total IBIL level (8.1 vs 10.3 μmol/L, p <0.001) in AR-S group were significantly lower than those in NC-S group. The mean total TBIL level (13.1 vs 15.7 μmol /L, p = 0.007), median total DBIL level (4.74 vs 5.3 μmol /L, p = 0.022), and mean total IBIL level (8.39 vs 10.3 μmol/L, p = 0.005) in AR2-S were significantly lower than those in NC-S group. The median total TBIL level (12.0 vs 15.7 μmol /L, p < 0.001), median total DBIL level (4.09 vs 5.3 μmol /L, p < 0.001), and median total IBIL level (7.91vs 10.3 μmol/L, p <0.001) in AR1-S were significantly lower than those in NC-S group. In addition, we found that there were 15 urine differential proteins related to bilirubin metabolism in AR2 and AR1. Their relative expression levels increased or decreased successively in NC group, normal IgE level group (AR2) and increased IgE level group (AR1).

Conclusions: Compared with healthy people, the levels of bilirubin metabolites in patients with allergic rhinitis were decrease in the blood. The levels of bilirubin metabolites in urine of patients with allergic rhinitis have changed. This result suggests that bilirubin may be a new target for AR diagnosis and treatment.

1. Background

Allergic rhinitis (AR) is a widespread atopic disease, which is characterized by symptoms of nasal pruritis, nasal congestion, clear rhinorrhea and sneezing. In addition to nasal symptoms, untreated AR patients may also suffer from post-nasal drip, Eustachian tube dysfunction, allergic conjunctivitis, chronic sinusitis, and allergic conjunctivitis [1,2,3].

Epidemiological studies have shown that 20% to 30% of adults and up to 40% of children are affected [4]. The number of patients with allergic rhinitis in the world has exceeded 500 million [5,6]. In recent decades,

with the rapid development of the global economy and urbanization, the incidence of AR has continued to increase [7,8]. It is extremely important to diagnose and treat AR patients as early as possible. A large number of inflammatory cells and inflammatory mediators participate in the pathogenesis of AR and cause acute or chronic inflammation [9,10].

In the past, bilirubin was considered to be a metabolite which is harmful to the human body. Bilirubin is often used as an important indicator to determine the severity of jaundice and to evaluate liver metabolic function. However, with the continuous in-depth research of bilirubin, more and more studies have shown that bilirubin not only has strong antioxidant properties in the body but also has anti-inflammatory and immune regulation effects [11]. Bilirubin can significantly reduce the risk of many inflammation and oxidative stress-related diseases [12,13,14,15], but its relationship with AR has not yet been reported.

Urine is the ultrafiltrate of blood which contains rich biological information. Changes in the composition of urine can reflect the body's metabolic state [16]. In addition, the retention and collection process of urine is a non-invasive operation, which is convenient and can be collected repeatedly. Urine has great clinical application value as a sample that reflects changes in the body. This study focused on the correlation between bilirubin metabolism and allergic rhinitis, and explored potential value of bilirubin-related metabolites in blood and urine in the assessment of AR disease.

2. Materials And Methods

2.1 Study Population

From March 2020 to June 2020, a total of 63 patients with allergic rhinitis according to the diagnostic and treatment principle for allergic rhinitis and a recommended scheme [17] were included in the allergic rhinitis (AR-S) group. The age was 38.8 ± 17.1 years old, including 21 males and 42 females.

According to the total serum IgE level, the patients were divided into AR patients with normal IgE level group (AR2-S group, IgE ≤ 125 IU / ml) and AR patients with elevated IgE level group (AR1-S group, IgE >125 IU / ml). There were 33 patients in AR2-S group, aged 40.19 ± 16.2 years, including 11 males and 22 females, and 30 patients in AR1-S group, aged 38.3 ± 18.3 years, including 10 males and 20 females. The NC-S group included 86 healthy people, aged from 22 to 80 years old, including 35 males and 51 females.

Ten subjects were randomly selected from each group (AR2-S, AR1-S and NC-S), and urine samples were collected for mass spectrometry analysis, which were AR2 group (aged 22~48 years), AR1 group (aged 22~53years) and NC group (aged 25~56 years). Each group including 5 males and 5 females.

The exclusion criteria for participants were as follows: (1) pregnant women; (2) chronic diseases such as chronic kidney disease, hypertension, diabetes, rheumatism and hepatitis; (3) history of somatic or psychiatric abnormalities in the medical records; (4) taking antihistamines, glucocorticoids and other drugs within one week and (5) concomitant medication history during the preceding 2 weeks. All of participants were permanent residents in the area and lived for more than 6 months. This study was

approved by the ethics committee of Beijing Shijitan Hospital, and the participants all gave informed consent, in accordance with the provisions of the Helsinki Declaration.

2.2 Samples Collection

According to a standard protocol to minimize the preanalytical bias, blood samples were collected from all subjects after overnight fasting. Midstream urine samples were collected in the morning into dry and clean containers from all the volunteers. Immediately after collection, urine samples were centrifuged at 4000r/min for 5minutes to remove cell debris and casts. Then we divided the supernatants into aliquots and froze them at -80°C refrigeration until use. Patients with medicine usage (corticosteroids, antibiotics, et al.) 2 weeks prior to the collections of the urine sample should be excluded. No urine samples exhibited hematuresis and urinary albumin/creatinine ratios (A/Cr) were less than 30mg/g. 10 urine samples in each group were mixed and analyzed three times.

2.3 Biochemical Measurements

Serum total IgE levels, total bilirubin (TBIL), direct bilirubin (DBIL) and indirect bilirubin (IBIL) were measured using protein analyzer (Siemens BNII, Siemens, Germany) and automatic biochemical analyzer (AU5800, Beckman Coulter Diagnostics, USA).

2.4 Urinary proteomics

Protein extraction and digestion

Firstly, the samples were diluted with lysis buffer to make the final concentration fall within the standard curve range. The lysis buffer was composed of 7 M urea (bio RAD), 2 m thiorea (sigma Aldrich) and 0.1% CHAPS (bio RAD). Diluted sample (5 ul), standard sample (5 ul) and protein quantitative dye (250 ul) were mixed in dark for 10 min. The standard curve was drawn according to the relationship between the absorbance value of each tube of standard and the concentration, and then calculate the sample concentration. Bradford method was used to measure the concentration of the extracted protein.

100 μg protein from each sample solution was placed in a centrifuge tube; the final volume was adjusted to 100 μ l with solution buffer. 5 μ l 200 mM Reducing Reagent was added into the centrifuge tube and incubated at 55°C for 1 h. Then 5 μ l 375 mM Iodoacetamide solution was added and incubated at room temperature for 30 min in the dark. Next, the liquid was transfer to 10 kd ultrafiltration tube, add 200 μ l 100 mM Dissolution Buffer. After centrifugation, the bottom solution was discarded. This step was repeated 4 times, and use 100 mM Dissolution Buffer to make up the volume to 100 μ L. Finally, 2.5 μ l trypsin was added to each sample, and the temperature was kept at 37 ℃ for 14 h.

TMT labeling

After thawing the TMT (Tandem mass tag) reagent at room temperature, add 41 μ l absolute ethanol into 0.8 mg TMT reagent per tube, shake for 5 min, and then centrifuge. 41 μ l TMT reagent was added

into 100 μ g (100 μ L / sample) of enzyme digested sample and reacted for 1 h at RT. 8 μ L 5% Quinching Reagent was added and incubated for 15 min to terminate the reaction. The mixed labeled samples were centrifuged to the bottom of the tube after vortex oscillation. The samples were stored after lyophilization.

Peptide identification by nano UPLC-MS/MS

The peptide fractions were suspended in 20 μ l buffer A (0.1% FA, 2% ACN) and centrifuged at 12,000 rpm for 10 min. 10 μ l of supernatant was injected into the nano UPLC-MS / MS system composed of Nanoflow HPLC system (EASY nLC 1000 system, Thermo Scientific) and Orbitrap fusion Lumos mass spectrometer (Thermo Scientific). The samples were loaded on an Acclaim PepMap100 C18 column for separation. The mass spectrometer operates in positive ion mode and the full MS scans were performed with 120000 resolution in the range of 300-1500 m/z. In MS / MS scanning, 20 most abundant multiple charged ions were selected for high energy collision dissociation fragmentation after a full MS scan. UniProt_HUMAN (2019.4.20) was used in this experiment. The MS / MS data were processed by proteome discoverer 1.4.

Protein identification

The protein was identified using the following parameters: (1) precursor ion mass tolerance: ± 15 ppm; (2) fragment ion mass tolerance: ± 20 mmu; (3) max missed cleavages: 2; (4) modification: static modification, dynamic modifications, carboxyamidomethylation of Cys residues; oxidation modification of Met residues. Data with $P \leq 0.05$ and difference rate ≥ 1.2 were selected for further analysis.

2.5 Bioinformatics analysis

After differentially expressed proteins were detected among NC group, AR2 group and AR1 group with a proteomics method, these data were submitted to the the Gene Ontology Consortium (http://geneontology.org/). Briefly, the dataset was analyzed to rank the proteins according to their associated biological process (BP), molecular function (MF) and cellular component (CC). The Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) was used to map proteins with differential expression levels in the pathway biological maps.

2.6 Statistical analysis

All data are presented as the mean ± standard deviation (SD). The Student's t test was used to evaluate differences among NC group, AR2 group and AR1 group. Kruskal Wallis test was used to compare non normal distribution among groups. Bonferroni correction test was used to compare the two groups. *P* value less than 0.05 were considered statistically significant (two-tailed). Statistical analyses were performed using SPSS 25.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.0 statistical software (GraphPad Software Inc., La Jolla, USA).

3. Results

3.1 Comparison of serum bilirubin parameters between AR-S and NC-S groups

The overall study population consisted of 149 subjects. The subjects in AR-S group had significantly lower mean total TBIL level (12.5 vs 15.7 μmol /L, p <0.001), lower median total DBIL level (4.4 vs 5.3 μmol /L, p <0.001), lower mean total IBIL level (8.1 vs 10.3 μmol/L, p <0.001) than those in NC-S group. (Table 1 and Figure 1).

Table1 Comparison of serum bilirubin parameters between AR-S and NC-S groups

	NC-S（n=86）	AR-S（n=63）	z/t	P
IgE（IU/mL）	19.5（11.2~40.9）	351.4（67.8~289）	-7.972	p＜0.001
TBIL（μmol/L）	15.7±4.28	12.5±3.8	4.715	p＜0.001
DBIL（μmol/L）	5.3（4.2~6.4）	4.4±1.4	-4.513	p＜0.001
IBIL（μmol/L）	10.3±2.95	8.1±2.6	4.567	p＜0.001

3.2 Comparison of serum bilirubin characteristics among NC-S group, AR2-S group and AR1-S group

The serum bilirubin characteristics among NC-S group, AR2-S group and AR1-S group studied are shown in Table2. The subjects in AR2 group had significantly lower mean total TBIL level (13.1 vs 15.7 μmol /L, p = 0.007), lower median total DBIL level (4.74 vs 5.3 μmol /L, p = 0.022), lower mean total IBIL level (8.39 vs 10.3 μmol/L, p = 0.005) than those in NC-S group. In addition, the subjects in AR1 group had lower median total TBIL level (12.0 vs 15.7 μmol /L, p < 0.001), lower median total DBIL level (4.09 vs 5.3 μmol /L, p < 0.001), lower median total IBIL level (7.91vs 10.3 μmol/L, p <0.001) than those in NC-S group. We found no significant differences in TBIL, DBIL and IBIL between AR2-S group and AR1-S group. The change trend of serum bilirubin characteristics among the three groups was shown in Figure 2.

Table2 Comparison of serum bilirubin parameters with three groups

	NC-S（n=86）	AR2-S（n=33）	AR1-S（n=30）	p value
IgE（IU/mL）	19.5（11.2~40.9）	70.2（24.4~107.5）	291.5（150.0~557.5）	<0.001
TBIL（μmol/L）	15.7±4.28	13.1±4.0	12.0±3.5	<0.001
DBIL（μmol/L）	5.3（4.2~6.4）	4.74±1.6	4.09±1.0	<0.001
IBIL（μmol/L）	10.3±2.95	8.39±2.7	7.91±2.6	<0.001

3.3 Comparison of urine differential protein among NC group and AR group

A total list of 2104 non-redundant proteins were measured with TMT-based mass spectrometry. We found 1614 differential proteins in AR1 compared with those in NC group, of which 1557 were up-regulated and 57 were down-regulated. In AR2 and NC groups, 1703 differential proteins were identified, including 1673 up-regulated and 30 down-regulated proteins. In addition, we found 418 differential proteins in AR1 compared with those in AR2 group, of which 97 were up-regulated and 321 were down-regulated (Table 3 and Figure 3).

Table 3 Comparison of the differential urine proteins among NC, AR2 and AR1 groups.

Comparisons	Upregulation	Downregulation	All
AR1 vs. NC	1557	57	1614
AR2 vs. NC	1673	30	1703
AR1 vs. AR2	97	321	418

3.4 Analysis of urine differential proteins related to bilirubin metabolism

This study mainly discusses the relationship between bilirubin metabolism and allergic rhinitis. We screened the urine proteins by bioinformatics analysis (GO and Kyoto Encyclopedia of Genes and Genomes analysis). Moreover, we found that there were 15 differential urine proteins related to bilirubin metabolism in the three groups, including 9 up-regulated proteins and 6 down-regulated proteins. Their relative expression levels increased or decreased successively in NC group, AR2 group and AR1 group. The protein information and relative expression values are shown in Table 4, figure 4 and Figure 5.

Table 4 Quantitative analysis of 15 differentially expressed proteins related to bilirubin metabolism among AR1, AR2 and NC groups.

ID	Description	NC	AR2	AR1	GO and KEGG
CD9_HUMAN	CD9 antigen	632.13	972.93	991.7	hsa:928,path:hsa04640,Hematopoietic cell lineage
HEM2_HUMAN	Delta-aminolevulinic acid dehydratase	20.23	109.37	116.93	GO:0004655,porphobilinogen synthase activity GO:0006783,heme biosynthetic process GO:0006782,protoporphyrinogen IX biosynthetic process GO:0010039,response to iron ion hsa:210,path:hsa00860,Porphyrin and chlorophyll metabolism hsa:210,path:hsa01100,Metabolic pathways
AMBP_HUMAN	Protein AMBP	38285.67	57106.8	57111.1	GO:0020037,heme binding GO:0042167,heme catabolic process
PERM_HUMAN	Myeloperoxidase	1380.87	2163.5	7667.97	GO:0020037,heme binding
NGAL_HUMAN	Neutrophil gelatinase-associated lipocalin	825.57	1823	3642.53	GO:0005506,iron ion binding GO:0006879,cellular iron ion homeostasis GO:0097577,sequestering of iron ion GO:0015891,siderophore transport
ACOC_HUMAN	Cytoplasmic aconitate hydratase	127.97	667.8	704.03	GO:0051538,3 iron, 4 sulfur cluster binding GO:0051539,4 iron, 4 sulfur cluster binding GO:0030350,iron-responsive element binding GO:0046872,metal ion binding GO:0006879,cellular iron ion homeostasis GO:0010040,response to iron(II) ion
CATA_HUMAN	Catalase	106.03	238.83	462.87	GO:0020037,heme binding GO:0020027,hemoglobin metabolic process
RGMC_HUMAN	Hemojuvelin	35.8	172.67	189.7	GO:1990459,transferrin receptor binding GO:0006879,cellular iron ion homeostasis GO:0055072,iron ion homeostasis
CDO1_HUMAN	Cysteine dioxygenase type 1	26.67	62.73	68.97	GO:0008198,ferrous iron binding
CSF1_HUMAN	Macrophage colony-stimulating factor 1	2257.07	2046.27	1840.03	GO:0030097,hemopoiesis hsa04640,Hematopoietic cell lineage
KIT_HUMAN	Mast/stem cell growth factor receptor Kit	81.63	47.63	40	GO:0035162,embryonic hemopoiesis GO:0050673,epithelial cell proliferation GO:0030218,erythrocyte differentiation GO:0038162,erythropoietin-mediated signaling pathway GO:0002244,hematopoietic progenitor cell differentiation GO:0035701,hematopoietic stem cell migration GO:0030097,hemopoiesis path:hsa04640,Hematopoietic cell lineage
NEO1_HUMAN	Neogenin	775.4	728.9	715.7	GO:0055072,iron ion homeostasis
VAS1_HUMAN	V-type proton ATPase subunit S1	299.17	225.1	188.53	GO:0055072,iron ion homeostasis
NECT1_HUMAN	Nectin-1	165.2	120.93	96.97	GO:0055072,iron ion homeostasis
HEPC_HUMAN	Hepcidin	292.5	275.57	243.43	GO:0097690,iron channel inhibitor activity GO:0060586,multicellular organismal iron ion homeostasis GO:1904255,negative regulation of iron channel activity GO:1904039,negative regulation of iron export across plasma membrane GO:0034760,negative regulation of iron ion transmembrane transport GO:0036017,response to erythropoietin GO:0010039,response to iron ion

GO:1990641,response to iron ion starvation;

4. Discussion

Allergic rhinitis (AR) is a response against inhaled allergens mediated by immunoglobulin (IgE), which causes inflammation driven by type 2 helper (Th2) cells. The main pathogenesis of AR is type I hypersensitivity mediated by IgE [18]. Allergens can activate T cells to increase the number of Th2 cells and secrete IL-4 to induce B cells to proliferate and differentiate into plasma cells, which produce IgE. The IgE combines with receptors on the surface of mast cells and basophils to make the body in a sensitized state. When the same allergen enters the body again, it will combine with the IgE and degranulate the target cells, release a variety of biological inflammatory mediators and cause an inflammatory response, which can cause nasal and extra nasal symptoms in AR patients [19].

Bilirubin is a metabolite of hemoglobin and heme. Bilirubin is mainly metabolized in the liver, including the absorption of bilirubin in the blood by hepatocytes, the transport of bilirubin in hepatocytes, the conversion of unconjugated bilirubin to conjugated bilirubin and excretion with bile [20,21,22]. In addition, hemoglobin is composed of heme and globin. Heme is an iron porphyrin compound. The biological processes of red blood cells, iron and porphyrins also play an important role in the metabolism of bilirubin.

In this study, serum bilirubin levels with AR patients and healthy control group were measured. The differential proteins in urine of the three groups were identified and quantitatively analyzed by combining TMT labeling with LC-MS / MS. As a result, we found that serum bilirubin levels in allergic rhinitis patients were significantly lower than NC groups. Besides, we also found that serum bilirubin levels in allergic rhinitis patients with elevated IgE levels were significantly lower than those with normal IgE levels and NC groups. In this study, the standard deviation of bilirubin levels was higher, which may be due to the greater biological variation of bilirubin [23]. All in all, by comparing the bilirubin levels in the serum of patients with AR and normal controls, we found that the bilirubin levels in the serum of patients with AR may be reduced. Urine is the ultrafiltrate of blood. How will the levels of bilirubin metabolism-related substances in urine change between patients with allergic rhinitis and normal controls?

Interestingly, through the bioinformatics analysis of GO and KEGG, we found 15 differential proteins in urine samples closely related to bilirubin metabolism. For example, Neutrophil gelatinase-associated lipocalin, Cytoplasmic aconitate hydrataseis, Hemojuvelin, Cysteine dioxygenase type 1, Neogenin, V-type proton ATPase subunit S1, Nectin-1and Hepcidin involved biological processes related to iron metabolism. Protein AMBP, Myeloperoxidase and Catalase involved biological processes related to heme metabolism. CD9 antigen, Macrophage colony-stimulating factor 1 and Mast/stem cell growth factor receptor Kit involved biological processes related to hematopoiesis, Delta-aminolevulinic acid dehydratase is closely related to porphyrin metabolism. Their relative expression levels increased or decreased successively in NC group, normal IgE level group (AR2) and increased IgE level group (AR1).

Combined with the changes in bilirubin-related metabolites in serum and urine, we speculate that bilirubin is closely related to allergic rhinitis. Some studies have shown that bilirubin can regulate the transcription process of the inflammatory mediator IL-2 and then inhibit the activity of T cells [24]. Bilirubin can also play an antioxidant role by down regulating the level of intracellular reactive oxygen species and scavenging free radicals [25]. Besides, studies have shown that when the bilirubin level is at a higher level within the normal physiological range, its protective effect on the body is stronger. As an endogenous anti-inflammatory and antioxidant, bilirubin can be consumed in inflammation and oxidative stress caused by allergic reactions, thereby reducing the damage of inflammation and oxidative stress to the body [26,27].This was consistent with our research results.

5. Conclusion

In conclusion, compared with healthy people, the levels of bilirubin in patients with allergic rhinitis were decrease in the blood. The levels of bilirubin metabolites in urine of patients with allergic rhinitis have changed. These results provide valuable information for further research on the relationship between bilirubin and allergic rhinitis. The bilirubin may be a new target for AR diagnosis and treatment. However, the specific mechanism of bilirubin and its metabolite-related products in allergic rhinitis needs further research and exploration.

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of Beijing Shijitan Hospital, and the participants all gave informed consent, in accordance with the provisions of the Helsinki Declaration.

Consent for publication

All the authors consent to the publication of the manuscript.

Availability of data and material

All the data in the manuscript are available from the corresponding author and first author.

Competing interests

All authors declare that they have no competing interests.

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Authors' contributions

LN collected patients' clinical information, analyzed the data and wrote the manuscripts. WJ and QS performed the detection and proteomics data processing. ZM obtained the fund, designed the experiments and revised the manuscripts. All authors read and approved the final manuscript.

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Figures

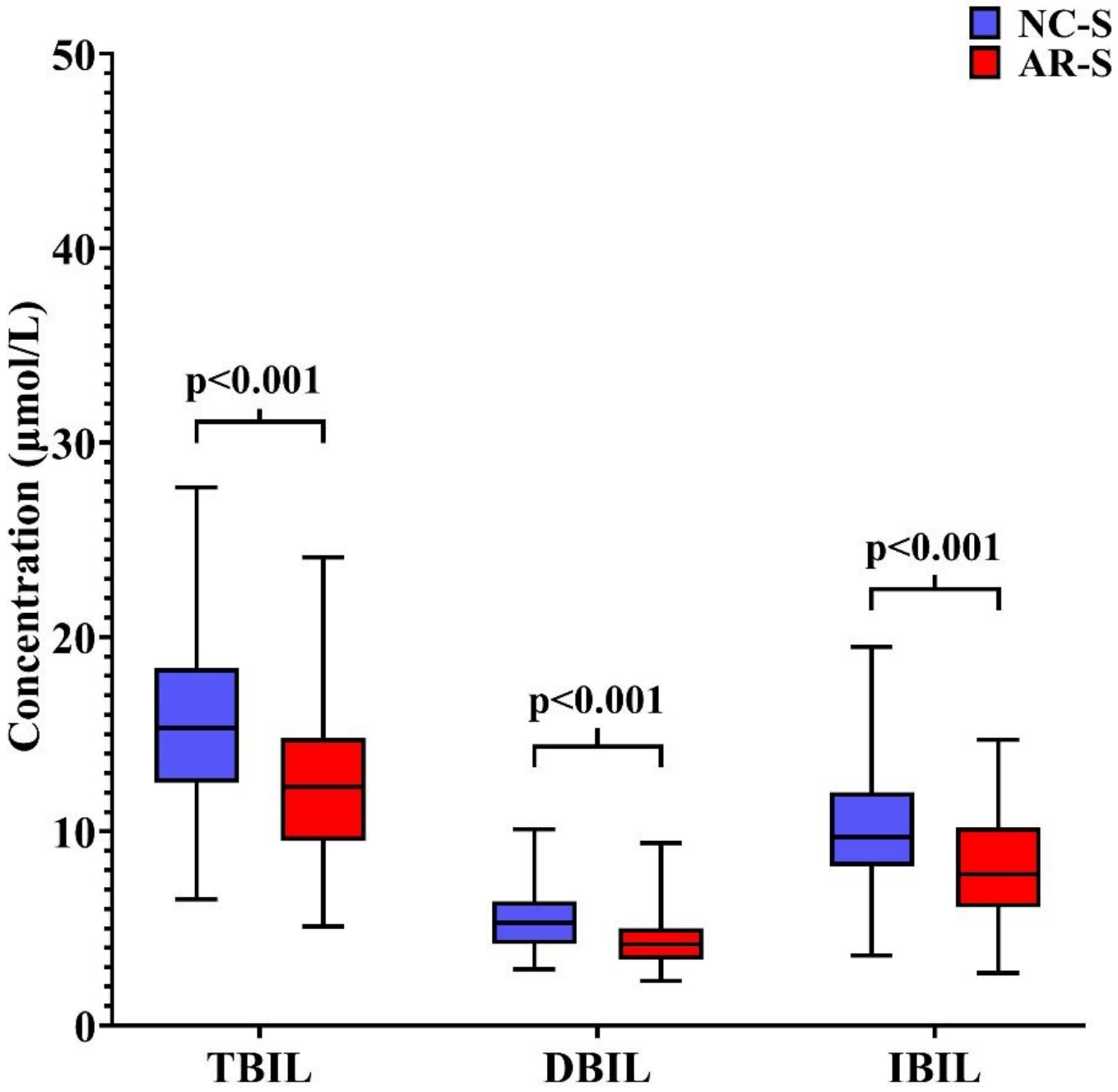

Figure 1

Comparison of serum bilirubin parameters between AR-S and NC-S groups

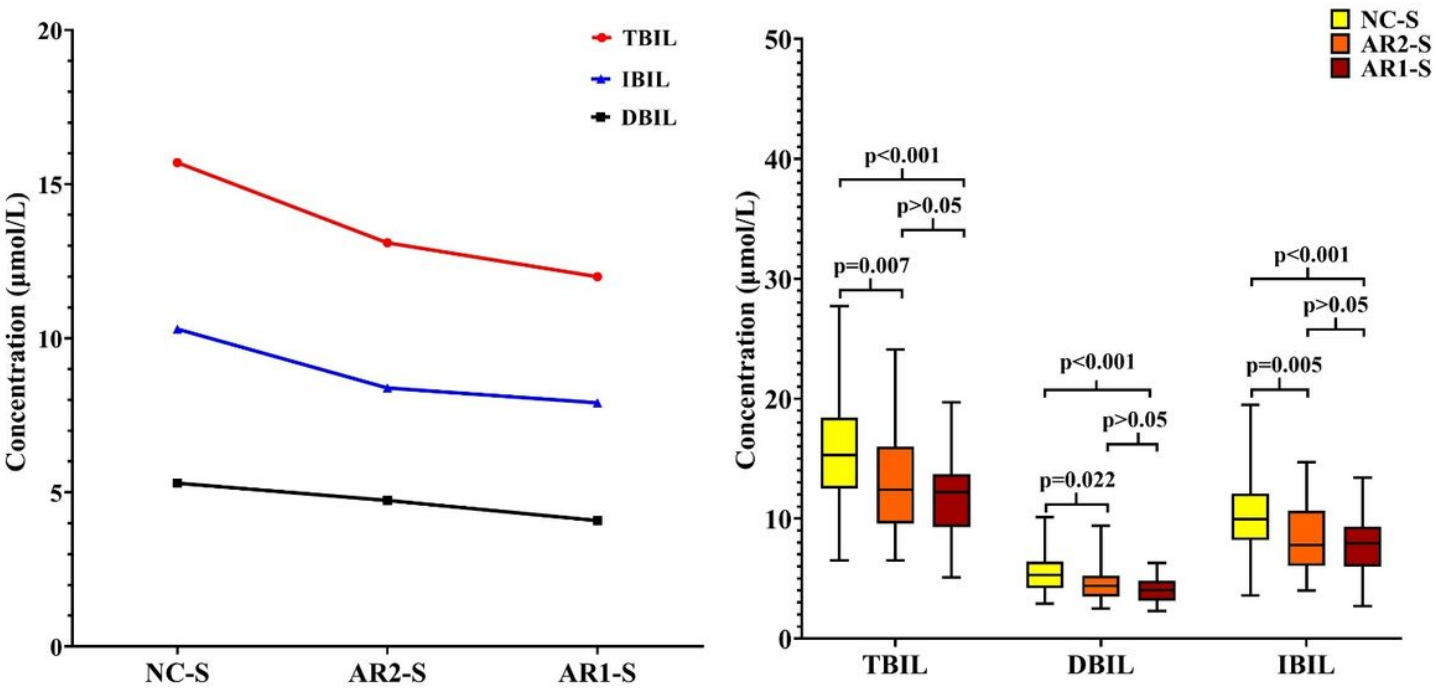

Figure 2

Comparison of serum bilirubin parameters with three groups

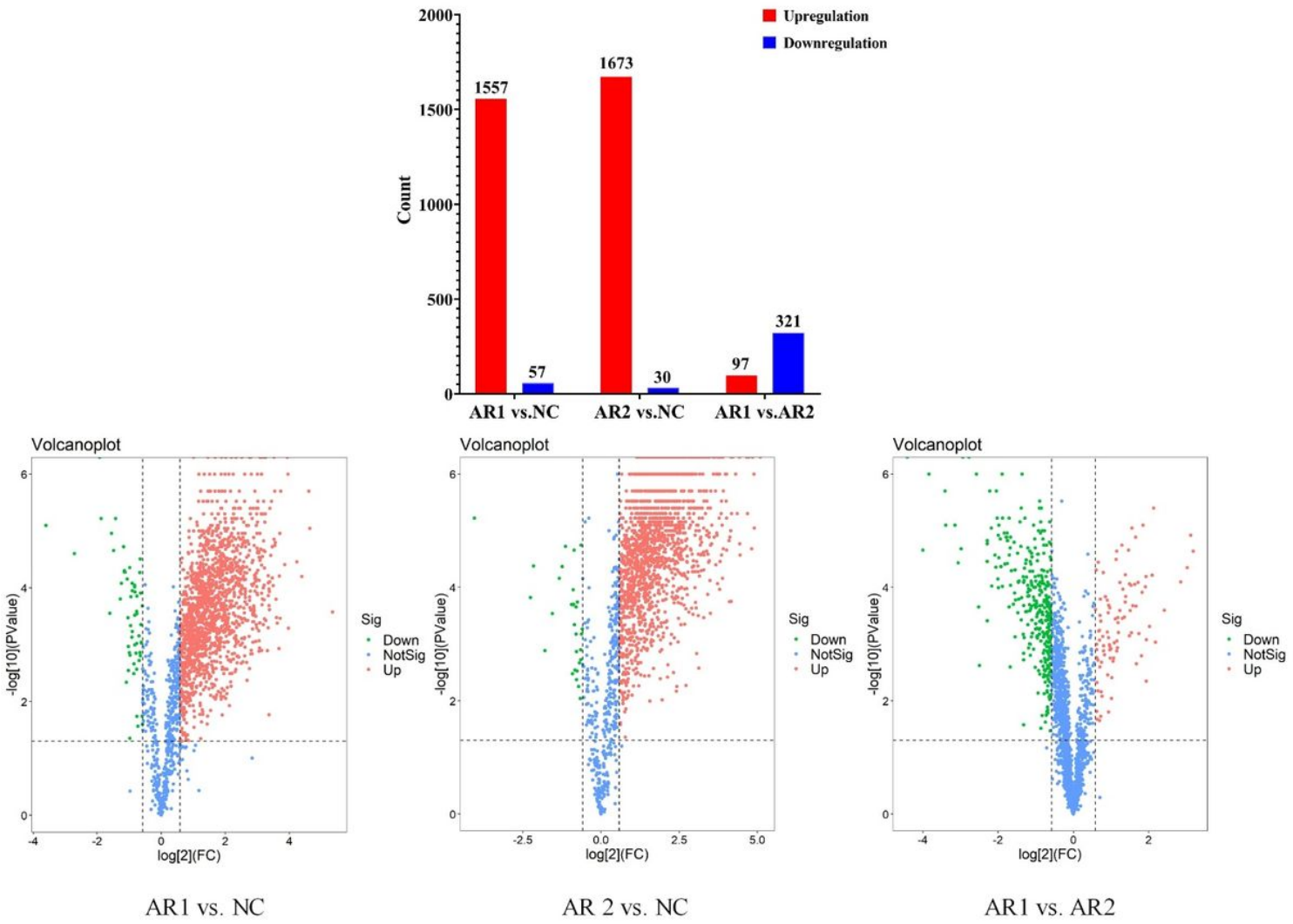

Figure 3

Differential urine proteins among AR1, AR2 and NC groups

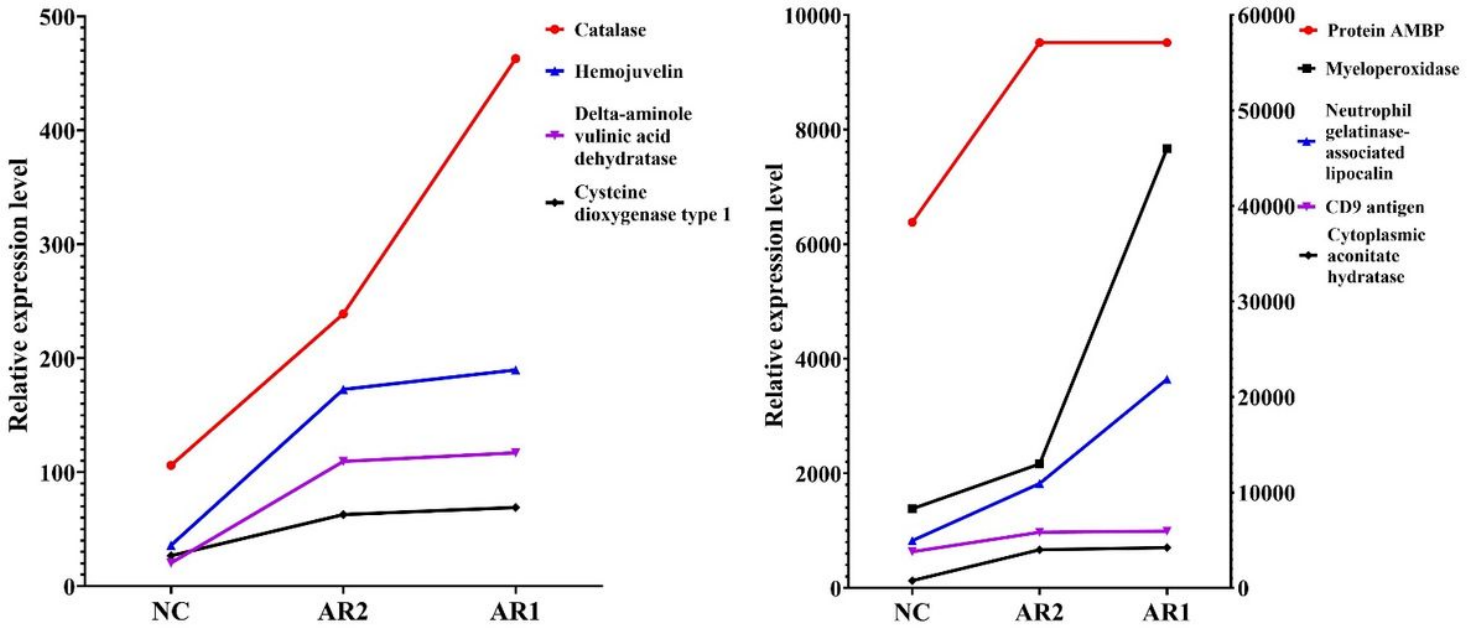

Figure 4

The relative fold changes comparison of 9 up-regulated proteins among NC group, AR2 group and AR1 group.

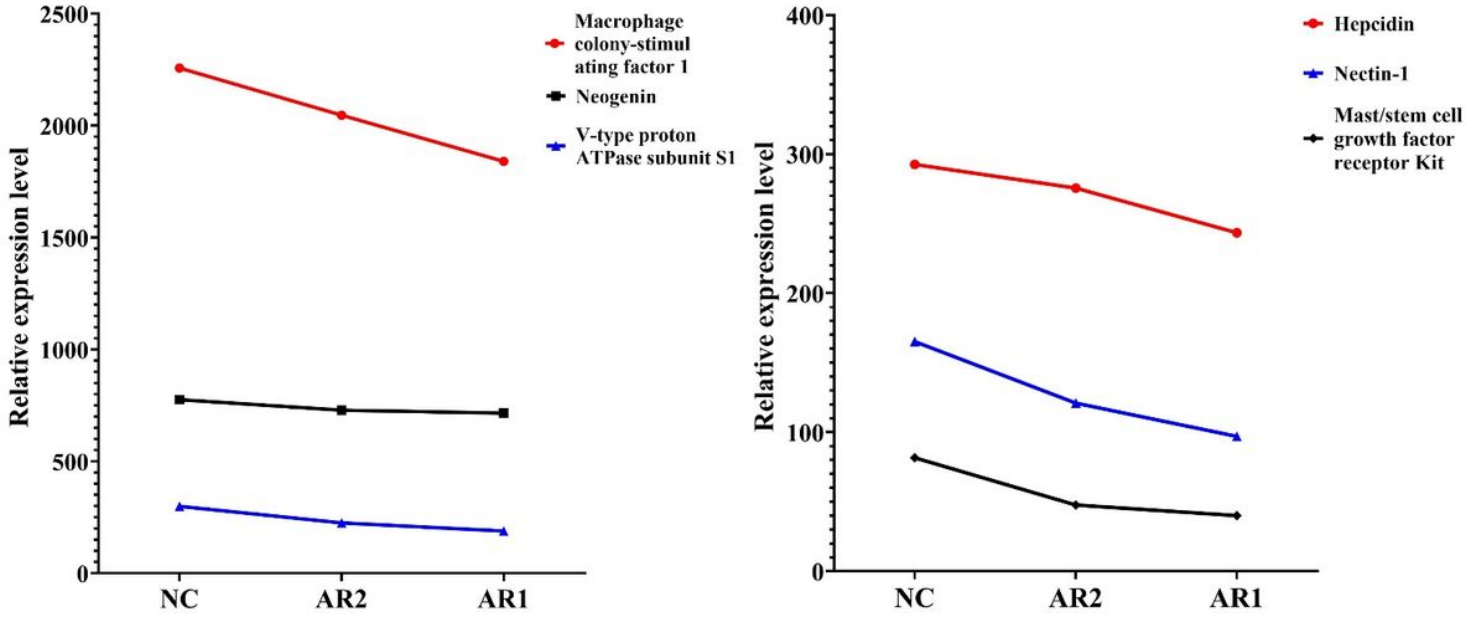

Figure 5

The relative fold changes comparison of 6 down-regulated proteins among NC group, AR2 group and AR1 group.