Proteomics to Metabolomics: A New Insight Into the Pathogenesis of Hypertensive Nephropathy

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Research

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Abstract

Background

Hypertensive nephropathy (HN) is a high burden disorder and a leading cause of end-stage renal disorder. In spite of huge investigations, the underlying mechanisms are yet largely unknown. Systems biology is a promising approach to provide a comprehensive insight towards this complex disorder.

Methods

Protein expression profiles of kidney tubule and cortex sub-compartments were retrieved from the PRIDE database and the quality of the datasets were assessed using principal component analysis (PCA) and hierarchical clustering. Differentially expressed proteins (DEPs) were detected and their attributed metabolites were enriched and their interactions were assessed in multi-layer networks. Moreover, considering the DEPs and the predicted metabolites, key biomedical phenomena with a leading role in HN pathogenesis were proposed.

Results

Amino acid and purine metabolisms are the most prominent alteration in kidney cortex whereas dysregulation of energy hemostasis is a key pathogenic mechanism in tubule. Besides, actin cytoskeleton disorganization is an enriched pathway in both anatomical areas.

Conclusion

The proteomics profiles of kidney sub-compartments were analyzed using a top-down approach to infer the main pathogenic processes. The constructed holistic map of HN can be exploited to propose novel therapeutic strategies.

Introduction

As a common cause of diverse non-communicable disorders, hypertension has posed a remarkable challenge to human health (1). Hypertensive nephropathy (HN) is a progressive disorder regarded as the second cause of end-stage renal disease (2). The current therapeutic strategies are insufficient due to the lack of comprehensive knowledge on the underlying mechanisms of this complex disorder (3). The generation of omics data in line with the holistic approach of systems biology offers a unique opportunity to approximate this complexity (4).

In this research, various integrative strategies have been employed to reanalyze two proteome profiling datasets previously generated from the kidney cortex and tubule of a rodent model of HN (5)(6). Although

proteomics datasets have provided valuable clues about the alteration of a very important layer of biomolecules, they are most insightful when inspected in their interactions with other molecular types (7). Therefore, the multi-layer networks of key interactions in kidney sub-compartments were provided to construct a bigger picture of the molecular events. It was found that, metabolic processes are essential components in the map of HN molecular pathogenesis.

Methods

Proteomics data acquisition

Proteomics datasets of (PXD002106, PXD012889) were achieved from an experimental study by Kenneth et.al. In these two datasets, the protein expression was explored in kidney tubules (PXD002106 dataset) and inner and outer cortex of kidney (PXD012889 dataset) in two kidney one clip (2K1C) model. A sham-operated animal model was used for comparison. For retrieving datasets from the Proteomics Identification database (PRIDE) (8), two keywords of hypertensive nephropathy and kidney were searched.

Identification and quantification of proteins

MaxQuant v1.6 (9) was used to convert raw datasets to data tables. The search was carried out for the Uniprot_Mus musculus proteome database (January 26, 2019, 22,287 entities) by the software integrated search algorithm called Andromeda (10). Datasets were analyzed by the same following parameters: 1- Trypsin was specified as cleavage enzyme and up to 2 missed cleavages were allowed, 2- Carbamidomethylation was selected as a fix modification and protein N-terminal acetylation and methionine oxidation were selected as variable modifications, 3- Match between runs parameter was enabled, and 4- False discovery rate (FDR) was considered at 1% for protein and peptide identification. Default settings were used for Mass analyzer parameters.

Quality control assessment

Quality control of the datasets was assessed by principal component analysis (PCA) and hierarchical clustering through the Euclidian cluster approach. R software and ggplot2 package (11) were employed for performing the PCA and hierarchical clustering, as well as their visualization.

Differential expression analysis

Perseus version 1.6.2.2 (12) was used to analyze the label-free quantification (LFQ) intensity columns obtained by the preliminary analysis of MaxQuant. In this step, first, the identified irrelevant protein groups including contaminants, only identified by site and reverse proteins. Then, the data were transformed into the logarithmic scale (log2(x)); to specify groups, filter rows, and statistical analysis, categorical annotation rows were applied. The table rows were filtered based on 3 valid values; while for the imputation of missing values, the missing values from normal distribution under the imputation tab

were replaced in the software environment. Student T-test (permutation-based FDR ≤ 0.05) was carried out to identify the differentially expressed proteins (DEPs).

Metabolites prediction

To predict metabolites, which may have a connection with the differentially expressed enzymes, all the related metabolites were first identified by Metscape plugin version 3.0 (13) of Cytoscape version 3.7 (14). Using Ingenuity pathway analysis (IPA) version 49932394 (QIAGEN Inc.) (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis), the specific metabolites related to the specific enzymes in metabolic pathways were selected.

Pathway enrichment analysis

IPA was applied for core analysis. The directional changes were identified by the calculated z-score using the Ingenuity Knowledge Base. Z-score >2 was considered as a significant alteration in the activities. Fisher exact test was utilized to select significant pathways with adjusted P-value ≤ 0.05.

Protein-protein interaction network construction and functional enrichment analysis

Interactions between the DEPs were constructed using STRING database version:11.1 of Cytoscape CluePedia plugin version 1.5.5 (15). ClueGO plugin version 2.5.5 (16) was applied for the functional enrichment analysis considering FDR ≤0.05. The highly connected sites of the networks were determined by Molecular Complex Detection (MCODE) plugin (version 1.5.1) (17).

Metabolites-Proteins interaction network construction

MetScape plugin of Cytoscape was utilized to discover and construct correlations between the predicted metabolites and the related enzymes. The search in the MetScape was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. In this step, the pathway-based option was selected and the constructed network included compounds, reactions, enzymes, and genes.

Metabolites functional and pathway enrichment analysis

Metabolite set enrichment analysis and pathway analysis of predicted metabolites was carried out by MetaboAnalyst 4.0 (18). Out degree centrality was applied for pathway topology analysis. The Fisher exact test was considered as the over-representation analysis. The predicted metabolites were annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG) (19) and enrichment analysis was performed based on Small Molecule Pathway Database (SMPDB) (20).

Results

Considering the increasing trend in big data generation and rapid advancements in bioinformatics tools, re-analysis of pre-existed datasets for discovering new pathways and molecular keys in pathological conditions is of utmost importance. In this study, a systematic approach was adopted to generate a map

of key events during the progression of HN (Fig. 1). Different proteomics datasets were entirely explored and two datasets generated by Kenneth et al. were chosen for further analysis. The datasets were proteomics profiles of tubules (PXD002106), as well as the inner and outer cortex (PXD012889) in a mouse model of HN known as 2K1C. In this model, unilateral renal artery constriction with a clip decreased renal artery perfusion and consequently systemic hypertension. Based on these profiles, the investigators have underscored the pivotal roles of Periostin, Transgelin and Vimentin in HN pathogenesis.

To provide further insights into the molecular pathogenesis, the above-mentioned datasets were re-analyzed with a systemic unsupervised approach. Using MaxQuant, we identified 2074, 1925, and 2015 proteins (FDR ≤ 0.01) in the tubule, inner, and outer cortex sub-compartments, respectively (Supplementary Table 1). Since we have previously highlighted the inappropriate quality of the majority of omics datasets, quality control assessment was performed before further analysis (20) Accordingly, principle component analysis (PCA) was applied which showed that most samples were scattered according to their experimental groups, indicating the acceptable quality of the data (Fig. 2a). However, a few samples not following this segregation pattern were eliminated to enhance the quality of the data. The quality of datasets was also examined by hierarchical clustering which was in agreement with the PCA (Fig. 2b). In the following, Perseus was applied to determine the differentially expressed proteins (DEPs) among the identified proteins considering permutation-based FDR ≤ 0.05. The results indicated 166, 378, and 320 DEPs in the tubule, inner and outer cortex, respectively (Fig. 2c, Supplementary Table 2). In agreement with previous studies, the alteration range of DEPs in the cortex was higher than that in the tubule (5)(6) implying the significant involvement of cortex in this disease (Table 1).

To explore the functional role of DEPs, their protein classes were determined. Notably, a considerable fraction of the DEPs was enzymes, providing an initial clue on the key role of enzymatic pathways in the disease pathogenesis (Fig. 3). Furthermore, Gene ontology (GO) enrichment analysis was carried out on different levels, including biological process (BP), molecular function (MF), and cellular component (CC) (Fig. 3). In agreement with the above findings, a variety of metabolic pathways were enriched in all sub-compartments, especially in the cortex. Furthermore, terms related to the cytoskeleton were among the other significantly enriched terms.

To further validate the above findings on the role of metabolic processes pathway enrichment analysis was conducted on the DEPs utilizing IPA. According to the GO results, a considerable fraction of the enriched pathways was related to metabolic processes especially for the inner cortex (Fig. 4). Additionally, the Rho related signaling pathways were enriched in all sub-compartments. Notably, several previous studies have reported the role of these pathways in vasoconstriction and hypertension (21–23). Moreover, other significantly enriched pathways such as VEGF signaling, complement system, and pathway of inositol have been previously recognized to be involved in kidney failure and hypertension (24–28). “HIPPO signaling” is also among the enriched pathways whose role in HN has not yet been determined.

To identify the interaction map of the DEPs, PPI networks were constructed for all sub-compartments using Cytoscape CluePedia plugin. Since the central genes in networks are the key drivers of biological pathways and processes (29), the topology of the constructed networks was analyzed and central proteins were identified in terms of degree and betweenness centrality (Table 2). Noteworthy, a substantial number of the central DEPs, such as Kyat1, Decr1, Fbp1, Sdha are enzymes involved in metabolic pathways. This could further signify the critical role of metabolic processes in the progression of HTN. Moreover, two modules with the highest clustering coefficient scores were identified in each of the three networks to identify densely connected sub-graphs (Fig. 5).

In agreement with the above findings, the proteins in the modules of the inner cortex network mainly attributed metabolic processes and cytoskeleton organization. One of the outer cortex modules is also related to cytoskeleton .Notably, the other module of the outer cortex and one of the modules in the tubule network are highly rich in proteasome elements which is in line with the previous research on the renoprotective effects of proteasome inhibitors in HN (30).Remarkably, the other group of highly-connected proteins in tubule involved in energy metabolism which can be described by increased mitochondrial function in tubules in HN as also reported in previous animal models (31)(32).

To elucidate the interactions between proteins and other bio-molecules, especially metabolites, integrated networks composed of DEPs, unique enzymes, reactions, and metabolites were constructed (Fig. 6). For this purpose, among all DEPs, unique enzymes involving in specific pathways were identified and their related metabolites were added to explore the relationships between enzymatic and non-enzymatic proteins with the metabolic reactions and metabolites. Moreover, pathway and functional enrichment analyses were carried out for the predicted metabolites using MetaboAnalyst. These analyses underscored the importance of amino acid and purine metabolism as well as energy homeostasis in this disorder (Fig. 7).

As the above analyses propose the essential role of amino acid and purine metabolisms in the pathogenesis of hypertensive nephropathy, a few representatives of such processes were further investigated in more detail; Several enzymes in tryptophan degradation such as Kmo, Kyat1, Kynu, and Afmid are among downregulated DEPs. Moreover, key enzymes for valine degradation including Bckdha, Echs1, and Hibadh are downregulated. Also, nearly all enzymes in purine metabolism showed up-regulation in the examined datasets which led to the accumulation of urate as the end product of this pathway (Fig. 8). To provide a holistic view of the functional relationships between key dysregulated enzymes, the map of affected processes is depicted (Fig. 9).

Discussion

Hypertension is a global health problem and a major risk factor of non-communicable disorders such as cardiovascular and kidney diseases. Despite huge investigations, the complex underlying mechanisms of these disorders have not been comprehensively understood. However, the recent advancements of high-throughput technologies and holistic systems biology approaches have provided the opportunity to shed

light on these complexities. In this study, a systematic approach was adopted to re-analyze previously generated proteomics datasets on HN to provide clinically-valuable knowledge.

The proteomics datasets exploited in this study were originally produced by Kenneth et al using a rodent model of hypertensive nephropathy. The advantage of their approach is the examination of expression profiles in kidney sub-compartments separately. It was previously shown that the kidney cortex and medulla possess considerably different gene expression patterns and respond differently to stressors (33). Hence, profiling the whole kidney tissue can be misleading. Besides, high throughput techniques are vulnerable to a variety of confounding elements and technical errors leading to unsatisfying quality of a majority of available data. The datasets examined in this study were appropriately generated as shown by quality control measures. Also, considering the importance of holistic insight and integration of biological layers as a trending approach to generate inclusive maps for complex disorders, the multi-layer networks were constructed for the interactions of different elements in HN. Since an unsupervised approach into omics data is a crucial criterion for working with big data, we followed this approach which could be recognized as one of the important superiorities of this study.

Various methods such as pathway and GO enrichment, protein classification, and network analysis were employed to explore the functional role of DEPs. All these approaches coherently revealed the pivotal role of energy homeostasis, and metabolic processes in the pathogenesis of hypertensive nephropathy. Furthermore, the results indicate that actin cytoskeleton organization is involved in the response of all kidney sub-compartments to hypertension. Accordingly, a large and growing body of evidence has indicated the role of actin cytoskeleton in the progression of kidney diseases (34–36). Moreover, previous studies have highlighted Rho GTPase pathway as a key regulator of actin cytoskeleton in podocytes and vascular injury (37–39). In this regard, fasudil was used as a Rho-kinase in HN models (40). Notably, our results revealed the considerable dysregulation of this pathway in all sub-compartments. In addition, Cdc42 the main element of Rho GTPase pathway and a critical protein in the regulation of actin cytoskeleton is among the central nodes in the inner cortex network (41). Furthermore, proteasome degradation is another significant term observed in the tubule part. Interestingly, the potential role of proteasomes in regulating actin cytoskeleton was first demonstrated experimentally by Haarer et.al.in 2011 (42). Remarkably, analysis of the tubule network indicated a deep connection between proteasome components. Taken together, in line with previous investigations, our findings also underscore the role of Rho GTPase pathways and proteasomes in the progression of HN which is potentially mediated via actin cytoskeleton dysregulation.

Consistent with previous studies, our results underline the role of energy hemostasis in the pathogenesis of hypertensive nephropathy. Different elements in TCA cycle are among the DEPs in the cortex. However, the terms related to energy metabolism are mainly enriched in the tubule dataset which can be described to high energy demand of ion transporters. The proteins of NADH: ubiquinone oxidoreductase supernumerary subunits (NDUF) family are detected to be differentially expressed in the tubule dataset. Dysregulation of this protein family is shown to be linked with reactive oxygen species (ROS) production (43). Notably, the above discussed Rho GTPase pathway is also involved in ROS accumulation (44). In

agreement, glutathione mediated detoxification and glutathione redox reaction are enriched in the cortex section. It should be noted that a high-metabolic-rate organ such as the kidney is vulnerable to ROS overload and the consequent inflammatory response.

The regulation of metabolic processes has been long known as one of the key functions of the kidney. However, the role of these processes in the pathogenesis of HN is just recently investigated. Our results indicate that a majority of DEPs, especially in the cortex, are functionally attributable to the metabolism of amino acids in particular, tryptophan, methionine, and valine. Remarkably, a bundle of recent evidence indicates the attenuation of kynurenine enzyme activity and accumulation of tryptophan pathway metabolites in HN (44–46). Additionally, dysregulation of methionine metabolism is shown in this disorder (45). Cianciolo et al. also demonstrated that folate deficiency perturbs the metabolism of methionine and results in hyperhomocysteinemia in CKD (46). Accordingly, the downregulation of MTHFD1 was observed which is a key enzyme in folate metabolism. We observed that different enzymes in valine degradation are downregulated in the inner cortex. Moreover, this process was detected in the enrichment analyses as well. To the best of our knowledge, this is the first report on the involvement of valine degradation process in HN. Interestingly, a recent study by Rinchen et.al supported our findings. Using an animal model of HN, they found that although valine is not differentially expressed, acetylvaline a derivative of valine is reduced in kidneys (47). Altogether, it seems that the alteration of amino acid metabolism is a key role player in the progression of HN. However, the exact underlying mechanisms remain to be understood.

Our analysis is in favor of purine metabolism dysregulation and uric acid accumulation in HN. On the other hand, previous studies have shown that uric acidemia activates renin-angiotensin system (RAAS) via ROS accumulation which will finally result in systemic hypertension and potential kidney damage (48). Hence, it seems that purine metabolism dysregulation and uric acid overload form a vicious cycle worsening kidney damage in HN, this process seems a reasonable candidate for therapeutic targeting. Fortunately, there are safe drugs in the market for xanthine dehydrogenase inhibition whose repositioning for HN can be investigated in future trials. In agreement with this suggestion, allopurinol is being assessed for diabetic nephropathy (49)(50)

Taken together, based on a holistic integrative approach, this study agrees with previous investigations underscoring the impact of metabolic pathways dysregulation in HN pathogenesis. Hence, this disorder can be viewed as an “acquired error of metabolism.”. This insight will hopefully pave the way for the development of novel therapeutics.

Declarations

Availability of data and materials

The Datasets analyzed by the current study are available in the PRIDE repository

https://www.ebi.ac.uk/pride/archive/projects/PXD012889

https://www.ebi.ac.uk/pride/archive/projects/PXD002106

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Author’s contribution

- Study concept and design by **Yousof Gheisari**
- Acquisition of data by **Yasin Eshraghi**
- Analysis and interpretation of data by **Yasin Eshraghi**, **Maryam Abedi**, **Yousof Gheisari**
- Drafting of the manuscript by **Yasin Eshraghi**, **Yousof Gheisari**
- Critical revision of the manuscript for important intellectual content by **Yasin Eshraghi**, **Maryam Abedi**, **Yousof Gheisari**
- Study supervision by **Yousof Gheisari**

Ethics declarations

The Ethics Committee of Isfahan University of Medical Sciences approved the study and waived the need of informed written/verbal consent for this study.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Conflict of interest

There is no conflict of interest to declare.

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Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.

Figures

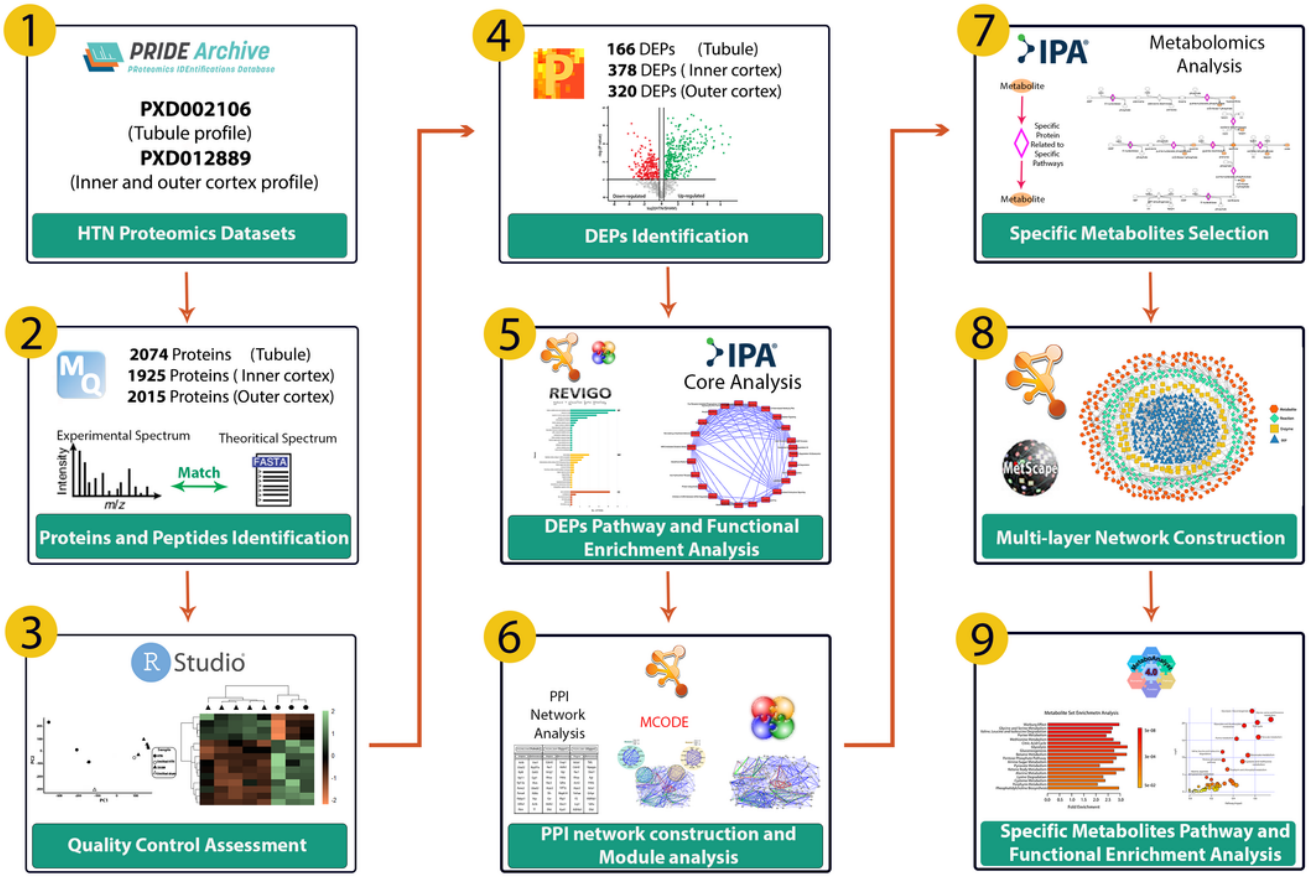

Figure 1

The schematic overview of present study. We employed a system biology approach to provide a comprehensive map of hypertensive nephropathy.

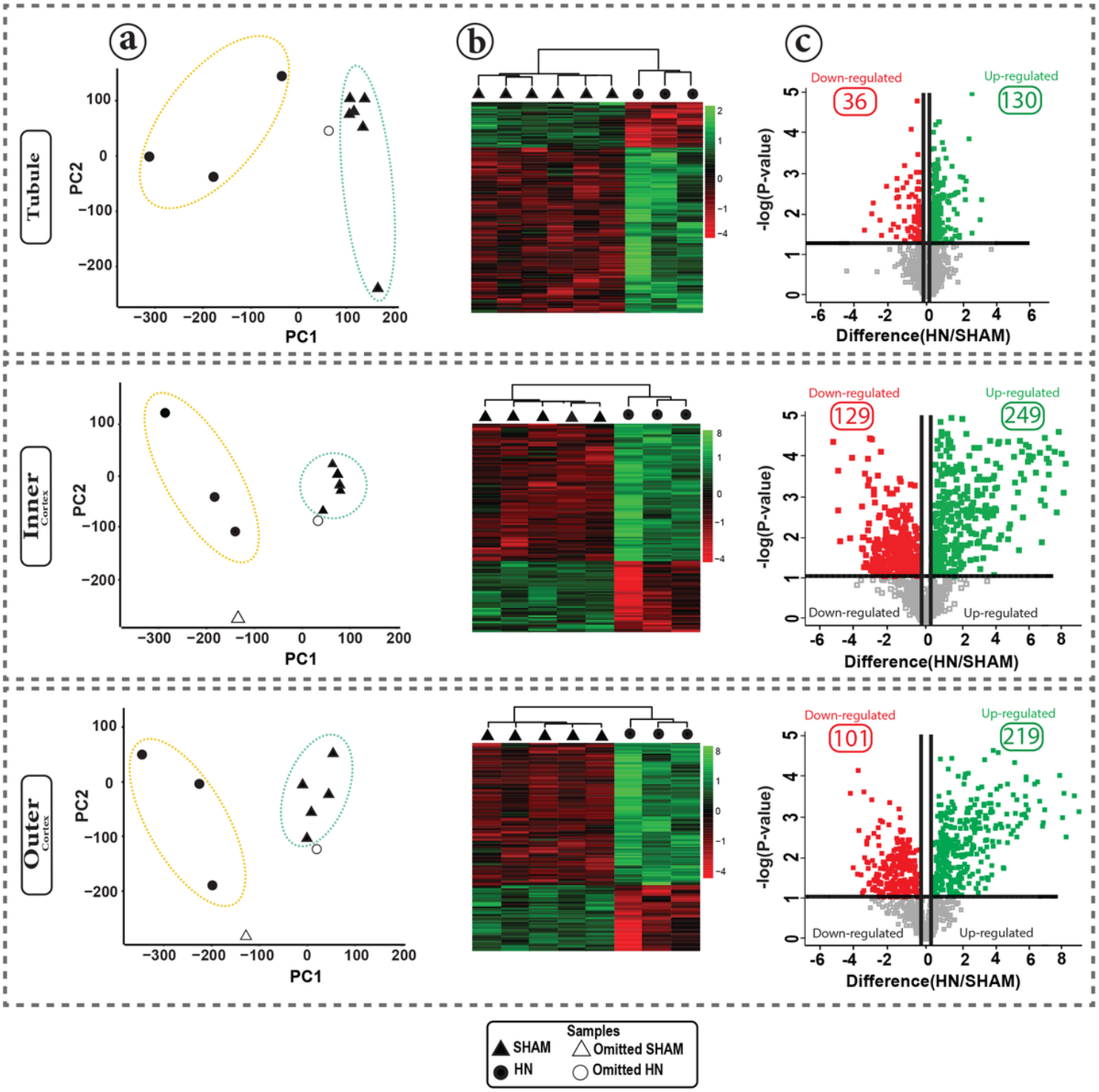

Figure 2

Datasets quality was assessed: Unsupervised quality control methods showed an appropriate segregation of samples based on study groups (a, b). Volcano plots of differentially expressed proteins with FDR<0.05 are shown. Up-regulated and down-regulated proteins depicted with green and red, respectively (c).

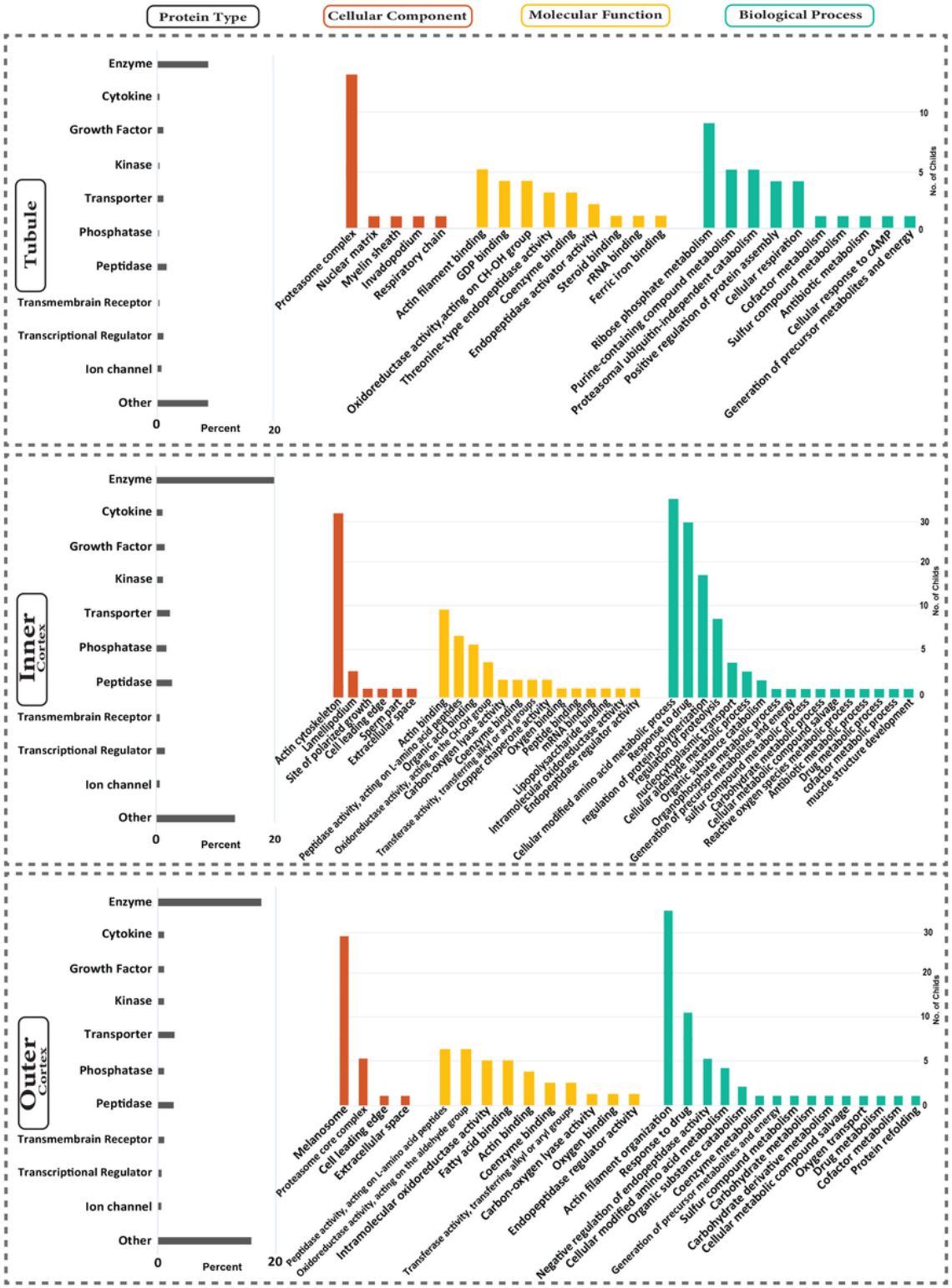

Figure 3

Gene ontology (GO) enrichment analysis determined key biological process involved in HN pathogenesis and protein classification were performed based on their types. GO molecular function analysis of differentially expressed proteins (DEPs) showed the majority of proteins are enzymes. GO enrichment analysis revealed the involvement of DEPs in metabolic process as well as actin cytoskeleton dysregulation. GO terms were summarized as parent terms and false discovery rate less than 0.05 was

considered as significant threshold. The biological process, molecular functions, and cellular components are depicted with green, yellow, and red respectively. The protein type section exhibits the classification of proteins concerned with their biological role.

Figure 4

Pathway enrichment analysis was performed for each dataset. The involvement of Rho GTPase signaling and actin cytoskeleton is highlighted in all kidney sub-compartments. Rho GTPase related terms, the metabolic process and actin related pathways are marked with one star, two stars and underline, respectively. Terms with positive and negative z-score are shown in orange and blue, respectively. Pathways with adjusted p-value ≤ 0.05 are shown

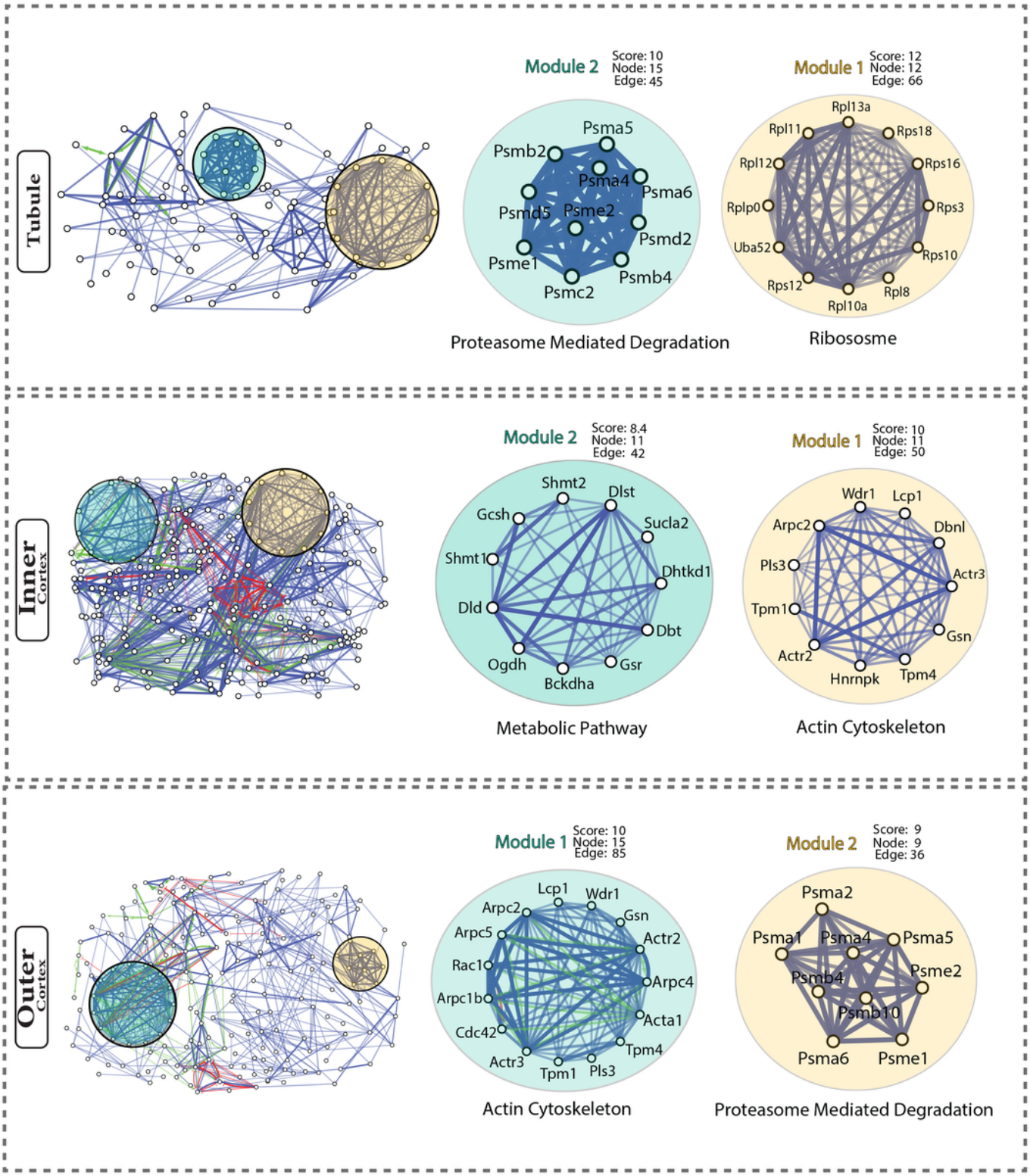

Figure 5

Modules were identified in interaction networks of DEPs. Proteins in dense modules of networks were related to metabolic process, actin cytoskeleton and proteasome degradation.

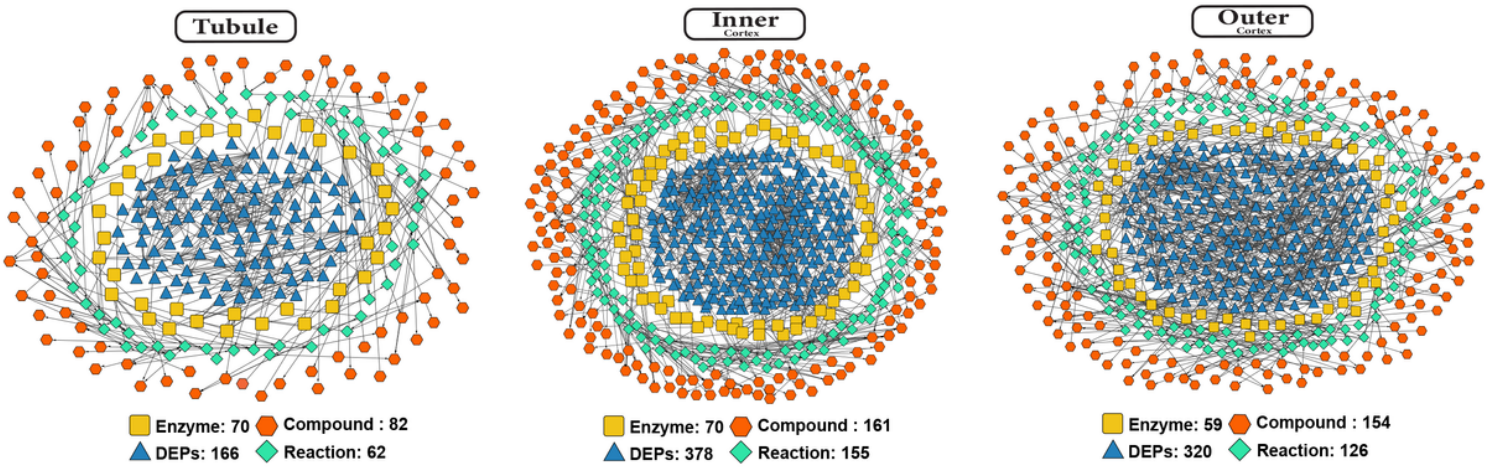

Figure 6

Multi-layer networks were constructed for kidney sub-compartments. For DEPs related metabolites were predicted and metabolic-enzyme-protein networks were constructed.

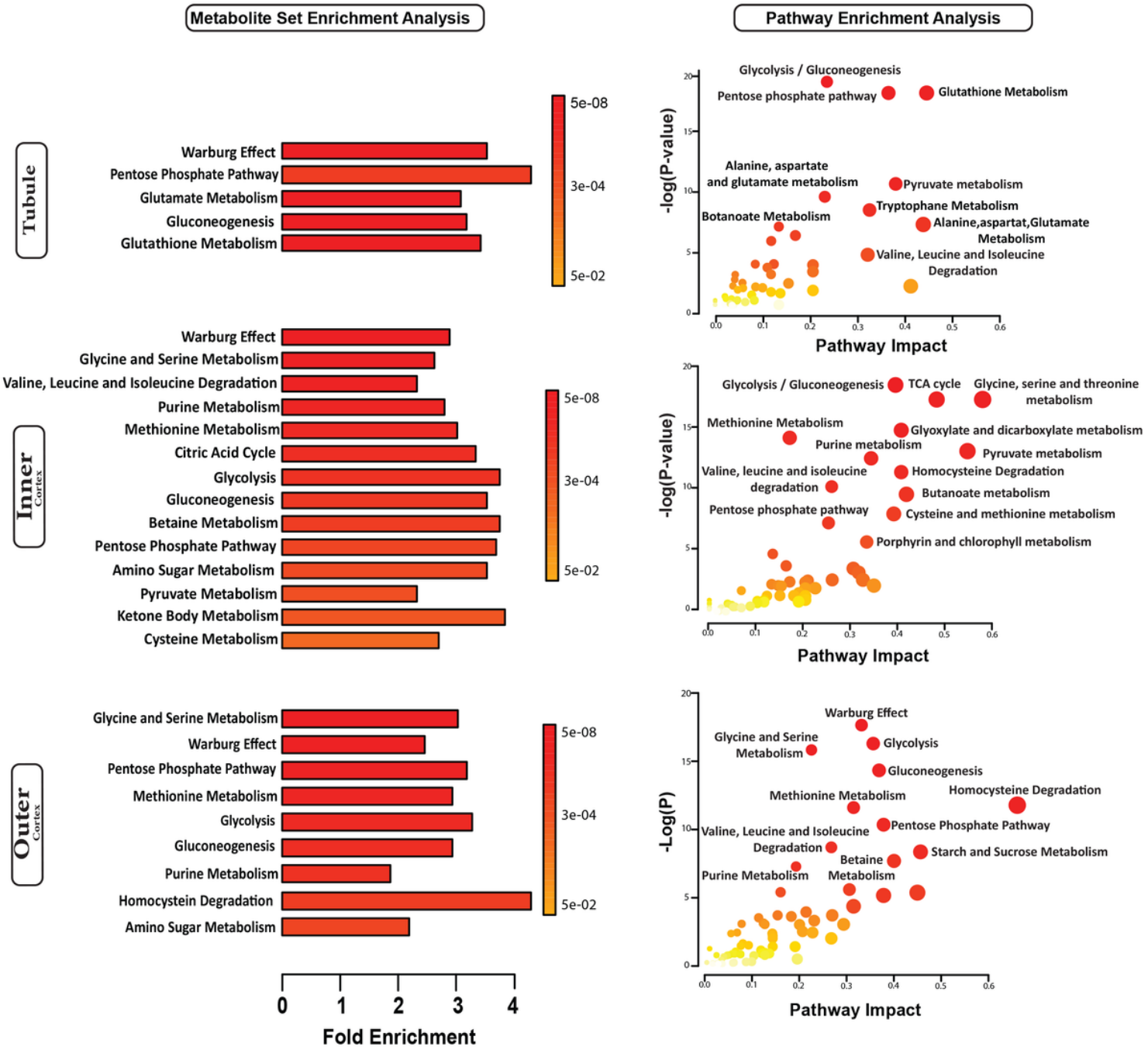

Figure 7

Functional enrichment analyses were performed for the metabolites. Enrichment analysis was carried out for predicted metabolites based on Small Molecule Pathway Database (SMPDB). Amino acids and nucleotide metabolisms are prominent enriched pathways related to the metabolites The altered metabolic pathways associated to each sub-compartment based on Kyoto Encyclopedia of Genes and Genomes. The color indicates adjusted p-value.

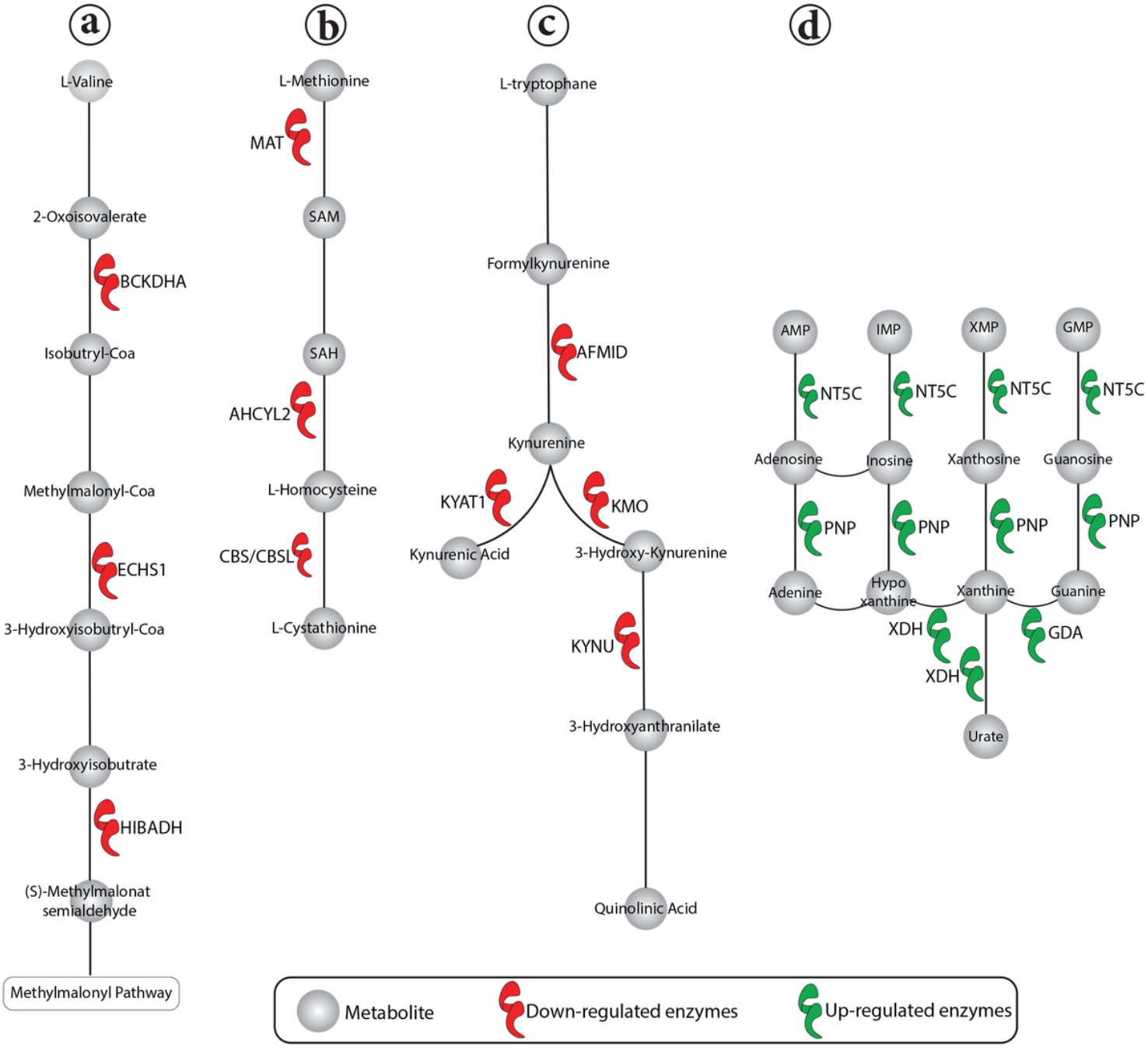

Figure 8

The key affected metabolic pathways. Valine (a), Methionine (b), Tryptophan (c) and purine (d) metabolisms are the key pathways that have been affected in HN pathogenesis.

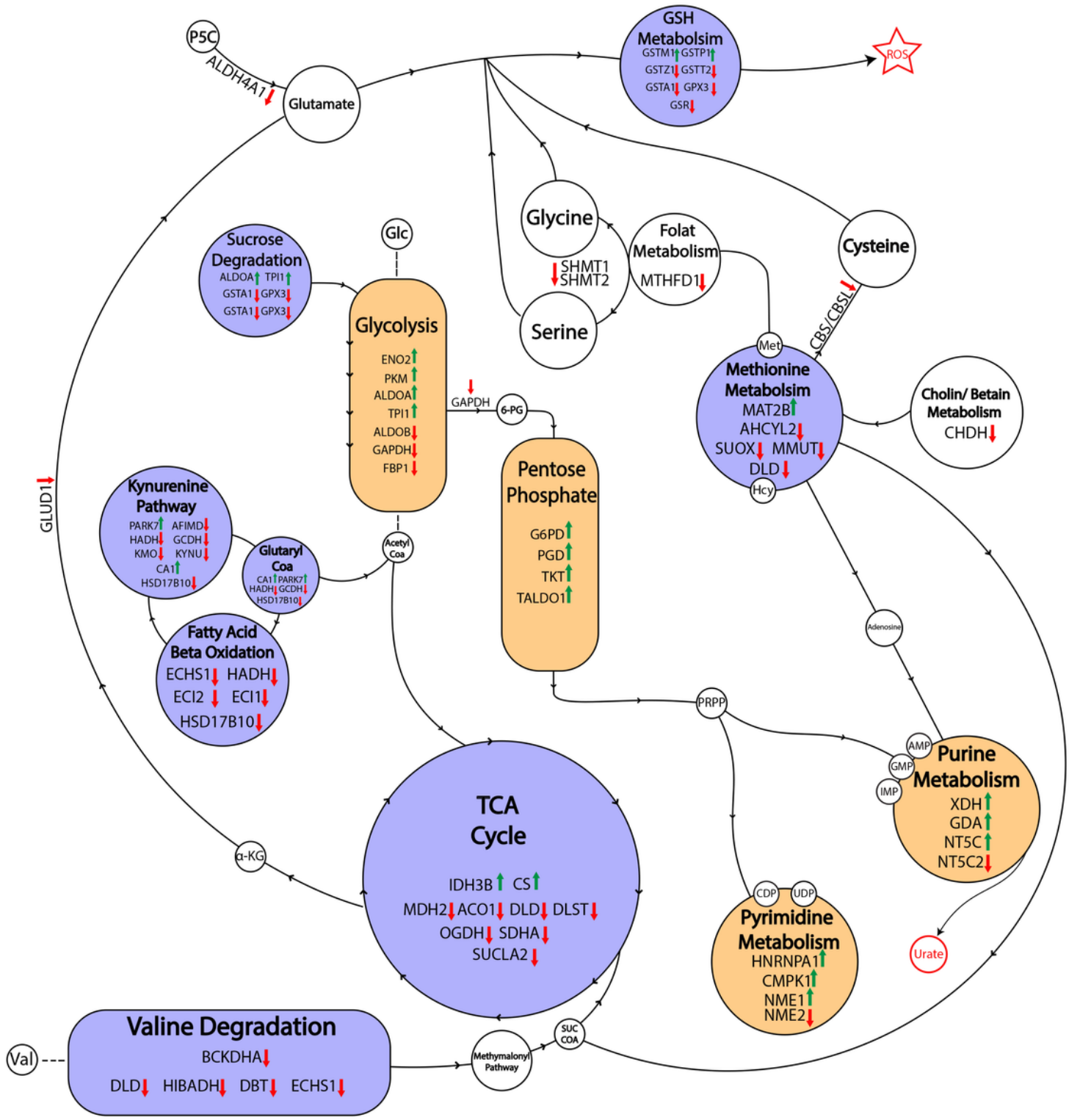

Figure 9

The schematic cross-talk of metabolic process in HN. A holistic metabolic map of key dysregulated enzymes, affected processes and their cross-talk is shown. Pathways with positive and negative z-score are shown in orange and blue, respectively

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.png
- Table2.png
- SUPP1MA05.xlsx
- SUPP2MA05.xlsx